Global Phosphoproteome Analysis Reveals Significant Differences Between Sporulated Oocysts of Virulent and Avirulent Strains of *Toxoplasma Gondii*

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Abstract

Background: The apicomplexan protozoan parasite *Toxoplasma gondii* is one of the most successful intracellular parasites capable of infecting warm-blooded animals. Several strains of *T. gondii* have been described and typed as virulent or avirulent based on their pathogenicity in mice, as well as sporulated oocysts of strains belonging to distinct *T. gondii* genotypes. Phosphorylation is a reversible form of protein post-translational modification (PTM) that influences many biological processes and is used by some apicomplexan parasites to facilitate manipulation of the host cells. Phosphoproteomic analysis of oocysts of *T. gondii* strains belonging to distinct genotypes may reveal mechanisms contributing to the differences in the virulence of this parasite at the posttranslational level.

Methods: In this study, the differences in the phosphoproteomic landscape of sporulated oocysts between virulent and avirulent strains of *Toxoplasma gondii* were examined using a global phosphoproteomics approach. Phosphopeptides from sporulated oocysts of the virulent PYS strain (Chinese ToxoDB#9) and the avirulent PRU strain (type II) were enriched by titanium dioxide (TiO₂) affinity chromatography and quantified using isobaric tag (iBT) approach. Motif analysis, GO enrichment, KEGG pathway analysis, STRING analysis and kinase related network analysis of phosphopeptides were conducted to discover distinct difference in sporulated oocysts between virulent and avirulent *T. gondii* strains.

Results: A total of 10,645 unique phosphopeptides, 8,181 nonredundant phosphorylation sites and 2,792 phosphoproteins were identified. We also detected 4,129 differentially expressed phosphopeptides (DEPs) between sporulated oocysts of PYS strain and PRU strain ($|\log_{1.5} \text{fold change}| > 1$ and $p < 0.05$), including 2,485 upregulated and 1,644 downregulated phosphopeptides. Motif analysis identified 24 motifs from the upregulated phosphorylated peptides including 22 serine motifs and two threonine motifs (TPE and TP), and 15 motifs from the downregulated phosphorylated peptides including 12 serine motifs and three threonine motifs (TP, RxxT and KxxT) in PYS strain when comparing PYS strain to PRU strain. Several kinases were consistent with motifs of overrepresented phosphopeptides, such as PKA, PKG, CKII, IKK, MAPK, EGFR, INSR, Jak, Syk, Src, Ab1. GO enrichment, KEGG pathway analysis and STRING analysis revealed DEPs significantly enriched in many biological processes and pathways. Kinase related network analysis showed that AGC kinase had the greatest connected peptides.

Conclusions: The present study revealed the global phosphoproteomic differences in sporulated oocysts between virulent and avirulent *T. gondii* strains of different genotypes. Abundant phosphopeptides in sporulated oocysts between virulent and avirulent *T. gondii* strains exhibited distinct difference in the conserved motifs, GO terms, enriched pathways and PPI networks. The kinase associated network analysis indicated the role of phosphorylation in the transformation of *T. gondii* kinases. AGC kinase was the most connected kinases peptides. These data provide new insight into the phenotypic differences in sporulated oocysts of virulent and avirulent *T. gondii* strains.

Background

The apicomplexan protozoan parasite *Toxoplasma gondii* is one of the most successful intracellular parasites capable of infecting warm-blooded animals [1]. Approximately one-third of the world human population have been estimated to be infected by this parasite [2]. Although most *T. gondii* infections in immuno-competent individuals are asymptomatic, severe or even fatal clinical illness can occur in immuno-compromised patients [3]. Infection with *T. gondii* during pregnancy can result in abortion and stillbirth, or hydrocephalus and retinochoroiditis in the survived neonates [4]. Several strains of *T. gondii* have been described and typed as virulent or avirulent based on their pathogenicity in mice [3, 5–6]. Significant phenotypic variations also exist between different strains of the most prevalent genotype Chinese 1 (ToxoDB#9) in animals and humans in China [7]. Despite the significant differences in virulence among strains of different *T. gondii* genotypes, the genetic difference deleted among these strains was only around 1% [8–9].

Understanding the molecular basis of the virulence mechanisms among the different genotypes of *T. gondii* can identify novel contributors to the significant genotype-related differences in the parasite's virulence. Proteomics approach revealed virulence-related genotypic differences, where strain-specific proteomic signatures were detected in the sporulated oocysts of virulent and avirulent strains belonging to distinct *T. gondii* genotypes [10]. Also, iTRAQ technology combined with titanium dioxide affinity chromatography revealed strain-specific variations in the phosphoprotein profile between tachyzoites of different *T. gondii* genotypes [11]. Phosphorylation is a reversible form of protein post-translational modification (PTM) that influences many biological processes and is used by some apicomplexan parasites to facilitate manipulation of the host cells [12–13]. Therefore, understanding the phosphoproteomic profiles of sporulated oocysts between virulent and avirulent strains of *T. gondii* is also essential because it enhances the understanding of the underlying molecular mechanisms contributing to *T. gondii* virulence.

In the present study, a global phosphoproteomics approach was used to analyze the phosphoproteomes of the virulent ToxoDB#9 (PYS) strain and the avirulent ToxoDB#1 (PRU) strain. We tested the hypothesis that phosphoproteomic analysis of oocysts of *T. gondii* strains belonging to distinct genotypes may reveal mechanisms contributing to the differences in the virulence of this parasite at the posttranslational level. Our data showed many differences in the phosphoproteomes of sporulated oocysts of two distinct *T. gondii* genotypes.

Methods

Production, Purification and Sporulation of Oocysts

The tachyzoites of the virulent PYS (ToxoDB#9 genotype) strain and the cysts of avirulent Prugniad (PRU; ToxoDB#1) strain were maintained in mice in our laboratory. 10^5 tachyzoites of PYS strain were intraperitoneally injected into 16-week-old female guinea pigs. One month later, guinea pigs were euthanized, and a specific-pathogen-free (SPF) kitten was inoculated with homogenate of the brain and muscle of guinea pigs. The kitten was examined by modified agglutination test (MAT) prior to the

inoculation in order to confirm that they aren't infected with *T. gondii*. After the infection, the kitten's feces were gathered daily and tested by a light microscope (Japan, Olumpus) for detection of the oocysts. For the PRU strain, cysts were routinely passaged via oral inoculation in six-week-old mice. Specific-pathogen-free mice was infected with 200 cysts through oral route and its feces was tested to detect the shedding of oocysts of the PRU strain as mentioned above.

Once oocysts of PYS strain and PRU strain were detected, they were isolated and purified from the kitten's feces by using caesium chloride (CsCl) centrifugation method [14]. Firstly, cat feces were blended with water and the fecal suspension was filtered using a 250- μ m pore size tea strainer. Afterward, the filtrates were centrifugated (60 g for 15 mins) and the sediment was rinsed three times with phosphate-buffered saline (PBS). Following the final wash, the supernatant was clarified, the pellet was suspended in 5 volumes of sucrose solution (1.15 specific gravity) and centrifugated for 10 mins at 350 g in order to concentrate oocysts. The oocysts were collected from the supernatant, blended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0) and purified applying a discontinuous CsCl density gradient method as previously described [15-16]. Finally, oocysts within the opaque-to-white layer were harvested and washed twice with 0.85% saline followed by resuspension in PBS and storage at 4 °C. For sporulation, the suspension of oocysts was centrifugated at 350 g and the pelleted oocysts were resuspended in 2% H₂SO₄ (sulfuric acid) to induce sporulation on a shaker in an aerobic condition for 7 days at ambient temperature. After two washes in PBS, the sporulated oocysts were suspended in sulfuric acid and stored at 4°C until use. Prior to use, sulfuric acid in sporulated oocysts suspension were removed by three washes in PBS and sporulated oocysts were ultimately resuspended in PBS.

Extraction and Digestion of Protein

Total protein was extracted from at least three biological replicates of mature oocysts of PYS strain and PRU strain ($\approx 10^7$ per each biological replicate). The sporulated oocyst's suspension was centrifugated at 350 g for 15 min followed by suspension of the pelleted oocysts in lysis buffer (20 mM Tris-HCl, 0.2% SDS, 7 M urea/2 M thiourea, 10 mM DTT, pH 8.5) supplemented with phosphatase inhibitors (PhosSTOP, Roche) and phenylmethylsulfonyl (PMSF, 1 mM, Thermo Scientific). The sporulated oocysts within the suspension were lysed by sonication on ice (2 sec ON/3 sec OFF pulses for 5min) and the suspension was pelleted (25,000 *g* for 20 min at 4°C) to eliminate lysate debris. Subsequently, the supernatant was deoxidized and alkylated by dithiothreitol (DTT, 10 mM, 60 min at 56°C) and iodoacetamide (55 mM IAM for 45 min at ambient condition), respectively. The Bradford assay was used to measure the protein concentration. To digest the protein extracted from each biological replicate/sample 300 μ g trypsin (Promega) were added at enzyme-to-protein ratio of 1:40 at 37°C for 24 hr. The enzymatic reaction was terminated by adding formic acid to a final concentration of 0.5% (v/v). Finally, samples were desalted using Strata X solid-phase extraction columns (Phenomenex) and dried utilizing a SpeedVac, before being used for iBT labeling.

iBT Labeling

The dried peptides were labeled using 6-plexed iBT reagent (BGI-Shenzhen, Shenzhen, China) as per the manufacturer's instructions. Briefly, the peptides were mixed with labeling reagent blended in 50 μ l isopropyl alcohol followed by an incubation at ambient temperature for 2 hr. Then, a Sep-Pack C₁₈ Cartridges (Waters) was used to desalt and concentrate the labelled peptides. The labelled peptides were stored at -80 °C until used for enrichment of phosphopeptides. Proteins of sporulated oocysts of PYS and PRU strains were labelled with iBT tags as follows: PYS strain (114, 115N and 115C) and PRU strain (116N, 116C and 117).

Enrichment of Phosphopeptides Utilizing TiO₂ Beads

TiO₂ (Titanium dioxide, GL Sciences, Tokyo, Japan) beads were used to enrich the labelled phosphopeptides. Before enrichment, 1 ml loading buffer (65% ACN /2% glutamic acid /2% TFA) was used to suspend TiO₂ beads with peptides-to-beads ratio of 1:4 for 10 min. Afterwards, the labeled peptides were mixed with the TiO₂ suspension and the mixture was agitated on a rotator for 1 hr at 37 °C. Then, the mixture was centrifugated at 12,000 g for 5 min, and the sediment was rinsed three times with 2 ml loading buffer (12,000 g for 5 min). After the final wash, the pellet was dissolved with 600 μ l elution buffer and the mixture was agitated for 20 min succeeded by a pellet at 12,000 g for 1 min. The pellet was rinsed with 500 μ l elution buffer using the same agitation and centrifugation conditions mentioned above. Finally, the supernatant from the both centrifugations, which contained phosphopeptides enriched by TiO₂, were collected and vacuum-dried for LC-MS/MS analysis.

LC-MS/MS

Utilizing a High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific Pierce), the samples' fractions were isolated according to the manufacturer's recommendation. Initially, 300 μ l 0.1% TFA was added to the samples to dissolve the enriched peptides, which were laded into the equilibrated fractionation spin column. After enriched peptides binding to the resin, the column was washed with sterile water by centrifugation at low speed in order to desalt the enrich peptides. Then, the bound peptides were eluted from the spin columns and harvested into six different fractions by a volatile high-pH elution which included a gradient of accelerative acetonitrile concentrations. Finally, fractions were respectively collected and vacuum-dried by centrifugation. After fractionation, the Q Exactive Mass Spectrometer (Thermo Scientific) conjugated with HPLC (Phenomenex columns Gemini-NX 3u C18 110A 150*2.00mm) was used to perform the LC-MS/MS analysis. Samples dissolved in 20 μ l buffer A (2% ACN, 0.1% FA) were laded into a C18 trap column at a speed of 8 μ l/min for 4 min. The samples were eluted by a linear gradient of buffer B (95% ACN, 0.1% FA), which was running at 300 nl/min and commenced from 5% buffer B, and then raised into 35% buffer B within 45 min. Within 5 min, the linearizing gradient of buffer B gradually increased into 60%, followed by an increase into 80% buffer B in 2 min. After a retention of 80% buffer B for 2 min, the concentration of buffer B decreased into 5% within 1 min and 5% buffer B was kept for 10 min. The spectra of first-grade MS involved in the following parameter: scan scope = 350 to 1500 m/z; resolution = 70,000; maximum injection time = 40 ms; AGC target = 3e6. As for the second grade MS, spectra were obtained during in the resolution of 17,500 with 40

ms maximum injection time and maximum injection time were 1e5, in which the 20 top precursors were screened out in the mode of HCD (high-energy collisional dissociation).

Database Search

The Proteome Discoverer 1.4 (Thermo Fisher Scientific) combined with a Mascot search engine (version 2.3, Matrix Science) was used to search *T. gondii* database ([http://www.toxodb.org/common/downloads/release-10.0/Toxoplasma gondii ME49/fasta/data/](http://www.toxodb.org/common/downloads/release-10.0/Toxoplasma_gondii_ME49/fasta/data/)) and identify phosphopeptides. Parameters used in the search were set as follow: enzyme = trypsin; peptide mass tolerance = 20 ppm; fragment mass tolerance = 0.05 Da; fixed modification = IBT-10plex (N-term), carbamidomethyl (C), IBT-8plex (K); variable modification = oxidation (M), acetyl (protein N-term), deamidated (NQ); phosphorylation (S/T/Y) maximum missed cleavage = 2. In order to filter the results, p value was set as $p \leq 0.05$ in order to identify and quantify phosphopeptides, and phosphosites with a phosphoRS probability not less than 0.75 were deemed as reliable phosphosites. With regard to differentially expressed phosphopeptides (DEPs), those with a fold-change >1.5 or <0.75 were determined as upregulated or downregulated DEPs, respectively. Hierarchical clustering method was applied to group the DEPs and heat maps were created by the software Multiple Experiment Viewer (Mev, version 4.9.0).

Bioinformatic Analysis

The online software Motif-X (<http://motif-x.med.harvard.edu/>) was applied to search the phosphorylation motifs of phosphopeptides in the present study. Phosphopeptides with the length of ± 7 amino acids neighboring the phosphorylation site were analyzed using the Motif-X software for searching conserved motifs adjacent to phosphorylation sites. Motifs identified were graphically shown with logo-like representation. The functions of the DEPs were sorted into molecular function (MF), biological process (BP) and cell component (CC) using Gene Ontology (GO) analysis (<http://www.geneontology.org>). KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was conducted to annotate the DEPs to pathways present in the KEGG database (<http://www.genome.jp/kegg/>). The significantly enriched pathways were displayed as bubble charts. STRING (Search Tool for the Retrieval of Interacting Genes/Protein, <http://string-db.org/>) database was used to survey processes affected by phosphorylation and to study interaction networks of the DEPs participate in.

Interactors whose confidence were higher (combined score ≥ 0.7) were displayed in protein–protein interaction (PPI) networks exhibited by Cytoscape (version 3.0.2).

Correlation between Phosphopeptides and *T. gondii* Kinase Peptides

The HMMER (<http://hmmer.org>) was used to search the Pfam 31.0 database with the purpose of identifying sequences of the phosphorylated proteins that are related to kinases in the Pfam 31.0 database [17]. An array of phosphorylated peptides matched to protein kinase and protein kinase domains was obtained. Afterward, Pearson correlational analysis was performed to connect kinase-matched phosphorylated peptides with total phosphorylated peptides in our dataset. This analysis was

conducted based on the DEPs ratio values per each strain. Only phosphorylated peptide with correlations > 0.998 were selected. DEPs with $\log_{1.5}$ fold change values $-0.75 \leq \log_{1.5} \leq 1.50$ were excluded. Cytoscape 3.6.0 (<https://cytoscape.org>) was used to visualize the network.

Prediction of Protein Interactions Enabled or Disabled by Phosphorylation

To characterize promising protein interactions enabled or disabled by phosphorylation, Mechismo [18] was used to predict phospho-enabled and phospho-disabled protein interactions. Because of the lack of *T. gondii* proteomes in Mechismo, the yeast and human orthologs of the phosphorylated proteins identified in our analysis were retrieved using the eggNOG-mapper ([PMC4702882] <http://eggnogdb.embl.de/#/app/emapper>). In brief, relevant Orthologous Groups (OG) were firstly extracted and the yeast and human orthologs among these groups were found out utilizing BioPython tools (<http://biopython.org/>). We then applied Clustal Omega [19] to align each orthologous sequence to the corresponding *T. gondii* protein and characterized the equivalent phosphorylation sites in the human or yeast proteins. Finally, they were input to Mechismo (<http://mechismo.russelllab.org/>) to characterize orthologous that enabled or disabled interactions in the human and yeast network.

Results

Phosphorylation Profiles of *T. gondii* Oocysts

In an effort to reveal genotype-specific differences in the phosphoproteomes of sporulated oocysts of *T. gondii*, we performed a comparative phosphoproteomic profiling of sporulated oocysts between virulent PYS strain and avirulent PRU strain. In total, we identified 10,645 unique phosphopeptides, 8,181 nonredundant phosphorylation sites and 2,792 phosphoproteins from sporulated oocysts of *T. gondii* virulent PYS strain and avirulent PRU strain with a FDR (false-discovery rate) < 0.01 for phosphopeptide and phosphoRS probability > 0.75 for phosphorylation site. The 8,181 phosphorylation sites consisted of 7,051 phosphoserine (86.27%), 1,092 phosphothreonine (9.28%), and 38 phosphotyrosine (0.42%) (Fig. 1a). Out of the 10,645 phosphopeptides, 10,206 (95.88%) phosphopeptides had one phosphorylation site, 426 phosphopeptides (4.00%) had double phosphorylation sites, 12 phosphopeptide (0.11%) possessed 3 triple phosphorylation sites and 1 phosphopeptide (0.01%) possessed quadruple phosphorylation sites (Fig. 1b). The number of phosphopeptides matched with 1 spectrum, 2 spectra, 3 spectra, 4 spectra, 5 spectra, and ≥ 6 spectra were 6, 201, 2, 124, 897, 462, 304, and 657, respectively (Fig. 1c). Additionally, the number of phosphoproteins harboring 1, 2, 3, 4, 5 and ≥ 6 phosphorylation sites were 1,031, 550, 323, 208, 124 and 367, respectively (Fig. 1d). Among the total phosphoproteins, 99% phosphoproteins had CV (Coefficient of variation) value $< 50\%$ among three replicates (Fig. 2).

Phosphopeptides Quantification and Clustering Analysis

The quantitative analysis identified 4,129 differentially expressed phosphopeptides (DEPs) between sporulated oocysts of PYS strain and PRU strain ($|\log_{1.5} \text{fold change}| > 1$ and $p < 0.05$), including 2,485 upregulated and 1,644 downregulated phosphopeptides (Fig. 3a, Additional file 1: Table S1 and Additional file 2: Table S2). Among these DEPs, 1,541, 665, 205, 61 and 13 phosphoproteins increased by > 1 , 2, 3, 4, and 5 $\log_{1.5}$ fold changes. In contrast, 1,273, 269, 82, 16 and 4 phosphoproteins decreased by > 1 , 2, 3, 4 and 5 $\log_{1.5}$ fold changes (Fig. 3b). The result of hierarchical clustering analysis of DEPs between sporulated oocysts of PYS strain and PRU strain is shown in Fig. 4.

Motifs Analysis of Phosphorylation Sites of PYS Strain Versus PRU Strain

Phosphosite motifs are crucial amino acid sequences, which are involved in the recognition of substrate by the corresponding kinase [16–17]. To investigate the potential motifs of upregulated or downregulated phosphorylated peptides of sporulated oocysts between PYS strain and PRU strain, substantial preference of amino-acid residue in sequence from -7 to $+7$ surrounding the phosphorylation sites was analyzed by the motif-X software tool. Motif analysis identified 24 motifs from the upregulated phosphorylated peptides in PYS strain when comparing PYS/PRU, including 22 serine motifs (VSP, SPR, SPxG, SPT, PxSP, LxxxRxxS, RxxSP, SDxE, SP, LxRxxS, RxxSxG, RxxSD, SExE, RxxS, SDxD, SGxE, GSE, DSD, GS, KxxS, SxxS and SxE) and 2 threonine motifs (TPE and TP) (Fig. 5). In regard to motif analysis of downregulated phosphorylated peptides in PYS strain when comparing PYS/PRU, 12 serine motifs (SPxG, AxSP, LSP, ExxxxxSP, DxSP, SPG, SPS, SPV, SP, RxxSL, RxxS and SxxxxxK) and 3 threonine motifs (TP, RxxT and KxxT) were overrepresented (Fig. 6). Because each motif corresponding to one or several category kinases, kinases consistent with motifs of upregulated or downregulated phosphorylated peptides are listed in Table 1 and Table 2, respectively. These results reflect the difference in the substrate-recognition abilities and kinase secretory preference of sporulated oocysts between virulent PYS strain and avirulent PRU strain.

Table 1

Types of kinases represented by the motifs of up-regulated phosphor-peptides as determined by KinasePho.

Motif	Kinase Classes
VSP	PKA, PKB, PKG, CKI, IKK, CaM-II, ATM, MAPK, CDK, CDC2
SPR	PKA, PKB, PKG, CKI, CKII, IKK, ATM, MAPK, CDK, CDC2, Syk, Jak, INSR
SP.G	PKA, PKB, PKG, CKI, CKII, IKK, CaM-II, ATM, MAPK, CDK, CDC2, INSR
SPT	PKA, PKB, PKG, CKI, IKK, ATM, MAPK, CDK, CDC2
P.SP	PKA, PKB, PKG, CKI, IKK, ATM, MAPK, CDK, CDC2
L...R..S	PKA, PKB, PKC, PKG, IKK, MAPK, CKI, CKII, CaM-II, ATM, CDC2
R..SP	PKA, PKB, PKC, PKG, CDK, CDC2, MAPK, ATM, IKK, CKI, CKII, CaM-II, INSR
SD..E	PKA, PKB, PKC, PKG, CKI, CKII, ATM, MAPK, IKK, CaM-II, EGFR, Src, CDC2
SP	PKA, PKB, PKC, PKG, CKI, CKII, ATM, MAPK, IKK, CaM-II, CDK, CDC2, Jak, INSR, Syk, Src, Ab1
L.R..S	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM
R..S.G	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, INSR, Src
R..SD	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDC2, ATM, MAPK, INSR, EGFR, Syk
SE.E	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, EGFR, INSR, Syk
R..S	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, INSR, Syk
SD.D	PKA, PKG, CKI, CKII, IKK, CDC2, ATM, MAPK, INSR
SG.E	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, INSR, Syk
GSE	PKA, PKG, CKI, CKII, IKK, CaM-II, CDC2, ATM, MAPK, Syk
DSD	PKA, PKC, PKG, CKI, CKII, IKK, ATM, MAPK
GS	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, INSR, Syk, Src, Ab1
K..S	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, EGFR, INSR
S..S	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, INSR, Syk, Src, Ab1
S.E	PKA, PKC, PKG, CKI, CKII, IKK, CDK, CDC2, ATM, MAPK, INSR, Syk, Other_MDD

Abbreviations:

PKA: cAMP-dependent Protein Kinase; PKB: Protein Kinase B; PKC: Protein Kinase C; PKG: cGMP-dependent Protein Kinase; CKI: Casein Kinase I; CKII: Casein Kinase II; IKK: I κ B Kinase; CaM-II: Calmodulin-dependent Protein Kinase II; ATM: Ataxia Telangiectasia Mutated Kinase; MAPK: Mitogen-Activated Protein Kinase; CDC2: Cell division Cycle Protein Kinase p34; CDK: Cyclin-Dependent Kinase; INSR: Insulin Receptor; EGFR: Epithelial Growth Factor Receptor; Syk: Cytoplasmic Tyrosine Kinases; Jak: Janus kinase; Src: Sarcoma gene kinase; Ab1: Abelson kinase .

Motif	Kinase Classes
TPE	PKA, PKB, PKC, PKG, CKI, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, INSR, Syk, Src, Ab1
TP	PKA, PKB, PKC, PKG, CKII, IKK, CaM-II, CDK, CDC2, ATM, MAPK, INSR
<i>Abbreviations:</i>	
PKA: cAMP-dependent Protein Kinase; PKB: Protein Kinase B; PKC: Protein Kinase C; PKG: cGMP-dependent Protein Kinase; CKI: Casein Kinase I; CKII: Casein Kinase II; IKK: IkappaB Kinase; CaM-II: Calmodulin-dependent Protein Kinase II; ATM: Ataxia Telangiectasia Mutated Kinase; MAPK: Mitogen-Activated Protein Kinase; CDC2: Cell division Cycle Protein Kinase p34; CDK: Cyclin-Dependent Kinase; INSR: Insulin Receptor; EGFR: Epithelial Growth Factor Receptor; Syk: Cytoplasmic Tyrosine Kinases; Jak: Janus kinase; Src: Sarcoma gene kinase; Ab1: Abelson kinase .	

Table 2

Types of kinases represented by the motifs of down-regulated phosphor-peptides as determined by KinasePho.

Motif	Kinase Classes
SP.G	PKA▯PKB▯PKC▯PKG▯CKI▯CKII▯IKK▯CDK▯CDC2▯ATM▯MAPK▯Ab1
A.SP	PKA▯PKB▯PKC▯PKG▯CKI▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK▯INSR▯Syk▯Src▯Ab1
LSP	PKA▯PKB▯PKC▯PKG▯CKI▯CKII▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK
E.....SP	PKA▯PKB▯PKG▯CKI▯CKII▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK
D.SP	PKA▯PKG▯IKK▯CaM-II▯CDC2▯ATM▯MAPK▯Jak
SPG	PKB▯PKC▯PKG▯CKI▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK
SPS	PKA▯PKB▯PKC▯PKG▯CKI▯CKII▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK▯INSR▯Syk
SPV	PKA▯PKB▯PKC▯PKG▯CKI▯CKII▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK▯INSR▯Ab1
SP	PKA▯PKB▯PKC▯PKG▯CKI▯CKII▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK▯EGFR▯INSR▯Syk▯Src
R..SL	PKA▯PKB▯PKC▯PKG▯CKI▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK
R..S	PKA▯PKB▯PKC▯PKG▯CKI▯CKII▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK▯EGFR▯INSR▯Jak▯Syk▯Src
S.....K	PKA▯PKB▯PKC▯PKG▯CKI▯CKII▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK▯INSR▯Jak▯Syk
TP	PKA▯PKB▯PKC▯PKG▯CKI▯CKII▯IKK▯CaM-II▯CDK▯CDC2▯ATM▯MAPK▯Jak
R..T	PKA▯PKB▯PKC▯PKG▯IKK▯CaM-II▯CDC2▯MAPK▯EGFR▯INSR▯Src▯Ab1
K..T	PKA▯PKC▯PKG▯CKI▯IKK▯CDK▯MAPK
<i>Abbreviations:</i>	
PKA: cAMP-dependent Protein Kinase; PKB: Protein Kinase B; PKC: Protein Kinase C; PKG: cGMP-dependent Protein Kinase; CKI: Casein Kinase I; CKII: Casein Kinase II; IKK: IkappaB Kinase; CaM-II: Calmodulin-dependent Protein Kinase II; ATM: Ataxia Telangiectasia Mutated Kinase; MAPK: Mitogen-Activated Protein Kinase; CDC2: Cell division Cycle Protein Kinase p34; CDK: Cyclin-Dependent Kinase; INSR: Insulin Receptor; EGFR: Epithelial Growth Factor Receptor; Syk: Cytoplasmic Tyrosine Kinases; Jak: Janus Kinase; Src: Sarcoma Gene Kinase; Ab1: Abelson Kinase.	

Functional Enrichment Analysis

To uncover the differences in the functions of the differentially expressed phosphoproteins (DEPs) of sporulated oocysts between PYS strain and PRU strain, GO enrichment analysis was performed. The enriched GO terms stratified into three GO categories (BP, CC and MF) are listed for upregulated and downregulated phosphoproteins (Fig. 7a-7b). The top five significantly enriched GO terms under BP for upregulated DEPs included catalytic activity, binding, transporter activity, structural molecule activity and transcription. In contrast, catalytic activity, binding, transporter activity, structural molecule activity and

signal transducer activity were the top five enriched GO terms under BP for downregulated phosphoproteins.

In regard to the CC category, the five enriched GO terms of upregulated phosphoproteins were cell part, cell, organelle, membrane and macromolecular complex, whereas cell part, cell, organelle, macromolecular complex and membrane were the top five significantly enriched GO terms for the downregulated phosphoproteins. Regarding the MF of upregulated phosphoproteins, metabolic process, cellular process, localization, biological regulation and regulation of biological process were the top five enriched GO terms. In contrast, the five mostly enriched GO terms for downregulated phosphoproteins included metabolic process, cellular process, biological regulation, localization and regulation of biological process.

The co-expression networks of GO terms belonging to the three functional categories were constructed using the R Igraph package in order to investigate the level of association among GO terms. The significantly enriched GO terms in the network of upregulated phosphoproteins in PYS when comparing PYS/PRU are shown in Additional file 3: Figure S1. In the MF category, six GO terms, including pseudouridine synthase activity, 2-alkenal reductase [NAD(P)] activity, metal ion transmembrane transporter activity, calcium ion transmembrane transporter activity, calcium-transporting ATPase activity, and motor activity, were significantly enriched. Also, in the CC category, six GO terms, including extracellular region, protein complex, membrane part, membrane protein complex, coated membrane, and membrane coat, were significantly enriched.

In the BP category, 40 GO terms were significantly enriched, including biological regulation, regulation of biological process, cell cycle process, response to stress, transmembrane transport, G-protein coupled receptor signaling pathway, glycerolipid metabolic process, glycerophospholipid metabolic process, phosphatidylinositol metabolic process, lipid phosphorylation, phosphatidylinositol phosphorylation, organophosphate metabolic process, aromatic compound catabolic process, cellular nitrogen compound catabolic process, heterocycle catabolic process, nucleobase-containing compound catabolic process, organic cyclic compound catabolic process, negative regulation of biological process, negative regulation of cellular process, regulation of cellular component organization, regulation of organelle organization, RNA modification, pseudouridine synthesis, regulation of metabolic process, regulation of cellular metabolic process, regulation of primary metabolic process, regulation of biosynthetic process, regulation of nitrogen compound metabolic process, regulation of cellular biosynthetic process, regulation of cellular amide metabolic process, regulation of macromolecule metabolic process, regulation of RNA metabolic process, regulation of macromolecule biosynthetic process, regulation of RNA biosynthetic process, regulation of cellular macromolecule biosynthetic process, regulation of nucleic acid-templated transcription, regulation of translation, regulation of gene expression, regulation of transcription, and posttranscriptional regulation of gene expression.

The significantly enriched GO terms in the network of downregulated phosphoproteins in PYS when comparing PYS/PRU are shown in Additional file 4: Figure S2. In the MF category, 17 GO terms, including

binding, small molecular binding, nucleoside phosphate binding, nucleotide binding, pattern binding, polysaccharide binding, starch binding ligase activity, transferase activity, oxidoreductase, kinase regulatory activity, protein kinase regulatory activity, antiporter activity, cation antiporter activity, proton antiporter activity, translation initiation factor activity and translation factor activity, were significantly enriched. Only three GO terms (cell, cell part and intracellular) in the CC category were enriched. However, in the BP category there were 21 significantly enriched GO terms, including locomotion, developmental process, cellular developmental process, anatomical structure morphogenesis, anatomical structure development, tissue development, nervous system development, response to external stimulus, regulation of phosphorus metabolic process, regulation of phosphate metabolic process, regulation of phosphorylation, regulation of protein phosphorylation, regulation of protein kinase activity, regulation of kinase activity, macromolecule metabolic process, pyruvate metabolic process, cellular carbohydrate biosynthetic process, cellular amide metabolic process, peptide metabolic process, peptide biosynthetic process, and translation metabolic process establishment of localization and transport.

Compared to PRU strain, more upregulated phosphoproteins were enriched in GO terms such as transcription regulator activity, macromolecular complex, immune system process, biological adhesion in PYS strain. However, more downregulated phosphoproteins were enriched in GO terms such as signal transducer activity, developmental process, reproductive process, reproduction, locomotion and pigmentation. These results clearly show significant differences in the biological functions of the DEPs of sporulated oocysts between PYS strain and PRU strain.

KEGG Pathway Analysis

Enzymes with phosphorylation sites can regulate key signal pathways in several organisms [11]. To better understand the signal pathways controlled by the phosphorylated proteins and the function of the DEPs, DEPs in sporulated oocysts between PYS strain and PRU strain were annotated and mapped against in the KEGG pathway database. As shown in Figs. 8, 39 and 18 DEPs were significantly enriched in RNA transport and phosphatidylinositol signaling system, respectively.

Protein-Protein Interaction (PPI) Analysis

Using Cytoscape software, the PPI networks (combined score ≥ 0.9) were built in order to identify the mechanisms regulated by phosphorylation and to determine the related functional clusters of the DEPs of sporulated oocysts between the virulent and avirulent *T. gondii* strains. The PPI network of upregulated and downregulated phosphoproteins between PRU strain and PYS strain included 344 nodes and 670 interactor edges (Fig. 9). Several major hubs were identified, including ribosome biogenesis protein BOP1 (TGME49_301390), nucleolar protein NOP5 (TGME49_205510), eukaryotic initiation factor-2 (TGME49_313230), translation initiation factor 2 beta (TGME49_235540), eukaryotic translation initiation factor 2 gamma subunit (TGME49_235970), translation elongation factor 2 family protein (TGME49_205470), ribosomal protein RPL6 (TGME49_313390), ribosomal protein RPL4 (TGME49_309120), and ATP-dependent RNA helicase (TGME49_312280). Several functional clusters were identified in the PPI network, including DNA replication or RNA transcription, proteasome,

metabolism, RNA splicing or translation, ribosomal proteins, molecular chaperones, tRNA related molecular, adapter, coatomer and kinases or enzymes.

Kinase Related Networks

Given that phosphorylation influences the activity of the phosphorylated protein that function as a kinase, potential peptides whose phosphorylation is related to the respective kinase were identified by performing a correlation analysis (Additional file 5: Table S3). After retaining phosphopeptides with $> \sim 1.5$ -fold change abundance, 2 phosphorylated kinases were identified and were associated with 58 phosphopeptides. Positive correlation and negative correlation indicate that kinase phosphorylation positively or negatively impacts its action. These correlations suggest potential differences in kinases and associated substrates of sporulated oocysts between the two *T. gondii* strains. Among the first cluster of phosphorylated peptides, peptides related to *T. gondii* AGC kinase A0A125YVN2 (connected with 50 phosphorylated peptides) connected most kinase peptides. The second cluster of phosphorylated peptides encompassed 6 proteins: zinc finger A0A125YGX1, TgCatPRC2 A0A151H897, possible RNA-binding protein Q1JTD1, DNA-directed RNA polymerase subunit S8F5G5, non-specific serine/threonine protein kinase S8F5G5 and the hub protein kinase is histone kinase SNF1 S8F5G5 (Fig. 10). The GO annotation of the most connected kinases peptides (AGC kinase) showed GO terms associated with nucleotide binding, ATP binding, metabolic process, protein serine/threonine kinase activity and phosphorylation, in the MF category.

Potential Phosphorylation-Dependent Interactions

Based on the *T. gondii* protein interaction network, the putative function of the different phosphorylation sites in our dataset were sought to identify and the effects of mutation or phosphorylation on the interaction between two phosphorylated proteins (enable or disable) were predicted. Using Mechismo and Pearson correlational analysis, a total of 147 interactors of the *T. gondii* orthologs, enabled or disabled by phosphorylation of the identified sites, were identified (Additional file 6: Table S4). The 9 interacting partners were annotated back to *T. gondii* proteins and were found to influence interactors in *T. gondii* (Fig. 10). The T6 and T12 phosphosites of *T. gondii* A0A151H503 gene were found to influence 7 interactions and phosphosite S10 of *T. gondii* A0A151GZW7 gene influenced 2 interactions.

Discussion

In this study, we used iBT-based mass-spectrometry approach combined with TiO₂ affinity chromatography to profile the phosphoproteomic landscape of sporulated oocysts of virulent (Chinese ToxoDB#9, PYS strain) and avirulent strains (type II, PRU strain) of *T. gondii*. Our results are consistent with previous phosphoproteomic studies in eukaryotic organisms [22–24]. We identified 10,645 phosphopeptides, 2,792 phosphoproteins and 8,181 phosphorylation sites, including 9,574 (86.27%) phosphoserine (pSer), 1,477 (9.28%) phosphothreonine (pThr), and 47 (0.42%) phosphotyrosine (pTyr) in the sporulated oocysts of *T. gondii* virulent PYS strain and avirulent PRU strain (Fig. 1). This finding is

anticipated because most *T. gondii* proteins containing serine kinase domains, such as ROP5, ROP16, ROP18 and TgSR3, play roles in the replication, invasion and virulence of this parasite [25–27]. Threonine phosphorylation was associated with serine phosphorylation in several virulence factors of *T. gondii*, such as ROP18 and type 2C serine-threonine phosphatase and can play role in the invasion and replication of *T. gondii* [28–29]. Although tyrosine phosphorylation was underrepresented compared to serine and threonine phosphorylation in the apicomplexan parasites, it contributes to important signaling pathways, such as the MAPK pathway [13, 30].

Motifs

Motif analysis identified 24 motifs for the upregulated phosphorylated peptides, and 15 motifs for the downregulated phosphorylated peptides in PYS strain compared to PRU strain (Fig. 5 and Fig. 6). The motif's function is to recognize the corresponding substrate by kinase, and every motif corresponds to one or several enzymes [20–21, 31]. Therefore, different motifs extracted from upregulated and downregulated phosphorylated peptides not only show the differences in the kinase expression of sporulated oocysts between PYS stain and PRU strain, but also reflect the discrepancy in the substrate-recognition abilities of sporulated oocysts between PYS and PRU strains.

Our results showed that these motifs are consistent with some traditional kinases (PKA; PKG; CK II; IKK; CDC2; MAPK), which play roles in the egress, motility, cell cycle, and stress response of *T. gondii* [32–35]. Some less prevalent kinases were also consistent with these motifs, such as EGFR, INSR, Jak, Syk, Src, Ab1. The protein kinase epidermal growth factor receptor (EGFR) is found on cell surface in the Apicomplexan parasite *Neospora caninum*. The *N. caninum* EGFR can be activated by NcMIC3 which also contains four EGF domains, or human EGF. Interestingly, the activation of EGFR in *N. caninum* enhanced the proliferation of tachyzoites [36]. INSR can participate in the insulin-like growth factor signaling pathway and was identified as a good candidate for parasitic RTK inhibition [37–39].

Syk kinases, identified in invertebrates, regulate the proliferation, differentiation, and survival of various types of cells. Among Syk kinase family, tyrosine kinase 4 (TK4) is a kinase expressed in *Schistosoma mansoni* and *S. japonicum*, which plays a pivotal role in the development of the testis and ovaries in *S. mansoni* and regulates the gametogenesis of *S. japonicum* [40]. The fact that Src inhibitors can affect the actin cytoskeleton of *Entamoeba histolytica* and inhibit the entry of *Trypanosoma cruzi* provides new targets for designing new drugs against protozoa [41–42]. The Abl family kinases not only can play a role in the signaling translation from growth factor and adhesion receptors into cytoskeletal rearrangements, but also can facilitate some processes, such as endocytosis, viral and bacterial uptake, autophagy, and IgG-mediated phagocytosis. Also, this kinase was shown to facilitate *Leishmania amazonensis* amastigote uptake by macrophages [43]. The important role of these kinases in parasites implies the potential role of phosphorylated protein in the sporulated oocyst of *T. gondii*, which warrants further investigation.

GO and KEGG analysis of the phosphorylated proteins

GO analysis revealed that transcription regulatory activity, macromolecular complex, immune system process, biological adhesion GO terms were enriched in more upregulated phosphoproteins in PYS strain when comparing PYS/PRU. However, the GO terms signal transducer activity, developmental process, reproductive process, reproduction, locomotion and pigmentation were more enriched in downregulated phosphoproteins. These results show the difference in the transcription regulation and developmental regulation of sporulated oocysts between PYS strain and PRU strain. Phosphorylation of translational regulators was associated with the reprogramming of the transcriptome of higher organisms [44]. Our results indicate that this post-translational protein modification exists in sporulated oocysts of *T. gondii*. The glideosome-associated protein with multiple-membrane spans (GAPM3) is a part of glideosome, a macromolecular complex consisting of adhesive proteins [45], which was an upregulated DEP in PYS strain when comparing PYS/PRU in our data. Substrate-dependent locomotion known as gliding motility is a unique form which is essential for invading host cells and traversing the environment of *T. gondii* [46]. Several *Toxoplasma gondii* MyoA motility complex were found to be phosphorylated in our results, for example myosin A, myosin C, myosin F, myosin G and myosin J. Of which, phosphorylated peptide of myosin F was downregulated in PYS stain when comparing PYS/PRU. As for the results of KEGG pathway analysis, phosphatidylinositol signaling system is a significantly enriched pathway. This pathway was deemed as crucial constitutive signals that regulate biochemical organelle identity at eukaryotic cellular membranes, as well as autophagy and endocytosis in protist parasites such as *T. gondii* [47–48].

Protein-Protein Interaction (PPI) Networks

Among the functional clusters identified in the PPI network, some well-known clusters (e.g. DNA replication or RNA transcription, metabolism, RNA splicing or translation, ribosomal proteins, molecular chaperones, tRNA related molecular, and kinases or enzymes) consist of phosphorylated proteins not only were identified, but also the unusual clusters, such as proteasome, adapter, coatomer, were included. The main function of proteasome is proteolytic removal of damaged proteins and this large intracellular protein complex has a crucial role in the pathogenesis, intracellular growth and replication of *T. gondii* [49–50]. The presence of proteasome cluster in the PPI network demonstrates that phosphorylation of proteins in the proteasome might contribute to the pathogenesis, intracellular growth and replication of *T. gondii*. The adaptor complex of all eukaryotes is composed of receptor/adaptor proteins that mediate the recruitment of dynamin-related protein, the key player of mitochondrial fission, to the mitochondrial outer membrane (MOM) at ER/actin precontracted fission sites [51]. So, this complex is critical for the reconstitution of the mitochondria membrane. The endodyogeny of tachyzoites of *T. gondii* is tightly linked with duplication of the mitochondrion [51]. Although there was no evidence that the division of sporulated oocysts of *T. gondii* was associated with phosphorylation of adaptor proteins, the essential of phosphorylation of adaptor proteins for its function is an interesting event to be survey. Coatomer coated complex (COP) is a type of transport vesicles, which mediates both selective and non-selective transport within the eukaryotic cell including the eukaryotic protozoan *T. gondii*. In fact, *T. gondii* has seven subunit homologues and the size of these homologues were different [52–53]. Our analysis showed that four subunits of COP were phosphorylated, including alpha, beta, gamma and zeta. This high percentage of

phosphorylation in the subunits of COP indicates that phosphorylation may be necessary for the function of COP and transport of vesicles of *T. gondii*.

Kinase-Protein Interactions

Kinase phosphorylate substrates in order to modulate activity and interactions of proteins. In our study, specific parasite peptides associated with the phosphorylated peptides were identified and the potential kinase-protein interactions were predicted. The parasite kinase (AGC kinase) had the greatest connected peptides. AGC kinase represents a category conserved kinases among eukaryotic genomes, including cyclic AMP [cAMP]-dependent protein kinase 1 (protein kinase A), cGMP-dependent protein kinase (PKG) and protein kinase C, which served various physiological functions, such as cell growth, metabolism, differentiation, and cell death [54]. This kinase was widely expressed in Apicomplexan parasites and contribute to the pathogenesis of parasites [55]. Our data revealed that AGC kinase of sporulated oocyst of *T. gondii* had regulatory roles in nucleotide binding, ATP binding, metabolic process, protein serine/threonine kinase activity and phosphorylation, indicating the important role of AGC kinase in the biology of *T. gondii* sporulated oocysts.

Conclusions

The present study revealed the global phosphoproteomic differences in sporulated oocysts between virulent and avirulent *T. gondii* strains of different genotypes using the iBT technology combined with TiO₂ affinity chromatography. Abundant phosphopeptides in sporulated oocysts between virulent and avirulent *T. gondii* strains exhibited distinct difference in the conserved motifs, GO terms, enriched pathways and PPI networks. The kinase associated network analysis indicated the role of phosphorylation in the transformation of *T. gondii* kinases. AGC kinase was the most connected kinases peptides. These data provide new insight into the phenotypic differences in sporulated oocysts of virulent and avirulent *T. gondii* strains.

Abbreviations

Ab1: Abelson kinase; ACN: acetonitrile; AGC: automatic gain control; ATM: Ataxia Telangiectasia Mutated Kinase; BOP: ribosome biogenesis protein; CaM-II: Calmodulin-dependent Protein Kinase II; CDC2: Cell division Cycle Protein Kinase p34; CDK: Cyclin-Dependent Kinase; CKI: Casein Kinase I; CKII: Casein Kinase II; COP: coatamer coated complex; CsCl: caesium chloride; CV: coefficient of variation; DEPs: differentially expressed phosphopeptides; DTT: dithiothreitol; EGFR: Epithelial Growth Factor Receptor; FA: formic acid; FDR: false-discovery rate; GO: Gene Ontology; HPLC : high-performance liquid chromatography; IAM: iodoacetamide; iBT: isobaric tag; IKK: I kappaB Kinase; INSR: Insulin Receptor; iTRAQ: isobaric tag for relative and absolute quantitation; Jak: Janus kinase; KEGG: Kyoto Encyclopedia of Genes and Genomes; LC-MS: liquid chromatography-mass spectrometry; MAPK: Mitogen-Activated Protein Kinase; MAT: modified agglutination test; MOM: mitochondrial outer membrane; MS/MS: tandem mass spectrometry; NOP: nucleolar protein; OG: Orthologous Groups; PBS: phosphate-buffered saline;

PKA: cAMP-dependent Protein Kinase; PKB: Protein Kinase B; PKC: Protein Kinase C; PKG: cGMP-dependent Protein Kinase; PPI: protein–protein interaction; PTM: post-translational modification; ROP: rhoptry protein; SPF: specific-pathogen-free; Src: Sarcoma gene kinase; STRING: Search Tool for the Retrieval of Interacting Genes/Protein; TFA: trifluoroacetic acid; TiO₂:Titanium dioxide; TK: tyrosine kinase; Syk: Cytoplasmic Tyrosine Kinases.

Declarations

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Ethical approval and consent to participate

All animal experiments were strictly performed according to the instructions set forth in the Animal Ethics Procedures and Guidelines of the People's Republic of China. Animals were obtained from the Laboratory Animal Center of Lanzhou Veterinary Research Institute (LVRI). All animal experiments were inspected and granted by the Animal Administration and Ethics Committee of LVRI, Chinese Academy of Agricultural Sciences. Earnest endeavors was conducted to reduce animal affliction and to minimize the quantity of animals used in the experiment.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the findings of this article are included within the article. All the mass spectrometry data have been deposited to the ProteomeXchange Consortium with identifier PXD017032.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZXW, HME, XQZ and XLS conceived and designed the research. ZXW performed the experiments, analyzed the data and wrote the paper. RSH and XLS contributed reagents/materials/analysis tools. ZXW, HME and XQZ critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Figures

Figure 1

Summary of phosphoproteomic parameters of sporulated oocysts between virulent PYS strain and avirulent PRU strain of *Toxoplasma gondii*. A, The distribution of phosphorylation on different residue types (pSer-serine, pThr-threonine, and pTyr-tyrosine) identified by TiO2. B, Distribution of single-, double-, triple- and quadruple- phosphorylated sites showed that the majority of phosphorylated peptides have only single phosphorylation site. C, Distribution of phosphorylated peptides matched by single spectrum

and multiple spectra. The majority of phosphorylated peptides were matched by single spectrum. D, The numbers of phosphorylated sites on the identified phosphorylated proteins. The number of phosphorylated proteins with several phosphorylated sites decreases with the increase in the number of the phosphorylated sites.

Figure 2

Reproducibility evaluation of phosphorylated peptides based on the value of Coefficient of variation (CV). The values of CV were indicated by percentage on the X-axis. The left and the right Y-axes represent the number of phosphopeptides and the cumulative percentage of phosphopeptides, respectively.

Figure 3

Quantification of phosphorylated peptides. A, Volcano plot of phosphorylated peptides. The X-axis label indicates phosphopeptides log2 fold change and the Y-axis label represents the $-\log_{10}$ P value of phosphopeptides. Upregulated and downregulated differentially expressed phosphoproteins (DEPs) were represented by red or green circles, respectively. Dashed curves represent (+/-) 1.5-fold change. B, Distribution of differentially expressed phosphopeptides (DEPs) of sporulated oocysts between PYS

strain and PRU strain. The X-axis label represents values of log1.5 fold changes and the Y-axis label represents the quantity of phosphopeptides. Upregulated and downregulated DEPs were indicated by red and green colors, respectively.

Figure 4

Hierarchical clustering heat map of differentially expressed phosphopeptides (DEPs). Expression values of DEPs were log1.5-transformed.

Figure 5

Identification of phosphorylation-specific motif within the upregulated DEPs using the Motif-X software. The frequency of amino acids appearing at specific positions were indicated by the height of the logos. The acidic and basic residues were represented in blue and red colors, respectively.

Figure 6

Identification of phosphorylation-specific motif within the downregulated DEPs using Motif-X software. The frequency of amino acids appearing at specific positions were indicated by the height of the logos. The acidic and basic residues were represented in blue and red colors, respectively.

Figure 7

GO analysis of differentially expressed phosphoproteins (DEPs). The X-axis label represents the number of DEPs. The Y-axis label indicates GO terms of (A) upregulated and (B) downregulated phosphoproteins in PYS vs. PRU, respectively.

Figure 8

KEGG pathway enrichment analysis of differentially expressed phosphoproteins (DEPs) in PYS vs. PRU. The Y-axis label represents the significantly enriched KEGG pathways and the X-axis label shows the rich factor. The rich factor indicates the number of DEPs to the number of total phosphoproteins in the pathway. Higher rich factors indicate higher enrichment. The p value and number of phosphoproteins in the pathways correspond to the color and size of the node, respectively.

Figure 9

Phosphoprotein interaction network (PPI, combined score ≥ 0.9) of the DEPs between PYS strain and PRU strain. The nodes denote DEPs and the edges represent interactors between DEPs. The color of edge represents the combined score of interactors.

Supplementary Files

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