The International Journal of Biochemistry & Cell Biology 81 (2016) 32–43

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Specific histone modifications play critical roles in the control of encystation and antigenic variation in the early-branching eukaryote *Giardia lamblia*

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ARTICLE INFO

Article history:
Received 10 June 2016
Received in revised form
29 September 2016
Accepted 17 October 2016
Available online 19 October 2016

Keywords:
Histone marks
Cellular differentiation
Gene regulation
Histone methylases
Histone deacetylases
HDAC
Sirtuin

ABSTRACT

During evolution, parasitic microorganisms have faced the challenges of adapting to different environments to colonize a variety of hosts. *Giardia lamblia*, a common cause of intestinal disease, has developed fascinating strategies to adapt both outside and inside its host's intestine, such as trophozoite differentiation into cyst and the switching of its major surface antigens. How gene expression is regulated during these adaptive processes remains undefined. *Giardia* lacks some typical eukaryotic features, like canonical transcription factors, linker histone H1, and complex promoter regions; suggesting that post-transcriptional and translational control of gene expression is essential for parasite survival. However, epigenetic factors may also play critical roles at the transcriptional level. Here, we describe the most common post-translational histone modifications; characterize enzymes involved in these reactions, and analyze their association with the *Giardia*'s differentiation processes. We present evidence that NAD^+-dependent and NAD^+-independent histone deacetylases regulate encystation; however, a unique NAD^+-independent histone deacetylase modulate antigenic switching. The rates of acetylation of H4K8 and H4K16 are critical for encystation, whereas a decrease in acetylation of H4K8 and methylation of H3K9 occur preferentially during antigenic variation. These results show the complexity of the mechanisms regulating gene expression in this minimalistic protozoan parasite.

1. Introduction

Giardia lamblia is a non-invasive parasite that inhabits the upper small intestine of humans (Adam, 2001). This protozoan belongs to the earliest diverging branch of the eukaryotic line of descent and is considered an excellent model to study evolutionary aspects of basic biochemical and cellular processes (Lujan and Svärd, 2011). *Giardia* possesses a small and compact genome of ~12 Mb containing nearly 9000 ORFs (Morrison et al., 2007). As other protozoa, *Giardia* lacks canonical transcription factors and other gene regulatory elements (Carranza and Lujan, 2010). It is possible that some of these molecules escape bioinformatic identification due to their primitive condition or, more likely, epigenetic factors may be playing essential roles in the regulation of gene expression by altering chromatin structure (Croken et al., 2012).

Giardia presents a simple life cycle: the environment-resistant cysts and the disease-causing vegetative trophozoites (Adam, 2001). Between these developmental stages *Giardia* needs to rapidly adapt to establish an infection in a new host. *Giardia* differentiation into cysts (encystation) consists of the developmentally-regulated expression, transport and assembly of

Abbreviations: ORF, open reading frame; CWPs, cyst wall proteins; HDAC, histone deacetylase; HAT, histone acetyl transferase; IFA, immunofluorescence assay; VSP, variant-specific surface protein; mAb, monoclonal antibody; qPCR, quantitative polymerase chain reaction; ChIP, chromatin immunoprecipitation; VSG, variable surface glycoprotein.

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http://dx.doi.org/10.1016/j.biocel.2016.10.010
1357-2725/

cyst wall proteins (CWPs) and a particular carbohydrate polymer (Lujan et al., 1997). The *Giardia* cyst wall protects the parasite from the harsh environmental conditions it has to endure outside the host's intestine (Adam, 2001; Lujan et al., 1997). When a putative host ingests water or food contaminated with *Giardia* cysts, a signal transduction cascade triggered by the low pH of the stomach allows the release of trophozoites in the upper small intestine (Ellis et al., 2003). Once in the small intestine, trophozoites produce a symptomatic infection or remain unnoticed (Adam, 2001).These events require a high degree of coordination to avoid the release of the trophozoites inside the stomach (which could be detrimental) or in the large intestine, where the nutrient levels turn the infection unlikely (Lujan et al., 1997).

Antigenic variation is a mechanism that allows parasite evasion from the host's immune response, enabling the establishment and maintenance of chronic and/or recurrent infections (Deitsch et al., 2009). Antigenic variation in *Giardia* consists of a clonal phenotype variation involving surface antigens called Variant-specific Surface Proteins or VSPs (Nash, 2002; Prucca et al., 2011). *Giardia* possesses approximately 200 different VSP genes (Adam et al., 2010; Morrison et al., 2007), but only one VSP is expressed at any point in time on the surface of individual trophozoites (Nash, 2002). Changes in the VSP expression also occur during the encystation process (Svärd et al., 1998; Einarsson et al., 2016). We have previously shown that the regulation of VSP expression is controlled post-transcriptionally by an RNAi-like mechanism (Prucca et al., 2008). Additionally, Wang's group showed that miRNAs could be implicated in the control of VSP expression at the translational level (Saraiya et al., 2011).

Why just one VSP mRNA bypasses this silencing mechanism, accumulates in the cytoplasm, and is finally translated into the protein that will be expressed on the trophozoite surface remains an open question (Prucca et al., 2011). How *Giardia* switches the expressed antigen to another from its genomic repertoire is also unknown (Prucca et al., 2008). Nevertheless, early nuclear run-on experiments, which demonstrated that many VSP are simultaneously transcribed, also showed variations of the transcription level of individual VSP genes (Prucca et al., 2008), suggesting that the concentration of each VSP transcript may influence the selection of the one that avoids RNAi degradation (Prucca et al., 2008). Accordingly, it can be assumed that epigenetic factors may be involved in such regulation. However, how changes in chromatin influence gene expression during encystation and antigenic variation has not been studied in detail (Kulakova et al., 2006; Sonda et al., 2010).

Epigenetic factors influence the local chromatin architecture allowing or preventing the accessibility of gene expression factors. Some of the best known epigenetic mechanisms are methylation of DNA and post-translational modification of histones (Kouzarides, 2007). *Giardia* has two copies of the histones H2A, H2B, H3 and three of H4, but no putative homologue of the linker histone H1 has ever been found (Wu et al., 2000; Yee et al., 2007). Just a few histone modifications have been mentioned in earlier reports (Dawson et al., 2007), but no broad studies have been performed regarding these aspects of the *Giardia* biology.

Among post-translational modifications of histones, acetylation and methylation of lysine residues in histone H3 and H4 are critical to the regulation of chromatin structure and gene expression (Vaquero, 2009). The steady-state levels of acetylation depend on the activity of histone acetyltransferases (HAT) and histone deacetylates (HDAC). According to the requirement of the cofactor nicotinamide adenine dinucleotide (NAD^+), histone deacetylases can be classified into NAD^+-independent deacetylases (or simply HDAC) and NAD^+-dependent deacetylases (Sirtuins or Sir2-like, for *Silent* Information Regulator 2) (de Ruijter et al., 2003; Frye, 2000; Yang and Seto, 2008). The HDACs have important roles in DNA repair and replication, cell cycle control, apoptosis and other functions (de Ruijter et al., 2003; Yang and Seto, 2008). Sirtuins have different functions, substrates, and subcellular localizations (Frye, 2000; Vaquero, 2009). Sirtuins modify chromatin structure through deacetylation of histones, particularly H4K16ac (Vaquero et al., 2007). Recently, histone deacetylases have gained considerable attention because of their function as metabolic sensors, mediating environmental stress responses (Fulco et al., 2003; Vaquero and Reinberg, 2009). This link between the environment and chromatin modifications allows detection of rapid environmental changes and effective adaptive processes, such as those that *Giardia* confronts during its life cycle.

In this work, we characterized the presence of the most common eukaryotic histone modifications associated with euchromatin, heterochromatin and intermediate stages during *Giardia* proliferation and differentiation. We also characterized, localized, and inhibited two protein families with deacetylase activity: the NAD^+-independent HDAC and the NAD^+-dependent Sirtuins, reproducing and complementing earlier reports (Sonda et al., 2010). Additionally, we determined that specific histone deacetylation/methylation events play critical roles during encystation and antigenic variation of this important human parasite.

2. Materials and methods

2.1. Bioinformatics analysis

Known histone deacetylase domains were used to search homologous sequences using GenBank™ and the GiardiaDB (www.giardiadb.org/giardiadb). The identification of nuclear localization sequences was made using NLS Mapper (http://nls-mapper.iab.keio.ac.jp/), EPipe server (http://www.cbs.dtu.dk/services/EPipe/), and PSORT II (http://psort.hgc.jp/). For phylogenetic analyses, multiple alignments (MUSCLE) were used into the SeaView. Phylogenetic trees were computed using PhyML.

2.2. G. lamblia *cultures and treatment with deacetylase inhibitors*

Trophozoites of isolate WB, clone 1267, clone 9B10 and clone A6 were cultured in TYI-S-33 medium (Keister, 1983). Encystation of trophozoites was induced as described (Lujan et al., 1996). For antigenic variation experiments, a *Giardia* clone expressing a unique VSP was obtained by serial dilution and selection by immunofluorescence assays (IFAs) using mAb that recognizes specific VSPs. Antigenic variation was induced according to Torri et al. (unpublished data). Monolayers of trophozoites were induced either to encyst or to switch in the presence of different concentrations of Trichostatin A, Sodium Butyrate or Nicotinamide. Harvested cells were used for IFA or lysed in RIPA buffer for 30 min and quantified for subsequent immunoblotting.

2.3. Histone immunoblotting and peptide competition assays

Histones were isolated using the classical acid extraction method (Shechter et al., 2007). Many anti-histone modification antibodies from different sources were tested. Only those obtained from Abcam (anti-H3K4m1, anti-H3K4m2, anti-H3K4m3, anti-H3K9m3, and anti-H3K9ac) and from Millipore (anti-H4K8ac, anti-H4K16ac) showed a good level of detection and clear specificity. In all cases, each antibody was pre-incubated with a peptide that has the corresponding amino acid modification prior to the immunoblotting assays. The immunoblotting experiments were run in duplicate: one with the antibody pre-incubated with the corresponding peptide and another with a control antibody not pre-incubated with the peptide. The presence of negative bands in