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Identification of Acetylated Proteins in *Borrelia burgdorferi*

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Abstract

Posttranslational modification (PTM) of proteins has emerged as a major regulatory mechanism in all three domains of life. One emerging PTM is N ϵ -lysine acetylation—the acetylation of the epsilon amino group of lysine residues. N ϵ -lysine acetylation is known to regulate multiple cellular processes. In eukaryotes, it regulates chromatin structure, transcription, metabolism, signal transduction, and the cytoskeleton. Recently, multiple groups have detected N ϵ -lysine acetylation in diverse bacterial phyla, but no work on protein acetylation in *Borrelia burgdorferi* has been reported. Here, we describe a step-by-step protocol to identify N ϵ -lysine acetylated proteins in *B. burgdorferi*.

Keywords

Borrelia burgdorferi; Posttranslational modification; Protein acetylation; Immunoprecipitation; Acetylated lysine protein

1 Introduction

Posttranslational modifications (PTM) control protein structure, stability, and function. One PTM is N ϵ -lysine acetylation. In eukaryotes, N ϵ -lysine acetylation is an abundant PTM that affects thousands of proteins in diverse processes [1–4]. N ϵ -acetylation is also abundant in bacteria [5–15]. As much as 15–20% of the *Escherichia coli* and *Bacillus subtilis* proteomes are acetylated [8, 11, 12, 15].

There are two mechanisms for N ϵ -lysine acetylation. The standard mechanism for N ϵ -acetylation is enzymatic; a lysine acetyltransferase (KAT) transfers the acetyl group from acetyl-coenzyme A to the ϵ -amino group of a deprotonated lysine. This is the mechanism used by KATs to acetylate lysines in the unstructured N-termini of eukaryotic histones. KATs from diverse bacteria have been identified and some have been characterized [16–22]. A novel non-enzymatic mechanism has been reported in *E. coli*. In this mechanism, acetyl phosphate (acP) donates its acetyl group directly to the ϵ -amino group of a deprotonated lysine with no involvement of an enzyme [8, 12]. The result is the same as the enzymatic mechanism, acetylation of the N ϵ -amino group of a lysine. Non-enzymatic, acP-dependent acetylation is not confined to *E. coli*; a similar story is unfolding in *B. subtilis* [15]. Acetyllysines are quite stable; however, they can be enzymatically reversed by lysine deacetylases (KDACs) [23–25].

Here, we describe a step-by-step protocol designed to identify Ne-lysine acetylated proteins from *B. burgdorferi* using co-immunoprecipitation followed by MS/MS analysis.

2 Materials

1. BSKII [26]: 9.8 g CMRL-1066, 6 g HEPES, 5 g glucose, 5 g neopeptone, 2.5 g TC yeastolate, 2.2 g NaHCO₃, 0.8 g sodium pyruvate, 0.7 g sodium citrate, 0.4 g N-acetyl glucosamine, 50 g BSA. Adjust the pH to 7.5 with 10 M NaOH, and add ddH₂O up to 1 l. Add 64 ml heat-inactivated rabbit serum and perform filter sterilization.
2. RIPA buffer [27]: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS. Add 1 mM PMSF immediately prior to use.
3. 1× lysis Buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100. Add 1 mM PMSF immediately prior to use.
4. Anti-acetylated lysine (AcK) protein antibodies: anti-acetylated lysine protein antibody from Cytoskeleton and Cell signaling are used for immunoprecipitation and Western blot, respectively.
5. Protein G PLUS agarose: from Santa Cruz.
6. HRP-conjugated goat anti-rabbit IgG: from Santa Cruz.
7. ECL: Enhanced chemiluminescence (ECL) Western blotting substrate from Fisher scientific.
8. 2× SDS-PAGE sample buffer: 100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 200 mM β-mercaptoethanol.
9. 1× SDS-PAGE gel running buffer: 3.02 g Tris, 14.4 g glycine, 1 g SDS. Add ddH₂O up to 1 l.
10. Coomassie Brilliant Blue staining solution: 0.725 g Coomassie Brilliant Blue, 143 ml methanol, 143 ml acetic acid. Add ddH₂O up to 1 l.
11. Coomassie Brilliant Blue destaining solution: 100 ml methanol, 100 ml acetic acid. Add ddH₂O up to 1 l.
12. 1× Transfer buffer: 2.8 g Tris base, 2.9 g glycine, 200 ml methanol. Add ddH₂O up to 1 l.
13. 1× PBST (phosphate-buffered saline containing 0.05% Tween 20): 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, 0.5 ml Tween-20. Add ddH₂O up to 1 l.
14. Blocking buffer: PBST containing 10% milk.
15. Washing buffer: PBST containing 1% milk.

3 Methods

3.1 Growth of *B. burgdorferi*

1. Resurrect the spirochetes by inoculating from the stocks at -80°C into 2 ml BSKII.
2. Incubate the culture at 37°C with 5% CO_2 until it reaches $1 \times 10^7/\text{ml}$.
3. Re-inoculate the spirochetes into 50 ml of BSKII at the concentration of $10^4/\text{ml}$ and harvest the spirochetes at late log phase.

3.2 Whole Cell Lysate Preparation [28]

1. Harvest the spirochetes by centrifugation at $4000 \times g$ 4°C for 30 min.
2. Wash the spirochetes twice with ice-cold PBS.
3. Resuspend the spirochetes ($\sim 5\text{--}7 \times 10^8$) in 500 μl RIPA buffer.
4. Sonicate the cells for 30 s.
5. Centrifugation at $14,000 \times g$ 4°C for 15 min.
6. Save the supernatant.

3.3 Preclear the Cell Lysate [28] (See Note ¹)

1. Add 1 μg normal mouse Ig G to 1.5 mg cell lysates (1 ml).
2. Incubate the samples at 4°C for 2 h with rotation.
3. Add 100 μl protein G agarose beads.
4. Incubate the samples at 4°C for 2 h with rotation.
5. Centrifuge at $500 \times g$ at 4°C for 5 min.
6. The supernatant is precleared cell lysate ready for IP.

3.4 Immunoprecipitation [28]

1. Add 15 μl of mouse anti-AcK monoclonal antibody from Cyto-skeleton (AAC01) to the precleared cell lysates (*see Note* ²).
2. Incubate the samples at 4°C with rotation for at least 4 h or O/N.
3. Add 50 μl of 50% Protein G agarose beads slurry and incubate at 4°C for 4 h or O/N with gentle rocking (*see Note* ³).

¹The purpose of the preclearing step is to reduce the amount of nonspecific contaminants in the cell lysate, and to remove proteins with high affinity to Protein G or Protein A-agarose beads prior to immunoprecipitation.

²Not all antibodies can be used in immunoprecipitation. One should optimize the effect of immunoprecipitation by testing different antibodies, as well as different incubation conditions (for example, under native or denaturing conditions).

³Immobilized Protein A or Protein G is critical to the success of the immunoprecipitation. Protein G PLUS-Agarose is suitable for immunoprecipitation of mouse IgG1, IgG2a, IgG2b and IgG3, rat IgG1, IgG2a, IgG2b and IgG2c, rabbit or goat polyclonal Abs, and human IgG1, IgG2, IgG3 and IgG4. Protein A-Agarose is suitable for immunoprecipitation of mouse IgG2a, IgG2b, and IgA, rabbit IgG, and human IgG1, IgG2 and IgG4. Protein A/G PLUS-Agarose is suitable for immunoprecipitation of mouse IgG1, IgG2a, IgG2b, IgG3 and IgA, rat IgG1, IgG2a, IgG2b and IgG2c, rabbit or goat polyclonal Abs, and human IgG1, IgG2, IgG3 and IgG4.

4. Pellet the beads by centrifugation at 4 °C $500 \times g$ for 5 min.
5. Discard the supernatant.
6. Wash the beads five times with lysis buffer, each for 10 min.
7. Resuspend the beads with 50 μ l of 2 $\times$ SDS-PAGE sample buffer.
8. Heat the sample to 95–100 °C for 5 min.
9. Centrifuge the samples at $500 \times g$ RT for 5 min.
10. Save the supernatant for SDS-PAGE.

3.5 SDS-PAGE

1. Load 5 and 20 μ l of the supernatant on 15% SDS-PAGE gel for Coomassie Brilliant Blue staining and Western blot, respectively.
2. Run the gel at 15 mA/gel until the bromophenol blue reaches the bottom of the gel.
3. The gel can be used for either Coomassie Brilliant Blue staining or Western blot.

3.6 Coomassie Brilliant Blue Staining

1. Soak the gel in Coomassie brilliant blue staining solution for 40 min with gentle shaking.
2. Transfer the gel into Coomassie Brilliant Blue destaining solution and destain until the background is clear (Fig. 1a).
3. Cut off the band(s) of interest for the identification of the acetylated protein by MS/MS analysis.

3.7 Western Blot

1. After SDS-PAGE, transfer the proteins from the gel(s) to nitrocellulose (NC) membrane(s) at 80 V 4 °C for 2 h.
2. Soak the membrane(s) in blocking buffer at RT for 1 h or 4 °C overnight with gentle shaking.
3. Probe the membrane(s) with rabbit anti-acetylated lysine protein antibody from Cell signaling (1:1000) at RT for 1 h.
4. Wash the membrane(s) three times at RT with washing buffer, each for 5 min.
5. Probe the membrane(s) with HRP-conjugated goat anti-rabbit IgG (1:2000) at RT for another 1 h.
6. Wash the membrane(s) three times as described above.
7. Detect the acetylated lysine protein (s) by ECL (Fig. 1b).

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Fig. 1. Co-immunoprecipitation of acetylated lysine proteins from *B. burgdorferi* B31A3. **(A)**, Coomassie stained gel. **(B)**, Western blot using anti-acetylated lysine protein antibody. AcK denotes the band corresponding to a major acetylated lysine protein in *B. burgdorferi*. *I* input (whole cell lysates); *U* unbound; *IP* immunoprecipitation; *M* protein markers