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Protein phosphorylation during *Plasmodium berghei* gametogenesis

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Abstract

Plasmodium gametogenesis within the mosquito midgut is a complex differentiation process involving signaling mediated by phosphorylation, which modulate metabolic routes and protein synthesis required to complete this development. However, the mechanisms leading to gametogenesis activation are poorly understood. We analyzed protein phosphorylation during *Plasmodium berghei* gametogenesis *in vitro* in serum-free medium using bidimensional electrophoresis (2-DE) combined with immunoblotting (IB) and antibodies specific to phosphorylated serine, threonine and tyrosine. Approximately 75 protein exhibited phosphorylation changes, of which 23 were identified by mass spectrometry. These included components of the cytoskeleton, heat shock proteins, and proteins involved in DNA synthesis and signaling pathways among others. Novel phosphorylation events support a role for these proteins during gametogenesis. The phosphorylation sites of six of the identified proteins, HSP70, WD40 repeat protein msi1, enolase, actin-1 and two isoforms of large subunit of ribonucleoside reductase were investigated using TiO2 phosphopeptides enrichment and tandem mass spectrometry. In addition, transient exposure to hydroxyurea, an inhibitor of ribonucleoside reductase, impaired male gametocytes exflagellation in a dose-dependent manner, and provides a resource for functional studies.

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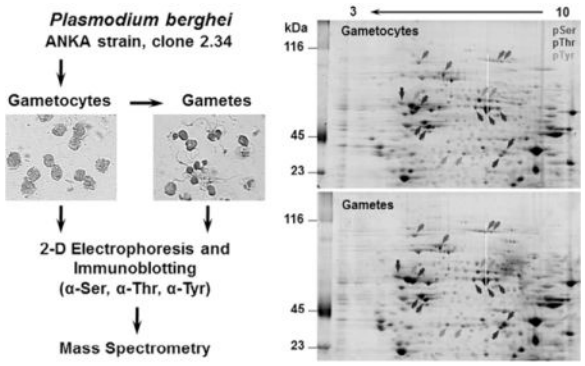
GRAPHICAL ABSTRACT



Keywords

Gametogenesis; *Plasmodium berghei*; 2-DE immunoblotting; Protein phosphorylation; Mass spectroscopy

1. Introduction

Malaria is caused by apicomplexan protozoan *Plasmodium* species and is a major contributor to worldwide mortality and morbidity (http://www.who.int/topics/malaria/). These parasites have a complex life cycle alternating between mosquito vectors and vertebrate hosts. In the vertebrate, gamete-precursor parasite stages (gametocytes) develop and are transferred to the mosquito midgut with the bloodmeal when females feed on infected vertebrates. Gametocytes rapidly exit erythrocytes and transform into gametes; male gametocytes undergo three rounds of genome replication and mitotic division, resulting in the release of eight highly-motile flagellated microgametes, whereas female gametocytes differentiate into macrogametes (Kuehn and Pradel, 2010; Ngwa et al., 2013). Gametes fertilize and give rise to zygotes that transform into motile ookinetes, which actively penetrate the mosquito midgut epithelium and develop into oocysts. Thousands of sporozoites formed inside oocysts migrate to the salivary glands and are transmitted during feeding on new vertebrate hosts (Kappe et al., 2004).

Protein phosphorylation and dephosphorylation are reversible post-translational modifications that play key roles in many cellular processes (Johnson, 2009). Protein phosphorylation can affect several key properties of proteins, including their activity, interaction with other proteins or sub-cellular localization. This modification occurs in response to intra- and extracellular signals, and participates in signal transduction, metabolism, differentiation and regulation of the cell cycle (Graves and Krebs, 1999; Webb and Miller, 2013). Furthermore, modifications catalyzed by kinases and phosphatases play a vital role in development of apicomplexan parasites (Solyakov et al., 2011).

Two independent genomic analyses identified in *P. falciparum* 86 and 99 genes encoding putative protein kinases corresponding to 1.1–1.6% of the total coding genes (Anamika et al., 2005; Ward et al., 2004). Sixty-five of these are related to the eukaryotic protein kinase

(ePK) family and a group of PKs involved in mitogen-activated protein kinase (MAPK) pathways (Doerig et al., 2008). Although the absence of “classical” tyrosine protein kinases (TyrK) is notable, and further because phosphorylation in tyrosines has been documented, a dual-specific activity of protein kinases has been proposed (Doerig, 2004).

Due to the biological importance of phosphoproteins, those of intra-erythrocytic stages of *P. falciparum* have been studied using several strategies including bidimensional electrophoresis (2-DE) followed by immunoblotting and phosphoprotein enrichment (Wu et al., 2009); IMAC phosphopeptides enrichment (Treeck et al., 2011); IMAC and TiO_2 phosphopeptides enrichment (Solyakov et al., 2011); strong anion exchange and TiO_2 phosphopeptides enrichment (Lasonder et al., 2012); isobaric labeling and IMAC phosphopeptides enrichment (Pease et al., 2013), all of these coupled to LC–MS/MS. Recently, a pipeline for analysis of phosphoproteomic data generated using collision induced dissociation (CID) and electron transfer dissociation (ETD) was applied to resolve the phosphoproteome of schizonts (Collins et al., 2014).

Information on the role of protein phosphorylation in the sexual stages of life cycle of malaria parasites is emerging. Two classic examples of protein kinases involved in parasite gametogenesis have been described: calcium-dependent protein kinase (PbCDPK4) and mitogen-activated protein kinase (Pbmap-2). PbCDPK4 involved in *P. berghei* gametogenesis is activated by an increase of intracellular Ca^{2+} (Billker et al., 2004). This kinase regulates genome replication in microgametocytes, and mutants lacking it do not produce microgametes and fail to infect mosquitoes. Pbmap-2 controls the formation of male gametes at cytokinesis (Tewari et al., 2005). Microgametes lacking Pbmap-2 progress through DNA replication but are blocked from forming motile axonemes. The target proteins are unknown. The cell-division cycle protein 20/CDC20 homolog 1(CDC20/CDH1) ortholog in *Plasmodium* is an important regulator of mitosis during male gametogenesis, and this protein is phosphorylated in asexual and sexual stages, with the level of modification higher in activated gametocytes and ookinetes (Guttery et al., 2012).

We tested herein the feasibility of conducting exflagellation of *P. berghei in vitro* in the absence of serum in the culture medium in order to obtain samples suitable for protein analysis. 2-DE coupled with immuno-blotting (IB) using specific antibodies specific to phosphorylated amino acids (Ser, Tyr and Thr) was used to identify phosphorylation changes in proteins. TiO_2 enrichment and tandem mass spectrometry were used to look for specific amino acid residues in the proteins whose phosphorylation levels are modified during gametogenesis. This information contributes to the understanding of the molecular events involved in parasite sexual stage development.

2. Materials and methods

2.1. Parasites and culture medium

This study was approved by the CINVESTAV’s Institutional Bioethical Committee for Care and Handling of Laboratory Animals (UPEAL-Protocol 013-02) following the Mexican law for humanitarian housing and management (NOM-062-ZOO-1999).

The *P. berghei*, gametocyte-producing ANKA strain, clone 2.34 (kindly provided by R. E. Sinden, Imperial College, UK) was used. Parasites were obtained as described previously (Rodriguez et al., 2002). Briefly, BALB/c male mice were injected intra-peritoneally with 6 mg/ml of phenyl hydrazine (Sigma-Aldrich, St. Louis, Mo, USA) to induce reticulocyte production. Mice were inoculated 3 days later with 10^8 *P. berghei*-infected mouse erythrocytes and were injected on days 3 and 4 post-infection with 1 mg/ml of pyrimethamine (Sigma-Aldrich, St. Louis, Mo, USA) to eliminate asexual stages. Giemsa-stained tail-blood smears were prepared 24 and 48 h after pyrimethamine treatment to assess gametocytemia.

Mice were bled using a heparinized syringe (30 U/ml blood) to obtain gametocyte-infected erythrocytes and the blood was diluted immediately 1:4 with pre-warmed (37 °C) RPMI 1640 medium (pH 7.2) (this temperature prevents gametogenesis) and passed through a CF-11 column (cellulose powder) (Whatman, Maidstone, UK) to deplete white blood cells. Cells were collected and layered onto a 15% HistoDenz cushion (Sigma-Aldrich) in a buffer A (10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.4), and centrifuged at 500 × *g* for 25 min. The interface containing gametocyte-infected erythrocytes was collected and washed with buffer A. The gametocyte-infected erythrocytes were diluted immediately 1:5 with RPMI 1640 medium (pH 8.3) (Gibco, Paisley, UK) to produce extracellular gametes. Activated gametocytes were cultured at 19–21 °C for 13–18 min in RPMI 1640 medium (pH 8.3) with or without fetal bovine serum (FBS) to obtain extracellular gametes.

2.2. Exflagellation assays and ookinete culture

The production of gametes and ookinetes in culture in the absence of FBS was evaluated by adding 5 μl of *P. berghei*-infected blood into 50 μl of RPMI 1640 medium, pH 8.3 and incubating at 19–21 °C. The number of exflagellation centers was counted after 15 min under a phase contrast microscope. The numbers of ookinetes were assessed after 24 h in blood Giemsa-stained smears (Torres et al., 2005). The percentages ± SD of exflagellation centers and ookinetes in cultures without FBS were compared to control groups (medium RPMI 1640 supplemented with FBS).

2.3. Treatment with hydroxyurea

Gametocytes were treated with hydroxyurea (HU), an inhibitor of ribonucleoside reductase, in the absence of FBS to analyze its effects on the development of gametocytes to gametes. Briefly, gametocytes were grown in RPMI 1640 medium pH 7.2 without FBS and containing different concentrations of HU (50, 75 and 100 mM; Sigma-Aldrich, 98% purity) for 1 h at 37 °C. Subsequently pH and temperature were changed to induce gametogenesis by removing the initial RPMI 1640 medium, and parasites were incubated in fresh RPMI 1640 medium pH 8.3 without FBS at 19 °C for 15 min. Exflagellation centers were counted immediately as before. A similar assay without HU was performed as a control. Data are presented as mean ± standard deviation of the mean (SEM). Experiments were repeated three or more times in duplicate.

2.4. Two-dimensional gel electrophoresis (2-DE)

Parasite samples were suspended in sample buffer [7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer pH 3–10 (GE Healthcare Life Sciences) and 40 mM dithiothreitol (DTT)], and protease (Complete, Roche Diagnostics, Mannheim, Germany) and phosphatase (PhosStop, Roche Diagnostics) inhibitor cocktails were added. Parasites were lysed by freeze/thawing, centrifuged at 16,000 × *g* at 4 °C for 15 min and supernatants were recovered. The protein samples were extracted and desalted using 2-D Clean-Up kit (GE Healthcare, UK) to eliminate contaminants. Proteins were solubilized in 2-DE rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer pH 3–10 and 65 mM DTT) with protease and phosphatase inhibitors added and centrifuged at 16,000 × *g* for 10 min. A 2D Quant Kit (GE Healthcare, UK) was used to quantify proteins; 250 μg of protein per sample (or as indicated) were used for 2-DE. 2-DE was performed using a dedicated system (GE Healthcare, UK); the isoelectric focusing (IEF) was conducted on precast Immobiline Drystrips pH 3–10 NL, 7 cm (GE Healthcare) rehydrated overnight with the samples using an Ettan IPGphor 3 Unit (GE Healthcare) following the manufacturer's instructions. Focused IPG strips were equilibrated in 10 ml equilibration solution (75 mM Tris–HCl, pH 8.8, 6 M urea, 29.3% glycerol, 2% SDS) with 1% DTT for 10 min, and 10 ml equilibration solution with 4.5% iodoacetamide for 10 min. The equilibrated strips were loaded on 10% SDS–PAGE for second dimension resolution. Independent triplicates of experiments were conducted to assess reproducibility of results.

2.5. SYPRO Ruby and Coomassie Blue staining for total proteins

Protein analysis by 2-DE was conducted in parallel on three sets of gels. Following the electrophoresis, the first set of gels was fixed and stained with SYPRO Ruby dye (Invitrogen, USA) following the manufacturer's instructions. Briefly, gels were washed twice with 10% methanol/7% acetic acid for 30 min and one rinse with water for 10 min. Images of gels were recorded in an Ettan DIGE Imager system (GE Healthcare, UK) using a 280/450 nm excitation/emission. A second set of gels was stained with Bio-Safe Coomassie Stain (Bio-Rad, USA) and registered in an ImageQuant LAS4000 System (GE Healthcare Bio-Sciences AB, USA). A third group of gels was blotted on nitrocellulose for analysis with anti-phospho-residue antibodies.

2.6. Immunoblotting with phospho-specific antibodies

2-DE gels were electrotransferred onto Hybond ECL nitrocellulose membranes (GE Healthcare, UK). Membranes were blocked with 3% bovine serum albumin (BSA) in TBS containing 1% Tween-20 (TBS-T) at room temperature for 2 h before being probed with rabbit anti-phosphoserine specific polyclonal antibody (61–8100, Invitrogen Life Technologies, CA, USA, diluted 1:1000) or mouse anti-phosphothreonine specific monoclonal antibody (13–9200, Invitrogen, diluted 1:1000), incubating overnight at 4 °C. Goat anti-mouse IgG antibody (H + L) HRP (AP308P, Millipore, Temecula, CA, USA; diluted 1:80,000) and a goat anti-rabbit IgG–HRP (65–6120, Invitrogen; diluted 1:50,000) conjugates were used to localize antibody–antigen complexes. Mouse anti-phosphotyrosine-HRP monoclonal antibody (03–7720, Invitrogen; diluted 1:3000) also was used. Signal detection was performed using the Supersignal West Pico Chemiluminescent Kit (Thermo

Scientific, Rockford, USA) in a luminescent image analyzer ImageQuant LAS 4000 system (GE Healthcare).

2.7. In-gel trypsin digestion and protein identification by MS/MS

Individual spots of 2-DE-resolved phosphorylated proteins were trypsin-digested in gel for mass spectrometry analysis. In-gel digestion was performed by removing the Coomassie Blue dye from gel pieces, reducing the proteins with DTT and then alkylating them with IAA. Polypeptides were digested overnight at 37 °C with sequencing-grade trypsin (25 ng/μl; Promega, USA). The supernatant was removed and the gel pieces were treated with protein extraction solution [50% acetonitrile (ACN)/0.5% trifluoroacetic acid (TFA)] for 10 min at room temperature, then vortex-mixed and centrifuged at 14,000 × *g* for 30 s. The supernatants were collected and protein digests were concentrated/desalted using a solid-phase extraction modified protocol with C18-ZipTips (Millipore Corporation, Bedford, MA, USA). Peptides were eluted in 15 μl of 50% ACN/0.1% TFA.

Sample digests were analyzed to obtain partial sequences using an AB Sciex MALDI TOF/TOF 5800 System (Applied Biosystems, Framingham, MA, USA) at the Mass spectrometry Facility, Department of Chemistry, University of California Irvine, CA, USA. MS/MS spectra were analyzed using the Paragon Algorithm (Protein Pilot Software, Applied Biosystems) against the UniProt Knowledgebase (UniProtKB)/SwissProt) as reported (Ruiz-Rosado et al., 2013) for *Plasmodium* species plus contaminant protein databases. Search parameters were adjusted for carbamidomethylation at cysteine as a fixed modification, and oxidation of methionine as variable modifications. A confidence interval >95% was used for protein identification (Unused ProtScore >1.3). Other groups of protein spots were analyzed by ESI–LC–MS/MS, at the Protein Core Facility of the Columbia University Medical Center, New York, NY, USA as described (Cázares-Raga et al., 2014). Briefly, selected protein spots were processed as before and were analyzed using a Micromass Hybrid Quadrupole/time of flight mass spectrometer (Micromass QTOF, Ultima, Waters, USA) equipped with an LC Packing nanoflow and operating in positive ion mode. Raw data files were processed using the MassLynx ProteinLynx software and Mascot (http://www.matrixscience.com/) and the NCBInr database for *Plasmodium* species plus contaminant protein databases. Mascot search parameters of the tryptic peptides were adjusted as before considering fixed and possible variable modifications.

2.8. TiO_2 enrichment of phosphopeptides

Selected protein spots were subjected to phosphopeptide enrichment using the Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment kit (Thermo Scientific, Rockford, Il, USA) following the manufacturer's protocol. Briefly, individual protein spots were excised from 2-DE Coomassie Blue stained gels, and then the gel pieces were further diced into ~1 mm^3 cubes for digestion in gel. Each spot was destained, reduced, alkylated and digested into peptide fragments with trypsin (25 ng/μl; Promega) overnight at 37 °C. The gel pieces containing peptide samples were sonicated, supernatants were collected, concentrated and desalted and eluted in 80% ACN/0.1% formic acid using HyperSep C18 SPE columns (Thermo Scientific). After adding formic acid to a final concentration of 2%, samples were added directly to TiO_2 magnetic beads, which were conditioned previously in binding buffer,

mixed carefully and incubated on a magnetic plate using a magnetic stand, at room temperature for 1 min. The supernatant was removed and the beads were washed with washing buffer. The beads with phosphopeptides were resuspended with 30 μl of elution buffer and incubated at room temperature for 10 min. The supernatant containing eluted phosphopeptides was removed, concentrated and suspended in 5% ACN/1% formic acid.

2.9. Phosphopeptide identification by LC–MS/MS

Phosphopeptides were analyzed using tandem mass spectrometry (LC–MS/MS) in a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, San Jose, CA, USA), interfaced with a nano-ultra performance liquid chromatography system (Easy *n*LC, Thermo Scientific) under optimized conditions at UC Davis Proteomics Core Facility (University of California Davis, CA, USA). To search for phosphorylation modifications, the raw spectra files were processed using the X!Tandem search engine and manual verification. X!Tandem was set up to search the UniProt *Plasmodium sp.* database and contaminant protein sequences. The analysis was performed with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 20 PPM (Rigor et al., 2011). Carbamidomethylation of cysteine residues was included as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine, acetylation of the N-terminus and phosphorylation of serine, threonine and tyrosine were included as variable modifications, specified in X! Tandem. Peptides of minimum seven amino acids and maximum of three missed cleavages were allowed for the analysis. Phosphorylated sites were considered with a probability cutoff of >75%. UniProt with Gene Ontology (GO) annotation was used to assign the identified phosphoproteins into biological process, molecular function, and cellular component categories. Predictions of serine, threonine, and tyrosine phosphorylation sites in phosphoproteins and surrounding ±6 amino acids were made using the NetPhos 2.0 Server.

3. Results

3.1. Differentiation of gametes and ookinetes in RPMI 1640 medium without FBS

Gamete samples prepared in RPMI 1640 medium supplemented with 20% fetal bovine serum, analyzed by 2-DE and stained with Coomassie blue showed abundant bovine serum albumin identified by MS (data not shown) (Fig. S1, upper right panel). This complicated further analysis of gamete and ookinete development so we developed a protocol for parasite culture in RPMI 1640 medium without FBS. The formation of exflagellation centers (between 13 and 18 minutes) and the yield of ookinetes (24 h) under these conditions were similar to those obtained using standard medium (Fig. 1), which demonstrated that gametes were functionally normal and could be used for subsequent analysis. 2-DE protein profiles of differentiated gametes in the absence of FBS were obtained (Fig. S1, lower right panel), and the proteins did not show any evidence of degradation or serum protein contaminants. Furthermore, the patterns were similar, with minor changes, to gametocyte profile (Fig. S1, upper panel).

3.2. Analysis of phosphorylated proteins of gametocytes and gametes from P. berghei

We analyzed proteome-wide changes in phosphorylation in gametocytes and differentiated gametes in parasites cultured for 15 min in the absence of FBS using 2-DE and the immune-

blot approach. First, total proteins of gametocytes and gametes were stained with a phosphoprotein specific stain, ProQ Diamond (Fig. S2); 130 and 120 putatively phosphorylated proteins were observed in gametocytes and gametes, respectively. Although the number of protein spots observed in gametes was lower than in gametocytes, some protein spots in gametes showed increased intensity including a typical horizontal string of spots ~100 kDa in a pI range of 5.7–7.0, whereas in gametocytes this protein group showed a pI range more basic (6.0–6.6), which is supportive of the conclusion that there are different levels of phosphorylation of the same proteins (Fig. S2). After staining with ProQ Diamond, total proteins in the gels were revealed with Sypro Ruby stain (Fig. 2). Approximately 710 protein spots with a mass range of 200–20 kDa were distinguished in the gels. Several spots showed variation in the protein abundance, among them a group of protein spots ~90 kDa with pI 7.0–7.5 (white ovals), which were more intensely staining in gametes. Furthermore, polypeptides of ~25 kDa/pI 3.8, 45 kDa/pI 5.5, 46 kDa/pI 6.0, 47 kDa/pI 7.0 and 50 kDa/pI 4.9 were present in gametocytes and absent in gametes (Fig. 2, white arrows in gametocytes and white circles in gametes). Interestingly, a group of protein spots in gametocytes forms a line at ~100 kDa in a pI range of 6.0–6.6, whereas in gametes the pI range shifted to 5.7–7.0 (pI changes are evidenced by a vertical white line, which is centered on another protein spot from 50 kDa and pI 6.2) (Fig. S3).

We analyzed protein phosphorylation following a 2-DE immuno-blot strategy. We used antibodies directed against the three majorly phosphorylated amino acids, phosphoserine (pSer), -phosphothreonine (pThr) and -phosphotyrosine (pTyr) antibodies while preventing phosphatase activities with an inhibitor cocktail in all processing. The analysis revealed ~75 Ser/Thr/Tyr phosphorylated proteins with an apparent mass range of 100–20 kDa, which changed their phosphorylation levels during gametogenesis (Fig. 3). It is interesting to note that contrasting total proteins stain with the immune-blot results, the Ser-phosphorylated proteins were low-abundance proteins.

Serine phosphorylation was analyzed using a polyclonal antibody (Fig. 3, top panels). Interestingly the group of proteins of 100 kDa/pI 5.7–7.0, which changed the spot distribution in the pI range during gametogenesis, also showed a noticeable increase in intensity with anti-phosphoserine antibody at the gamete stage (top right panel, spots 3 and 4). In addition, two proteins of 110 kDa/pI 4.8 and 70 kDa/pI 5.5) were labeled mainly in gametocytes (spots 1 and 2, respectively; top left panel).

Threonine phosphorylation was analyzed using a monoclonal antibody (Fig. 3, middle panels). Several protein spots of ~50 kDa with pI 4.6 and 4.8 (spots 5 and 6), and with pI 6.2 (spot 8) were labeled more intensely in gametes (right panel) than in gametocytes (left panel). Spot 9 (pI 6.5) was absent in gametocytes and detected lightly in gamete samples. Also, two protein spots of ~37 kDa with pI 6.6 and 6.7 (spots 12 and 13) were labeled mainly in gametes. In addition, two polypeptides of ~45 kDa with pI 5.2 and 5.3 (spots 10 and 11, respectively) were labeled mostly in gametocytes (Fig. 3, middle panels).

The changes in the tyrosine phosphorylation between gametocytes and gametes were analyzed using a specific monoclonal antibody (Fig. 3, lower panels). The results showed that two protein spots of 55 kDa with pI 6.0 and 6.2 (spots 14 and 15, respectively) were

marked; spot 14 showed greater intensity in gametes and spot 15 diminished at this same stage. Also, a protein spot of ~55 kDa with pI 4.9 (spot 16) was labeled more intensely in gametes. Two protein spots of 30 kDa with 5.6 and 5.7 (spots 17 and 18) showed greater intensity in gametocytes than in gametes (Fig. 3, lower panels).

3.3. Identification of phosphoproteins by MS/MS

Eighteen protein spots with differential phosphorylation levels in gametocyte and gamete preparations were analyzed by tandem MS (Table 1). Twenty-three proteins were identified, among which were five proteins corresponding to stress proteins: Endoplasmin putative (HSP90), heat shock protein 70 (HSP70), two T-complex protein beta subunit putative isoforms (TPC_beta) and a disulfide isomerase (PDI) precursor. Four proteins are involved in DNA synthesis (two isoforms of ribonucleoside reductase (RNR) large subunit, WD-40 repeat protein MSI1, and histone-binding protein n1/n2), six cytoskeleton proteins (alpha, beta and gamma tubulins and actin, actin-1 and actin-2), three signaling proteins [guanine nucleotide-binding protein (G protein), one corresponding to receptor for activated c kinase, putative (RACK1) of *P. berghei* (gi:675225715), two ADP-ribosylation factor GTPase-activating protein (ArfGAP) isoforms], three metabolic proteins (three enolase isoforms) and one ribosomal protein. In addition, one protein identified as uncharacterized and its function unknown showed 99% identity with the p1/s1 nuclease putative of *P. berghei* (gi: 675227026), which is involved in the salvage of preformed purines through the hydrolysis of either 3′-nucleotides or nucleic acids. Protein functional classification was assigned using Gene Ontology resources (Fig. 4).

Ten of the identified proteins showed increased phosphorylation in gametocytes including HSP90, HSP70, actin-1/actin-2, histone-binding protein putative, a T-complex protein beta subunit putative isoform, and two ADP-ribosylation factor GTPase-activating protein isoforms. Ten additional phosphorylated proteins showed greater intensity in gametes: two RNR large subunit isoforms, WD40 repeat protein MSI1, α and β tubulin isoforms, two enolase isoforms, G Protein putative, p1/s1 nuclease putative and a TPC_beta subunit putative isoform. The proteins of this study, which have been also identified as phosphorylated in previous studies, are grouped in Table S1.

3.4. Identification of phosphorylation sites by TiO_2 enrichment and LC–MS/MS

A strategy using highly selective metal-dioxide-bound magnetic microspheres for phosphopeptide enrichment, combined with tandem mass spectrometry was used to identify the phosphorylation sites in six of the identified phosphoproteins recognized by specific anti-pSer, -pThr or -pTyr antibodies, based mainly on the phosphorylation level and comparisons were made with the immuno-blot results. Of the six phosphoprotein spots selected for this analysis, HSP70 and RNR (two isoforms) are found phosphorylated at serine in both methods (Table 2). However, both the WD40 repeat protein MSI1 and actin were phosphorylated in threonine in the IB analysis but mass spectrometry detected the phosphorylated residues at serine and tyrosine, respectively. However, MSI1 showed a slight reaction with the anti-serine antibody and actin with the anti-tyrosine in the IB analysis.

3.5. Effect of hydroxyurea on gametogenesis

The RNR enzyme was interesting because it had noticeable changes in serine phosphorylation and variation of pI during gametogenesis. Hydroxyurea (HU), an inhibitor of DNA replication, acts by the inhibition of this enzyme. Treatment of gametocyte preparations with HU impaired male gametocyte exflagellation in a dose-dependent manner. Exflagellation centers diminished to 50% compared to the controls in the presence of 50 mM for 1 h HU (Fig. 5).

4. Discussion

Phosphorylation of serine, threonine and tyrosine plays significant roles in cellular signal transduction and in modifying multiple protein functions. Signaling pathways that regulate *Plasmodium* gametogenesis are poorly studied. This study describes changes in the phosphorylation of proteins that occur during gametogenesis of *P. berghei*. These results were obtained using a protocol to obtain highly-pure gametocyte and gamete samples in serum-free culture medium, avoiding major contaminants for proteomic analysis. This method enabled proteomic analysis. Therefore, this simple method of induction of gametogenesis provides the basis for further analyses in the field of cellular and molecular biology of this development stage of *Plasmodium*. We observed ~75 phosphoproteins, 18 of which showed differences in phosphorylation levels and were identified by tandem mass spectrometry. Various studies report more than one protein in a spot from 2-DE, however, the parameters used in our analysis for tandem mass spectrometry show only five (6, 8, 10, 11 and 13) of the 18 spots comprise more than one protein. These results also showed that only four proteins (HSP90, alpha tubulin, actin and p1/s1 protein) from gels stained with ProQ Diamond and Sypro Ruby showed light differences in relative abundance, supporting the hypothesis that the observed changes in their phosphorylation (2-DE immunoblots) are due to kinase/phosphatase activity and not to protein concentration. In addition, phosphorylated sites from six proteins were localized, and although the analysis started from protein spots, the use of highly selective TiO_2-phospho-enrichment method was suitable for high-performance phosphopeptide enrichment and identification of phosphorylated sites by tandem mass, with localization probabilities greater than 0.75. Most phospho-sites confirmed previous results obtained by immunoblotting and *in silico* predictions. Previous phosphoproteome studies in *P. falciparum* parasites from intra-erythrocytic stages used several methods of phosphoprotein enrichment (IMAC or TiO_2) combined with mass spectrometry-based approaches (LC–MS/MS) and identified numerous phosphoproteins (about 1900) and unique phosphorylated sites (about 6300), including tyrosine phosphorylation sites (Collins et al., 2014; Lasonder et al., 2012; Treeck et al., 2011).

Our work provides an initial phosphoproteome characterization of an under-studied process, gametogenesis, of the life cycle of malaria parasite. Anti-pTyr residues recognized protein spots and a p-Tyr residue was identified in actin. The detection of phosphorylated Tyr is intriguing because of the reports of the lack of gene encoding protein tyrosine kinases in *Plasmodium* species (Doerig, 2004). However, our data are consistent with the previous reports in these findings (Collins et al., 2014; Lasonder et al., 2012; Treeck et al., 2011), including that Pfnek3 (NIMA-like kinase), which displayed both serine/threonine and

tyrosine kinase activities in autophosphorylation reactions as well as in phosphorylation of myelin supplied as an exogenous substrate (Low et al., 2012).

Seventy-five proteins showing serine, threonine and/or tyrosine phosphorylation were detected by 2-DE IB in gametocytes and gametes of *P. berghei*, of which 23 were identified. Six gel spots were reanalyzed by TiO_2 phosphopeptides enrichment followed by MS/MS, in order to search for specific phosphorylated residues. The residues found showed some differences with those obtained by antibodies recognition; it may be due to the small amount of sample recovered from gel spots allowing for the possibility that it could be more phosphorylated residues in these proteins. Although, it is important to note that all of the phosphorylated proteins detected by IB have residues that were confirmed as phosphorylated by MS analysis, it was not possible in all cases to match the IB detected residue with those found by MS. Interestingly several of the proteins found as phosphorylated at gametogenesis process in this work also have been found phosphorylated in intra-erythrocytic life cycle stages of *P. falciparum* (Table S1) (Collins et al., 2014; Lasonder et al., 2012; Pease et al., 2013; Treeck et al., 2011).

A group of protein spots of ~100 kDa, corresponding to the large subunit of ribonucleoside reductase (RNR; gi:68070011) exhibited a marked change in the proportion of species present in gametes compared to gametocytes. RNR is a heterotetrameric enzyme composed of two large (R1) and two small (R2) subunits that synthesize the four dNTPs required for DNA replication and repair (Nordlund and Reichard, 2006). Reports from other organisms confirm that large and small subunits of the RNR can be phosphorylated on serine residues (Chang et al., 2008; Conner, 1999) as revealed in our work for PbRNR1. In addition, RNRs also showed a significant change in serine phosphorylation levels, supporting the conclusion that changes in phosphorylated isoforms could be related to regulation during gametogenesis. Also, semi-quantitative RT-PCR showed that RNR1 is expressed in both gametocytes and gametes (data not shown). It is consistent with the DNA synthesis happening in this developmental phase and should be confirmed that it occurs during male gamete production. The full-length DNA sequences in *P. falciparum* corresponding to the large (PfR1) and small (PfR2) and a second copy of the small subunit gene, PfR4, have been identified and the transcript and protein expression of the three subunits during erythrocytic development have been observed mainly in trophozoite stage (Bracchi-Ricard et al., 2005; Rubin et al., 1993). RNR1, RNR2 and RNR4 orthologs (PbR1: PBANKA_061160, PbR2: PBANKA_103660 and PbR4: PBANKA_121420) have been found in *P. berghei* but not been studied.

Hydroxyurea is a specific inhibitor of RNR, it interrupts DNA synthesis and stops parasite intra-erythrocytic maturation (Holland et al., 1998; Rubin et al., 1993). It has been demonstrated that RNR inhibition leads to a reduction of dNTP pools and the stalling of replication forks (Osborn et al., 2002). Here we confirmed that HU-treatment decreases exflagellation, supporting a role for RNR during male gametogenesis when the parasite undergoes high levels of DNA synthesis. Recently, RNR has been proposed as a target for drugs that inhibit its activity in the parasite (Munro and Silva, 2012). In spite of HU has been repeatedly used as a specific RNR inhibitor, the utilized doses are in a wide range depending on the experimental model and, in high doses, genotoxic effects have been reported

(Osterman Golkar et al., 2013), leading to the consideration that side-effects should be evaluated.

Two heat shock proteins, HSP70 and HSP90, are molecular chaperones whose functions are modulated by co-chaperones and post-translational modifications and are responsible for the production of signal transduction proteins and cell cycle regulators (Muller et al., 2013; Soroka et al., 2012; Truman et al., 2012). Both HSP70 and HSP90 were recognized by anti-pSer antibody gametocytes and three serine phosphorylation sites on HSP70 were located (S106, S585 and S588). Additionally a threonine site also was detected (T587). Recently, co-immunoprecipitation experiments and *in silico* studies performed in *P. falciparum* suggest that a complex formed by the heat shock organizing protein (Hop), HSP70 and HSP90, is present in trophozoite stage (Gitau et al., 2012; Hatherley et al., 2015). HSP70 identified in this work contains C-terminally located EEVD motif that interacts with Hop. HSP90 and HSP70 are potential antimalarial drug targets (Botha et al., 2011; Gitau et al., 2012).

The ProQ Diamond stain and anti-pThr and anti-pTyr antibodies detected in this study phosphorylation in α and β tubulin and actin-1 and actin-2 proteins, but we were not able to confirm Thr-phosphorylated sites by mass spectrometry in these proteins. However, it has been observed that in many organisms the dynamic microtubule changes are regulated by the phosphorylation of tubulin at serine and threonine residues (Fourest-Lieuvin et al., 2006; Wang et al., 2014). In *P. falciparum*, α-tubulin II is expressed in the sexual stages of the parasite, specifically in the male gametes (Rawlings et al., 1992), and it is located in the microgamete axoneme, a structure important for the motility of the parasite. In contrast, α-tubulin II is expressed in *P. berghei* in male gametocytes and also in the asexual stages (Kooij et al., 2005). Furthermore, a Tyr-phosphorylated site (Y363) was identified in both actin-1 and actin-2, as *in silico* prediction. We do not know the role that phosphorylation has during gametogenesis. In *P. berghei* it has been observed that both actin-1 and actin-2 isoforms are expressed in gametocytes; however, actin-2 is expressed specifically in gametocytes, particularly in male forms where it is essential (Deligianni et al., 2011).

A group of three proteins corresponding to enolase were also detected by anti-pThr antibody, however the identified phosphorylation site was serine (S429). Enolase is a multifunctional protein, with roles as metabolic enzyme, and other cellular processes (Diaz-Ramos et al., 2012). Enolase is present in the cytoplasm, nucleus and membrane, food vacuoles and cytoskeleton in different stages of *P. yoelii*, and its phosphorylation varies with its subcellular localization (Bhowmick et al., 2009; Shevade et al., 2013). Enolase on the ookinete surface can function as ligand for mosquito midgut epithelial receptors during invasion by *P. falciparum* (Ghosh and Jacobs-Lorena, 2011; Hernández-Romano et al., 2011), as plasminogen receptor generating plasmin that degrades the extracellular matrix surrounding the targeted host cell, thereby facilitating pathogen invasion (Ghosh et al., 2011). The differential phosphorylation and resulting isoforms of enolase found in this study could be regulating its function during gametogenesis, but more studies are required to demonstrate their role in this process.

Multicopy suppressor of IRA (msi1) and histone-binding protein n1/n2, involved in chromatin assembly, which are important for gene expression (Dumbliauskas et al., 2011;

Kleinschmidt et al., 1986), were also identified with changing phosphorylation level in gametes. MSI1 is a scaffold protein that contains a series of WD40 repeats and has a pleiotropic role in chromatin assembly and regulation of signaling pathways.

The G proteins play a central role in signal transduction and many other cellular processes (Hall, 2012; Oldham and Hamm, 2008). A putative *P. chabaudi* G protein ortholog identified corresponds to the *P. berghei* scaffold protein RACK1 (PbRACK1) and it was detected mainly by anti-pTyr antibody. Although its Tyr phosphorylation sites were not located by mass spectrometry, it has been reported that Tyr residues phosphorylated in RACK are required for interaction with protein partners to regulate signal transduction in mammalian cell lines (Chang et al., 2001; Kiely et al., 2008, 2009). Also, taking advantage of RACK1 is a very conserved protein, preliminary IB experiments with an anti human RACK1 confirmed the expression of PbRACK1 in gametocytes and gametes (data not shown). A RACK1 ortholog has been characterized in *P. falciparum* and its constitutive expression in asexual stages supports its role in the regulatory processes of malaria parasite life cycle (Madeira et al., 2003). In addition, it has been observed that PfRACK1 directly inhibits InsP3-mediated Ca^{2+} signaling in mammalian cells, interfering with host cell signaling pathways, which may be an important mechanism for parasite survival (Sartorello et al., 2009). Additional work is necessary to outline the RACK1 function during the gametogenesis process in *Plasmodium sp*.

Two T-complex protein β subunit isoforms were identified as having phosphorylated tyrosine residues. T-complex protein β subunit is encoded in humans by the *CCT2* gene. This gene encodes a molecular chaperone that is a member of the chaperonin containing TCP1 complex (CCT), also known as the TCP1 ring complex (TRiC) (Valpuesta et al., 2002). CCT2 protects newly-formed proteins of other molecules (Brackley and Grantham, 2009). The complex acts as chaperone to other proteins and helps to fold the microfilaments in the cell in order to construct the cytoskeleton (actin and tubulin) (Yebenes et al., 2011).

Translational repression is a regulatory mechanism occurring in mammalian reproductive cells (Flemr and Svoboda, 2011), and in malaria parasites it has been demonstrated as an important mechanism regulating the synthesis of proteins during host switching: from gametocyte to ookinete (Saeed et al., 2013); sporozoites until liver invasion (Hanson and Mair, 2014; Silvie et al., 2014); members of LCCL protein family transcribed in gametocytes but translated until ookinetes (Saeed et al., 2013); and Pfs25 and Pfs28 which are regulated by Puf family RNA-binding proteins (Miao et al., 2013). DOZI and CITH proteins are proteins required for the stability of repressed messengers for translational repression in *Plasmodium* sexual forms; using a RIP-CHIP analysis a *P. berghei* catalog of messengers associated to these repressors (repressome) was reported (Guerreiro et al., 2014). It was interesting to compare the phosphorylated proteins described in this work with the molecules present in the repressome because phosphorylation is a regulatory mechanism regulating the activity of synthesized proteins. Interestingly, only the RNR large subunit was found associated to both CITH and DOZI proteins, suggesting the presence of RNR repressed messengers. In our 2-DE analysis, RNR showed major changes both in quantity and in phosphorylation. Considering the importance of DNA synthesis during gametogenesis, major regulatory mechanisms could be applied to this important activity. On

the other hand, comparing the 23 proteins that we found changing their phosphorylation condition with those described for proteomes of male gamete (Talman et al., 2014), and both sexes gametocytes (Khan et al., 2005), it is not possible to propose with this information their participation in sex-specific regulatory mechanisms.

5. Conclusions

2-DE immunoblotting revealed differential phosphorylation between gametocytes and gametes of ~75 proteins during gametogenesis of *P. berghei*. Twenty-three of these proteins were identified by LC–MS/MS and, TiO_2 enrichment, and tandem mass spectrometry of six phosphoproteins allowed the identification with high confidence of some phosphorylation sites in gametocytes and gametes. Not all of the expected residues were detected, most likely as a result of the difficulty of obtaining enough specific proteins in the samples. The identified phosphoproteins are involved in DNA synthesis, protein folding, cytoskeleton, and signal transduction that could play a role during the gametogenesis process, in which the phosphorylation is a regulatory mechanism. This work adds important phosphorylation information on protein regulation in gametogenesis and helps to set the basis for understanding *Plasmodium* development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.exppara.2015.05.010.

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HIGHLIGHTS

- *Plasmodium berghei* gametes fertilized and developed *in vitro* to ookinetes in a serum-free medium.
- At least 75 proteins changed phosphorylation status during gametogenesis.
- Phosphorylations of serine, threonine and tyrosine were found.
- Proteins identified by mass spectrometry are involved in DNA synthesis, cytoskeleton and other processes.

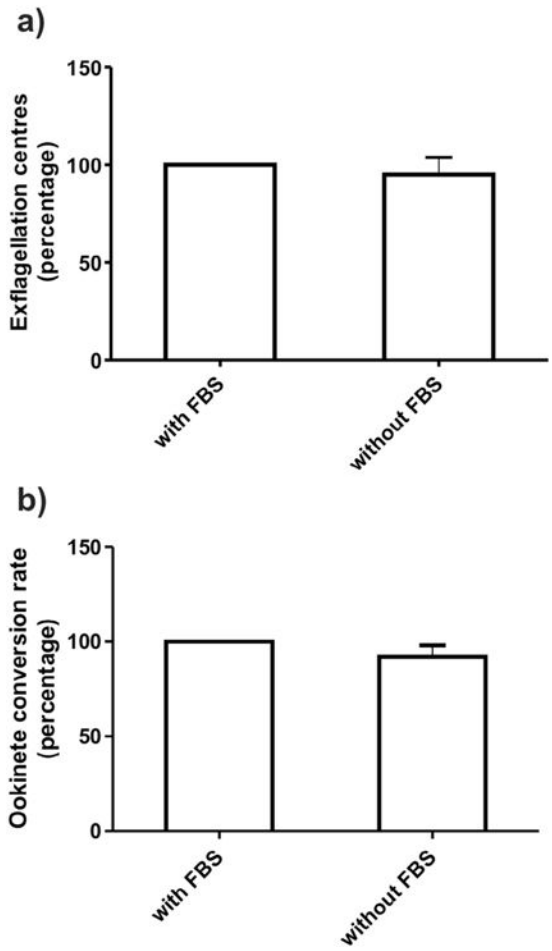

Fig. 1.

Gametogenesis and ookinete production in RPMI 1640 medium with and without FBS. *Plasmodium berghei* gametocyte-infected mouse blood was cultured in RPMI 1640 medium with and without FBS for 13–18 min to determine the numbers of exflagellation centers (a) and 24 hours to determine the number of ookinetes (b) in fields corresponding to 10,000 erythrocytes in Giemsa-stained smears. N = 5 and bars correspond to ±SD.

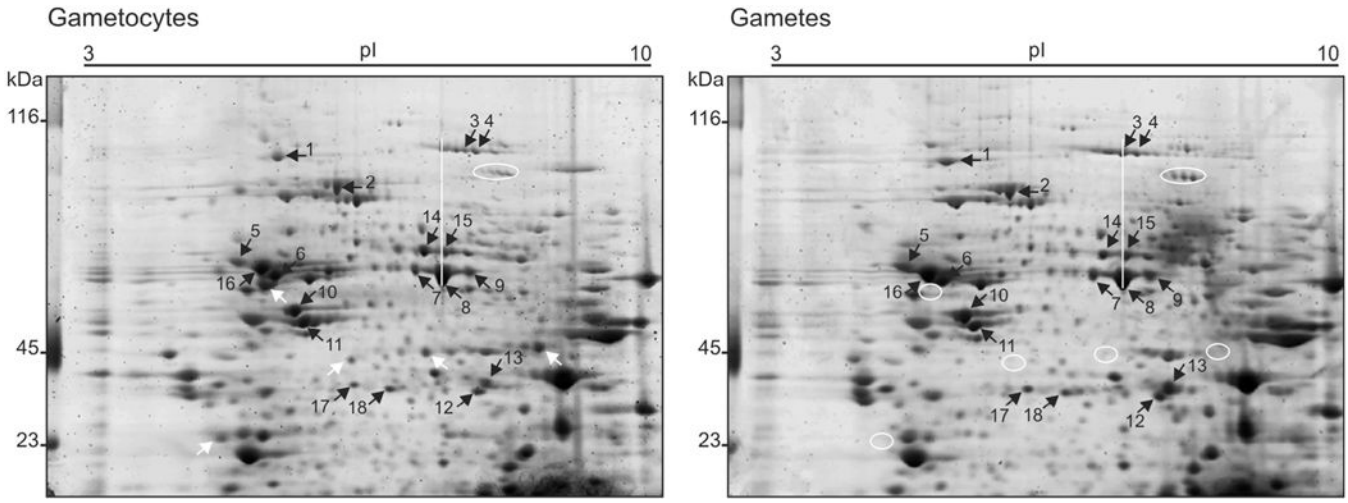

Fig. 2.

Two-dimensional gel electrophoretic analysis of *P. berghei* gametocytes and gamete proteins. Proteins of gametocytes and gametes differentiated *in vitro* in RPMI 1640 medium without FBS were resolved on Immobiline DryStrips (pH 3–10 NL, 7 cm) and second dimension was performed using 10% SDS–PAGE. The gels were stained with SYPRO Ruby. Molecular mass markers (kDa) are indicated to the left. Isoelectric points (pI) are indicated at the top. White arrows indicate polypeptides present in gametocytes but not in gametes, indicated by circles in the gamete gel. Black arrows indicate polypeptides common to both preparations that change in phosphorylation state and were identified by MS. The vertical white line is a reference to situate pI changes in the ~100 kDa protein group.

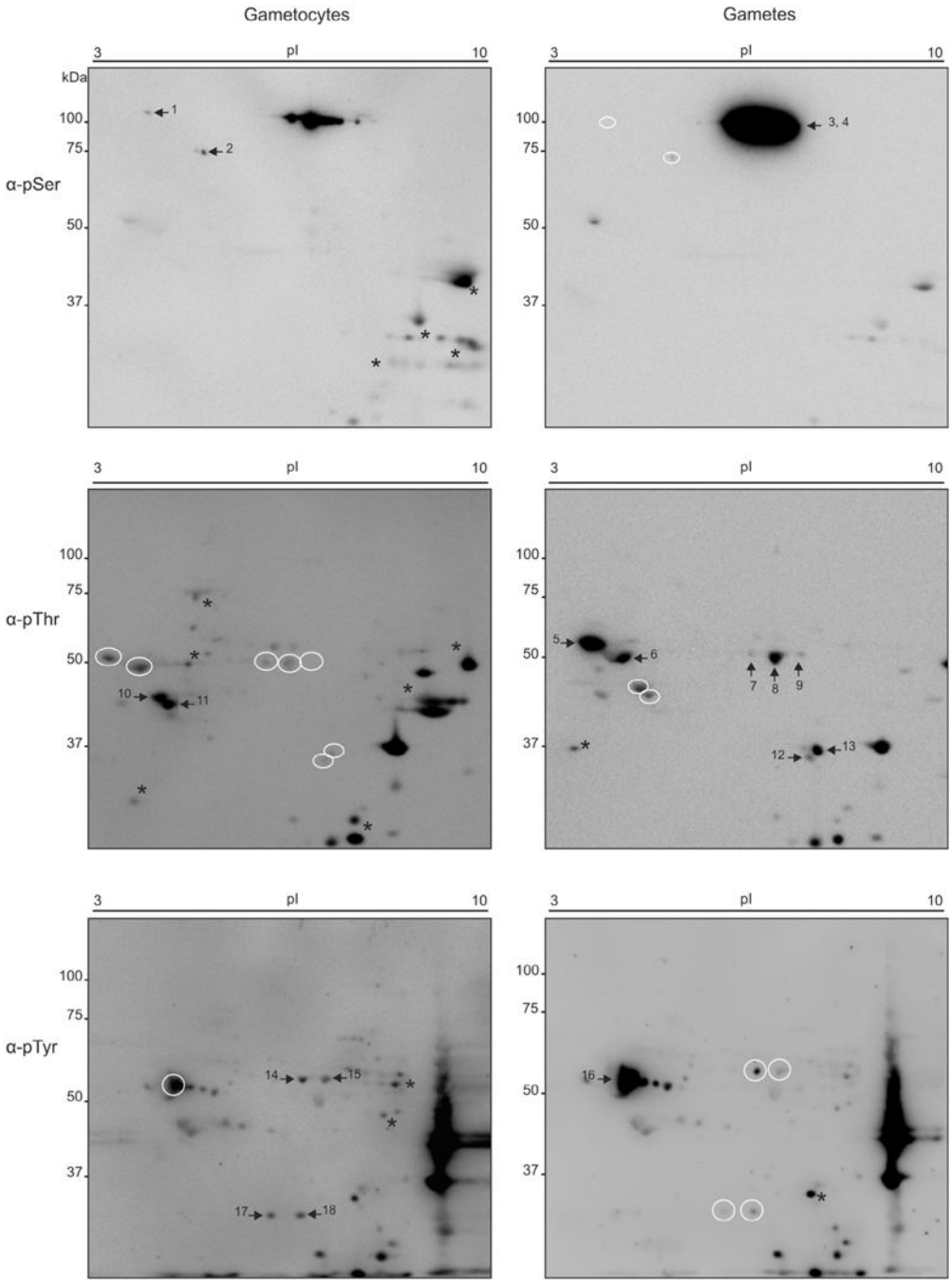

Fig. 3.

Proteins differentially phosphorylated in gametocytes and gametes. Replicated 2-DE gels were transferred onto nitrocellulose membranes and probed with antibodies that recognize phosphorylated serine, threonine or tyrosine residues. Top panels, pSer – proteins; middle panels, pThreo – proteins; lower panels, pTyr – proteins, as indicated. Black arrows indicate polypeptides with differential phosphorylation identified by MS/MS (Table 1). The white circles indicate the absence or decrease in phosphorylation and asterisks indicate the rest of the observed changes.

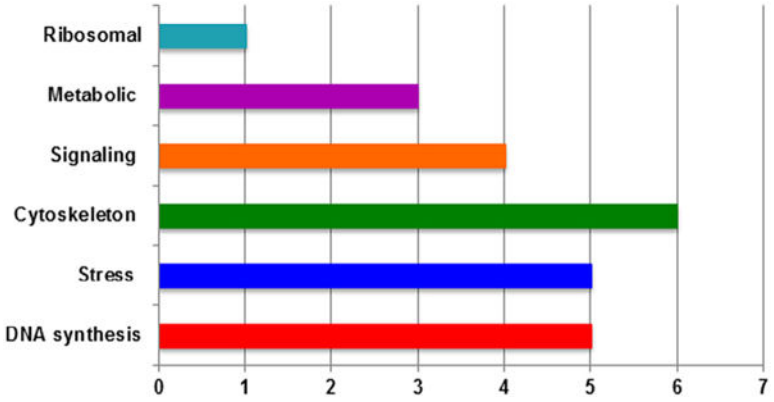

Fig. 4.

Functional annotation of the identified *P. berghei* phosphorylated proteins in biological process. All proteins were detected using anti-pSer, pThr or pTyr antibodies by 2-DE/IB. Categories were obtained from the Gene Ontology/annotations of biological process at *Plasmodium* DB.

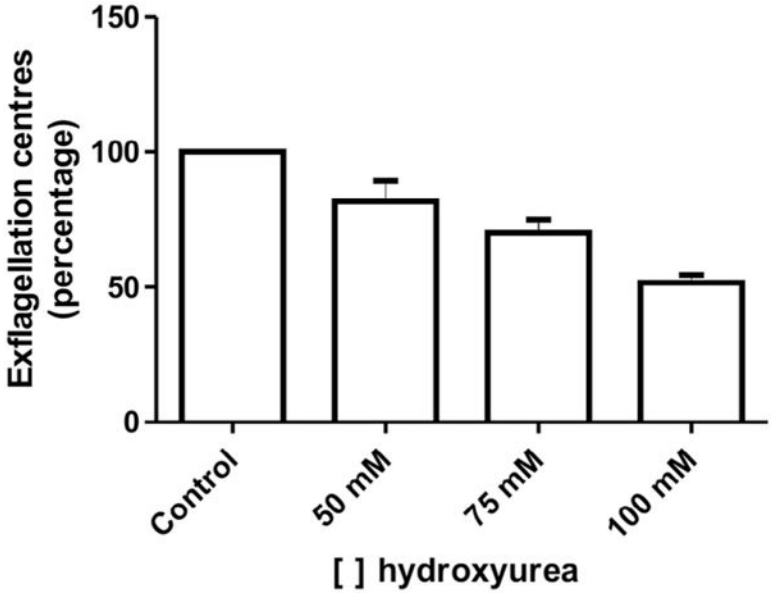

Fig. 5.

Effect of hydroxyurea on the gametogenesis of microgamete. Purified gametocytes were incubated with different concentrations of hydroxyurea for 1 hour in RPMI 1640 medium pH 7.2 at 37 °C. Subsequently the pH was changed by removing the RPMI 1640 medium. To induce gametogenesis, parasites were incubated in RPMI 1640 medium pH 8.3 at 19 °C; after 15 minutes exflagellation centers were counted. Experiments were performed in triplicate.

Table 1

Identified proteins with changed phosphorylation levels during *in vitro* gametogenesis of *P. berghei*.

Spot num.	Accession num. NCBI	Unused Prot score[a]	Protein [species]	Peptides matched/%coverage	Theoretical MW/pI	Observed MW/pI	Phosphorylated residue[c]/phase
1	gi|675229094	17.88	Endoplasmin putative [*P. berghei*]	8/31.4	93.24/5.31	110/4.8	S/Gametocytes
2	gi|74935931	24.67	Heat shock protein 70 [*P. berghei*]	12/25	75.14/5.51	70/5.5	S/Gametocytes
3	gi|68070011	1255[b]	Ribonucleoside diphosphate reductase, large subunit [*P. berghei*]	34/42	97.05/6.97	100/6.2	S/Gametes
4	gi|68070011	1188[b]	Ribonucleoside diphosphate reductase, large subunit [*P. berghei*]	29/39	97.05/6.97	100/6.4	S/Gametes
5	gi| 75011989	10.43	Wd-40 repeat protein msi1 [*P. yoelii*]	5/16.6	50.70/4.92	50/4.6	T/Gametes
6	gi| 67649703	419	Alpha tubulin [*P. berghei*]	12/30	50.48/4.90	50/4.8	T/Gametes
	gi|68070467	325	Disulfide isomerase precursor [*P. berghei*]	8/17	55.72/5.44		
7	gi|74987958	8.69	Enolase [*P. berghei*]	4/23	49.02/6.10	50/5.9	T/Gametes
8	gi|74987958	22.84	Enolase [*P. berghei*]	12/45.5	49.02/6.10	50/6.2	T/Gametes
	gi|74929959	2.98	Tubulin gamma chain [*P. falciparum*]	1/5.3	51.64/6.04		
9	gi|74987958	10.95	Enolase [*P. berghei*]	5/26.5	49.02/6.10	50/6.5	T/Gametes
10	gi|74882334	23.19	Actin-1 [*P. yoelii*]	14/44.7	41.88/5.21	43/5.2	T/Gametocytes
	gi|74989223	55.59	Actin-2 [*P. berghei*]	3/11	42.65/5.07		
11	gi|68064003	19.68	Actin [*P. berghei*]	8/39.4	41.90/5.05	42/5.3	T/Gametocytes
	gi|75012030	1.52	Histone-binding protein n1/n2 [*P. yoelii*]	1/3.3	38.67/4.82		
12	gi|74979254	15.05	Guanine nucleotide-binding protein, putative [*P. chabaudi*]	7/40.6	35.72/6.34	35/6.6	T/Gametes

Spot num.	Accession num. NCBI	Unused Prot score[a]	Protein [species]	Peptides matched/%coverage	Theoretical MW/pI	Observed MW/pI	Phosphorylated residue[c]/phase
13	gi|74985398	14.81	Uncharacterized protein [*P. berghei*]	7/45.4	38.79/6.30	37/6.7	T/Gametes
	gi|74843605	9.37	Ribosomal phosphoprotein P0 [*P. berghei*]	2/24.1	34.86/6.17		
14	gi|74878250	5.25	Putative T-complex protein beta subunit (fragment) [*P. yoelii*]	2/11.8	59.35/5.87	54/6.0	Y/Gametocytes
15	gi|74976730	21.68	T-complex protein beta subunit, putative [*P. chabaudi*]	12/38.9	58.93/5.83	54/6.2	Y/Gametocytes
16	gi|74879261	33.86	Tubulin beta chain [*P. yoelii*]	22/60.7	49.78/4.76	50/4.9	Y/Gametes
17	gi|68073955	651[b]	ADP-ribosylation factor GTPase-activating protein [*P. berghei*]	14/50	33.05/6.09	30/5.6	Y/Gametes
18	gi|68073955	695[b]	ADP-ribosylation factor GTPase-activating protein [*P. berghei*]	15/59	33.05/6.09	30/5.7	Y/Gametes

[a]The MS/MS spectra were analyzed using Paragon algorithm against the Uniprot/SwissProt plus contaminant protein database.

[b]Mascot score calculated using NCBInr database for *Plasmodium* species plus contaminant protein databases. Spot number refers to the spot number indicated in Fig. 2.

[c]S, T and Y refer to serine, threonine and tyrosine, respectively detected by immunoblotting.

Table 2

Phosphorylated sites of identified phosphoproteins during gametogenesis.

Protein num.	Protein	pRes 2D/IB[a]	Phosphopeptides	pRes site	Site position in protein	XCorr	Charge	MH+ [Da]
2	HSP70	S	**s**GIEEKPMIEVVYQGEK	S1	S106	2.82	3	2032.9561
			sV**ts**ILEWLEKNQLAGKDEYEAK	S1	S585	2.69	3	2731.3159
				S4	S588			
				T3	T587			
3	RNR1	S	LP**s**SSEGDQLK	S3	S10	3.90	2	1240.5470
			RLP**ss**SEGDQLKK	S4	S10	3.06	3	1525.7234
			TD**s**GKIFDDGIKR KTD**s**GKIFDDGIK	S5	S11	3.62	3	1531.7165
			TP**s**GKPIQTMYVLNR	S3	S22	3.06	2	1503.7091
			RTP**s**GKPIQTMYVLNR	S4	S22	3.66	3	1801.8552
			EV**s**RE**t**I**st**E**s**TVTQNACPLR	S3	S35	3.86	3	1957.9592
				S4	S35	2.44	3	2459.1161
				S3	S817			
				S8	S822			
				S11	S825			
				T6	T820			
				T9	T823			
4	RNR1	S	LP**s**SSEGDQLKK	S3	S10	3.15	2	1368.6400
			RLP**s**SSEGDQLKK	S4	S10	2.67	2	1524.7405
			TD**s**GKIFDDGIK	S3	S22	3.33	2	1375.6132
			KTD**s**GKIFDDGIK	S4	S22	5.25	3	1503.7083
			RTP**s**GKPIQTMYVLNR	S4	S35	1.81	3	1958.9339
			TP**s**GKPIQTMYVLNR	S3	S35	3.60	2	1800.8707
			EV**s**RE**t**I**st**ESTVTQNAcPLRR	S3	S817	1.99	3	2614.2261
				S8	S822			
				T6	T820			
				T9	T823			
5	Wd-40 RP msi1	T	RK**s**NALDEACLELSEEP**t**NEEIMK	S3	S12	2.10	3	2903.2699
				T18	T27			

Protein num.	Protein	pRes 2D/IB[a]	Phosphopeptides	pRes site	Site position in protein	XCorr	Charge	MH+ [Da]
8	Enolase	T	IE**Es**LGANGSFAGDK	S4	S429	2.18	2	1575.6497
10	Actin-1	T	EE**y**DESGPSIVHR	Y3	Y363	2.65	2	1597.6529
	Actin-2		EE**y**DESGPSIVHR	Y3	Y363	2.65	2	1597.6529

[a]Phosphorylated residue recognized by immunoblotting. S, T and Y refer to serine (Ser, S), threonine (Thr, T) and tyrosine (Tyr, Y). **s**, **t** and **y** refer to the phosphorylated forms.