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Identification of new palmitoylated proteins in *Toxoplasma gondii*

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ABSTRACT

Protein palmitoylation has been shown to be an important post-translational modification in eukaryotic cells. This modification alters the localization and/or the function of the targeted protein. In recent years, protein palmitoylation has risen in importance in apicomplexan parasites as well. In *Toxoplasma gondii*, some proteins have been reported to be modified by palmitate. With the development of new techniques that allow the isolation of palmitoylated proteins, this significant post-translational modification has begun to be studied in more detail in *T. gondii*. Here we describe the palmitoylome of the tachyzoite stage of *T. gondii* using a combination of the acyl-biotin exchange chemistry method and mass spectrometry analysis. We identified 401 proteins found in multiple cellular compartments, with a wide range of functions that vary from metabolic processes, gliding and host-cell invasion to even regulation of transcription and translation. Besides, we found that more rhoptry proteins than the ones already described for *Toxoplasma* are palmitoylated, suggesting an important role for this modification in the invasion mechanism of the host-cell. This study documents that protein palmitoylation is a common modification in *T. gondii* that could have an impact on different cellular processes.

1. Introduction

Protein palmitoylation refers to the post-translational addition of palmitoyl-Coenzyme A to cysteine residues of certain proteins [1]. This reversible modification has been shown to play key roles in regulating sub-cellular localization (reviewed in [2]), trafficking [3], enzymatic activity [4,5], protein–protein interaction [6] and gene expression [7,8].

In *Toxoplasma gondii*, the causative agent of toxoplasmosis, N-terminal palmitoylation has been shown to play a key role in the localization of proteins to the parasite's inner membrane complex (IMC), pellicle and rhoptry membranes. It has been reported that predicted myristoylation and palmitoylation sites of the *i*nner membrane complex *s*ub-compartment *p*rotein (ISP)-1, -2, and -3 are important to target these proteins to the IMC since site-directed mutagenesis alters the localization from the IMC to the cytosol [9]. It is important to highlight that these proteins are involved in cell-daughter formation. ISP-4 only contains one predicted palmitoylation site which seems to be sufficient to target this protein to the IMC [10]. Similarly, the myosin light chain 2 (MLC2), a protein that is part of the motility system, has been reported to be anchored to the pellicle likely by N-terminal palmitoylation [11]. More recently, it has been demonstrated that the small heat shock protein 20 (TgHSP20) depends on N-terminal palmitoylation for proper IMC localization [12]. Furthermore, N-terminal myristoylation and palmitoylation are responsible for the attachment of the enzyme cGMP-dependent protein kinase (PKG) to the pellicle [13], and it has been demonstrated that an *a*rmadillo *r*epeat *o*nly protein (ARO) depends on myristoylation and palmitoylation for its localization on the cytosolic face of rhoptry membranes [14]. Additionally, a recent publication has described that the microneme protein AMA1 is palmitoylated and that its palmitoylation status regulates its own release and the release of other invasion-related proteins [15].

Supporting the importance of protein palmitoylation in the invasion process, it has been shown that inhibition of palmitoylation by 2-bromopalmitate alters invasion as well as the gliding mechanism in *T. gondii* [16]. Furthermore, inhibition of depalmitoylation enhances those same two processes [17]. This suggests that more proteins than the ones found to date must be targeted by palmitoylation.

Interestingly, it has been reported that *T. gondii* possesses all the machinery required to add palmitate on a subset of selected proteins since it expresses 18 palmitoyl-acyltransferases (TgPATs) with different localizations, 16 of which are found in the tachyzoite stage and some are unique to apicomplexan organelles important for the invasion of host-cells [18].

Although many important biological aspects of *T. gondii* are affected by palmitoylation, the identity of the proteins affected by this modification is starting to be uncovered [15]. As such, a *T. gondii*'s palmitoylome

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provides key information to start unraveling the relevance of protein palmitoylation in this parasite's biology.

In the present study, isolation of palmitoylated proteins using acyl-biotin exchange strategy (ABE) was coupled to mass spectrometry analysis. This approach led to the identification of 401 proteins displaying a wide array of functions and localizations. Some of them were previously described palmitoylated proteins and most of them were novel palmitoylated proteins. Interestingly, localization of some rhoptry proteins as well as the rhoptries themselves seem to be palmitoylation-dependent. Thus, our work provides further evidence of the importance of protein palmitoylation in the biology of *T. gondii*.

2. Materials and methods

2.1. Antibodies and reagents

Specialized and common reagents were from Sigma, unless specified. Dulbecco's Modified Eagle Medium, penicillin and streptomycin were from Life Technologies (CABA, Argentina). N-ethylmaleimide (NEM), streptavidin-agarose and HPDP-biotin were from Thermo Scientific (IL, USA). Complete protease inhibitor cocktail tablets were from Roche Diagnostics Corporation (IN, USA). ECL Plus was from GE Biosciences (UK). The serum anti-Ty was kindly provided by Dr. Dubremetz (Université de Montpellier, France) as well as anti-ROP4, -ROP5, -ROP7 monoclonal antibodies. Anti-AMA1 antibodies were generously provided by Dr. Ward (University of Vermont, USA).

2.2. Toxoplasma and host-cell cultures

T. gondii tachyzoites of the RH Δhxgprt strain [19] were used throughout the study. Parasites were maintained by serial passage on confluent monolayers of human foreskin fibroblasts (HFFs) in Dulbecco's Modified Eagle Medium supplemented with 10% *v*/*v* bovine serum albumin (BSA), 100 I.U. (international units)/ml penicillin and 100 μg/ml streptomycin. Tachyzoites were then physically separated from host cells by passage through a 27G syringe needle and purified from host cell debris using a 3.0 μm filter before use [20].

2.3. Acyl-biotin exchange method on total parasite lysates

ABE of whole parasite lysates was mainly carried out as described by Wan and colleagues [21] with the following modifications. Briefly, parasites were purified by 3.0 μm polycarbonate filter and a total of 1–5×10^9 parasites were used for the assay. Parasites were resuspended in 4 ml of lysis buffer containing 10 mM NEM and sonicated 15″ on/off for 10 periods. Then the concentration of NEM was adjusted to 2 mM for overnight treatment. The rest of the procedure was performed as described [21].

2.4. Separation and digestion of proteins

Protein samples were separated by 12% SDS-PAGE. The resultant gel was stained with Coomassie Brilliant Blue R-250. Each lane of the gel was completely cut into individual slices. Each band was then cut into 1 mm^3 cubes and further destained with three washes of 50 mM NH_4HCO_3 in 50% CH_3CN with 10 min incubations. Each group of gel cubes was then dehydrated in CH_3CN for 10 min and dried in a Speed Vac. Protein samples were reduced by dithiothreitol (DTT) and alkylated by iodoacetamide [22]. A solution of 10 ng/μl trypsin in 50 mM NH_4HCO_3 was used to re-swell the gel pieces completely at 4 °C for 30 min, followed by a 37 °C digestion overnight. A small amount of 10% formic acid was then added to stop the digestion. The sample was then centrifuged at 2800 ×*g*, and the supernatant was collected for LC–MS/MS.

2.5. LC–MS/MS analysis

Five microliters of tryptic peptide samples were loaded onto the LC microcapillary column (12 cm × 100 μm inner diameter) packed with C18 reversed-phase resin (5 μm particle size; 20 nm pore size; Magic C_{18}AQ, Michrom Bioresources Inc.), and separated by applying a gradient of 3–60% acetonitrile in 0.1% formic acid for 45 min at a flow rate of 500 nl/min after the flow is split to waste. The flow rate was controlled by a 1000 psi back pressure regulator (IDEX Health & Science LLC, Oak Harbor, WA) which connected flow to waste. The nanospray ESI was fitted onto a linear quadrupole ion trap mass spectrometer (Thermo Electron, San Jose, CA) that was operated in a collision-induced dissociation mode to obtain both MS and tandem MS (MS/MS) spectra. Mass spectrometry data were acquired in a data-dependent acquisition mode, in which a full MS scan from m/z 400–1700 was followed by 10 MS/MS scans of the most abundant ions.

2.6. Protein identification

Obtained MS spectra were searched against the *T. gondii* ToxoDB (v 26; www.toxodb.org) protein database using Proteome Discovery 1.4 (Thermo Electron, San Jose, CA). The workflow includes Spectrum Files, Spectrum Selector, Sequest search nodes followed by Target Decoy PSM Validator. The search parameters permitted a 2 Da peptide MS tolerance and a 1.0 Da MS/MS tolerance. Oxidation of methionine (M) and carboxymethylation of cysteines (C) were allowed as variable modifications. Up to two missed tryptic peptide cleavages were considered. The proteins, which False Discovery Rate (FDR) is less than 1% at the peptide level. All identified proteins, even the ones with one peptide matched, had been listed in the supplements.

2.7. Data analysis and bioinformatics

Palmitoylated proteins were classified according to all 3 gene ontology (GO) categories (molecular function, biological process and cellular component) taking into account all GOs provided by ToxoDB [23]. Conserved domains in the identified proteins were predicted by Prosite [24] and BLASTp. Predicted presence of signal peptide or transmembrane domains were determined by SignalP 4.1 Server [25] and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) respectively. For subcellular localization predictions, PSORT II [26] was used, except for those cases where localization to a *Toxoplasma* specific organelle or compartment was already determined and annotated in ToxoDB. Presence of potentially lipidated residues was predicted by: CSS-Palm 3.0 [27] for palmitoylated cysteines, Myristoylator [28] for N-terminal myristoylated glycines and PrePS [29] for prenylated motifs. GO and metabolic pathway enrichment analyses, as well as comparison between *T. gondii* and *Plasmodium falciparum* proteins were performed using the informatics tools provided by ToxoDB and EuPathDB respectively.

2.8. Treatment with 2-bromopalmitate (2-BP)

For western blot, immunofluorescence and electron-microscopy studies, treatment was performed using 12.5 μM 2-BP which was shown to inhibit palmitoylation while not causing gross cell morphology alterations [16]. For immunofluorescence studies monolayers of HFF cells grown on glass slides were infected with *T. gondii* tachyzoites in 24-well dishes. After 10 min on ice, parasites were allowed to invade for 1 h at 37 °C. Then, the cells were washed twice with PBS and the media was changed by DMEM containing 1% *v*/*v* BSA fatty acid-free and 12.5 μM 2-BP. The infected cells were incubated for 16–18 h at 37 °C, then were subjected to indirect immunofluorescence studies. For transmission electron-microscopy (TEM), the treatment was similar except it was performed in T25 culture flasks, and after incubation with 2-BP, cells were fixed and scraped as described in the EM section.