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A quantitative multiplexed mass spectrometry assay for studying the kinetic of residue-specific histone acetylation

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ABSTRACT

Histone acetylation is involved in gene regulation and, most importantly, aberrant regulation of histone acetylation is correlated with major human diseases. Although many lysine acetyltransferases (KATs) have been characterized as being capable of acetylating multiple lysine residues on histones, how different factors such as enzyme complexes or external stimuli (e.g. KAT activators or inhibitors) alter KAT specificity remains elusive. In order to comprehensively understand how the homeostasis of histone acetylation is maintained, a method that can quantitate acetylation levels of individual lysines on histones is needed. Here we demonstrate that our mass spectrometry (MS)-based method accomplishes this goal. In addition, the high throughput, high sensitivity, and high dynamic range of this method allows for effectively and accurately studying steady-state kinetics. Based on the kinetic parameters from *in vitro* enzymatic assays, we can determine the specificity and selectivity of a KAT and use this information to understand what factors influence histone acetylation. These approaches can be used to study the enzymatic mechanisms of histone acetylation as well as be adapted to other histone modifications. Understanding the post-translational modification of individual residues within the histones will provide a better picture of chromatin regulation in the cell.

1. Introduction

Histones are highly basic proteins that organize DNA in eukaryotic cells. This compact DNA-histone conformation limits accessibility to the DNA. Post-translational modification (PTM) of histones modulates DNA accessibility, which is one of the mechanisms that regulates gene transcription and DNA repair [1–3]. However, different modifications, or even the same modification found on a different site, can lead to different functions in cells. For example, acetylation on lysine 5 of H4 (H4K5) is related to histone deposit in many eukaryotes [4]. H3K56 acetylation is involved in DNA damage repair [3], while H3K14 acetylation is important for gene transcription *in vivo* [5]. In addition, aberrant regulation of lysine acetylation not only alters gene activation but also has been shown to correlate with human diseases [6–9]. Thus, determining both the location and quantity of acetylation on histones is important to characterize how genes are regulated in response to DNA damage.

Lysine acetyltransferases (KATs) catalyze histone acetylation, which is the transfer of an acetyl group from acetyl-CoA to the lysine residues of a histone [10]. While histones usually have a positive charge, the addition of an acetyl group to a lysine residue results in neutralization of this charge, which in turn contributes to a decreased histone-DNA or nucleosome–nucleosome interaction. This increases the accessibility of DNA to enzymes, allowing for initiation of transcription, DNA replication, and DNA damage repair [1–5]. However, many KATs, such as p300 and Gcn5, are able to acetylate multiple lysine residues on histones and different acetylation sites can lead to different down stream effects [11–13]. Regarding this multiplexing ability, the acetylation specificity and selectivity of a KAT becomes adjustable by different factors such as chaperone complex or the addition of KAT activators/inhibitors. Note that specificity is the ability of a KAT to acetylate a specific residue on histones, while selectivity is the efficiency of a KAT to acetylate one site relative to another. Therefore, in order to understand the contribution to the histone acetylation by a particular KAT with or without the corresponding factors, we require a multiplexed technique to detect each potential site of histone acetylation simultaneously.

Although under ideal conditions conventional site-specific antibody methods can provide high specificity for detection of histone

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modifications, the drawback to this technique is that one antibody can only measure one modification of one location at a time and could be difficult to quantitate. In addition, varying quality of antibodies and the potential for epitope occlusion when utilizing antibodies may cause errors for quantitative measurements. These problems make it less feasible to have accurate quantification via antibody assays, not to mention how time consuming and arduous such a process would make the measurement of multiple residues and multiple samples from kinetic assays. While the use of radioactive or fluorescence methods can meet the criterion of being high throughput [14,15], it is only capable of measuring the total amount of acetylation, not site-specific amounts and are not capable of measuring histone modifications in cells. The approach we present herein has the advantage of being able to quantitate histone acetylation at multiple sites on multiple proteins at the same time and the label free nature of this approach allows for the ability to also quantitate modifications on histones extracted from cells.

To overcome these limitations, we have developed a label-free quantitative mass spectrometry (MS)-based method that is able to quantitate acetylation at all known sites of histone H3 and H4 in a single run [16,17]. Because we use a tandem MS, we can utilize the mode of selected reaction monitoring (SRM) to gain sensitivity and selectivity for peptide analysis. Briefly, SRM is used to detect the decomposition reactions (product ions) of the selected ions that are characteristic of individual peptides (parent ions). Thus, we are able to monitor specific parent-ion-to-product-ion transitions that are both unique to the peptides of interest and to the sites of modification. Here we describe the workflow for performing the kinetic analysis of a KAT, sample preparation for MS detection, and data analysis (Fig. 1). While our work allows examining the histone acetylation patterns of KATs on the histone monomer and tetramer, in a broader sense, this MS-based method can be applied to studying PTMs of different histone conformations (e.g. nucleosome) by those multi-targeting enzymes, and can provide a rapid and accurate workflow for the determination of kinetic parameters of such enzymes.

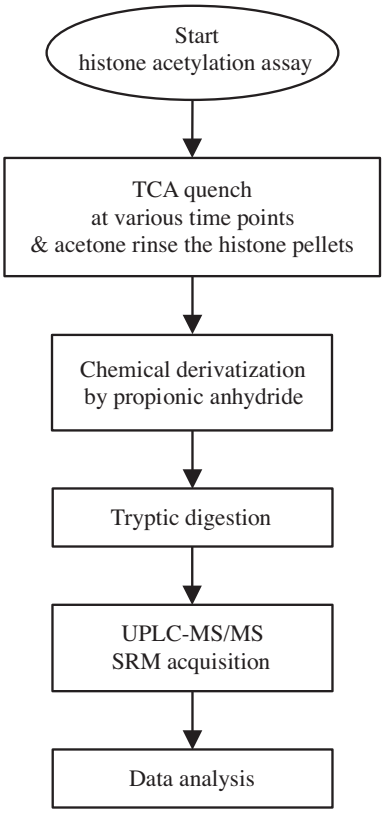

Fig. 1. Experimental flow chart of the multiplexed MS-based assay.

2. Materials and methods

2.1. Steady-state experimental setup for histone acetylation

All Chemicals were purchased from Sigma–Aldrich (St. Louis, MO) or Fisher (Pittsburgh, PA) and the purity at least meets LC/MS grade. Ultrapure water was generated from a Millipore Direct-Q 5 ultrapure water system (Bedford, MA). Recombinant histone H3 and H4 were purified and provided from the Protein Purification Core at Colorado State University. H3/H4 was refolded from purified H3 and H4 using previously published methods [18,19]. KATs (e.g. p300, CBP, and Rtt109) were also prepared and purified following the reported procedures [16,20,21]. Protein molecular weight and purity was confirmed through SDS-PAGE with Coomassie stains. The concentrations of purified KATs and histones were determined by UV absorbance and calculated from the extinction coefficients [22,23].

To conduct steady-state kinetics with histone titration (0.15–10.3 μM), our enzyme concentrations need to be much less than substrate (histones) concentrations while using saturating acetyl-CoA concentration (200 μM). On the other hand, to conduct steady-state kinetics where we titrate acetyl-CoA (0.1–20 μM), we make substrate (acetyl-CoA) concentrations much larger than enzyme concentrations while saturating histones (10 μM). All kinetic assays were conducted under the identical buffer condition (100 mM HEPES buffer (pH 6.8) and 0.08% Triton X-100 at 37 °C). Note that we need to adjust the enzyme amount (2–18 nM) and/or sampling time to ensure that the collected samples analyze the initial acetylation rates for each individual. To fulfill steady-state assumptions (i.e. that we are measuring acetylation events that occurs before more than 10% of the total substrate is acetylated), 5–8 different time points of each substrate concentration should be collected. In addition, substrate concentrations ranging from 0.25–5-fold of the Michaelis constant (K_m) should be used to sufficiently analyze steady-state kinetics [24].

2.2. Quench steps for enzyme kinetics

An efficient quenching reagent should immediately stop the acetylation at each time point and is key to achieving the accuracy of a kinetic assay. However, considering the incompatibility of numerous surfactants with MS detection, they cannot be used as a quench reagent. Thus, we examined the quenching efficiency of three different reagents (trichloroacetic acid (TCA), isopropanol, and acetone), which are compatible with MS detection. We found that 4 volumes of 100% TCA for 30-min incubation (on ice) was the most efficient quench procedure [17], which had no observable acetylation detected. However, there was maximum 2% and 5% acetylation found with isopropanol and acetone quench, respectively, for over-night 4 °C incubation. Therefore, at varying time points, the collected samples were quenched with at least 4 volumes of 4 °C TCA and cooled on ice for 30 min. Each precipitate was then washed twice with 150 μL acetone (−20 °C). By doing so, excess salts and acetyl-CoA are removed, and individual samples can easily dry for either further processes or storage at −80 °C.

2.3. Chemical derivatization and tryptic digestion of histones

There is a dilemma when selecting a protease to digest histones for MS analysis; that is, not all proteolytic enzymes are suitable for