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Original article

Phosphosite-specific regulation of the oxidative-stress response of *Paracoccidioides brasiliensis*: a shotgun phosphoproteomic analysis

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Abstract

Paracoccidioides brasiliensis, a thermally dimorphic fungus, is the causative agent of paracoccidioidomycosis, a systemic mycosis that is widespread in Latin America. This fungus is a facultative intracellular pathogen able to survive and replicate inside non-activated macrophages. Therefore, the survival of *P. brasiliensis* inside the host depends on the ability to adapt to oxidative stress induced by immune cells, especially alveolar macrophages. For several years, reactive oxygen species (ROS) were only associated with pathological processes. Currently, a plethora of roles for ROS in cell signaling have emerged. We have previously reported that low ROS concentrations cause cell proliferation in the human pathogenic fungus *P. brasiliensis*. In the present report, we investigated the influence of phosphorylation events in that process. Using a mass spectrometry-based approach, we mapped 440 phosphorylation sites in 230 *P. brasiliensis* proteins and showed that phosphorylation at different sites determines fungal responses to oxidative stress, which are regulated by phosphatases and kinases activities. Furthermore, we present additional evidence for a functional two-component signal transduction system in *P. brasiliensis.* These findings will help us to understand the phosphorylation events involved in the oxidative stress response.

Keywords: Oxidative stress; Post-translational modification; Phosphorylation; *Paracoccidioides brasiliensis*

1. Introduction

Paracoccidioides brasiliensis is a thermally dimorphic fungus and the major causative agent of paracoccidioidomycosis (PCM), an important human disease in Latin America. PCM has a multitude of clinical presentations, from cutaneous to systemic forms, and can spread to various tissues, especially in the lung [1]. The first line of defense confronted by *P. brasiliensis* during host invasion is the lung-resident macrophages. The capability of this fungus to cause disease is dependent on several host immune response factors, such as reactive oxygen species (ROS) production by macrophages [2]. For several years, ROS were thought to be only a product of the stress response, but many other functions in cell signaling have been discovered for these molecules. By inducing post-translational modifications, ROS can regulate the activity of enzymes and determine cell fate [3].

Interest in how *P. brasiliensis* responds to oxidative stress has emerged recently [4]. Although many proteins have been suggested to be involved in oxidative stress [4], little is known about the phosphorylation events that control these and related processes. Protein phosphorylation, one of the most important and well-characterized post-translational modifications, plays

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a key role in eukaryotic signal transduction and has been implicated in several processes, such as the cell cycle, adhesion, survival, proliferation and differentiation [5]. Phosphorylation at specific serine, threonine, and tyrosine residues in proteins, such as kinases and phosphatases, are becoming major drug targets for a wide variety of diseases [6].

Co-culturing *Candida albicans* with macrophages increases the level of protein phosphorylation in macrophages, and these phosphorylated proteins are related to inflammation and the oxidative stress response [7]. In *Saccharomyces cerevisiae*, HOG-MAPK pathway-dependent phosphorylation of 6-phosphofructo-2-kinase in response to increased environmental osmolarity has been reported as a critical event for adaptation and survival [8], suggesting an active role for phosphorylation events in this context. In contrast, the *P. brasiliensis* isocitrate lyase is inhibited by phosphorylation induced by the presence of glucose [9]. Therefore, phosphorylation dynamically regulates protein activity under different fungal contexts.

Mass spectrometry-based approaches have been employed successfully to identify phosphorylation sites in microorganisms [10–13]. Here, we performed a shotgun phosphoproteomic analysis of *P. brasiliensis* under different oxidative conditions and mapped relevant phosphorylation sites that determine cell responses.

2. Materials and methods

2.1. Strain, media and solutions

The *P. brasiliensis* strain *Pb18* was used in this study. Yeast extract peptone dextrose modified medium (mYPD) (0.5% yeast extract, 1% casein peptone, and 0.5% glucose, pH 6.7) or RPMI medium 1640 (Gibco, Carlsbad, CA, USA) was used to cultivate yeast cells, which were cultured at 37 °C. Colony forming unit (CFU) counts were performed on supplemented BHI plates (Becton Dickinson Company) containing 5% fetal bovine serum, ampicillin (100 IU/mL) and streptomycin (100 mg/mL). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise mentioned.

2.2. H_2O_2 treatment and total protein extract preparation

P. brasiliensis yeast cells were grown in mYPD broth for 5 days at 37 °C, shaking at 150 rpm, then incubated in RPMI medium for 24 h. Cells were washed with PBS and resuspended in medium containing 0.1, 1 or 10 mM H_2O_2 for 5 h at 37 °C, shaking at 150 rpm. For growth curve, yeast cells were stimulated, separated, cultured on BHI and incubated at 37 °C for 10 days. Medium without stimulus was used as a control, and fungal viability was evaluated by CFU counts.

Total protein extracts were prepared with the protocol previously described for the preparation of yeast lysate [14], with modifications. Briefly, 700 μL of ice-cold lysis buffer (8 M urea, 75 mM NaCl, 50 mM Tris, pH 8.2, 1 mM PMSF, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 10 mM sodium pyrophosphate) and glass beads (425–600 μm, Sigma–Aldrich, St. Louis, MO, USA) were added to samples. A Mini Beadbeater (BioSpec Products Inc., Bartlesville, OK, USA) was used at maximum speed for four 90 s cycles to disrupt yeast cells. Microcentrifuge tubes were adapted onto 15-mL tubes and spun at 1000 × *g* for 3 min at 4 °C. Lysates were homogenized and centrifuged at 15,000 × *g* for 10 min at 4 °C; then, the supernatant was recovered. Protein concentrations were estimated using the Bradford reagent and bovine serum albumin as a standard in accordance with manufactory's instruction (BioRad, California, USA). Protein electrophoresis was carried out using 12% SDS-polyacrylamide gels and proteins were stained with Coomassie brilliant blue G-250.

2.3. Immunoblotting analysis

Total cell lysates (50 μg/mL) were submitted to electrophoresis on 10% or 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Thermo Scientific, Waltham, MA, USA). Blots were probed using specific monoclonal antibodies against pSer/pThr and pTyr (Cell Signaling Technology, Danvers, MA, USA) at a 1:1000 dilution. After incubation with the appropriate HRP-conjugated secondary antibodies (at 1:2000 dilution), blots were developed using the Super Signal system (Thermo-Pierce – Rockford, IL, USA). Image acquisition and densitometry were carried out using a chemiluminescence documentation system (UVITEC, Cambridge, UK).

2.4. Phosphatase activity

Phosphatase activity was determined as previously described [15], with modifications. Briefly, 100 μg of total protein extract was incubated with 5 mM *p*-nitrophenylphosphate (*p*NPP) in 15 mM HEPES, pH 5.0, for 1 h at room temperature in the dark. The reaction was stopped by adding 1 mM NaOH, and absorbance was measured at 405 nm using an ELISA micro-plate reader (Biotek Instruments, Winooski, VT, USA). The phosphatase activity was measured in quintuplicate and repeated three times with similar results. Data were normalized by negative control without *p*NPP.

2.5. Protein digestion and peptide desalting

Protein reduction, alkylation and trypsin digestion were performed as previously described [14]. Briefly, 2 mg of total protein extract were reduced with 5 mM dithiothreitol, alkylated by 15 mM iodoacetamide and digested with proteomics grade trypsin (1/250 enzyme/substrate) (Sigma–Aldrich, St. Louis, MO, USA) overnight at 37 °C. Trypsin digestion was stopped by acidifying samples with trifluoroacetic acid. For desalting, cleanup and concentration of peptide mixtures, C18 stop-and-go-extraction tips (StageTips) manufactured in our laboratory were assembled and used as previously described [16].