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Global profiling of lysine acetylation in human histoplasmosis pathogen *Histoplasma capsulatum*

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ABSTRACT

Histoplasma capsulatum is the causative agent of human histoplasmosis, which can cause respiratory and systemic mycosis in immune-compromised individuals. Lysine acetylation, a protein posttranslational protein modification, is widespread in both eukaryotes and prokaryotes. Although increasing evidence suggests that lysine acetylation may play critical roles in fungus physiology, very little is known about its extent and function in *H. capsulatum*. To comprehensively profile protein lysine acetylation in *H. capsulatum*, we performed a global acetylome analysis through peptide prefractionation, antibody enrichment, and LC–MS/MS analysis, identifying 775 acetylation sites on 456 acetylated proteins; and functionally analysis showing their involvement in different biological processes. We defined six types of acetylation site motifs, and the results imply that lysine residue of polypeptide with tyrosine at the −1 and +1 positions, histidine at the +1 position, and phenylalanine (F) at the +1 and +2 position is a preferred substrate of lysine acetyltransferase. Moreover, some virulence factors candidates including calmodulin and DnaK are acetylated. In conclusion, our data set may serve as an important resource for the elucidation of associations between functional protein lysine acetylation and virulence in *H. capsulatum*.

1. Introduction

Histoplasma capsulatum, the causative agent of human histoplasmosis, is a major cause of respiratory and systemic mycosis, especially in immune-compromised individuals (Graybill, 1988). Histoplasmosis typically manifests as a benign respiratory infection and even entirely asymptomatic. In some individuals, especially immunocompromised persons, *H. capsulatum* readily disseminates, causing significant morbidity and mortality (Kauffman, 2008). Histoplasmosis is a common endemic mycosis throughout most of the world, which is endemic in the mid-western United States (such as in the Mississippi and Ohio River valleys) and Central America (Kauffman, 2007). Sporadic cases of autochthonous histoplasmosis have been found in China, which was traditionally considered non-endemic for *H. capsulatum*. It is noteworthy that most cases of histoplasmosis occurred in regions through with the Yangtze River flows (Cao et al., 2010, Ge et al., 2010, Pan et al., 2013). *H. capsulatum* thrives in soil in mycelial (mold) form, as with most other dimorphic fungal pathogens, conversion to a unicellular haploid yeast form occurs following inhalation and exposure to the warmer temperature of the respiratory tract (Eissenberg and Goldman, 1991).

Recent genetic approaches have begun to reveal the regulatory machinery that governs the conversion of Histoplasma to the yeast form and expression of the gene set involved in pathogenesis with the assumption that virulence factors are components unique to the yeast-phase (Batanghari and Goldman, 1997, Colonna-Romano et al., 1998, Hwang et al., 2003). Some virulence factors of *H. capsulatum* yeasts were identified to subvert or avoid activating macrophage antifungal defenses (Holbrook and Rappleye, 2008). Histoplasma HSP60 localized to the cell surface of yeast cells and functions as adhesin for macrophage binding and phagocytosis (Habich et al., 2006). The β-glucan polysaccharides of all fungal cell walls were related to macrophages recognition, reactive oxygen compounds production and proinflammatory cytokines secretion

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(Robinson et al., 2006, Willment and Brown, 2008). Histoplasma yeasts effectively conceal their immunostimulatory β-glucan signatures underneath a cell wall layer composed of α-glucan which was involved in the virulence of Histoplasma, as genetic loss of the α-(1,3)-glucan covering through mutation or RNAi of α-glucan synthase (AGS1) severely attenuates Histoplasma virulence (Rappleye et al., 2004). Secreted factor YPS3 and calcium-binding protein CBP play an important role in the virulence of Histoplasma yeast (Bohse and Woods, 2007), as YPS3 can decrease the pathogen burden in the internal organs of infected mice and CBP can impair the intracellular growth of Histoplasma yeast and attenuate the ability to colonize the lung (Sebghati et al., 2000). However, the pathogenesis of *H. capsulatum* remains largely unknown.

The reversible lysine acetylation in proteins is now recognized as a common posttranslational modification (PTM) in both prokaryotes and eukaryotes (Thao and Escalante-Semerena, 2011, Xie et al., 2012). Since the lysine acetylome revealed in mammalian cells (Kim et al., 2006), global acetylation in eukaryotes has been reported and several biochemical studies showed that lysine acetylation may influence various cellular processes including metabolic pathway and transcriptional regulation (Rardin et al., 2013, Still et al., 2013, Weinert et al., 2011). Recent advances in antibody-based affinity enrichment and high sensitive MS-based proteomics have made contributions to the global analysis of lysine acetylation in bacteria including *Mycobacteria tuberculosis* (Liu et al., 2014, Xie et al., 2015), *Escherichia coli* (Yu et al., 2008), *Salmonella enteric* (Wang et al., 2010b), *Bacillus subtilis* (Kim et al., 2013), *Geobacillus kaustophilus* (Lee et al., 2013), *Erwinia amylovora* (Wu et al., 2013), and *Thermus thermophilus* (Okanishi et al., 2013), *Saccharopolyspora erythraea* (Huang et al., 2015), *Streptomyces roseosporus* (Liao et al., 2014), *Pseudomonas aeruginosa* (Ouidir et al., 2015). Despite the popular studies of lysine acetylation in bacteria, the progress of lysine acetylome in fungus is relative limited and only one fungus species *Saccharomyces cerevisiae* have been examined.

In this study, we investigated the first acetylproteome of the *H. capsulatum* using a high-resolution mass spectrometry-based proteomics approach. Combining the affinity immuno-separation of acetylated peptides with nano-HPLC–MS/MS analysis, we identified a total of 775 unique lysine acetylation sites on 456 proteins. Bioinformatics analysis showed that lysine-acetylated proteins are mainly involved in metabolic processes. Moreover, several sequence motifs including $K^{ac}Y$, $K^{ac}F$, $K^{ac}H$, $K^{ac}*F$, YK^{ac} and $K^{ac}***R$ were identified. Furthermore, a total of 116 acetylation sites on 86 proteins were also found to be succinylated, suggesting extensive overlap between acetylation and succinylation in this fungus. This first global acetyaltion profiling of *H. capsulatum* provides a basis for future interrogation of the roles of these acetylated proteins.

2. Materials and methods

2.1. Strain culture and protein extraction

The total steps including protein extraction, peptides digestion, acetylated peptides enrichment, MS identification, data processing and bioinformatics analysis are described as our previous article (Xie et al., 2015). The *Histoplasma capsulatum* NAm1 cultured cells were harvested and washed twice with cold phosphate-buffered saline (PBS), then lysed in 8 M urea supplemented with 1 mM DTT, 2 mM EDTA, protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem), and HDAC inhibitor (30 mM nicotinamide, 50 mM sodium butyrate, 3 μM Trichostatin A). This was then sonicated with 12 short bursts of 10 s intervals followed by 30 s intervals for cooling. Unbroken cells and debris were removed by centrifugation at 4 °C for 10 min at 20,000 g. Protein content in supernatant was defined with 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions and precipitated with 20% trichloroacetic acid overnight at 4 °C. The resulting precipitate was then washed three times with ice-cold acetone. The air-dried precipitate was resuspended in 100 mM NH_4CO_3 and then digested with trypsin (Promega) at an enzyme-to-substrate ratio (1:50) at 37 °C for 12 h. The tryptic peptides were reduced with 5 mM dithiothreitol for 45 min at 56 °C and then alkylated with 15 mM iodoacetamide at room temperature for 30 min in complete darkness. The reaction was finally terminated with 15 mM cysteine for 30 min at room temperature. To ensure complete digestion, additional trypsin at an enzyme-to-substrate ratio (1:100) was added, and the mixture was incubated for an additional 4 h. The digested peptides were freeze-drying in a SpeedVac (Thermo Scientific).

2.2. Enrichment of lysine acetylated peptides and liquid chromatography electrosprayionization tandem mass spectrometry (LC-ESI–MS/MS) analysis

The trypic digest was redissolved in NETN buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, pH 8.0) and incubated with anti-acetyllysine agarose beads (PTM Biolabs) at 4 °C overnight with a gentle oscillation. After incubation, the beads were carefully washed three times with NETN buffer, twice with ETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0) and once with water. The bound peptides were eluted from the beads by 1% trifluoroacetic acid and dried in the SpeedVac. Prior to HPLC/MS/MS analysis, the obtained peptides were rinsed with C18 ZipTips (Millipore) according to the manufacturer's instructions.

Peptides were resuspended in buffer A (0.1% FA, 2% ACN) and centrifuged at 20,000 g for 2 min. The supernatant was transferred into a sample tube and loaded onto an Acclaim PepMap 100 C18 trap column (Dionex, 75 μm × 2 cm) by EASY nLC1000 nanoUPLC (Thermo Scientific) and peptides were eluted onto an Acclaim PepMap RSLC C18 analytical column (Dionex, 50 μm × 15 cm). A 34 min gradient was run at 300 nl/min, starting from 5% to 30% B (80% ACN, 0.1% FA), followed by 2 min linear gradient to 40% B, 2 min to 80% B, and maintenance at 80% B for 4 min.

Peptides were subjected to nanospray ionization (NSI) source followed by tandem mass spectrometry (MS/MS) in Q Exactive (Thermo Scientific) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using 25% Normalized Collision Energy (NCE) with 4% stepped NCE. Ion fragments were detected in the Orbitrap at a resolution of 17500. A data-dependent procedure that alternated between one MS scan followed by 15 MS/MS scans was applied for the top 15 precursor ions above a threshold ion count of 4E4 in the MS survey scan with 2.5 s dynamic exclusion. The electrospray voltage applied was 1.8 kV. Automatic gain control was used to prevent overfilling of the ion trap; 2E5 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800 Da.

2.3. Data Processing

The identification of protein and acetylation site was performed by MaxQuant with integrated Andromeda search engine (v. 1.3.0.5). Tandem mass spectra were searched against Uniprot Histoplasma capsulatum protein database concatenated with reverse decoy database and protein sequences of common contaminants. Trypsin/P was specified as cleavage enzyme allowing up to 3 missing cleavages, 4 modifications per peptide and 5 charges. Mass error was set to 6 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethylation on Cys was specified as fixed modification and oxidation on Met, acetylation on Lys and acetylation on protein N-terminal were specified as variable modifications. False