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Protein phosphorylation during *Plasmodium berghei* gametogenesis

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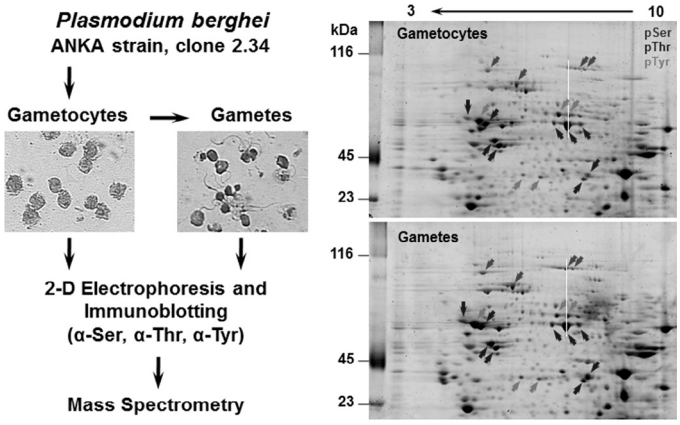
HIGHLIGHTS

- *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.

GRAPHICAL ABSTRACT



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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

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(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 TiO_2 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.

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