The Developmental Phosphoproteome of *Fasciola Gigantica* by Shotgun

Ming Pan

Yangzhou University

Shao-Yuan Bai

Yangzhou University

Jing-Zhi Gong

Yangzhou University

Dan-Dan Liu

Yangzhou University

Qi-Wang Jin

Yangzhou University

Jian-Ping Tao

Yangzhou University

Si-Yang Huang (✉ siyang.huang@hotmail.com)

Yangzhou University

Research

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Abstract

Background: Protein phosphorylation plays key roles in a variety of essential cellular processes. *Fasciola gigantica* is a tropical liver fluke causing hepatobiliary disease fascioliasis, leading to public health threats and heavy economic losses. Although the genome and protein kinases of *F. gigantica* provided novel insights to understand the molecular biology and etiology of this parasite, there is scant knowledge of protein phosphorylation events in *F. gigantica*.

Results: In this study, we characterized the phosphoproteome of *F. gigantica*. A total of 1030 phosphopeptides and 1005 phosphorylation sites were identified in 635 *F. gigantica* phosphoproteins. The bioinformatic analyses and homologous comparisons of these phosphoproteins identified their features participating in cellular processes including metabolic regulation and signal transduction.

Conclusions: To the best of our knowledge, this work performed the global phosphoproteomic analysis in *F. gigantica*, which provides a valuable resource to facilitate the development of novel interventions targets in *F. gigantica* and related nematodes in the future.

Background

As a tropical liver fluke, *Fasciola gigantica* (*F. gigantica*) is a parasitic food-borne trematode causing hepatobiliary disease-fascioliasis, leading to extensive liver tissue damage and cirrhosis [1]. Combining with *Fasciola hepatica* (*F. hepatica*) infection, fascioliasis is endemic worldwide infecting 17 million people from 61 countries, and 180 million people are at risk of infection [2, 3]. Meanwhile, these trematodes infect approximately 600 million domestic ruminants with a significantly negative effect on their live weight gains, causing profound damage to animal productivity and economic losses in excess of US\$3 billion per year [4, 5]. As the only drug effective against the infection, triclabendazole (TCBZ) was recently approved to be used by the FDA and there are currently no therapeutic alternatives to treat human fascioliasis. However, someone treated with TCBZ still continue excreting eggs through feces despite a standard care regimen [6]. Additionally, emerging hybridization and introgression between *F. gigantica* and *F. hepatica* make the prevention and control of fascioliasis more difficult [7].

Post-translational modifications (PTMs) are biochemical changes to regulate the modified proteins, which are indispensable for their functional diversities [8]. Therein, as one of the most important PTMs, reversible phosphorylation at serine, threonine and tyrosine residues plays key roles in a variety of cellular processes including signal transduction, membrane transport and metabolic regulation [9, 10]. Protein kinases and protein phosphatases are enzymes that catalyze the protein phosphorylation/dephosphorylation processes via addition and removal of phosphoryl groups to substrates [11, 12]. Particularly, protein kinases from *Schistosoma* and *Leishmania* as well as *Toxoplasma* had been identified as potential drug targets against these zoonotic parasites [13–15]. For *F. gigantica*, the draft genome (size: 1.04–1.13 Gb) was assembled with a high degree of genomic polymorphism. Using DNA library construction and sequencing as well as bioinformatics analysis, a total of 20858 genes were

predicted and 455 protein kinases were identified in 2020 [16, 17]. However, due to the limitations in genome annotation and functional study of these kinases, we have scant knowledge of protein phosphorylation events in *F. gigantica*. Furthermore, recent progress has been made in the global phosphoproteomics of different worms [11, 12, 18], which made contributions to understanding the complex biology of these organisms and developing new antiparasitic candidates.

In the present study, to obtain a comprehensive understanding of phosphorylation in *F. gigantica*, we used phosphopeptides enrichment method and LC-MS/MS analysis to define the first global phosphoproteomic of *F. gigantica*. Here, in total 1005 phosphosites from 635 *F. gigantica* proteins were discovered. Combining with functional annotations and biological connections, this work provides an overview and discusses current knowledge on phosphorylation of *F. gigantica*, that enables to develop new therapeutics to control fascioliasis.

Methods

Parasite preparation

Fasciola gigantica were isolated from the bile ducts of naturally infected buffaloes, washed thoroughly with phosphate-buffered saline (PBS, pH 7.4), and then incubated in RPMI 1640 medium at 37 °C for 1 h. The flukes were frozen in liquid nitrogen and stored at -80 °C until use.

Protein extraction and digestion

Protein extraction was obtained from three biological repeats from the frozen samples. Each sample was ground into powder and suspended in a lysis buffer (8 M urea in 100 mM triethyl ammonium bicarbonate, pH 8.5) with 1% protease inhibitor cocktail (Merck, Denmark). Subsequently, the sample was ultrasonicated on ice at 30 s bursts for 10 min. The lysate debris were eliminated by centrifugation at 12000 g for 30 min at 4 °C, and individual supernatants were collected and conducted to determine the protein concentration by a bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific, USA). Protein digestion was carried out as previously described [18, 19], with some modifications. Briefly, 300 µg protein sample was reduced with 20 mM dithiothreitol at 56 °C for 30 min, followed by alkylation with 100 mM iodoacetamide at room temperature for 30 min, then dissolved into urea solution with the addition of 25 mM NH_4HCO_3 . Finally, the digestion process was conducted in a trypsin (Promega, USA) solution (6 µg trypsin in 40 µL 100mM NH_4HCO_3).

Phosphopeptides enrichment

After enzymatic hydrolysis, the peptide solution was lyophilized in vacuum and dissolved in a 2,5-dihydroxybenzoic acid (DHB) buffer [20]. Phosphopeptides were enriched using a titanium dioxide (TiO_2) enrichment method [11, 21], with some modifications. Briefly, TiO_2 beads (ThermoFisher Scientific, USA) were mixed with peptides and incubated for 40 min at room temperature, and then eluted with 1%

ammonia solution and 30% acetonitrile. Subsequently, phosphopeptides were freeze-dried and then dissolved with 20 μ L 0.1% formic acid (FA). Therein, 6 μ L sample was analysed for LC-MS/MS analysis.

LC-MS/MS

LC-MS/MS analysis was performed using the Q Exactive Mass spectrometer (ThermoFisher Scientific, USA) with a nanoliter flow rate HPLC liquid system-EASY-nLC1000 (ThermoFisher Scientific, USA) according to the manufacturer's instructions.

The tryptic peptides were injected into the enrichment column EASY column SC001 traps 150 μ m*20 mm RP-C18 (ThermoFisher Scientific, USA) at an isocratic flow of 5 μ L/min of 2% CH₃CN containing 0.1% FA (solvent A) for 6 min, and then separated by the analytical column EASY column SC200 150 μ m*100 mm RP-C18 (ThermoFisher Scientific, USA) at an isocratic flow of 400 nL/min. The eluents were abovementioned solvent A and 84% CH₃CN in 0.1% FA (solvent B). The gradient of solvent B was as follows: (i) 0-110 min, 0%-55% solvent B; (ii) 110-118 min, 55%-100% B; (iii) 118-120 min, maintaining at 100% B. Subsequently, the elution products were analyzed by the Q Exactive Mass spectrometer. The spectra of first-grade MS (MS1) were acquired during the scan scope of 300-1800 m/z with a 2.0 kV electrospray voltage, a resolution of 70000, an automatic gain control (AGC) target of 3e⁶ and a maximum IT time of 50 ms. The spectra of second-grade MS (MS2) were obtained using the following parameter: a resolution of 17500; a maximum injection time of 40 ms; an AGC target of 1e⁵. In addition, the mode of MS2 spectra was high-energy collisional dissociation (HCD) and the normalized collision energy of which was set as 30 eV.

Data analysis

All MS/MS data were analyzed using Maxquant1.3.0.5 implementing a Mascot search engine (Matrix Science, London, UK version 2.6.1). The mascot was set up to search the database P17429_fasciola_hepatica_33454_20170815.FASTA (total number of sequences: 33,454; download link: <http://www.uniprot.org>). The search parameters were set as follows: (i) enzyme: trypsin; (ii) missed cleavage sites: set to 2; (iii) a fixed modification: carbamidomethyl cysteine; (iv) variable modifications: an oxidation of methionine (M), acetylation of protein N-term, phosphorylation of serine (S), threonine (T) and tyrosine (Y). For proteins and peptides, the filter parameter false discovery rate (FDR) was set as $\leq$ 0.01.

Bioinformatic analysis

BLAST analysis was performed using Blast2GO (<http://www.blast2go.com>), and the Gene Ontology (GO) analysis was performed to identify functional phosphoproteins as previously described, which could be roughly summarized as Blast, GO mapping, GO annotation (<http://www.geneontology.org>) and InterProScan (<http://www.ebi.ac.uk/interpro/>) [22, 23]. The InterPro database was utilized for the inspection of each category of phosphoproteins.

In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (<http://www.genome.jp/kegg/>) were carried out to identify the enriched pathways. The classified pathways were shown in the hierarchical categories according to the data from the KEGG website.

Results

Identification of phosphorylated proteins and peptides

Using TiO₂-dependent phosphopeptides enrichment method and LC-MS/MS analysis as well as Mascot search, a total of 635 phosphoproteins of *F. gigantica* representing 1030 phosphopeptides with 1005 phosphosites were identified in this study, with FDR ≤ 0.01 in phosphopeptide level and localization probability ≥ 0.75 in phosphorylation site level (Fig. 1A). These phosphoproteins represent 3% of proteins in recently database of *F. gigantica* [16]. Therein, 30.9% (196/635) phosphoproteins contained ≥ 2 phosphopeptides, while BN1106_s323B000258 (myosin heavy chain 6/7) and BN1106_s378B000167 (hypothetical protein) contained more than 10 phosphopeptides (Fig. 1A, Additional file 1: Table S1). Furthermore, a number (n = 25) of phosphoprotein kinases that covered 5.5 % of total kinases of this parasite [17] was identified, including mitogen-activated protein kinase (MAPK) and calcium/calmodulin-dependent protein kinase (CAMK/CDPK) as well as serine/threonine/tyrosine-protein kinase etc. Out of the identified phosphopeptides, 249 (24.2%) were found phosphorylated at ≥ 2 phosphosites and most (82.5%) peptides contain ≥ 10 amino acids (Fig. 1A, Additional file 2: Table S2). The identified 1005 phosphosites consisted of 858 (85.4%) serine phosphorylation, 138 (13.7%) threonine phosphorylation and 9 (0.9%) tyrosine phosphorylation (Fig. 1B, Additional file 3: Table S3). The phosphoserine, phosphothreonine or phosphotyrosine modification ratios found in this work were similar to those previously described in other organisms [11, 24, 25], and most (88.4%) phosphosites located at the second amino acid of the peptides or after the position (Fig. 1A, Additional file 3: Table S3).

GO enrichment analysis

Using the automatic annotation tool Blast2GO online, functional annotations were conducted to characterize the identified phosphoproteins in this project. By way of blast, mapping and annotation as well as inspection, the GO enrichment analysis showed that these phosphoproteins involved in a wide variety of biological processes (BP) including cellular, metabolic or single-organism processes and biological regulation, localization, cellular component organization or biogenesis as well as in response to stimulus (Fig. 2, Additional file 4: Table S4), which played important roles in protein phosphorylation [18]. Regarding the subcellular localization of the phosphoproteins, the GO annotations based on level 2 and level 5 inferred that these proteins were found predominantly in cellular components (CC) like membranes, organelles and nuclei. Interestingly, only a small portion of them were predicted to located in extracellular regions (Fig. 2, Additional file 5: Table S5), which made the finding reliable as extracellular components were not typically regulated by phosphorylation. According to the localization, the molecular functions (MF) of phosphoproteins was predominantly (51.3%) associated with term “binding” based on level 2, followed by different activities such as catalytic activity (40.6%) and both transporter activity and

structural molecule activity that occupied a small proportion (Fig. 2, Additional file 6: Table S6). In total, we found that half (317/635) of the phosphoproteins were annotated with at least one GO category (BP, CC or MF) and one third of them were annotated with at least two GO categories (Additional file 7: Table S7).

KEGG pathway annotation

To determine whether the identified phosphoproteins function independently or in collaboration with other proteins in related biological or signaling pathways, KEGG database was carried out to annotate these phosphoproteins, which helped us understand their potential functions. The KEGG annotation inferred that tight junction, spliceosome and RNA transport were found to be the most enriched pathways of the phosphoproteins, of which each one contains 15 identified proteins (Fig. 3, Additional file 8: Table S8). PI3K-akt signaling pathway, oxytocin signaling pathway, cAMP and cGMP-PKG signaling pathway as well as hippo and insulin signaling pathway were ranked in the top 20 of KEGG pathway annotation. Additionally, parts of phosphoproteins were also predicted to participate in the focal adhesion and protein processing as well as carbon metabolism (Additional file 8: Table S8). In terms of phosphoproteins, we found that 30.4% (193/635) of them were annotated with at least one KEGG pathway and 16.9% was annotated with at least two KEGG pathways (Additional file 8: Table S8). Therein, metabolic enzymes such as adenylate cyclase 9 (ADCY9) and hexokinase (HK), and protein kinases including p21-activated kinase 1 (PAK1), Rho-associated protein kinase 2 (ROCK2), mitogen-activated protein kinase 1/3 (MAPK1/3) and serine/threonine-protein kinase mTOR, as well as effectors like calmodulin (CALM) and heat shock protein 90 (HSP90) were predicted to be annotated in more than 10 KEGG pathways (Additional file 9: Table S9).

Comparison with phosphoproteins of other parasites

According to KEGG database, we compared abovementioned phosphoproteins of *F. gigantica* annotated in multiple pathways with other parasites including protozoa and helminth in order to identify possible connections between the biological processes and the phosphoproteins of these parasites. Based on recent reports (Table 1), calcium/calmodulin-dependent protein kinase (CAMK4/CDPK4) and 14-3-3 protein of *Plasmodium falciparum* participated in controlling the parasite invasion and regulating the assembly of signaling complexes, respectively [26, 27]. In *Leishmania*, mitogen activated protein kinase 1 (MAPK1) and heat shock protein 90 (HSP90) played important roles in stress regulation and nascent protein synthesis [28, 29]. Additionally, the metabolic enzymes acted as sensors to regulate the metabolites and metabolic flux, contributing to parasite growth [30, 31]. In short, the relationship between these proteins of different parasites (Table 1) will help us better understand the phosphoprotein profile of *F. gigantica*.

Table 1
Recent researches on the functions of phosphoproteins in other parasites

Phosphoproteins		Functions	Parasites	References
Metabolic enzymes	hexokinase (HK)	a glucose sensor to regulate the glycolysis flux and ATP production	<i>Trypanosoma brucei</i>	[31]
	pyruvate dehydrogenase (PDH E1 α)	contributes to parasite growth via enhanced fatty acid synthesis	<i>Toxoplasma gondii</i>	[30]
Protein kinases	calcium/calmodulin-dependent protein kinase 4 (CAMK4/CDPK4)	controls parasite motility and host cell invasion	<i>Plasmodium falciparum</i>	[26]
	Mitogen activated protein kinase 1 (MAPK1)	acts in regulation of stress machinery through post-translational modifications	<i>Leishmania donovani</i>	[28]
Effectors	Calmodulin	declines the plasticity of high-activity neurons	<i>Caenorhabditis elegans</i>	[44]
	14-3-3	binds phosphorylated PKAr and CDPK1 to mediate the assembly of signaling complexes	<i>Plasmodium falciparum</i>	[27]
	Heat shock protein 90 (HSP90)	regulates nascent protein synthesis	<i>Leishmania mexicana</i>	[29]

Discussion

As a food-borne trematode, *F. gigantica* is responsible for the hepatobiliary disease fascioliasis. The pathogenicity of *F. gigantica* is closely related to the massive proliferation of parasites, which brings mechanical stimulation and toxin to the host [1]. In terms of parasite growth and signal transduction, protein phosphorylation or dephosphorylation catalyzed by specialized protein kinases or phosphatases of *F. gigantica* played crucial roles in corresponding cellular processes, which helps maintain the cell homeostasis in parasites [17]. However, the phosphoproteomic profile of *F. gigantica* remains unclear. As such, to study the profile is of great significance for exploring the roles of these proteins in the growth and development of parasite, which could also help to screen new drug targets against *F. gigantica*.

In this work, to the best of our knowledge, a global phosphoproteomic profile of *F. gigantica* were performed by shotgun proteomics. In total, we identified 635 phosphoproteins of *F. gigantica* representing 1030 phosphopeptides with 1005 phosphosites (Fig. 1A). A number of phosphorylated enzymes were identified, including metabolic enzymes and protein kinases as well as phosphatases. Therein, as drug candidates, cathepsin L1 (CL1) and glutathione S-transferase (GST) of *F. gigantica* could be efficiently inhibited by chalcones and two antiparasitic agents (thymoquinone and curcumin), respectively [32, 33],

and both of them had been confirmed as single or combination vaccines against both *F. hepatica* and *F. gigantica* with good efficacy [3]. Wherefore, concentrating on phosphorylated enzymes recognized in this study, we can find more effective targets and open new avenues to treat fascioliasis. In addition, phosphorylated effectors such as 14-3-3 and thioredoxin with good immuno-reactivity were also identified [34, 35]. However, the phosphorylation processes catalyzed by gelsolin and calreticulin as well as tropomyosin had never been reported before, and BN1106_s378B000167 in particular deserved to mention as a result of hyper-phosphorylation (14 identified phosphopeptides) despite without any annotation, showing that BN1106_s378B000167 is unique in *F. gigantica* and can be used for subsequent functional studies and drug design in the future. The identified 1005 phosphosites consisted of 858 (85.4%) serine phosphorylation, 138 (13.7%) threonine phosphorylation and 9 (0.9%) tyrosine phosphorylation. Previous studies had found the mutations in important phosphosites might result in the termination of the phosphorylation, leading to disorders of biological processes [18]. Moreover, the modification ratios found in this work seemed to be consistent with previously studies in other organisms [11, 24, 25], so these phosphosites can provide references for future research on phosphorylation process in *F. gigantica*.

Subsequently, GO functional annotations (level 2) containing BP, MF, and CC categories were conducted to characterize the identified phosphoproteins. The top 3 in the BP category were involved in cellular process and metabolic process as well as single-organism process, highlighting the correlation between phosphorylation process and bioenergetics in *F. gigantica*. The largest percentage of CC terms were involved in cell and cell part such as membranes, organelles and nuclei, showing intracellular components were predominantly regulated by phosphorylation. Based on the localization, the MF were involved in binding and catalytic activities, indicating critical roles of phosphorylation in the structural and functional regulation of *F. gigantica* [36].

According to KEGG annotation, tight junction, spliceosome and RNA transport were found to be the most enriched pathways of the phosphoproteins. In addition, signaling pathways (PI3K-akt and oxytocin), protein processing and the regulation of actin cytoskeleton, as well as carbon metabolism were ranked in the top 10 of KEGG pathway annotation (each one contains ≥ 10 identified proteins), which seemed to be roughly consistent with the phosphoproteomic annotation in other parasites [11, 12]. For phosphoproteins, nearly 7% of them indicating multiple roles in different biological processes were predicted to be annotated in more than 5 KEGG pathways. Therein, malate dehydrogenase (MDH) of *F. gigantica* was a single cytosolic enzyme to catalyze the reversible oxidation of malate to oxaloacetate using NAD^+ . Been as an isoenzyme, the pattern of MDH in *F. gigantica* and *F. hepatica* were the same due to similar relative mobilities [37]. Further study found the superimposition structure model of FgMDH and human MDH showed overall structural similarity in the active site loop region, however, the conformation of the residues was different [38]. Combining with our annotations on the functions of FgMDH in metabolic pathways such as pyruvate metabolism, cysteine and methionine metabolism, glyoxylate and dicarboxylate metabolism, the FgMDH can be used in future drug design for the treatment of fascioliasis. P21-activated protein kinase (PAKs) had long been established to play important roles in vital cellular

functions such as proliferation, survival and motility. Emerging evidence showed host PAK1 increased cell survival during the stage of virus infections [39], and recent study found PAK1 enhanced macrophage activation, resulting in promoting of Th17 cell response during *Schistosoma japonicum* infection [40], both of which guide the future research on the immunomodulation roles of host PAK1 and parasitic PAK1 in *Fasciola* infection. Calmodulins (CaMs) were involved in fundamental processes including the phosphorylation of protein kinases, gene transcription and calcium transport [41]. In *Schistosoma mansoni*, calmodulins had been implicated in egg hatching, miracidial transformation and larval development. While in *F. hepatica*, the FhCaMs functioned as a Ca^{2+} modulator was proved to be important for the growth and movement of juvenile fluke [42, 43]. Recent research clarified calmodulin of *Caenorhabditis elegans* was implicated in the plasticity impairment of high-activity neurons with age, indicating a different but novel role in neuronal activity [44]. Furthermore, others phosphoproteins of *F. gigantica* annotated in more than 10 KEGG pathways were compared with other parasites (Table 1).

Conclusions

In this work, we defined the phosphoproteome of a tropical liver fluke by a phosphorylation-specific enrichment technique. From the mass spectrometry and data analysis, we identified several phosphoproteins and predicted their functions, which enhanced our knowledge of key biological processes in *F. gigantica*. With a view towards future exploration into the phosphoproteomics of this parasite at different life-cycle stages, new effective intervention targets will be developed for the treatment of fascioliasis.

Declarations

Ethics approval and consent to participate

The experiments were performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. All experimental animal procedures and protocols (Approval No. 14567) were approved by the Institutional Animal Care and Use Committee of Yangzhou University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Higher Education Institutions/Veterinary Medicine; The funders were not involved in the study design; collection, analysis and interpretation of the data; or in writing the manuscript.

Authors' contributions

SYH and MP conceived and designed the study. MP, SYB, JZG, and DDL performed the laboratory analyses. QWJ and JPT analyzed the data. All authors critically appraised and interpreted the results. MP drafted the first version of the manuscript. All authors provided feedback on the manuscript, and read and approved the final version.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article and its Additional files.

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Figures

A

Categories (Phosphorylation)	Total number	Remarks
Proteins	635	30.9 % ≥ 2 peptides
Peptides	1030	24.2 % ≥ 2 sites
Sites	1005	88.4 % ≥ 2 nd position of peptide N-terminal

B

Figure 1

Overall characteristics of the phosphoproteome of *F. gigantica*. (A) General description about remarks of *F. gigantica* phosphoproteins, phosphopeptides and phosphosites. (B) Distribution of identified phosphosites on serine, threonine and tyrosine.

Figure 2

Gene Ontology (GO) term distribution of *F. gigantica* phosphoproteins in three categories (biological process, molecular function and cellular component). GO annotation and categorization were performed using Blast2GO.

Figure 3

Enrichment analysis of top 20 KEGG pathway of *F. gigantica* phosphoproteins.

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