Journal of Microbiology, Immunology and Infection (2013) 46, 366—373

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.e-jmii.com

ORIGINAL ARTICLE

Phosphoproteome profiling of the sexually transmitted pathogen *Trichomonas vaginalis*

Yuan-Ming Yeh [a,b], Kuo-Yang Huang [a,c], Ruei-Chi Richie Gan [d,e], Hsien-Da Huang [e], Tzu-Chien V. Wang [a,b], Petrus Tang [a,c,d,*]

[a] *Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[b] *Department of Molecular and Cellular Biology, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[c] *Molecular Regulation and Bioinformatics Laboratory, Department of Parasitology, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[d] *Bioinformatics Center, Chang Gung University, Taoyuan, Taiwan*
[e] *Institute of Bioinformatics and Systems Biology, National Chiao Tung University, Hsinchu, Taiwan*

Received 23 May 2012; received in revised form 13 June 2012; accepted 10 July 2012

KEYWORDS
Phosphoproteome;
Titanium dioxide enrichment;
Trichomonas vaginalis

Background/Purpose(s): Trichomoniasis caused by *Trichomonas vaginalis* is the most common non-viral sexually transmitted infection. Morphological transformation from the trophozoite stage to the amoeboid or pseudocyst stage is crucial for *T. vaginalis* infection and survival. Protein phosphorylation is a key post-translational modification involved in the regulation of several biological processes in various prokaryotes and eukaryotes. More than 880 protein kinases have been identified in the *T. vaginalis* genome. However, little is known about the phosphorylation of specific proteins and the distribution of phosphorylated proteins in different stages of the morphological transformation of *T. vaginalis*.
Methods: To obtain a more comprehensive understanding of the *T. vaginalis* phosphoproteome, we analyzed phosphorylated proteins in the three morphological stages using titanium dioxide combined with LC-MS/MS.
Results: A total of 93 phosphopeptides originating from 82 unique proteins were identified. Among these proteins, 21 were detected in all stages, 29 were identified in two different stages, and 32 were stage specific.

* Corresponding author. Molecular Regulation and Bioinformatics Laboratory, Department of Parasitology, College of Medicine, Chung Gung University, 259 Wenhwa 1st Road, Kweishan, Taoyuan 333, Taiwan.
E-mail address: petang@mail.cgu.edu.tw (P. Tang).

1684-1182/$36 Copyright © 2012, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. All rights reserved.
http://dx.doi.org/10.1016/j.jmii.2012.07.010

Conclusion: Identification of stage-specific phosphorylated proteins indicates that phosphorylation of these proteins may play a key role in the morphological transformation of *T. vaginalis*.
Copyright © 2012, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. All rights reserved.

Introduction

Trichomonas vaginalis is the causative agent of one of the most common sexually transmitted diseases, trichomoniasis.[1] Approximately 5 million cases of trichomonal vaginitis are reported annually in North America. Worldwide, over 170 million cases of trichomoniasis are reported each year. Infection is acquired primarily through transmission of trophozoites by direct sexual contact, although neonatal infection has also been reported.[2] Trichomoniasis is associated with adverse pregnancy outcomes, enhanced predisposition to HIV infection and risk of cervical neoplasia.[3,4]

A unique feature of the *T. vaginalis* life cycle is the reversible morphological transformation from the trophozoite stage to the amoeboid or pseudocyst stage. The parasite multiplies in the vagina or prostate of infected hosts in the trophozoite form. When it attaches to the surface of vagina epithelial cells, the trophozoite transforms to an amoeboid stage and induces acute inflammation of the vagina, resulting in a yellowish-green foul-smelling discharge in some females. Transformation to the amoeboid stage is crucial for establishment and maintenance of *T. vaginalis* infection in the vagina. Several proteins that mediate adherence, such as surface proteins (AP120, AP65, AP51, AP33, AP23),[5,6] cysteine proteinases,[7] glyceraldehyde 3-phosphate dehydrogenase,[8] and lipophosphoglycan,[9] have been identified. Increasing evidence demonstrates that *T. vaginalis* can survive in the form of a pseudocyst under unfavorable environmental conditions, such as nutrient deprivation, the presence of drugs or dramatic changes in temperature.[10] In this form, the parasite becomes non-motile and the flagellae are internalized, but a true cyst wall is not formed. Recent studies indicate that the pseudocyst stage is reversible and represents a defense mechanism against conditions detrimental to trichomonad survival.[11,12] This pseudocystic stage is probably responsible for transmission of trichomoniasis via non-sexual contact. At present, how transformation between the trophozoite, amoeboid and pseudocyst stages is controlled and regulated in *T. vaginalis* remains largely unknown.

The modulation of protein phosphorylation through the antagonistic effects of protein kinases and protein phosphatases is a major regulatory mechanism for most cellular processes. Protein kinases are one of the major classes of enzymes involved in signal transduction and signaling networks. Many growth factors, cytokines and antigens initiate signaling events through protein phosphorylation, leading to cellular responses such as gene expression, changes in cell shape and migration, entry into the cell cycle, and proliferation. Protein phosphorylation is a major mechanism by which external stimuli are transformed into intracellular signals to which cells respond. It has been estimated that 30—50% of intracellular proteins are phosphorylated at some point in their lifetime.[13] The *T. vaginalis* kinome comprises more than 880 eukaryotic protein kinases and 40 atypical protein kinases, making it one of the largest kinomes identified to date.[14] Large-scale gene expression analysis using expressed sequence tags showed that 451 kinase genes (50% of the kinome) are expressed. At present, it is not known if any of these kinases participate in the transformation of trophozoites into amoeboids or pseudocysts. An alternative approach to explore the function of these kinases in the transformation process is to investigate the phosphoproteome of *T. vaginalis* and to use the resulting data set as a basis for deducing the kinase—substrate interaction network. In the present study, we elucidated the expression profile of phosphoproteins in the trophozoite, pseudocyst and amoeboid stages of T. *vaginalis* using a reverse-phase LC/MS/MS system.

Methods

Cell culture

T. vaginalis isolate ATCC30236 (JH 31A#4) was maintained in YIS medium, pH 5.8, containing 10% heat-inactivated fetal calf serum at 37 °C.[15] The number of viable cells was determined based on hemocytometer counts using Trypan blue exclusion. Trophozoites grown to the late logarithmic phase with more than 90% viable cells were harvested for further study. The pseudocyst stage was induced by incubating trophozoites in late logarithmic phase on ice for 4 hours. The amoeboid stage was induced by growing trophozoites in late logarithmic phase in fibronectin-coated T-75 flasks for 3 hours at 37 °C.

Preparation of cell extracts, digestion of protein mixtures and enrichment of phosphopeptides

T. vaginalis cells from trophozoites, pseudocysts and amoeboids were harvested by centrifugation at 3000 rpm for 15 minutes and were washed there times in normal saline. Cell pellets were resuspended in lysis buffer (8 M urea, 4% CHAPS) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) at a cell density of approximately 1.2×10^8 cells/mL. Cells were disrupted by ultrasonication (XL2000, Misonix Inc., Farmingdale, NY, USA) in an ice bath for eight cycles, each consisting of 10 seconds of sonication followed by a 10-second break. After centrifugation at 4 °C and 13,000 rpm for 15 minutes, impurities in the supernatant were removed using a 2D cleanup kit (GE Healthcare, Taipei, Taiwan). For tryptic digestion, the protein mixtures were diluted in 50 mM ammonium bicarbonate (Sigma, St. Louis, MO, USA) and digested with sequencing-grade trypsin (1:50 w/w; Promega, Madison, WI, USA) at 56°C for 1 hour. The digested lysate was reduced with 25 mM NH_4HCO_3 (Sigma) containing 10 mM dithiothreitol (DTT; Sigma) at 37°C for 30 minutes and then alkylated with 55 mM iodoacetamide (Sigma) at room temperature for 30 minutes. After further reduction with

10 mM DTT at room temperature for 30 minutes, the protein mixtures were digested with sequencing-grade trypsin (20 mg/mL; Promega) at 37°C overnight. The *T. vaginalis* phosphopeptides were enriched using a PHOS-TRAP phosphopeptide enrichment kit (Perkin Elmer, Waltham, MA, USA) according to the manufacturer's protocol.

LC/MS/MS

The enriched phosphopeptides from trophozoites, pseudocysts and amoeboids were separated using a gradient system on a 10-cm analytical C18 column (inner diameter, 75 μm) with a 15-μm tip (New Objective, Woburn, MA, USA) as previously described.[16] Analytes in the eluent were detected with an online LTQ-Orbitrap linear ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA).

Sequence database search and data analysis

The resulting MS/MS spectra for the phosphoproteome were used to search reference amino acid sequences downloaded from the TrichDB database (release 1.2; http://eupathdb.org/eupathdb/) using the X! Tandem (CYCLONE release)[17] and MASCOT search engines (Matrix Science, London, UK; version 2.2.07). For protein identification, mass tolerance of 2 Da was permitted for intact peptide masses and 1 Da for fragment ions, with allowance for two missed cleavages in the trypsin digest, oxidized methionine (+15.99 Da) and phosphorylation (S, T, Y, +79.98 Da) as potential variable modifications and carbamidomethyl cysteines (C, +57.05 Da) as fixed modifications. The experimental data were searched and merged with different search engine results.

Results

T. vaginalis phosphoproteome

To determine the profile of phosphoproteins expressed in cells of the three different *T. vaginalis* stages, total protein lysates were extracted from trophozoites, pseudocysts and amoeboids and subjected to in-solution tryptic digestion

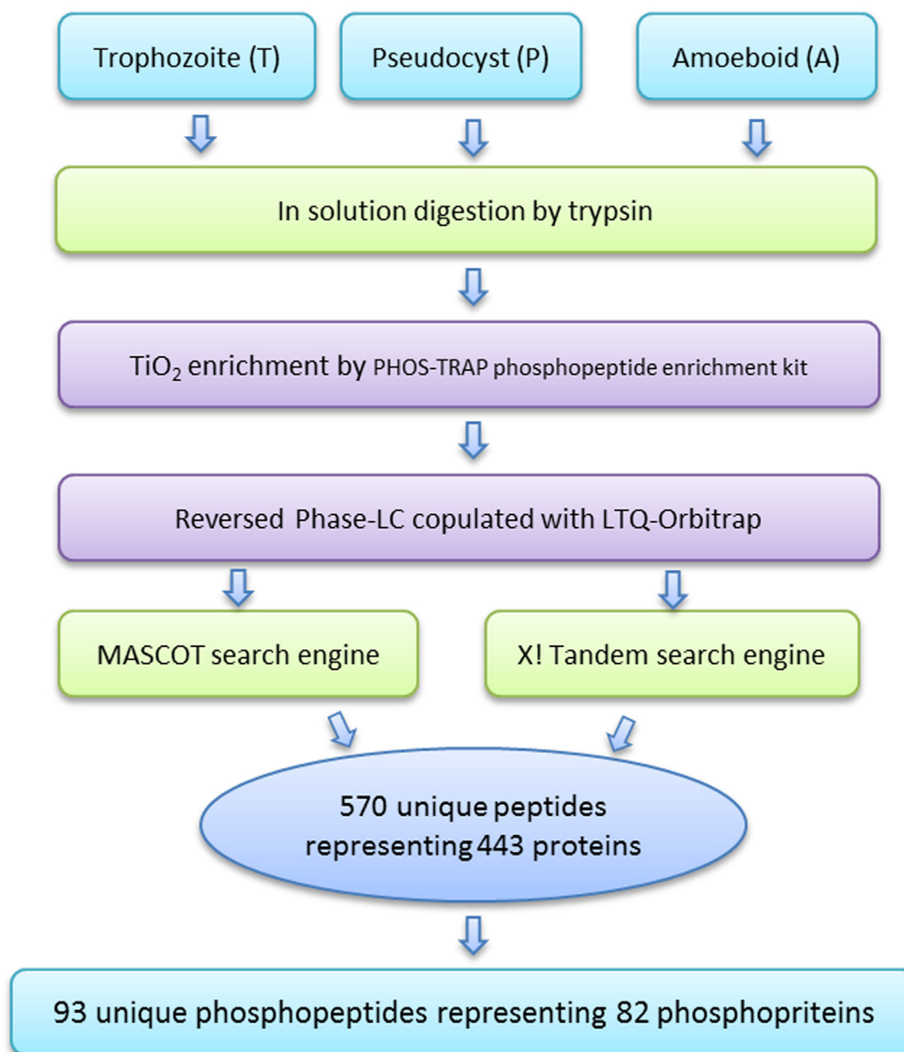

Figure 1. Flowchart of the experimental work. A summary of experimental procedures for identification of phosphorylated proteins from three different stages of *T. vaginalis*.

Table 1 Summary of the *T. vaginalis* phosphoproteome

GB Accession	Peptides [1]	T [2]	P [3]	A [4]	S [5]	T [6]	Y [7]	$ [8]	Protein [9]
TVAG_057130	R.SVSPVIANK.K				1	0	0	1	conserved hypothetical protein
TVAG_057360	R.FISTDNINR.T				1	0	0	1	uridine cytidine kinase I, putative
TVAG_065750	K.QFDNDSMDTVEK.T				1	0	0	1	sucrose transport protein, putative
TVAG_116380	K.SSDEQLVTFR.S				0	1	0	1	conserved hypothetical protein
TVAG_119570	K.APQSQPDLFAPQQQK.Q				1	0	0	1	conserved hypothetical protein
TVAG_150170	K.SSSLPAILNK.N				1	0	0	1	ormdl proteins, putative
TVAG_185160	K.TLQSQIDAR.R				1	0	0	1	60S ribosomal protein L19, putative
TVAG_192930	K.ETVEENSFVSQR.G				1	0	0	1	hypothetical protein
TVAG_202020	K.ANISEGDDDAILSR.A				1	0	0	1	neurofilament triplet M protein, putative
TVAG_202020	R.VLLSDDEDDVPK.K				1	0	0	1	neurofilament triplet M protein, putative
TVAG_256840	R.AYQEAGSGINQLSQESK.E				1	0	0	1	14-3-3 protein sigma, gamma, zeta, beta/alpha, putative
TVAG_260790	R.TASFQDTDLK.A				1	0	0	1	glucokinase, putative
TVAG_277150	K.QNPEELTDQELK.T				0	1	0	1	conserved hypothetical protein
TVAG_346750	K.DGFGSFDNVK.F				1	0	0	1	conserved hypothetical protein
TVAG_348330	K.NASLNTLSHK.V				1	0	0	1	glycogen phosphorylase, putative
TVAG_354150	K.LAGENASESNAEDDAALER.L				1	0	0	1	trichohyalin, putative
TVAG_407690	K.INEASVSYISTR.S				0	0	1	1	CMGC family protein kinase
TVAG_407690	K.INEASVSYISTR.S				1	0	0	1	CMGC family protein kinase
TVAG_474360	R.EETEQYATQLNNSVQER.D				1	0	0	1	centrosomal protein of 135 kDa, putative
TVAG_478270	K.ALVNDDESDDEDLQGK.K				1	0	0	1	conserved hypothetical protein
TVAG_479600	K.GNNDQEDSEVEPDPDVPK.K				1	0	0	1	conserved hypothetical protein
TVAG_498830	K.ISSDLQDNEDLAR.F				1	0	0	1	mannose-1-phosphate guanyltransferase, putative
TVAG_039020	K.ASGISDAEADNLEK.A	0			1	0	0	1	conserved hypothetical protein
TVAG_103680	K.NNTVLTIYFEQK.L	0			0	1	1	2	conserved hypothetical protein
TVAG_116180	K.DISNDDLISK.R	0			1	0	0	1	DNA excision repair protein ercc-6, putative
TVAG_169740	K.QNIVSPDSPAK.V	0			1	0	0	1	RAB 19, 41 and, putative
TVAG_175590	K.ITGDSITEIPSFVGLK.K	0			0	1	0	1	conserved hypothetical protein
TVAG_251650	R.MLSDGSSYSESR.N	0			2	0	1	3	conserved hypothetical protein
TVAG_271090	R.ITNQDGTITEK.I	0			0	2	0	2	conserved hypothetical protein
TVAG_321580	K.DPQVSLLIDDLNK.I	0			1	0	0	1	conserved hypothetical protein
TVAG_350140	K.GDTEQGSALILTNGSTQYK.V	0			0	1	0	1	conserved hypothetical protein
TVAG_353380	K.QDISGS*M*NVDEITSK.L	0			1	0	0	1	ADP-ribosylation factor, arf, putative
TVAG_364550	K.SPSIPQLLNK.N	0			1	0	0	1	ordml, arthropod, putative
TVAG_405400	K.SAQSTSSVLISTQDSIK.Q	0			0	1	0	1	adaptin, alpha/gamma/epsilon, putative
TVAG_499810	K.ALPELSEEDDADENQAK.N	0			1	0	0	1	conserved hypothetical protein
TVAG_126010	R.SDEAFDFSSSQQK.T		0		1	0	0	1	conserved hypothetical protein
TVAG_342390	R.EELEGSEPMSER.T		0		1	0	0	1	DNA polymerase alpha catalytic subunit, putative
TVAG_353230	K.GLI*M*DDSTKK.V		0		1	1	0	2	vacuolar protein sorting-associated, putative
TVAG_440820	R.SDAASAASQSISDAAGK.A		0		1	0	0	1	nascent polypeptide associated complex alpha subunit, putative
TVAG_486750	K.TITSQDTTDPLPK.S		0		1	0	0	1	conserved hypothetical protein
TVAG_491100	K.EQTQEMEK.F		0		0	1	0	1	protein C9orf39, putative
TVAG_515920	K.IEDIDDSEDDELQ*M*R.T		0		1	0	0	1	conserved hypothetical protein
TVAG_120870	K.LDA*M*SSDDEVLK.A	0	0		2	0	0	2	calreticulin, putative
TVAG_021720	R.DYDDYDDYR.G			0	0	0	1	1	polyadenylate-binding protein, putative
TVAG_161820	R.ALSPFITPDK.Q			0	1	0	0	1	conserved hypothetical protein
TVAG_225330	K.LGEGTYGVVFK.A			0	0	1	0	1	CMGC family protein kinase
TVAG_340530	K.VADDSDEEPIEK.E			0	1	0	0	1	conserved hypothetical protein
TVAG_344540	R.LQQISQATLGAGDLK.T			0	0	1	0	1	maintenance of ploidy protein mob1, putative
TVAG_362760	K.VTSPPEPTTTNPK.A			0	1	0	0	1	conserved hypothetical protein
TVAG_376790	K.VGEQGLSIAFNPIT*M*AK.N			0	0	1	0	1	Clan SB, family S8, subtilisin-like serine peptidase
TVAG_390160	K.GSLNHSLSIEIEK.Y			0	1	0	0	1	conserved hypothetical protein
TVAG_483080	K.KPENEDSDEDIVK.F			0	1	0	0	1	fetal globin-inducing factor, putative
TVAG_578100	K.DVFDSDAII.-			0	1	0	0	1	conserved hypothetical protein
TVAG_008140	R.STTQLLPSLNDNSNNR.A	0		0	0	1	0	1	serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit epsilon isoform, putative
TVAG_096340	K.SSGSLAQDIQAIAK.Y	0		0	1	0	0	1	conserved hypothetical protein
TVAG_101840	R.FGIYYHLPK.G	0		0	0	0	2	2	conserved hypothetical protein
TVAG_139450	R.NSGGEPIPLIDDVK.S	0		0	1	0	0	1	Involucrin, putative
TVAG_140940	R.NESQSLSAPNSPNPR.R	0		0	2	0	0	2	conserved hypothetical protein
TVAG_143470	R.TLLEFGCNPNAK.E	0		0	0	1	0	1	ankyrin repeat-cotaining protein, putative
TVAG_147690	R.DYEDRSPVQSR.R	0		0	1	0	0	1	conserved hypothetical protein
TVAG_149260	K.YLNDVNDASDGAAQG*M*ANANR.T	0		0	1	0	0	1	ras GTP exchange factor, putative
TVAG_156120	K.FTDCQTNLAQKPK.S	0		0	0	1	0	1	conserved hypothetical protein
TVAG_174150	R.FIQQPASNYIQSR.Y	0		0	0	0	1	1	CMGC family protein kinase
TVAG_268110	K.DNEISQNSQAIDDSSK.H	0		0	1	0	0	1	conserved hypothetical protein
TVAG_311190	K.RPSQGIGDSPPISEK.E	0		0	1	0	0	1	conserved hypothetical protein
TVAG_346750	K.QSSTDGFGTFGEEPK.K	0		0	1	0	0	1	conserved hypothetical protein
TVAG_352370	K.TDEEIMDALEQANAK.K	0		0	0	1	0	1	ABC transporter, putative
TVAG_358370	R.MSEDSPFTNTVK.Q	0		0	1	1	0	2	axoneme-associated protein mst101, putative
TVAG_405400	K.SAQSTSSVLISTQDSIK.Q	0		0	1	0	0	1	adaptin, alpha/gamma/epsilon, putative
TVAG_435960	K.ACSVADIQEEV*M*PDTK.D	0		0	1	0	0	1	Clan CA, family C19, ubiquitin hydrolase-like cysteine peptidase
TVAG_453930	K.IEESSDDA*M*VPLR.E	0		0	1	0	0	1	conserved hypothetical protein
TVAG_486750	K.DI*M*D*M*LSAPSPK.V	0		0	1	0	0	1	conserved hypothetical protein
TVAG_003250	K.GIASTFSCASIK.D		0	0	1	0	0	1	eukaryotic peptide chain release factor GTP-binding subunit, putative
TVAG_019140	R.SGSFGMQLGEASR.S		0	0	1	0	0	1	conserved hypothetical protein
TVAG_035030	K.NLSEEDDELNPK.S		0	0	1	0	0	1	conserved hypothetical protein
TVAG_068570	R.NGSTEKK.C		0	0	1	0	0	1	conserved hypothetical protein
TVAG_082990	K.SRPSAADSAETTPDGK.L		0	0	1	0	0	1	hypothetical protein
TVAG_101260	K.IQSVDLTQPADNK.Q		0	0	1	0	0	1	Rab32, putative
TVAG_112950	K.ITTDDLEPLVPK.N		0	0	0	1	0	1	conserved hypothetical protein
TVAG_131820	K.LAQLDSDDEQIQER.K		0	0	1	0	0	1	transcription initiation factor TFIID subunit, putative
TVAG_204870	K.NGTSTQR.D		0	0	1	0	0	1	conserved hypothetical protein
TVAG_283750	R.ILVGLGTGPTYVAALK.I		0	0	0	1	0	1	conserved hypothetical protein
TVAG_295720	K.QESDDDIVVVK.R		0	0	1	0	0	1	nuclease sbcCD subunit C, putative
TVAG_310170	-.MSEPDSNLSDK.K		0	0	1	0	0	1	conserved hypothetical protein
TVAG_311220	R.LAFSVVAI*M*NKK.T		0	0	1	0	0	1	ribonuclease, putative
TVAG_363690	K.DGDGETALHITAAR.N		0	0	0	2	0	2	ankyrin repeat-cotaining protein, putative
TVAG_497440	K.EDTLASQLDESQTSEADR.G		0	0	1	0	0	1	conserved hypothetical protein

1. Peptides : MS/MS peptide. 2. T: *T.vaginalis* trophpzoite stage. 3. P: *T.vaginalis* pseudocyst stage. 4. A: *T.vaginalis* amoeboid stage. 5. S: Number of phospho-Serine sites.
6. T: Number of phospho-Threonine sites. 7. Y: Number of phospho-Tyrosine sites. 8. $: total phosphorylation sites. 9. Protein: protein description.

Am = *T. vaginalis* amoeboid stage; Ps = *T .vaginalis* pseudocyst stage; Tr = *T. vaginalis* trophozoite stage.

followed by TiO_2 enrichment and LTQ-Orbitrap MS analysis. The resulting MS and MS/MS spectra were searched against the TrichDB database (release 1.2) using the MASCOT and X! Tandem search engines. The experimental design is summarized as a flowchart in Fig. 1. To increase the accuracy of phosphopeptide identification, only phosphopeptides that were identified by both search engines were considered. A total of 93 unique peptides representing 82 proteins were retained for subsequent analysis. A summary of the phosphopeptides identified is presented in Table 1. There were 84 peptides with one phosphorylated site, eight peptides with two phosphorylated sites, and one peptide with three phosphorylated sites.

Fig. 2 shows the distribution of 82 phosphoproteins among the different stages of *T. vaginalis*. A total of 52, 59 and 41 phosphoproteins were expressed in the trophozoite, pseudocyst and amoeboid stages, respectively. Only 21 common enriched proteins were shared by the three stages; 32 proteins were stage specific, and 29 proteins were expressed in two different stages. The distribution of the phosphorylation sites identified is summarized in Fig. 3. The percentage of phosphorylated serine, threonine and tyrosine sites was 71.8%, 21.4% and 6.8%, respectively.

Gene ontology analysis

To obtain an overview of the functional distribution of phosphoproteins identified in the present study, gene ontology (GO) annotation terms were used to classify the phosphorylated proteins into three categories: biological process, cellular component, or molecular function. The 82 phosphorylated proteins identified were searched against the NCBI non-redundant (nr) protein database with a cutoff e-value of 10^{-3}. The GO annotation of these proteins was inferred from the results of a sequence homology search using Blast2GO software.[18]

A total of 41 of the phosphorylated proteins were annotated with at least one GO term. Some 28, 34 and 19 proteins were classified into the biological process, molecular function and cellular component categories, respectively. The distribution of level 2 GO terms in the three categories is summarized in Fig. 4.

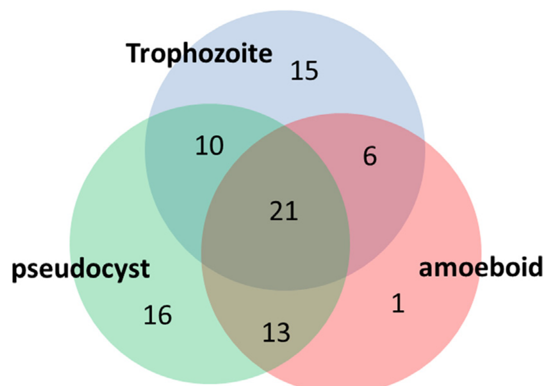

Figure 2. Distribution of enriched phosphorylated proteins in different stages of *T. vaginalis*. A total of 93 phosphopeptides representing 82 proteins enriched by TiO_2 affinity binding were identified in the trophozoite, pseudocyst and amoeboid stages of *T. vaginalis*. The number of enriched proteins in each stage is indicated. A complete list of the peptides identified is provided in Table 1.

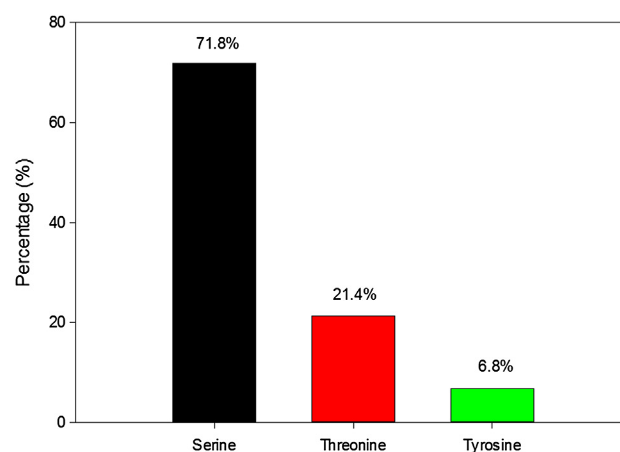

Figure 3. Percentage of serine, threonine and tyrosine phosphorylated sites in different stages of *T. vaginalis*. The distribution of substrate-specific phosphorylation sites identified in *T. vaginalis* is expressed as a percentage of the whole phosphoproteome.

Discussion

The 14-3-3 family is a group of highly conserved 30-kDa proteins expressed in a wide range of organisms and tissues. This protein family is involved in many biological processes, including regulation of the cell cycle, cell signaling, intracellular trafficking, transcription and cytoskeletal structure.[19] These regulatory networks are usually involved in phosphorylation of interacting proteins that show a distinct preference for a particular 14-3-3 isoform. A number of 14-3-3 sequences have been isolated from protists and characterized.[20] For example, 14-3-3 proteins have been identified in apicomplexan protozoans such as *Plasmodium falciparum*[21] and *Toxoplasma gondii*.[22] Expression of 14-3-3 proteins in *P. falciparum* begins during the ring stage, increases in young trophozoites, and declines thereafter. In *T. gondii*, 14-3-3 protein expression was found in feline enteroepithelial gametocyte stages, sporozoites, tachyzoites and bradyzoites. In addition, screening of cDNA libraries from *Entamoeba histolytica* and *Trypanosoma brucei* led to the identification of several 14-3-3 protein-like sequences.[22] Although 14-3-3 proteins have been identified in some parasitic organisms, the biological roles of these proteins in protists remain unclear and should be investigated further. Many researchers have speculated that 14-3-3 proteins play a role in signal transduction related to cellular processes such as proliferation, migration and morphological changes during the parasite life cycle. In the present study, we demonstrated that a 14-3-3 protein (TVAG_256840) can be phosphorylated in different stages of *T. vaginalis*, a result that has not been reported previously. These results suggest that 14-3-3 phosphorylation may be a crucial post-translational modification that allows *T. vaginalis* to adapt to diverse environments.

We previously established a proteome reference map for *T. vaginalis* by 2D electrophoresis combined with MALDI-

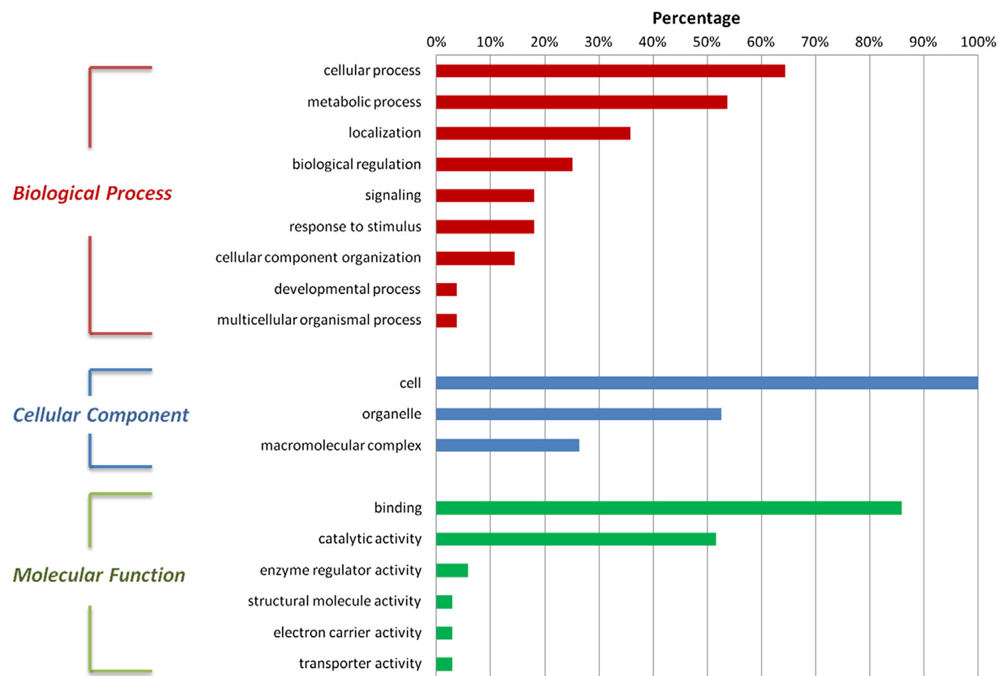

Figure 4. Gene ontology of putative phosphorylated proteins *in T. vaginalis*. Functional classifications of enriched *T. vaginalis* phosphoproteins based on biological process, cellular component and molecular function. The number of phosphoproteins identified in each gene ontology category is indicated on the right of the histogram.

TOF-MS.[23] Among the proteins identified, proteins related to carbohydrate metabolism represented the most abundant category, accounting for 24.7% of all proteins. This result implies that carbohydrate metabolism may be one of the most important physiological processes in *T. vaginalis*. Several glycolytic enzymes have been described in previous studies[24]; however, regulation of carbohydrate metabolism has not been elucidated in *T. vaginalis* or in other protists. In this study, we demonstrated that glucokinase (TVAG_260790) and glycogen phosphorylase (TVAG_348330) are phosphorylated in different stages of *T. vaginalis*, suggesting that regulation of glucose homeostasis is a crucial physiological process for *T. vaginalis* under different conditions. A previous study showed that glucokinase (GK) purified from rat liver can be phosphorylated on a serine residue by protein kinase A (PKA).[25] A later study confirmed that GK phosphorylation by PKA led to a concomitant decrease in GK activity.[26] Conversely, GK activation results in an increase in glucose 6-P concentration, which has a synergistic effect with glucose in promoting dephosphorylation and thus inactivation of glycogen phosphorylase (GP).[27] Moreover, defects in both GK activation and GP dephosphorylation may contribute to dysregulation of glucose concentrations, resulting in type 2 diabetes in humans. On the basis of previous observations, we postulate that *T. vaginalis* may have a similar regulatory system to modulate glucose concentrations. Phosphoproteins identified in all three stages of *T. vaginalis*, such as 14-3-3 protein and several glycolytic enzymes, have also been identified in other higher organisms, suggesting that these proteins are highly conserved and their phosphorylation signals are essential in different stages.

We identified several peptides that are phosphorylated only in the trophozoite stage (Table 1). Eukaryotic peptide chain release factors (eRF) (TVAG_003250) composed of eRF1 and eRF3 are associated with translation termination in eukaryotes.[28] eRF1 recognizes all three stop codons and induces hydrolysis of the peptidyl—tRNA bond. eRF3 carries four GTP-binding motifs associated with eRF1 to form a complex and stimulates eRF1 release activity in a GTP-dependent manner.[29] However, eRF3 may have non-translational functions, such as cell cycle regulation and cytoskeleton organization. Further functional characterization demonstrated that eRF3 depletion decreases the global translation rate and reduces TOR activity.[30]

It is currently thought that the pseudocyst stage is irreversible and represents a defense form. Hence, a better understanding of the regulation of pseudocyst formation may provide additional information for the development of new therapeutics against trichomoniasis.

The serine/threonine protein phosphatase 2A (PP2A) (TVAG_008140) family is a large family of heterotrimeric enzymes that are highly conserved in all eukaryotes. Studies have demonstrated that PP2A activity is crucial for cell cycle regulation, cell survival, embryonic development and stress responses.[31,32] More recently, attention has focused on the involvement of PP2A in tumorigenesis through regulation of cell transformation, which requires remarkable changes in cytoskeleton remodeling. Several cell types, such as neutrophils, hepatocytes, endothelial and epithelial cells, treated with PP2A inhibitors exhibit F-actin disorganization, cell rounding and loss of cell polarity, suggesting that PP2A has a role in cytoskeleton organization.[33] In addition, other findings in plants indicate that okadaic acid, a PP2A inhibitor, enhances the cold response in wheat, tomato and *Brassica napus* leaves, suggesting that PP2As act as native regulators of low-temperature responses.[34] Given the importance of PP2A in regulation

of the cytoskeleton and cold stress responses in other organisms, it would be worth studying the role of PP2A in the response to low temperature and in pseudocyst formation by *T. vaginalis*.

There have been many attempts to elucidate the roles of adhesion-related proteins in the pathogenesis of *T. vaginalis*; however, how the regulation of these proteins is not well understood. Calreticulin (CRT; TVAG_120870) is the only amoeboid-specific phosphorylated protein identified. It is a calcium (Ca^{2+})-binding protein with multiple functions and is predominantly located in the endoplasmic reticulum (ER). CRT is highly conserved in a diverse range of species.[35] In protists, the physiological and pathological significance of CRT remains to be defined. *Entamoeba histolytica* CRT (*Eh*CRT) is remarkably immunogenic in patients with invasive amoebiasis.[36] In addition, *Eh*CRT may play an important role in adapting to a new environment during the early stages of infection. In *Trypanosoma cruzi*, CRT translocates from the ER to the area of flagellum emergence and allows the parasite to recruit the complement component C1, which inhibits the classical pathway of human complement activation.[37] It has been proved that *T. cruzi* CRT is a novel virulence factor that promotes infectivity.[38] It has also been shown that CRT may regulate cellular adhesion and the expression of several adhesion-related proteins, such as vinculin and N-cadherin.[39] Another study revealed that CRT is regulated by protein kinase C (PKC).[40] One of the post-translational modifications induced by PKC activation is tyrosine phosphorylation in CRT. Since CRT is involved in cellular adhesion and even in virulence of some protists, characterization of CRT in *T. vaginalis* may shed light on the regulation of adherence and further our understanding of the pathogenesis of *T. vaginalis*. The stage-specific phosphoproteins unique to *T. vaginalis* may play a role in the signaling involved in morphological transformation of the parasite.

The distribution of phosphoproteins identified in the three stages implies that different signal transduction cascades are activated. GO analysis of 41 annotatable proteins revealed that most proteins are located in the cell and have a binding function. The phosphopeptides detected in different stages revealed possible roles in the regulation of phosphorylation-based signaling pathways. The phosphoproteomics data sets generated in the present study will serve as a foundation for further characterization of the functions of these phosphoproteins. This could ultimately help in the identification of potential drug targets for the treatment of *T. vaginalis* infection.

Acknowledgments

Funding for this project was provided by the National Science Council of Taiwan (NSC-100-2911-I-009-101) and a Chang Gung Medical Research Grant, Taoyuan, Taiwan (CMRPD1A0421).

References

1. Petrin D, Delgaty K, Bhatt R, Garber G. Clinical and microbiological aspects of *Trichomonas vaginalis*. *Clin Microbiol Rev* 1998;**11**:300–17.
2. McLaren LC, Davis LE, Healy GR, James CG. Isolation of *Trichomonas vaginalis* from the respiratory tract of infants with respiratory disease. *Pediatrics* 1983;**71**:888–90.
3. Cotch MF, Pastorek 2nd JG, Nugent RP, Hillier SL, Gibbs RS, Martin DH, et al. *Trichomonas vaginalis* associated with low birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group. *Sex Transm Dis* 1997;**24**:353–60.
4. Sorvillo F, Kerndt P. *Trichomonas vaginalis* and amplification of HIV-1 transmission. *Lancet* 1998;**351**:213–4.
5. Arroyo R, Engbring J, Alderete JF. Molecular basis of host epithelial cell recognition by *Trichomonas vaginalis*. *Mol Microbiol* 1992;**6**:853–62.
6. Moreno-Brito V, Yanez-Gomez C, Meza-Cervantez P, Avila-Gonzalez L, Rodriguez MA, Ortega-Lopez J, et al. A *Trichomonas vaginalis* 120 kDa protein with identity to hydrogenosome pyruvate:ferredoxin oxidoreductase is a surface adhesin induced by iron. *Cell Microbiol* 2005;**7**:245–58.
7. Hernandez H, Sariego I, Garber G, Delgado R, Lopez O, Sarracent J. Monoclonal antibodies against a 62 kDa proteinase of *Trichomonas vaginalis* decrease parasite cytoadherence to epithelial cells and confer protection in mice. *Parasite Immunol* 2004;**26**:119–25.
8. Lama A, Kucknoor A, Mundodi V, Alderete JF. Glyceraldehyde-3-phosphate dehydrogenase is a surface-associated, fibronectin-binding protein of *Trichomonas vaginalis*. *Infect Immun* 2009;**77**:2703–11.
9. Okumura CY, Baum LG, Johnson PJ. Galectin-1 on cervical epithelial cells is a receptor for the sexually transmitted human parasite *Trichomonas vaginalis*. *Cell Microbiol* 2008;**10**(10):2078–90.
10. Pereira-Neves A, Ribeiro KC, Benchimol M. Pseudocysts in trichomonads — new insights. *Protist* 2003;**154**:313–29.
11. Lipman NS, Lampen N, Nguyen HT. Identification of pseudocysts of *Tritrichomonas muris* in Armenian hamsters and their transmission to mice. *Lab Anim Sci* 1999;**49**:313–5.
12. Pereira-Neves A, Benchimol M. *Tritrichomonas foetus*: budding from multinucleated pseudocysts. *Protist* 2009;**160**:536–51.
13. Zolnierowicz S, Bollen M. Protein phosphorylation and protein phosphatases. De Panne, Belgium, September 19–24, 1999. *EMBO J* 2000;**19**:483–8.
14. Carlton JM, Hirt RP, Silva JC, Delcher AL, Schatz M, Zhao Q, et al. Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. *Science* 2007;**315**:207–12.
15. Diamond LS, Clark CG, Cunnick CC. YI-S, a casein-free medium for axenic cultivation of *Entamoeba histolytica*, related *Entamoeba*, *Giardia intestinalis* and *Trichomonas vaginalis*. *J Eukaryot Microbiol* 1995;**42**:277–8.
16. Wu CC, Hsu CW, Chen CD, Yu CJ, Chang KP, Tai DI, et al. Candidate serological biomarkers for cancer identified from the secretomes of 23 cancer cell lines and the human protein atlas. *Mol Cell Proteomics* 2010;**9**:1100–17.
17. Craig R, Beavis RC. TANDEM: matching proteins with tandem mass spectra. *Bioinformatics* 2004;**20**:1466–7.
18. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 2005;**21**:3674–6.
19. Aitken A. 14-3-3 proteins: a historic overview. *Semin Cancer Biol* 2006;**16**:162–72.
20. Siles-Lucas Mdel M, Gottstein B. The 14-3-3 protein: a key molecule in parasites as in other organisms. *Trends Parasitol* 2003;**19**:575–81.
21. Al-Khedery B, Barnwell JW, Galinski MR. Stage-specific expression of 14-3-3 in asexual blood-stage *Plasmodium*. *Mol Biochem Parasitol* 1999;**102**:117–30.
22. Koyama T, Ohsawa T, Shimada S, Omata Y, Xuan X, Inoue N, et al. A 14-3-3 protein homologue is expressed in feline

enteroepithelial-stages of *Toxoplasma gondii*. *Vet Parasitol* 2001;**96**:65—74.

23. Huang KY, Chien KY, Lin YC, Hsu WM, Fong IK, Huang PJ, et al. A proteome reference map of *Trichomonas vaginalis*. *Parasitol Res* 2009;**104**:927—33.
24. Wellerson Jr R, Kupferberg AB. On glycolysis in *Trichomonas vaginalis*. *J Protozool* 1962;**9**:418—24.
25. Ekman P, Nilsson E. Phosphorylation of glucokinase from rat liver in vitro by protein kinase A with a concomitant decrease of its activity. *Arch Biochem Biophys* 1988;**261**:275—82.
26. Munoz-Alonso MJ, Guillemain G, Kassis N, Girard J, Burnol AF, Leturque A. A novel cytosolic dual specificity phosphatase, interacting with glucokinase, increases glucose phosphorylation rate. *J Biol Chem* 2000;**275**:32406—12.
27. Agius L. Glucokinase and molecular aspects of liver glycogen metabolism. *Biochem J* 2008;**414**:1—18.
28. Chavatte L, Frolova L, Kisselev L, Favre A. The polypeptide chain release factor eRF1 specifically contacts the s_4UGA stop codon located in the A site of eukaryotic ribosomes. *Eur J Biochem* 2001;**268**:2896—904.
29. Frolova L, Le Goff X, Zhouravleva G, Davydova E, Philippe M, Kisselev L. Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. *RNA* 1996;**2**:334—41.
30. Chauvin C, Salhi S, Jean-Jean O. Human eukaryotic release factor 3a depletion causes cell cycle arrest at G1 phase through inhibition of the mTOR pathway. *Mol Cell Biol* 2007;**27**:5619—29.
31. Sontag E. Protein phosphatase 2A: the Trojan horse of cellular signaling. *Cell Signal* 2001;**13**:7—16.
32. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 2001;**353**:417—39.
33. Sontag JM, Sontag E. Regulation of cell adhesion by PP2A and SV40 small tumor antigen: an important link to cell transformation. *Cell Mol Life Sci* 2006;**63**:2979—91.
34. Pais SM, Tellez-Inon MT, Capiati DA. Serine/threonine protein phosphatases type 2A and their roles in stress signaling. *Plant Signal Behav* 2009;**4**:1013—5.
35. Michalak M, Robert Parker JM, Opas M. Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium* 2002;**32**:269—78.
36. Gonzalez E, de Leon Mdel C, Meza I, Ocadiz-Delgado R, Gariglio P, Silva-Olivares A, et al. *Entamoeba histolytica* calreticulin: an endoplasmic reticulum protein expressed by trophozoites into experimentally induced amoebic liver abscesses. *Parasitol Res* 2011;**108**:439—49.
37. Ferreira V, Valck C, Sanchez G, Gingras A, Tzima S, Molina MC, et al. The classical activation pathway of the human complement system is specifically inhibited by calreticulin from *Trypanosoma cruzi*. *J Immunol* 2004;**172**:3042—50.
38. Ramirez G, Valck C, Molina MC, Ribeiro CH, Lopez N, Sanchez G, et al. *Trypanosoma cruzi* calreticulin: a novel virulence factor that binds complement C1 on the parasite surface and promotes infectivity. *Immunobiology* 2010;**216**: 265—73.
39. Coppolino MG, Woodside MJ, Demaurex N, Grinstein S, St-Arnaud R, Dedhar S. Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. *Nature* 1997; **386**:843—7.
40. Castaneda-Patlan MC, Razo-Paredes R, Carrisoza-Gaytan R, Gonzalez-Mariscal L, Robles-Flores M. Protein kinase C is involved in the regulation of several calreticulin posttranslational modifications. *Int J Biochem Cell Biol* 2009;**42**:120—31.