Experimental Parasitology 132 (2012) 465–469

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Differential dissolved protein expression throughout the life cycle of *Giardia lamblia*

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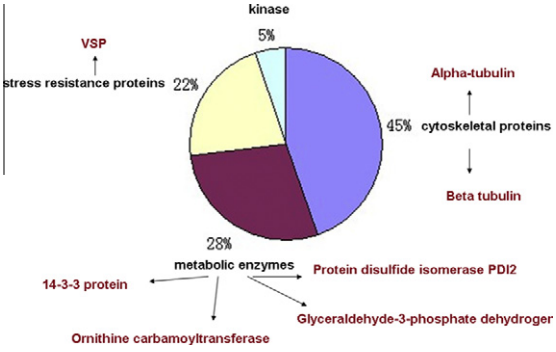
HIGHLIGHTS

- Sixty-three soluble proteins were detected from both trophozoites and cysts.
- Significant differences were detected from seven members among these 63 proteins.
- Differences of mRNA expression patterns also exist in four of these seven proteins.
- It is necessary to compare proteomes of trophozoites encystment at different stage.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 4 August 2012
Accepted 20 September 2012
Available online 8 October 2012

Keywords:
Giardia lamblia
Proteome
Cyst
Trophozoite

ABSTRACT

Giardia lamblia (*G. lamblia*) has a simple life cycle that alternates between a cyst and a trophozoite, and this parasite is an important human and animal pathogen. To increase our understanding of the molecular basis of the *G. lamblia* encystment, we have analyzed the soluble proteins expressed by trophozoites and cysts extracted from feces by quantitative proteomic analysis. A total of 63 proteins were identified by isobaric tags for relative and absolute quantitation (iTRAQ) labeling, and were categorized as cytoskeletal proteins, a cell-cycle-specific kinase, metabolic enzymes and stress resistance proteins. Importantly, we demonstrated that the expression of seven proteins differed significantly between trophozoites and cysts. In cysts, the expression of three proteins (one variable surface protein (VSP), ornithine carbamoyltransferase (OTC), β-tubulin) increased, whereas the expression of four proteins (14-3-3 protein, α-tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), protein disulfide isomerase 2 (PDI-2)) decreased significantly when compared with the levels of these proteins in trophozoites. The mRNA expression patterns of four of these proteins (OTC, α-tubulin, GAPDH, VSP) were similar to the expression levels of the proteins. These seven proteins appear to play an important role in the completion of the life cycle of *G. lamblia*.

1. Introduction

The protozoan parasite *Giardia lamblia*, which causes acute or chronic diarrhea in both humans and animals, is considered an important worldwide pathogen (Solaymani-Mohammadi and Singer, 2010). The parasite has two major stages in its life cycle. The trophozoite is the vegetative form and replicates in the small intestine, whereas the cyst is relatively inert, thereby allowing prolonged survival in a variety of environmental conditions (Adam, 2001). *G. lamblia* differentiation from the trophozoite to the cyst is the key step for the completion of the life cycle. So the study of these processes represents a target for the development of new molecular strategies and the design of transmission blocking agents to combat this parasite. Over the last two decades, *G. lamblia*

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http://dx.doi.org/10.1016/j.exppara.2012.09.014

encystation has been studied extensively from morphological, cell biological, biochemical, molecular and proteomic viewpoints, as the encystation can be induced in vitro by modulating physiological stimuli (Lauwaet et al., 2007). However, the precise mechanisms involved in *G. lamblia* encystation remain unresolved.

Proteomic profiling is a useful approach for obtaining a global overview of the proteins present in a system under different conditions and can aid in understanding the molecular determinants involved with pathogenesis and vaccine development (List et al., 2008). This approach has been used in parasite research and has provided valuable information describing the life cycle of parasites. The expression of proteins was examined using proteomic approaches during sporozoite excystation of *Cryptosporidium parvum* and eight proteins were identified that represent promising targets for the development of vaccines or chemotherapies that could block parasite entry into host cells (Snelling et al., 2007). The iTRAQ labeling method was used for a comparative proteomic analysis of salivary gland samples from infected or *Plasmodium* berghei-free mosquitoes and five secreted proteins were found to have altered levels when the parasite was present. These proteins represent potentially important proteins for characterizing the interaction between *Anopheles gambiae*, *Plasmodium* and the mammalian host (Choumet et al., 2007). Comparative proteomic analysis of trophozoites versus cysts generated in culture identified 20 proteins that were up-regulated, and this result provides information on encystation in *G. lamblia* (Kim et al., 2009). However, a comparison of the transcriptome of cysts generated in culture or extracted from feces revealed negligible overlap, raising the possibility of significant biological differences between the two types of cysts (Faghiri and Widmer, 2011). Additionally, no analyses of the proteomics of cysts obtained from infected animals have been performed. Here, we quantitatively analyzed the presence of isobarically-tagged proteins in trophozoites and cysts isolated from feces, which provides more information on encystation in *G. lamblia* and the relationship between cysts taken from culture or extracted from feces.

2. Materials and methods

2.1. Parasite cultures and cyst isolation

G. lamblia (Changchun strain, Assemblage D) trophozoites were maintained in a modified TYI-S-33 medium (Liu et al., 2008). Cysts of *G. lamblia* were collected from dog fecal matter that was experimentally inoculated with trophozoites. To recover high numbers of cysts, with minimal fecal and bacterial contaminants, cysts were recovered using feces purification with zinc sulfate and sucrose density gradients (Foreyt, 1989). Cyst viability was assessed by monitoring the exclusion of propidium iodide, as described previously (Sauch et al., 1991). Trophozoites and purified cysts were counted with a hemocytometer, pelleted by centrifugation and washed in PBS prior to protein extraction.

2.2. Protein extraction

Total protein for iTRAQ was isolated from trophozoites and cysts by five cycles of freeze/thawing and subsequent use of 1% sodium dodecyl sulfate. Samples were then sonicated with eight 20 s pulses (100 watts) on ice with 1 min intervals and were ultracentrifuged at 14 °C (20,000*g* for 20 min) to remove insoluble impurities. DTT (10 mM) was added to 100 μl of the supernatant and incubated for 1 h at 56 °C. Iodoacetamide (55 mM) was then immediately added to the sample and the solution incubated for another 45 min in the dark at room temperature. Proteins in the soluble lysate fractions were precipitated by mixing the lysates with four volumes of ice-cold acetone at −20 °C for 3 h and then centrifuged at 20,000*g* for 20 min. Pellets of the precipitated soluble proteins were re-suspended in 0.2% SDS, and the concentration was determined using a 2D-Quant kit (Jedelský et al., 2011).

2.3. iTRAQ labeling

Samples of trophozoites and cysts (100 μg of total protein each) were denatured, and cysteines were blocked as described in the iTRAQ protocol. Five micrograms of sequence grade modified trypsin (Promega, Madison, WI) was added into the sample and incubated at 37 °C for 36 h. The peptides were dried and resuspended in 30 μl of dissolution buffer provided in the iTRAQ kit. Each iTRAQ 8-plex reagent (Applied Biosystems, Foster City, CA) was dissolved in 70 μL isopropanol, mixed for 1 min and added to the corresponding sample as follows: reagents 113 and 121 were added to trophozoites and cysts, respectively. Labeled peptides from different samples were mixed and subjected to strong cation exchange (SCX) fractionation (Snelling et al., 2007).

2.4. SCX fractionation

The labeled samples were injected into a high performance liquid chromatography system (Shimadzu, Japan) with a SCX column (Luna 5u column, 4.6 mm I.D. × 250 mm, 5 μm, 100 Å; Phenomenex, Torrence, CA). The retained peptides were gradient eluted using buffer A (10 mM KH_2PO_4 in 25% acetonitrile, pH 3.0) and buffer B (2 M KCl, 10 mM KH_2PO_4 in 25% acetonitrile, pH 3.0). Thirty-eight fractions were collected when the gradient elution (0% B 0–30 min, 0–5% B 30–31 min, 5–30% B 31–46 min 30–50% B 46–55 min) was completed. Otherwise, a strata-X 33U polymeric reversed-phase column (Phenomenex) was used to remove the salt from the high salt concentration fractions. Finally, each fraction was redissolved in a 0.1% formic acid solution prior to injection into a reversed-phase Nanoliquid Chromatography/Tandem MS (nano LC–MS/MS) (Snelling et al., 2007).

2.5. Reversed-phase nano LC–MS/MS

The amount of peptides in each fraction was equalized before being injected into the Nano-LC system. Seventeen new fractions were obtained from the 38 peptide fractions by pooling fractions together to reduce peptide complexity. The samples were loaded onto a PepMap 100 C18 RP column (3 mm particle size, 15 cm long, 75 mm internal diameter; Dionex) and separated with a gradient of 5% (v/v) acetonitrile and 0.1% (v/v) TFA to 95% (v/v) acetonitrile and 0.1% (v/v) TFA over 100 min at a flow rate of 250 nl/min. The fractions were analyzed using a hybrid quadrupole/time-of-flight MS (MicroTOF-Q II, Bruker, Germany) with a nano electrospray ion source. The MS/MS scans from 50–2000 m/z were recorded. Nitrogen was used as the collision gas. The ionization tip voltage and interface temperature were set at 1250 V and 150 °C, respectively (Snelling et al., 2007).

2.6. Data analysis

All of the mass spectrum data were collected using the Bruker Daltonics micro TOF control, and processing and analysis were performed using the data analysis software (Bruker Daltonics, Bremen, Germany). The database of GiardiaDB was downloaded and integrated into the Mascot search engine version 2.3.01. All parameters were the same as previously (Besson et al., 2011). The tolerance settings for peptide identification in the Mascot searches were 0.05 Da for MS and MS/MS mass. Finally, the results of the Mascot searches were exported as a DAT FILE, normalized and quantified using the Scaffold version 3.0 software (Jedelský et al., 2011).