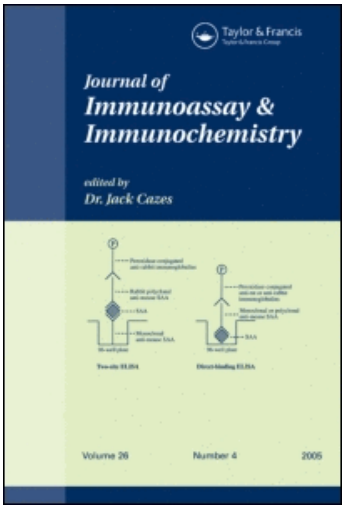

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Development of a PAN-Specific, Affinity-Purified Anti-acetylated Lysine Antibody for Detection, Identification, Isolation, and Intracellular Localization of Acetylated Protein

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Abstract: Acetylation on the lysine residue is an important event of posttranslational modification of proteins. In this study, we developed a simple method to produce and to affinity purify the specific anti-acetylated lysine polyclonal antibody, which is useful for the detection, identification, isolation, and intracellular localization of acetylated proteins on the lysine residues. We utilized the chemically acetylated hemocyanin of keyhole limpets (KLH) as an immunogen to raise the immune serum and to isolate the population of the acetylated lysine specific antibody using the immobilized acetylated lysine as immunoaffinity-ligand. The isolated antibody was tested to be useful for ELISA, immunoblotting detection, immunofluorescent localization, and affinity isolation of the acetylated proteins.

Keywords: Anti-AcK antibody, Acetylation, Affinity purification, ELISA, Immunoprecipitation

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INTRODUCTION

Posttranslational modification of proteins such as acetylation on the lysine residues is an important process of regulation of cellular functions. Transcriptionally active genes are usually associated with highly acetylated histones,[1,2] whereas transcriptional repression is associated with low levels of histone acetylation.[3,4] Acetylation of the lysines by histone acetylase removes their positive charge and results in an open chromatin structure, which facilitates gene transcription. The protein acetylation is mediated by a number of acetylases, which transfers the acetyl group from the acetyl-CoA to the substrate. According to the literature review,[5] there are at least three groups of acetylases, PCAF/GCN5, p300/CBP, and MYST proteins, all of which acetylate the histones and regulate the transcription of genes. In addition to the histones, a number of proteins such as p53 and E2F1 were also reported as the substrates of p300/CBP.[1,5] The process of histone acetylation is reversed with the enzyme called histone deacetylase (HDAC).[6–8] HDAC removes the acetyl groups from lysine, which reverse the process of histone acetylation and silences gene expression.

Dysregulation of the specificity of HDAC may be associated with neoplastic transformation. For instance, a variety of inhibitors of HDAC exhibited marked antitumor activity both in animal models and in preliminary clinical trials.[9,10] The HDAC inhibitor, phenylbutyrate, in combination with retinoic acid induced a complete remission in a patient with acute promyelocytic leukemia resistant to retinoid therapy.[11] In addition, combination treatment of the HDAC inhibitor trichostatin A (TSA) with the DNA methylation inhibitor 5-azadeoxycytidine resulted in a synergistic activation of the tumor suppressor gene p16CDKN2A.[12] Based on biological importance of the protein acetylation in gene transcription and cell transformation, we developed a simple method to generate an anti-acetylated lysine (anti-AcK) specific antibody, which could be used for detection, identification, isolation, and intracellular localization of the acetylated proteins.

EXPERIMENTAL

Materials

Acetic anhydride, pyridine, hemocyanin of keyhole limpets (KLH), bovine serum albumin (BSA), TSA, L-lysine agarose, 3,3′, 5,5′-tetramethylbenzidine (TMB), and Freund adjuvants (complete and incomplete) were purchased from Sigma (Oakville, ON, Canada). Goat anti-rabbit IgG agarose and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were from Immune-Chem (Burnaby, BC, Canada). Protein A sepharose and the enhanced chemiluminescence detection (ECL) kit were purchased from Amersham

(Little Chalfont, England), green fluorescent-CY2-conjugated goat anti-rabbit secondary antibody from Jackson ImmunoResearch Laboratories (West Grove, PA), mouse anti-p53 DO-1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-acetylated histone 3 from the Upstate Biotechnology (Waltham, MS).

Preparation of Acetylated KLH (AcKLH) and Immobilized Acetylated BSA

Acetic anhydride is a mild-acetylated reagent, which can acetylate primary amine under alkaline condition.[13] Therefore, we used the acetic anhydride to chemically acetylate the proteins. KLH (10 mg) or BSA (10 mg) was dissolved in 5 mL of 0.1 M $NaCO_3$ in a 15 mL tube. 200 μL of pyridine was added to the tube followed by 50 μL of acetic anhydride, and incubated for 4 hr at 30°C. The acetylation reaction was then stopped with 100 μL of saturated *Tris*-base. The pyridine was removed using 50 mL of G25 Sephadex gel filtration column. Approximately 15 mL of the protein fraction was collected. The AcKLH or BSA in the gel filtration fraction was then subjected to SDS-PAGE to confirm the status of acetylation. The acetylated protein is migrated slower than the non-acetylated protein, which can be visualized with Coomassie brilliant blue staining of the gel. The band of the acetylated BSA is approximately 4 kDa higher in molecular weight as compared with non-acetylated BSA, whereas the migration of the AcKLH was approximately 2 kDa higher in molecular weight than KLH.

Immunization

Approximately 2 mg of AcKLH was used to immunize two rabbits (New Zealand species, 3 kg body weight) with complete Freund adjuvant through subcutaneous injections. After two weeks of immunization, the rabbit was boosted with the AcKLH (250 μg/rabbit) with incomplete adjuvant every two weeks. Serum was collected after 6 weeks of immunization (after two boosts).

Preparation of Acetylated Lysine Affinity Matrix

An aliquot of 10 mL of lysine agarose was washed with 50 mL water/acetone (1 : 1, v/v) and then with 50 mL of distilled water. The lysine-agarose beads were then suspended in 10 mL of pyridine/distilled water (1 : 10, v/v). The acetylation reaction was initiated with the addition of 100 μL of acetic anhydride at 50°C for 4 hr in a fume hood. After the reaction, the agarose

beads were washed with 100 mL of acetone/distilled water mixture (1 : 1, v/v), followed by 500 mL of phosphate buffered saline (PBS). The acetylated lysine-agarose beads were stored at 4°C before use.

Affinity Purification of the Anti-AcK-Specific Antibody

An aliquot of 10 mL of the acetylated lysine-agarose was packed in a 50 mL glass column. After the column was washed with 50 mL of PBS and then 200 mL of the AcKLH, immune serum was applied to the column. The serum was reloaded to the column three times at room temperature. The flow rate of the column was controlled at approximately 4 mL/min. Then the column was washed with 200 mL of PBST (PBS containing 0.1% Tween 20) at flow rate of 5 mL/min. Before the elution of the antibodies, the column was washed one more time with 50 mL of 1 M NaCl to eliminate the possible non-specific, and ionic interactions. The bound antibodies on the column were then eluted with 0.2 N glycine buffer (pH 2.5) in three fractions (10 mL/fraction). Each fraction was neutralized with saturated *Tris*-base immediately and subjected to ELISA analysis.

ELISA

The 96-well plate was coated with 100 μL of the acetylated BSA (10 μg/mL in 0.5 M NaCl, 0.05 M Na_2CO_3) at room temperature overnight. The coated plate was blocked with 1.5% skimmed milk at room temperature for 20 min. Serial dilutions of the anti-AcK-specific antibody in each fraction were added to the plate and incubated at room temperature for 30 min. The bound anti-AcK antibodies were detected with HRP-conjugated goat anti-rabbit IgG (1 μg/mL in PBST) at room temperature for 30 min. After washing with PBST three times, the peroxidase substrate TMB (0.2 mg/mL in 0.05 M citrate buffer, pH 4.5) was added to the plate. The color reaction was stopped by addition of 20 μL of 2 N HCl in 30 min and the color signal was read at 450 nm.

Cell Culture and TSA Treatment

MMRU melanoma cells (kind gift from Dr. R. Byers, Boston University School) were maintained in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum (Canadian Life Technologies, Burlington, ON), 100 units/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 atmosphere at 37°C. Cells were treated with 200 ng/mL TSA for 24 hr prior to lysis or immunofluorescent staining.

Western Blot

Proteins from TSA-treated and untreated cell lysate were resolved with 12% SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was blocked with 3% BSA at room temperature for 20 min. Then the membrane was incubated with affinity-purified anti-AcK antibody (1 μg/mL in PBST with 0.5% BSA) at room temperature for 4 hr. After washing with PBST four times 5 min each, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1 μg/mL in PBST with 0.5% BSA) at room temperature for 2 hr. The ECL system was utilized for the blot signal detection.

Immunoprecipitation

Both TSA-treated and untreated cell lysate (5 mg/mL) were subjected to immunopreciptation using the affinity-purified anti-AcK antibody. In general, 10 μg of the affinity-purified anti-AcK antibody was initially incubated with 20 μL of the goat anti-rabbit IgG agarose or protein A agarose beads. Unbound antibodies were washed away with PBST. The anti-AcK antibody bound on the agarose beads was incubated with 100 μL of the cell lysates (approximately 500 μg crude proteins) at room temperature for 1 hr in a rotor shaker. The immune-complex in the agarose beads was washed with PBST four times to remove the impurity and then boiled in 50 μL of SDS-sample loading buffer which contains 2% SDS and 1% of mercaptoethanol. The boiled samples were resolved with 12% SDS-PAGE and transferred to nitrocellulose membrane. After blocking the membrane with 3% BSA, the immunoprecipitated proteins on the membrane were blotted with both anti-histone H3 and affinity-purified anti-AcK antibodies.

Immunofluorescent Staining

Melanoma MMRU cells were grown on 22 × 22 mm coverslips at a density of 2×10^4 cells/mL, and treated with TSA as described earlier. Cells were simultaneously fixed and extracted in 3.7% paraformaldehyde (v/v), 0.25% glutaraldehyde (v/v), and 0.5% Triton X-100 (v/v) in PEM buffer (40 mM PIPES, 2.5 mM EGTA, 0.5 mM $MgCl_2$, pH 6.9) for 10 min. Indirect immunolabeling was performed by inverting coverslips on antibody solutions (dissolved in PBS) at room temperature for 45 min and by washing with PBS three times 5 min between incubations. Cells were counterstained with Hoechst 33,258 (2 mg/mL stock diluted 1 : 3000 in PBS) and then mounted in Permount mounting media (Fischer Scientific) and visualized under a Zeiss Axioplan 2 microscope.

RESULTS AND DISCUSSION

The currently available anti-acetylation antibody is either whole immune serum or protein A fractionated antibody against acetylated proteins or affinity-purified antibody against acetylated peptide. Those antibodies are only for specific proteins or peptides with acetylated lysine residues. Since acetylation of proteins on the lysine residue is an important event of protein posttranslational modification, it would be important to have a single anti-acetylated lysine antibody to universally detect the unknown acetylated protein or proteins with unknown acetylated sites. We then used the chemically acetylated protein, AcKLH, as the immunogen and the acetylated lysine as affinity ligand to specifically isolate the anti-AcK antibody.

We used AcBSA as the immobilized antigen in ELISA plate to determine the titer of AcKLH immune serum. Since the protein sequence of BSA and KLH are completely different, the signal from the ELISA assay is considered to be specific to the acetylated group. High ELISA titer (approximately 1 : 80,000) was obtained from the serum of both rabbits after 6 weeks of immunization of the AcKLH (data not shown). We applied 200 mL of the AcKLH immune serum to the acetylated lysine affinity column, and approximately 5 mg of the highly purified anti-AcK antibody was obtained based on the semi-quantitative analysis using SDS-PAGE Coomassie staining technique. After one round of affinity column purification, very little anti-AcK antibody was left in the flow through serum, suggesting that the anti-AcK antibody has high affinity binding to aceylated lysine. However, the ELISA titer of the flow through serum determined by AcBSA as the immobilized antigen remained high ($>1:50{,}000$), suggesting that not all the antibodies binding to the AcBSA are specific anti-AcK antibody, presumably the immune serum contains antibodies against the epitopes with one or more amino acid residues in addition to the acetylated lysine.

The main goal of this study is to isolate the fraction of the antibodies which can universally recognize the acetylated lysine residues of any proteins and test its applications on the detection, quantitation, identifiaction, isolation, and intracellular localization of the acetylated proteins. As for detection of acetylated proteins, our results showed that the affinity-purified anti-AcK antibody from the immune serum against AcKLH did not recognize the non-acetylated BSA proteins as for both ELISA and Western blot analyses. However, this antibody can detect as little as 1.25 ng of acetylated BSA with a 5-sec exposure using the ECL detection system (Figure 1). The sensitivity may be higher with longer exposure time. The result suggested that the anti-AcK antibody could be utilized to detect acetylated proteins.

In order to examine if the anti-AcK antibody could detect the protein acetylation in a living cell, Western blot analysis was carried out on the MMRU cells treated with TSA, an inhibitor of HDAC, which inhibits the deacetylation of acetylated histones. It is well documented that inhibition

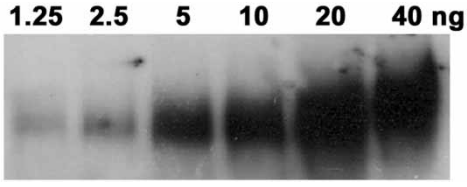

Figure 1. Detection of chemically acetylated BSA with anti-AcK antibody by Western blot analysis. 1.25–40 ng/lane of acetylated BSA was used.

of HDAC with TSA induces the accumulation of the acetylated histones.[14–16] Our results showed that the affinity-isolated anti-AcK antibody detected significantly stronger acetylation signal of 16 kDa band from TSA-treated cells compared to the non-TSA-treated control cells (Figure 2, right panel). This band corresponds to the molecular size of histone H3 (16 kDa). We then confirmed this band to be the histone H3 with the commercially available anti-acetylated H3 antibody that detects the 16 kDa band (Figure 2, left panel). Our anti-AcK antibody, however, showed much stronger signal than the anti-acetylated histone H3 antibody. The possible explanation is that histone H3 is acetylated on multi-lysine residues which provide multiple binding sites for the anti-AcK antibody, but only one binding site for the anti-acetylated histone H3 antibody.

Next, we carried out immunoprecipitation studies to evaluate if the affinity-purified anti-AcK antibody could be utilized for isolation of the acetylated proteins. The affinity-purified anti-AcK was pre-absorbed to the goat anti-rabbit IgG and then incubated with either TSA- or non-TSA-treated cell lysate for 1 h. The immunopreciptated samples were blotted with anti-AcK antibody and anti-histone H3 antibody. Figure 3A showed that at least two acetylated proteins were pulled down with the anti-AcK antibody from the TSA-treated samples (left panel). A strong band of 16 kDa and a weak band of 14 kDa were detected. The same acetylated proteins were detected in the crude cell lysate (right panel). We then blotted

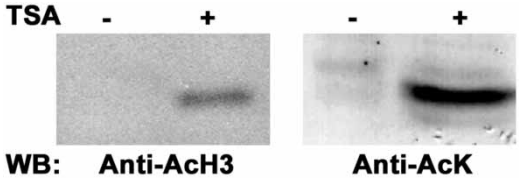

Figure 2. Detection of acetylated histone H3 in melanoma MMRU cells. Cells were treated with TSA at a concentration of 200 ng/mL for 24 h. 50 μg/lane of cell lysate was separated on a 12% SDS-PAGE and blotted with anti-AcH3 and anti-AcK antibodies. Untreated cells were used as negative controls.

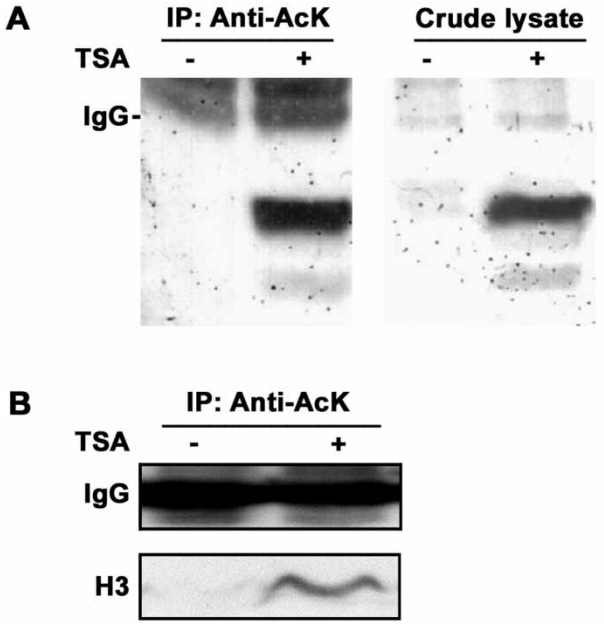

Figure 3. Immunoprecipitation of the acetylated proteins by the anti-AcK antibody. (A), 500 μg of cell lysate from both TSA-treated (200 ng/mL) or untreated MMRU cells were immunoprecipitated with 10 μg of anti-AcK antibody for 1 h at room temperature and the immune complex was resolved in a 12% SDS-PAGE, and blotted with the anti-AcK antibody. (B), Same cell lysates as (A) were immunoprecipitated with anti-AcK antibody and blotted with anti-histone H3 antibody.

the immunopreciptated samples with an anti-histone H3 antibody and found that the major 16 kDa band from the TSA-treated sample was histone H3 (Figure 3B). There was no histone H3 immunoprecipitated by anti-AcK antibody from the non-TSA-treated sample (left lane), suggesting that the anti-AcK antibody specifically binds to acetylated histone H3. These results suggest that the anti-AcK antibody is useful for purification of proteins with acetylated lysine residues by immunoprecipitation.

To study the biological functions of protein acetylation, it is often important to determine the intracellular localization of the acetylated proteins. We employed the immunofluorescent staining to test if the anti-AcK antibody could be used to detect the acetylated histones in the nucleus. Figure 4 shows that both the anti-AcK antibody and the commercially available anti-acetylated histone H3 antibody stained strongly in the nuclei of TSA-treated cells, but weakly in non-TSA-treated cells.

In summary, we show that a universal antibody against acetylated lysine can be generated using the AcKLH and immuno-affinity-purified with acetylated lysine as the immobilized antigen. Our data demonstrated that this

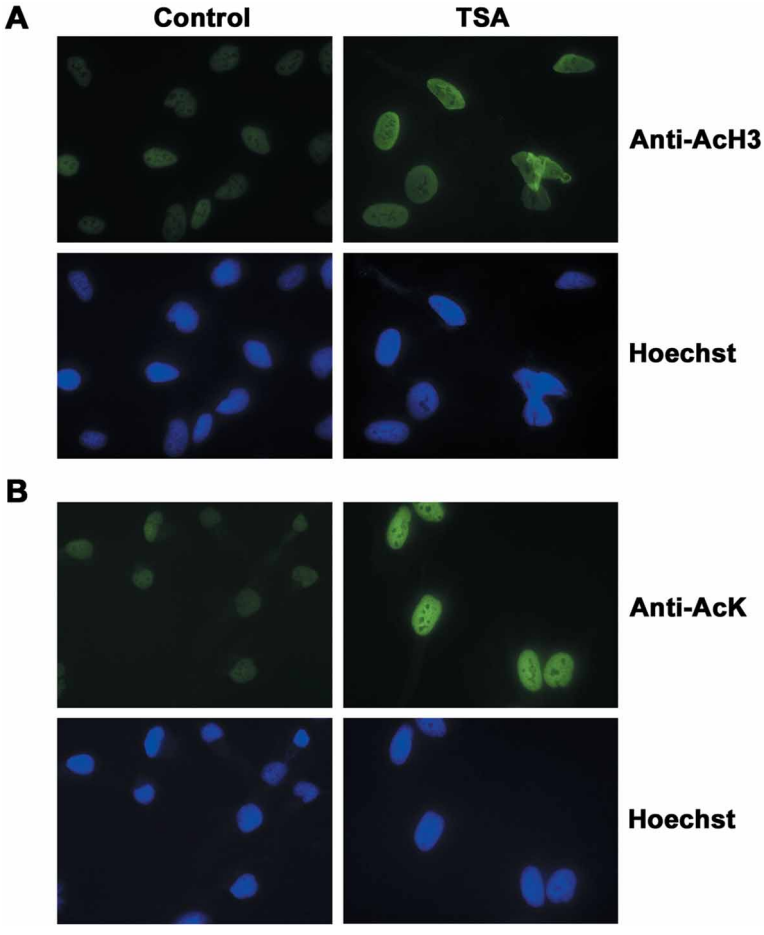

Figure 4. Immunofluorescent staining of nuclear localization of acetylated histones with acetylation antibodies. Control or TSA-treated (200 ng/mL) MMRU cells for 2 hr were stained with anti-acetylated histone H3 (AcH3) (A) and with anti-AcK (B) antibodies. Hoeschst staining was performed to detect the DNA in the nucleus.

antibody is useful for the detection, identification, isolation, and intracellular localization of acetylated proteins.

ABBREVIATIONS

AcK, acetylated lysine; BSA, bovine serum albumin; HDAC, histone deacetylase; KLH, hemocyanin of keyhole limpets; PBS, phosphate buffered saline; TSA, trichostatin A.

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