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Lysine acetylation stabilizes SP2 protein in the silkworm *Bombyx mori*

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ABSTRACT

Lysine acetylation (Kac) is a vital post-translational modification that plays an important role in many cellular processes in organisms. In the present study, the nutrient storage proteins in hemolymph were first found to be highly acetylated—particularly SP2 protein, which contains 20 potential Kac sites. Further results confirmed that lysine acetylation could stabilize and up-regulate the protein level of *anti*-apoptosis protein SP2, thereby improving the survival of H_2O_2-treated BmN cells and suppressing the apoptosis induced by H_2O_2. The potential mechanism involved in the inhibition of ubiquitin-mediated proteasomal degradation by crosstalk between lysine acetylation and ubiquitination. Our results showed that the increase in the acetylation level by TSA could decrease the ubiquitination and improve the protein level of SP2, indicating that lysine acetylation could influence the SP2 protein level through competition between ubiquitination and the suppression of ubiquitin-mediated proteasomal degradation, thereby stabilizing the protein. SP2 is a major nutrient storage protein from hemolymph for amino acid storage and utilization. The crosstalk between lysine acetylation and ubiquitination of SP2 might imply an important role of lysine acetylation for nutrient storage and utilization in silkworm.

1. Introduction

Lysine acetylation (Kac) regulates many cellular processes, particularly transcription through histone acetylation or deacetylation (Yang and Seto, 2007). Lysine acetylation is catalyzed by various acetyltransferases such as p300, CBP, and PCAF (Kouzarides, 2000). Histone acetylation was first reported to regulate many cellular processes, such as cell growth and apoptosis (Chan and La Thangue, 2001; Spange et al., 2009). Subsequently, lysine acetylation was found to play an extensive role in non-histone proteins. In the metabolic cycle, many more enzymes are acetylated, and their activities are coordinated by lysine acetylation (Zhao et al., 2010). However, for other proteins such as structural proteins, the function of lysine acetylation has been rarely reported. Recently, many studies have reported that lysine acetylation can increase the stability of proteins via competition for the site of lysine ubiquitination (Ge et al., 2009; Zhang et al., 2010).

Bombyx mori (*B. mori*) is an important economic insect for agriculture and model organism in biological research. The hemolymph of *B. mori* contains many nutrient storage and immunity-related proteins, showing important functions in the growth and development of the silkworm (Li et al., 2012; Zhang et al., 2014a). Many experiments have shown that silkworm hemolymph can resist insect and mammalian cell apoptosis, demonstrating *anti*-apoptosis activity (Choi et al., 2002; Rhee et al., 2002; Rhee and Park, 2000), and apoptosis-inhibiting components from hemolymph have also been identified, such as storage protein (SP) and 30 K proteins (Kim et al., 2003, 2001; Rhee et al., 2007). SP2 is a member of the SP protein family that can inhibit apoptosis induced by many factors, such as staurosporine and BmNPV (Yu et al., 2013). SP2 isolated from hemolymph in the silkworm can suppress HeLa cell apoptosis induced by STS (Rhee et al., 2007). Furthermore, SP2 is a pivotal nutrient storage protein in hemolymph. It is an arylphorin storage protein that is rich in phenylalanine and tyrosine and is involved in the formation of the epidermis (Yu et al., 2013). During silkworm growth and pupation, SP2 is broken down into amino acids, providing amino acids and energy for the

Abbreviations: TSA, Trichostatin A; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; PTM, post-translational modification; p300, histone acetyltransferases p300; CREB, cAMP-response element binding protein; CBP, CREB binding protein; PCAF, p300/CBP-associated factor; CHX, cycloheximide.

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subsequent development of the silkworm (Munn and Greville, 1969; Wheeler et al., 2000).

In the present study, using western blotting and nano-HPLC/MS/MS, nutrient storage proteins in hemolymph were first found to be highly acetylated—particularly the nutrient storage protein SP2 protein, which contains 20 potential Kac sites. We confirmed that the protein level of SP2 could be up-regulated by increased lysine acetylation, leading to the suppression of cell apoptosis induced by H_2O_2. Further results showed that lysine acetylation could influence the SP2 protein level through competition between ubiquitination and the suppression of ubiquitin-mediated proteasomal degradation, thereby stabilizing and up-regulating the protein. The crosstalk between lysine acetylation and ubiquitination in SP2 might imply a new mechanism for nutrient storage and utilization in insects.

2. Material and methods

2.1. Animals, cell culture and cell viability assay

Bombyx mori and the BmN cell line were maintained in our laboratory. Cells were grown in SF-900 II culture supplemented with 10% FBS at 27 °C. Hemolymph was collected on the 1st, 3rd, 5th, and 7th day in fifth instar larvae, the spinning stage and the 1st day in pupae, respectively, and was then lysed in cOmplete lysis-M (EDTA free). SDS-PAGE and Western blotting were used to detect the change of proteins. Cell viability was assessed by the MTT assay.

2.2. Kac peptide enrichment coupled with nano-HPLC/MS/MS

In the previous work, Kac peptide enrichment coupled with nano-HPLC/MS/MS was used to evaluate the sites of lysine acetylation (Nie et al., 2015). Briefly, The late larvae (day 4 of the fifth instar) and early pupae (day 2 after pupation) were frozen in liquid nitrogen, ground to a fine powder and treated with lysis buffer. Then, the extractions were used for Kac peptide enrichment experiments. iTRAQ was used to detect the level of lysine acetylation in proteins from *Bombyx mori*.

2.3. Antibodies and reagents

Protein G-Agarose, cOmplete lysis-M (EDTA free) and His6 antibody (1:5000) were purchased from Roche. Normal mouse IgG and *anti*-ubiquitin antibody (1:3000) were purchased from Cell Signaling Technology. *anti*-Lysine-acetylation antibody (1:3000) was purchased from PTM. *anti*-Beta-tubulin antibody (1:5000) and Tubastatin A were purchased from Beyotime Biotechnology. FuGENE 6 Transfection Reagent was purchased from Promega. MG132, CHX, C646 and anti-flag antibody (M2) were purchased from Sigma-Aldrich.

2.4. RNA extraction and real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) and Direct-zol RNA MiniPrep (zymo Research). cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche). To synthesize cDNA from mRNA, 1 μg of total RNA was added to the reverse transcription system. Real-time PCR was conducted using FastStart Universal SYBR Green Master (RoChe) and the ABI 7300 system. The primer sequences for real-time PCR were GTGCAGTACCAAAGCCGAGC (F) and CGAAACCGTGGCAATCAGAG (R). Additionally, actin A3 was used for normalization. The PCR conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s. The change in gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method.

2.5. Virus, plasmids and transfection

The pIEx-1-SP2 and *BmNPV-SP2* were prepared by our laboratory. Cell transfection was performed using FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's protocol.

2.6. TSA, C646, MG132 and CHX treatment in BmN cells

Twelve hours after the transfection of BmN cells with plasmid or infection of BmNPV-SP2, 50, 100, 200, 500, 1000 nM TSA, 10, 25, 50, 100 nM C646 were added to the cell culture. Forty-eight hours later, cells were collected and subjected to western blotting and immunoprecipitation analysis.

Twelve hours following the addition of 500 nM TSA, 100 ng/μL CHX was added to the cell culture. After CHX treatment for 6 h, cells were collected and subjected to western blot analysis of the changes in protein levels.

Twelve hours following the addition of BmNPV-SP2, then TSA and 10 μM MG132 were added to the culture. After 24 h, cells were collected and subjected to western blot analysis of the changes in protein levels.

2.7. Western blotting (WB)

Cells were lysed using cOmplete lysis-M EDTA free (Roche), separated by SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes (PVDF; Roche). The PVDF membrane was blocked by 5% BSA in TBST for 2 h at room temperature, the primary antibody was added, and the solution was incubated at room temperature for 2 h. After washing with TBST, secondary antibody (1:10,000) was added and incubated at room temperature for 2 h. Finally, the proteins were detected using Western HRP Substrate (Luminata, USA) and Tanon 5500 Hypersensitivity Chemiluminescence Analyzer.

2.8. Immunoprecipitation (IP) for the detection of acetylated and ubiquitinated SP2

His-SP2-over-expressing BmN cells were lysed, and the cell extracts were immunoprecipitated with Protein G-Agarose beads conjugated to the His6 antibody. The complexes were then analyzed by western blotting. Precipitated complexes were detected by WB with anti-acetylated lysine, anti-Ubiquitin and His6 antibody.

3. Results

3.1. SP2 and other nutrient-storage proteins in hemolymph are highly acetylated in Bombyx mori

Using iTRAQ and Kac peptide affinity enrichment coupled with nano-HPLC/MS/MS, we performed large-scale identification of Kac proteins in *Bombyx mori* (Nie et al., 2015). We found that the proteins in hemolymph were highly acetylated, including many nutrient-storage proteins, such as apolipophorin, vitellogenin, SPs, and 30 K proteins. We further validated the acetylation level of these proteins from hemolymph in the larval, spinning and pupal stages of *Bombyx mori* using anti-acetyl-lysine antibody (Fig. 1A). The results revealed that nutrient-storage proteins in hemolymph were highly acetylated, particularly SPs and 30 K proteins. SPs, including SP1, SP2 and SP3, were highly expressed from the fifth instar larval to the spinning stage; however, in pupae, only