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ORIGINAL PAPER

Site-Specific Acetyl Lysine Antibodies Reveal Differential Regulation of Histone Acetylation upon Kinase Inhibition

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Abstract Lysine acetylation regulates diverse biological functions for the modified proteins. Mass spectrometry-based proteomic approaches have identified thousands of lysine acetylation sites in cells and tissues. However, functional studies of these acetylation sites were limited by the lack of antibodies recognizing the specific modification sites. Here, we generated 55 site-specific acetyl lysine antibodies for the detection of this modification in cell lysates and evaluated the quality of these antibodies. Based on the immunoblotting analyses, we found that the nature of amino acid sequences adjacent to the modification sites affected the specificity of the site-specific acetyl lysine antibodies. Amino acids with charged, hydrophilic, small, or flexible side chains adjacent to the modification sites increase the likelihood of obtaining high quality site-specific acetyl lysine antibodies. This result may provide valuable insights in fine-tuning the amino acid sequences of the epitopes for the generation of site-specific acetyl lysine antibodies. Using the site-specific acetyl lysine antibodies, we further discovered that acetylation of histone 3 at four lysine residues was differentially regulated by kinase inhibitors. This result demonstrates the potential application of these antibodies in the study of new signaling pathways that lysine acetylation may participate in.

Keywords Acetylation · Acetyl lysine antibody · Hydrophilicity · Histone 3 · Kinase inhibitor

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Introduction

Post-translational modifications (PTMs) play important roles in regulating diverse cellular functions [1]. The ε-amine on the side chain of lysine residues can be modified with an acetyl group both enzymatically or non-enzymatically [2]. Acetyltransferases covalently add an acetyl group to the lysine side chain while deacetylases remove the conjugated acetyl groups from the modified proteins [3]. These two classes of enzymes dynamically regulate lysine acetylation in cells. Lysine acetylation can regulate chromatin structure, gene expression, metabolism, cell structure, and many other biological processes [4]. Dysregulation of lysine acetylation is associated with many diseases, such as cancer [5], cardiovascular diseases [6], and Alzheimer's disease [7]. Due to the strong connection between lysine acetylation and human diseases, these enzymes have become attractive molecular targets for drug discovery [8, 9].

High-throughput proteomic approaches have precisely identified thousands of acetylation sites in cells and tissues [10–13]. The functions of the majority of lysine acetylation

sites are mostly unknown although the roles of acetylation of histones and many transcription factors, such as p53, have been studied extensively [14, 15]. It has also been discovered that many proteins have multiple acetylation sites [10]. However, it is largely unknown whether these acetylation sites are regulated differentially or similarly in a cell signaling event. Application of antibodies that can specifically recognize the acetylated lysine sites in the context of neighboring amino acids will allow us to readily detect the relative acetylation level of these sites and their dynamic regulation in cell signaling events, leading to the elucidation of their biological functions. For many small molecular weight PTMs, such as phosphorylation, methylation, and acetylation, it is possible to generate antibodies that recognize site-specific modification in the context of neighboring amino acids although these modification moieties are relatively small.

Some principles in the selection of peptide sequences for antigen generation have been applied in the production of antibodies recognizing non-modified proteins. Usually, surface exposed and charged residues without strong secondary structures serve as good antigenic regions [16, 17]. Computational approaches have also been developed to study the correlation between epitopes and the specificity of the generated antibodies [18, 19]. Whether similar rules can be applied in the selection of epitopes for the site-specific PTM antibodies has not been explored. Generally, it is more difficult to generate site-specific PTM antibodies than to generate antibodies that recognize the corresponding non-modified proteins. This is because in the latter case, multiple peptide sequences at various locations of a protein may be used as epitopes, while in the former case, the modification site is fixed and, therefore, the selection of the amino acid sequences for the epitope is limited. In addition, it is difficult to estimate whether a small modification on a peptide could generate an antibody sufficiently different from that for the non-modified peptide during the immunization.

In order to study the relationship between the antigenic region and the quality of site-specific antibodies, we generated more than 50 polyclonal antibodies, which were designated for the recognition of the site-specific acetyl lysine modification. We used enzyme-linked immunosorbent assay (ELISA) and Western blotting to evaluate the specificity and the quality of these antibodies. Further analyses of the properties of the amino acids adjacent to the modification sites discovered some general principles for the selection of peptides to obtain acetyl lysine antibodies with high specificity. Western blotting analyses with four histone 3 acetylation antibodies recognizing modifications at different sites revealed that the acetyl lysine modification was differentially regulated upon kinase inhibition.

Materials and Methods

Materials

Amino acids for solid phase peptide synthesis (SPPS) were from Suzhou Tianma Specialty Chemicals Co. Wang Resin was from Tianjin Nankai Hecheng S&T Co. Anti-histone 3 antibody and secondary antibody were from Cohesion Biosciences. ECL chemiluminescent horseradish peroxidase (HRP) substrate was from Millipore. Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC), Sulfo-Link^{TM} coupling gel, bicinchoninic acid (BCA) protein assay, and keyhole limpet hemocyanin (KLH) were from Thermo Fisher. Bovine serum albumin (BSA) was from MP Biomedicals. 5,6-dichlorobenzimidazole riboside (DRB, an inhibitor for casein kinase I and II [20]) was from Sigma and roscovitine (Rosc, an inhibitor for cyclin-dependent kinases [21]) was from Selleck.

Synthesis, Characterization, and Conjugation of Acetyl Lysine Peptides

Peptides containing acetylated lysines were synthesized manually by SPPS procedure [22] using the Wang resin. During the peptide synthesis, all the amino acids used were Fmoc (N-α-(9-fluorenylmethyloxycarbonyl) modified amino acids whose functional groups at the side chains were protected. When the acetylation sites were incorporated into the designated sites of the peptides, Fmoc-lysine ($COCH_3$)–COOH instead of the *t*-butyloxy carbonyl (Boc) protected lysine, Fmoc-lysine (Boc)–COOH, was used. Only one acetyl lysine was incorporated into each peptide. An additional cysteine residue was added at the N-terminus of the acetyl peptides to facilitate their conjugation to the carrier. Peptides were cleaved from the resin with trifluoroacetic acid, precipitated, and purified in a high performance liquid chromatography (HPLC, LC-2010A, SHIMADZU). Twenty milligrams of each acetyl lysine peptide were obtained. The relative molecular weights of peptides were measured by electrospray ionization (ESI)-mass spectrometry (MS) (U3000 LC-MS, DIONEX) and the purity was analyzed in a reversed-phase analytical HPLC with a C18 column (Vydac, USA). The corresponding non-acetylated peptides were also synthesized and used for the evaluation of the acetyl lysine antibodies and for the removal of antibodies recognizing the non-acetyl lysine containing proteins.

The peptides were further conjugated to the carrier protein, KLH, with the N-terminal cysteine to obtain antigens according to a protocol described previously [23]. Briefly, KLH was first reacted with the N-hydroxylsuccinamide moiety in Sulfo-SMCC to form multiple functional groups.

The excess amount of Sulfo-SMCC was removed through a desalting column (GE Healthcare). Then, 3 mg of the functionalized KLH were further reacted with 3 mg of peptides. In this reaction, the cysteine residue at the N-terminus of peptides reacted with the maleimide moiety on KLH to obtain the antigen conjugated with acetyl lysine peptides. The unreacted maleimide moiety on KLH was blocked with free L-cysteine. The antigen was dialyzed three times against phosphate buffered saline (PBS) to remove small molecular reagents or reaction products. In total, 55 antigens were prepared from this work. The acetyl lysine peptides and the non-acetylated peptides were also conjugated to BSA for an initial evaluation of the antibodies.

Immunization and Antibody Production in Rabbits

Two New Zealand white rabbits (16 weeks old) that were raised in specific pathogen-free conditions were immunized with each antigen according to a similar method described previously [24]. Briefly, 200 µg of antigen were injected into the rabbits subcutaneously for the first immunization. Immunization was boosted three times with three week intervals. One week after the final immunization, 50 mL of blood were collected and antiserum was obtained after centrifugation. The animal protocol was approved by the institutional reviewer board.

Antibody Purification

The antiserum was purified through the affinity column coupled with the corresponding acetyl lysine peptides. First, SulfoLink™ coupling gel was equilibrated with the coupling buffer (50 mM Tris-HCl pH 8.5 and 5 mM EDTA). The acetyl and non-acetyl lysine peptides were separately conjugated to the gel in the coupling buffer at 4 °C overnight. The conjugated SulfoLink coupling gel was washed with the coupling buffer and the unreacted functional groups were blocked with 50 mM L-cysteine at room temperature for 2 h. The gel was washed sequentially with 1 M NaCl, PBS, 0.1 M glycine (pH 3.0), and PBS. The antiserum was then incubated with the gel conjugated with acetyl lysine peptides at 4 °C overnight. The gel was washed with PBST (PBS with 0.1% tween 20) and PBS. The purified antibody was eluted with 0.1 M glycine (pH 3.0) within 5 min and immediately neutralized to pH 7.4 with 1 M Tris-HCl (pH 7.5). The purified antibody was further passed through the SulfoLink coupling gel conjugated with the non-acetylated peptides and dialyzed against PBS three times (each for 4 h). It was then concentrated with PEG20,000 (Sinopharm Chemical Reagent Co.), mixed with glycerol at a 1:1 ratio, and stored at −20 °C. The concentration of the purified antibody (about 1 mg/mL) was determined by BCA protein assay.

ELISA for Antibody Screening

ELISA [25] was used for the initial evaluation of the quality of the acetyl lysine antibodies. The BSA conjugated with the acetyl lysine peptides or the non-acetylated peptides was immobilized on the surface of a 96-well plate overnight. The wells were washed three times with PBST and the purified antibodies were added in serial dilutions and incubated with the acetyl lysine antibodies. The wells were washed three times with PBST to remove the non-specific background binding. Secondary antibodies conjugated with HRP were further incubated in each well followed by extensive washing. Chemiluminescent HRP substrate was added to the wells. After 30 min incubation, 1 M H_2SO_4 was used to quench the reaction and the optical density (O.D.) was measured at 490 nm with a plate reader. The experiments were carried out four times.

Cell Culture and Drug Treatment

HEK293T cells were cultured in growth medium composed of high glucose DMEM (Hyclone), 10% FBS (Transgene), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco). HEK293T cells without treatment or treated with DMSO, DRB or Rosc (50 µM) for 12 h were washed with ice-cold PBS and lysed in the modified RIPA buffer on ice for 15 min [26, 27]. The cell lysates obtained after centrifugation were mixed with lithium dodecyl sulfate (LDS) sample buffer, heated at 98 °C for 10 min, centrifuged, and separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for immunoblotting analysis.

Western Blotting Analysis

Western blotting analysis was conducted according to a published procedure [28]. Briefly, 20 µg of whole cell lysate were separated on an SDS–PAGE. Proteins were transferred to immobilon PVDF membrane (Millipore) and the membrane was blocked with 5% non-fat milk in PBS for 1 h at room temperature. Each lane on the membrane was cut and incubated with corresponding antibodies for immunoblotting.

Three immunoblotting experiments were performed to characterize the site-specific acetyl lysine antibodies. The first blot was incubated with antibodies recognizing the non-modified proteins, and the second blot was incubated with the site-specific acetyl lysine antibodies. The third blot was incubated with the acetyl lysine antibodies that were pre-incubated with the corresponding acetyl lysine peptides. The blots were washed three times with PBST, incubated

with the secondary antibody, washed again four times with PBST and the bands were visualized with the immobilon ECL chemiluminescent HRP substrate in a Bio-Rad ChemiDoc imaging system. The blots were stripped and immunoblotted again for β-tubulin to show similar sample loading.

For the quantification, Western blotting experiments for three sets of samples prepared separately were performed and the images were recorded for the detection of histone 3 acetylation. The relative signal intensity for acetylated bands was quantified with ImageJ and normalized to total histone 3. Then signals for DRB-treated and Rosc-treated samples were further normalized to that from the DMSO-treated sample. Means along with standard error of measurements (SEMs) were plotted on the figure. Student's *t* test was used to obtain the *P*-value against the DMSO-treated sample.

Immunofluorescence

Immunofluorescence experiments were carried out using a previously published method [29]. Briefly, HeLa cells were grown to 80% confluency in a 24-well plate with growth medium same as HEK293T. Then cells were starved with DMEM without FBS for 24 h and treated with trichostatin A (Sigma, 400 nM) for 24 h in a 5% CO_2 incubator at 37 °C. Cells were further fixed with formalin and permeabilized with 0.1% Triton X-100 in TBS for 5 min and blocked with 3% BSA in PBS for 30 min at room temperature. Cells were washed three times with ice-cold PBS and probed with the primary antibody in 3% BSA in PBS overnight in a humidified chamber at 4 °C. Cells were washed again, incubated with a FITC-conjugated secondary antibody (Sigma) at room temperature in dark, and washed extensively. The images were taken under a fluorescence microscope with the excitation wavelength of 494 nm and the emission wavelength of 520 nm.

Bioinformatic Analysis of Amino Acid Sequences

Three amino acids from each side of the acetylated lysines were extracted from the peptide sequences. The relative frequency of each amino acid adjacent to the acetylated lysines was plotted with WebLogo (http://weblogo.berkeley.edu/logo.cgi) after sequence alignment [30, 31]. In the WebLogos, the height of each letter represents the frequency of the amino acids in the subset of the peptides used for antibody generation [32]. The amino acids were categorized into three groups: charged, hydrophilic, and hydrophobic residues. They were then analyzed for two sets of antibodies: specific and non - specific. The hydrophobicity was calculated based on the Hopp–Woods [18] and Kyte–Doolittle [19] methods for the lysine and the adjacent six amino acids in each peptide.

Results

Characterization of Acetyl Lysine Peptides from SPPS

Sequences of acetyl lysine peptides were extracted from proteins that were identified to contain lysine acetylation modification. The following principles were applied for the selection of the specific peptide sequences for antibody production if possible. (1) The acetylated lysines were chosen to be located at the middle of the peptides. (2) Cysteines were avoided to eliminate potential modifications, such as disulfide formation and oxidation, and to prevent the interference with peptide conjugation to the carrier protein. (3) The peptide sequence was slightly shifted to the N-terminus or the C-terminus to avoid proline. (4) Strong preference on secondary structures was avoided. (5) Peptides with high solubility were selected. (6) Conserved sequence in human and mouse species was selected. These criteria might increase the likelihood of generating successful antibodies.

Peptides were synthesized with the traditional SPPS method with an acetyl-modified Fmoc-lysine residue at the modification site. An additional cysteine was added to the N-terminus of the peptides. The synthesized peptides were cleaved, purified by HPLC, and analyzed with ESI-MS and reversed-phase analytical HPLC. The ESI-MS spectra of two representative peptides, acetyl-cytokeratin (K194) CQIKTLNNkFASFIDK and acetyl-HSP90 (K292) CIDQEELNkTKPIWTR (the lowercase k represents the acetylation site), were shown in Fig. 1. These peptides are doubly charged and the calculated relative molecular weights match the theoretical value. The analytical HPLC trace showed one major peak, indicating the high purity of resulting peptides (Supplementary Fig. S1).

Production and Purification of Polyclonal Acetyl Lysine Antibodies

The procedure for antibody production was schematically represented in Fig. 2. Briefly, the synthesized peptides were first conjugated to KLH using the N-terminal cysteine residue as the functional group and the conjugates were injected to rabbits for immunization. After three immunizations, the blood was collected from the rabbits and antiserum was obtained after centrifugation. The antiserum was further purified through the affinity beads covalently conjugated with the corresponding acetyl lysine peptides. The antibodies recognizing non-acetylated peptides were removed by passing the antibodies through the beads

conjugated with non-acetylated peptides. The purified antibodies were tested by ELISA for initial evaluation. Western blotting was carried out with cell lysates from different cell lines or animal tissues to detect endogenous proteins acetylated at the specific lysine residues.

Characterization of Acetyl Lysine Antibodies

Antibodies were first evaluated with ELISA. The BSA conjugated with the acetyl lysine peptides or the corresponding non-modified peptides was used for the ELISA measurement. The representative results for four acetyl lysine antibodies were shown in Fig. 3. All of them showed a decrease in O.D. after serial dilutions for the acetyl peptides, but almost a constant O.D. with a much smaller value for the non-acetylated peptides. This result indicated that the antibodies could specifically recognize the acetyl lysine peptides but not the non-modified peptides.

The acetyl lysine antibodies were further evaluated by Western blotting of cell lysates from different cell lines. Representative Western blotting images with four antibodies were shown in Fig. 4. In these images, each antibody specific for non-modified proteins detected a clear band at the expected molecular weight. Blotting the same sample with the acetyl lysine specific antibody on a different blot detected a clear band at the same location. In addition, when the acetyl lysine antibody was preincubated with the acetyl lysine peptides for competitive binding, the antibody was no longer able to detect the same band. Images with eight additional acetyl lysine antibodies were shown in Supplementary Fig. S2. Together with the ELISA experiment, these results demonstrated that the antibodies could specifically recognize endogenous lysine acetylation at the designated location and do not recognize modification at other sites.

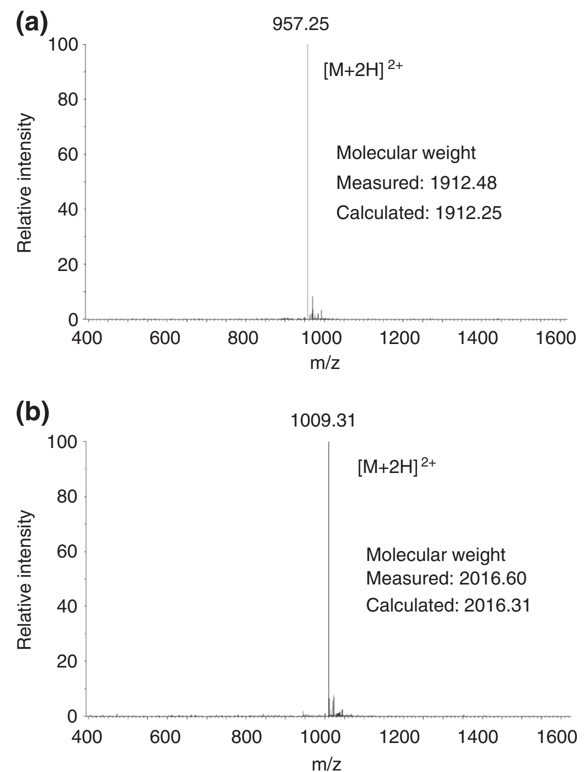

Fig. 1 ESI-MS analyses of two acetyl lysine peptides. The peptides were synthesized by solid phase peptide synthesis, purified by HPLC, and analyzed by ESI-MS. **a** ESI-MS trace for an acetyl-Cytokeratin (K194) peptide with a sequence of CQIKTLNNkFASFIDK. **b** ESI-MS trace for an acetyl-HSP90 (K292) peptide with a sequence of CIDQEELNkTKPIWTR. The lowercase k represents the acetylation site. The HPLC traces for the purified peptides were shown in Supplementary Fig. S1

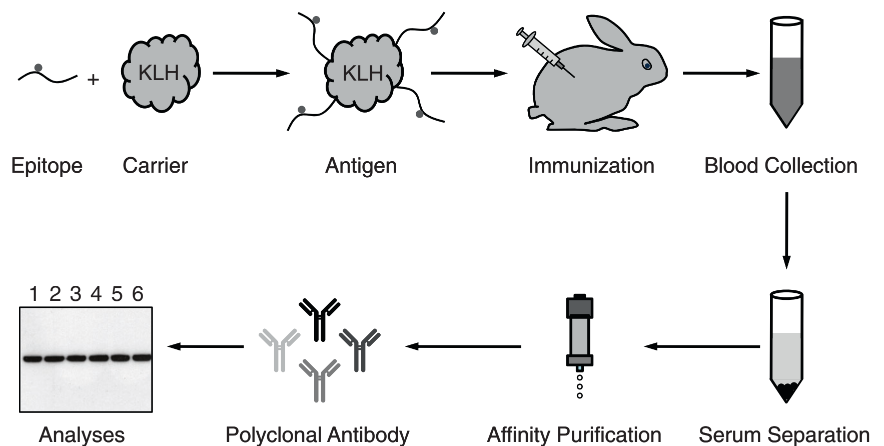

Fig. 2 Flowchart for the production of polyclonal acetyl lysine antibodies. The synthesized acetyl lysine peptides were first conjugated to keyhole limpet hemocyanin (KLH) for immunization in rabbits. Blood was collected and antiserum was obtained after centrifugation. The antibodies were further purified through an affinity column immobilized with the corresponding acetyl lysine peptides. Antibodies recognizing the non-acetylated peptides were removed by passing through beads coupled with non-acetyl peptides. The purified antibodies were further evaluated by ELISA and Western blotting analyses

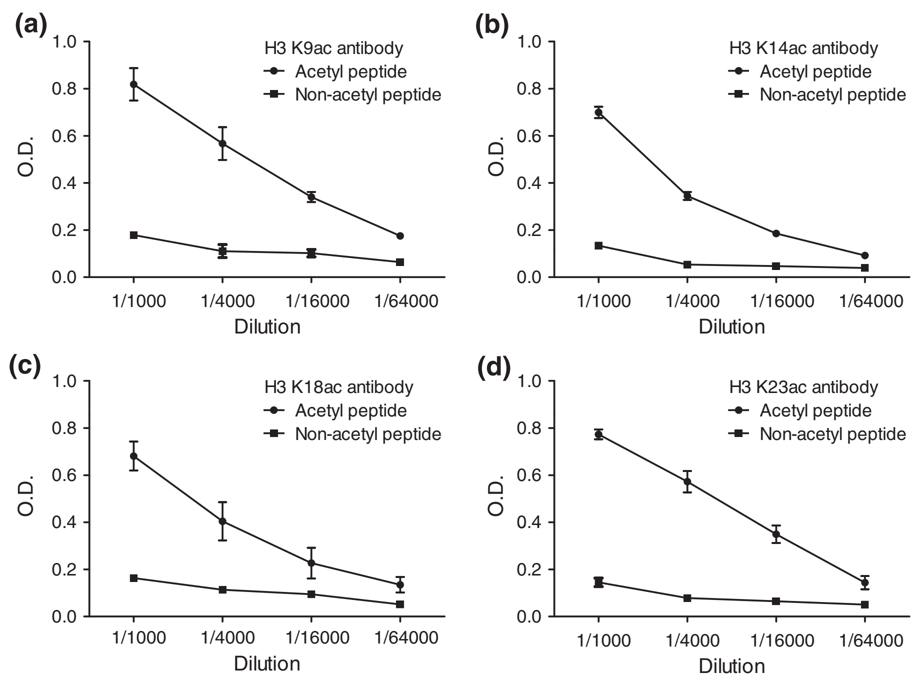

Fig. 3 ELISA analyses for four acetyl lysine antibodies. The antibodies were diluted as indicated and the optical density (O.D.) of the ELISA experiments with acetyl lysine peptides or non-acetyl peptides was measured at 490 nm. The antibodies shown here were histone 3 acetylation antibodies recognizing **a** H3 K9ac; **b** H3 K14ac; **c** H3 K18ac; **d** H3 K23ac. The experiments were repeated four times and the means and standard error of measurements (SEMs) were depicted in the plots. The first methionine is not counted during the numbering of amino acids for histone 3 in order to be consistent with previous publications

Based on the ELISA screen and the quality of Western blotting images, the antibodies were classified into two groups, "specific" and "non-specific". The following criteria were used to define a specific antibody. (1) The ELISA experiment detected the acetyl lysine peptides but not the corresponding non-acetylated peptide. (2) Only one distinct band was detected in the Western blotting of cell lysate or animal tissues with the acetyl lysine antibody. (3) The position of the band detected by the acetyl lysine antibody was at the location of the band when the same sample was blotted with an antibody recognizing the non-modified protein. (4) When the antibody was preincubated with the acetyl lysine peptide, no band was detected in the Western blotting. These stringent criteria (Table 1) can ensure that the acetyl lysine antibodies have high quality. However, these criteria may also lead to the exclusion of slightly suboptimal antibodies from the "specific antibody" group.

In our experiments, many antibodies did not detect endogenous lysine acetylation sites with satisfactory results on the Western blotting under the experimental conditions used in this work. These antibodies were defined as "non-specific" because they gave inconsistent results in Western blotting. In some cases, no distinct bands were detected at the expected molecular weight, multiple bands at different positions were detected, or the detected band at the expected location was not the most intense band. The detailed information about antibodies generated for 55 acetyl lysine peptides was shown in Supplementary Table S1. Among these, 24 antibodies were classified as "non-specific" antibodies and 31 as "specific" antibodies based on the aforementioned criteria.

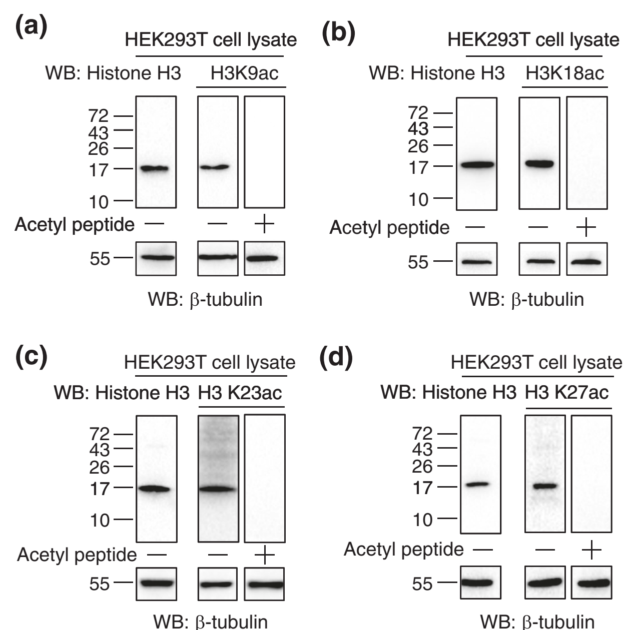

Fig. 4 Western blotting analyses of cell lysates with four representative acetyl lysine antibodies. Images for **a** histone H3 K9ac, **b** histone H3 K18ac, **c** histone H3 K23ac, and **d** histone H3 K27ac in HEK293T cell lysate. In each panel, the left image was blotted for the non-modified protein, the middle image blotted for the acetyl lysine modified proteins, and the right image blotted for the acetyl lysine modified proteins with antibodies pre-incubated with competitive acetyl lysine peptides. The presence of competitive acetyl lysine peptides in the antibody resulted in the loss of chemiluminescence signal in the Western blotting analysis of proteins containing the corresponding acetyl lysine. The experiments were carried out at least twice for each antibody. Immunoblotting of β-tubulin was performed simultaneously for three samples in each panel after stripping, which was used to show similar sample loading

Table 1 Criteria used for the evaluation of acetyl lysine antibodies

Measurement	Specific acetyl lysine antibodies	Non-specific acetyl lysine antibodies[a]
ELISA to detect acetyl lysine peptides	Signal detected	No signal detected
ELISA to detect non-acetyl lysine peptides	No signal detected	Signal detected
Western blotting	One specific band matching protein size	No band, multiple bands, or the most intense band does not match protein size
Western blotting with antibodies preincubated with acetyl lysine peptides	No signal detected	Signal detected

[a] Antibodies were considered as non-specific antibodies when one of the situations was detected during the measurements

In order to test whether the antibodies could be used for immunofluorescence experiments, we chose four acetyl histone 3 antibodies for immunostaining of HeLa cells. After cells were treated with an HDAC inhibitor, trichostatin A, and stained with acetyl histone 3 antibodies, we detected significant amounts of fluorescence signals for H3 K9ac, H3 K18ac, H3 K23ac, and H3 K122ac (Fig. 5, left images). However, when the antibodies were preincubated with the corresponding acetyl peptides, no fluorescence signals were observed (Fig. 5, right images), suggesting that the antibodies were highly specific for the recognition of acetylated histone 3 by immunofluorescence. In addition, the fluorescence signals detected were from the nucleus, consistent with its nuclear localization. This result demonstrated that the acetyl antibodies could be used for immunofluorescence studies.

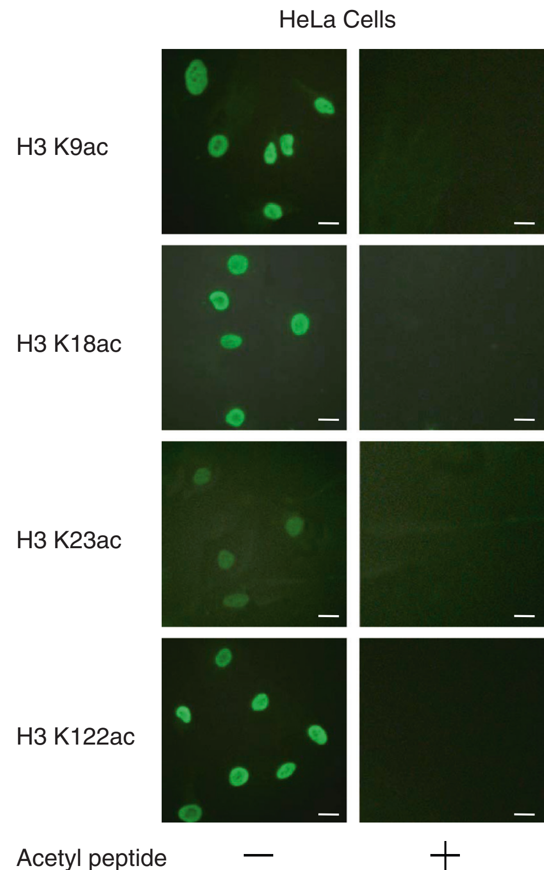

Fig. 5 Immunostaining of HeLa cells with acetyl histone 3 antibodies recognizing K9ac, K18ac, K23ac, or K122ac. The left and right images were obtained from immunostaining of cells with antibodies without and with preincubation of the corresponding acetyl histone peptides. Scale bar: 20 μm

Effect of Hydrophobicity and Charge State of the Acetyl Lysine Peptides on the Quality of Acetyl Lysine Antibodies

After obtaining these site-specific acetyl lysine antibodies, we sought to investigate whether the quality of the site-specific antibodies had any preference to the amino acid sequence adjacent to the modification sites. In general, the length of an epitope is about 5~11 amino acids. If the peptide is too short, the resulting antibodies may recognize multiple proteins containing the same epitope. If the epitope is too long, the probability of generating antibodies that do not recognize the modification site may be high. Here, we extracted three amino acids from each side of the modification sites to analyze the properties of these amino acids.

First, we calculated the percentage of each of the 20 amino acids in the extracted amino acid sequences from the epitopes used for the generation of two sets of antibodies (Fig. 6a). The results showed that the peptides that generated specific antibodies had a slight preference for D, E, K, N, P, and R adjacent to the modification sites. The peptides that produced non-specific antibodies had a slight preference for A, L, Q, T, V adjacent to the modification sites. The WebLogo of the alignment of the two sets of amino acid sequences highlighted similar results with specific antibodies preferring G, K, and R at the position adjacent to the modification sites (Fig. 6b).

We then classified the amino acids into three categories: charged, hydrophobic, and hydrophilic, and compared their percentages for the two sets of antibodies. The analyses showed a strong preference of charged residues for specific antibodies while the peptides generating non-specific antibodies had a higher percentage of hydrophobic residues (Fig. 7a). We further quantified the hydrophilicity based on the Kyte–Doolittle scale [19] for a span of six amino acids

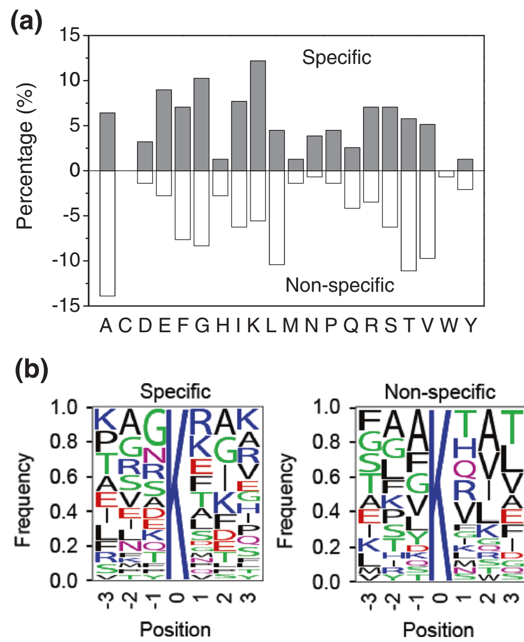

Fig. 6 Sequence analyses for the amino acids adjacent to the acetyl lysine modification sites. **a** Percentage of each amino acid adjacent to the modification sites from the specific and non-specific antibodies. **b** WebLogos of amino acid sequences adjacent to the modification sites

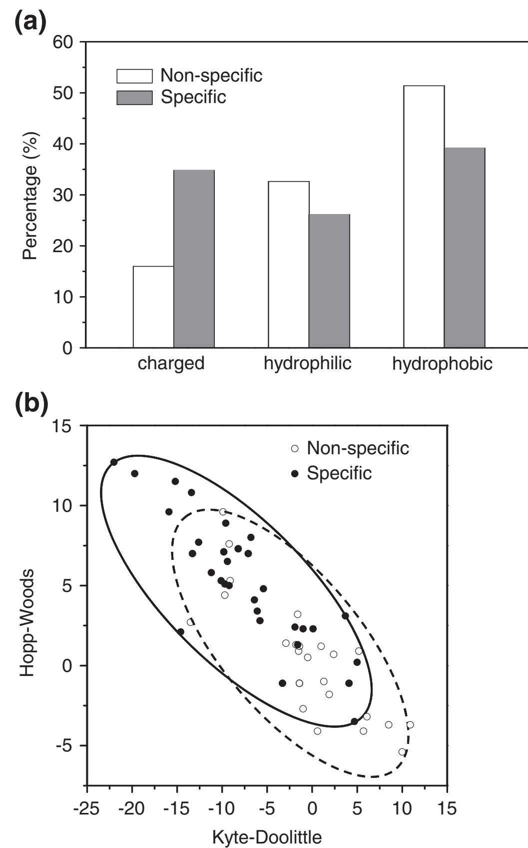

Fig. 7 Hydrophilicity analyses of acetyl lysine peptides used for antibody production. **a** The relative percentage of the charged, hydrophilic, and hydrophobic amino acids in the acetyl lysine peptides. **b** Hopp–Wood and Kyte–Doolittle plot of acetyl lysine peptides. The solid and dashed ellipses, used to guide the eyes, include points from specific or non-specific antibodies, respectively

adjacent to the acetylation sites and the lysine residue itself. The hydrophilic region has a negative value in the Kyte–Doolittle scale. In addition, we predicted the potential antigenic sites based on the Hopp–Wood scale [18], which basically represents a hydrophilic index. The apolar residues were assigned negative values and polar residues were assigned positive values. In general, the higher the Hopp–Wood value, the higher the hydrophilicity of the peptides. Therefore, the likelihood of being a good antigenic site is high. The calculation of hydrophilicity of the two sets of peptides showed that peptides generating specific antibodies had higher (positive) Hopp–Wood value and lower (negative) Kyte–Doolittle value (Fig. 7b). The result is in line with previous publications which predicted the antigenic regions for proteins [16, 18, 19].

Histone Acetylation is Differentially Regulated by Kinase Inhibitors

The next question we wanted to ask is whether the acetyl lysine antibodies can provide important information about lysine acetylation in cell signaling events. Our previous experiments demonstrated that kinase inhibitors, DRB (inhibits casein kinase I and II [20]) and Rosc (inhibits cyclin-dependent kinases [21]), induced apoptosis in OPM2 cells [27]. In addition, it has also been reported that DRB is a transcription inhibitor [33, 34], which might alter histone acetylation. Therefore, we sought to investigate whether DRB and Rosc could affect histone acetylation at various lysine residues. In order to see whether such effect is a general phenomenon, we examined the acetylation of histone 3 in HEK293T cells. Four site-specific acetyl lysine antibodies recognizing acetylated lysine at 9, 18, 23, and 122 on histone 3 were chosen for this experiment (Fig. 8a and b). Western blotting analyses showed that the acetylation of histone 3 at K9, K18, and K23 was significantly reduced after DRB treatment for 12 h. The acetyl modification at K9 and K18 was moderately reduced upon Rosc treatment. Most of them had P-values of <0.05 compared to the DMSO-treated samples. The only exception was that the P-value for the acetylation level of K23 on histone 3 of the Rosc-treated sample was not statistically significant due to the large variation in three independent experiments. However, when we examined the acetylation site (K122) at the C-terminus of histone 3, we found that neither DRB nor Rosc altered the acetylation level at this site. These results demonstrated that the acetylation of histone 3 at these sites was differentially regulated upon kinase inhibition.

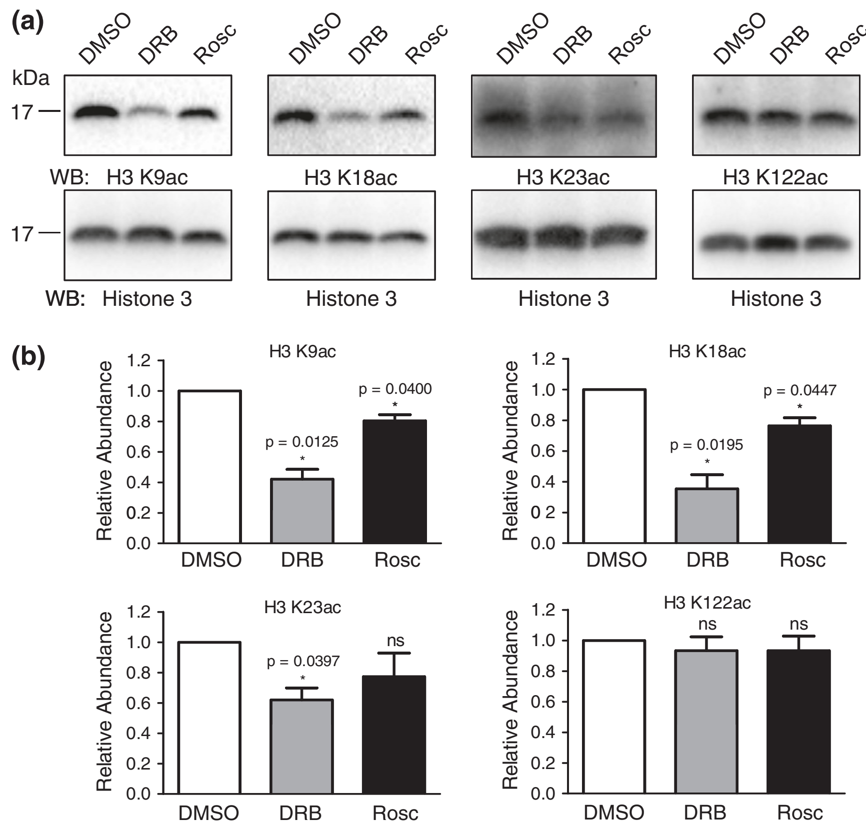

Fig. 8 Site-specific acetyl lysine antibodies revealed differentially regulated histone 3 acetylation in HEK293T cells upon kinase inhibition. Western blotting images (**a**) and quantification (**b**) of histone 3 acetyl lysines at K9, K18, K23, and K122 for cell lysates from HEK293T cells treated with DMSO or kinase inhibitors (50 µM DRB or Rosc) for 12 h. Histone 3 was used as a loading control for normalization. The quantification (**b**) was obtained from three biological replicates and the means along with standard error of measurement (SEM) were plotted in the figures. The *P*-value was calculated with Student's *t* test compared with the DMSO-treated samples ($^*P < 0.05$)

Discussion

Lysine acetylation plays important roles in regulating many cellular functions. High-throughput proteomics have identified thousands of acetylation sites in cells and animal tissues [10–12, 35]. Site-specific acetyl lysine antibodies are a wealth of resources for the functional study of this modification. Here, we tried to make polyclonal antibodies that recognize the acetyl lysine modification at specific sites. Our experiments successfully generated 31 antibodies that specifically recognize the endogenous lysine acetylation at the designated location. However, 24 of the 55 antibodies did not show high specificity to the acetylated proteins in complex cell lysates using standard Western blotting analysis. Amino acid sequence analyses revealed that the peptides which produced site-specific acetyl lysine antibodies contained a higher percentage of amino acids with charged, hydrophilic, small, or flexible side chains. This result may guide us to choose the optimized peptides for the generation of satisfactory acetyl lysine antibodies. Although the modification sites determine the amino acid sequences that can be used for the production of site-specific antibodies, it is still possible to slightly shift the peptide sequence to the amino or carboxyl terminus of the modification sites to optimize the above properties. This principle may also be used in the generation of site-specific antibodies other than lysine acetylation.

Our experiments may exclude some moderately specific antibodies due to the stringent criteria we used to define specific antibodies. These criteria may exclude antibodies that recognize the lysine acetylation with weak or non-specific bands in the Western blotting analyses. Antibodies with low affinity to the acetylated lysine may also have been missed in the standard Western blotting analyses due to the weak signal or the requirement of special procedures for Western blotting. Some proteins have multiple isoforms or splicing variants that contain the acetylation site with the same adjacent amino acid sequence. In this case, multiple bands may be detected on the blot due to their different molecular weights. Hence, our criteria may classify antibodies recognizing these sites as non-specific antibodies. The abundance and the occurrence of protein acetylation at

specific sites may be cell-type specific or regulated by different cell signaling events [36, 37]. Therefore, the cell lysates obtained under our experimental conditions may not contain all the acetylation sites recognized by the antibodies. Use of different samples may result in a thorough evaluation of the quality of the antibodies that were classified as "non-specific" in this work. The presence of other modifications adjacent to the acetylation sites may also prevent the recognition of lysine acetylation by the antibodies. In addition, the generation of antibodies may be affected by the immune response of individual animals used in the antibody production. In short, more antibodies in the list may be specific if the above situations were considered during the evaluation of antibody quality.

It is important to know whether acetylation at different sites of a protein is regulated differentially in a signaling pathway. By using four acetyl lysine antibodies recognizing different acetylation sites on histone 3 in immunoblotting, we showed that the acetylation levels at various sites of a protein were differentially regulated upon kinase inhibition. Our result in the regulation of H3 K9ac, H3 K18ac, and H3 K23ac showed that upon the addition of kinase inhibitors, which could cause apoptosis in cancer cells [38], the acetylation level of histone 3 at some N-terminal sites is reduced. This result is consistent with previous findings which revealed that the expression of oncoproteins increased the levels of H3 K9ac and H3 K18ac [39] and that the high level of H3 K18 acetylation was associated with aggressive tumor phenotypes and poor prognosis [40]. This also suggested that these acetylation modifications might have distinct roles in regulating gene expression. These experiments demonstrated the usefulness of these site-specific acetyl lysine antibodies for the study of lysine acetylation in signaling events.

In summary, we have successfully generated a panel of site-specific acetyl lysine antibodies. Through the evaluation of the antibody quality and analyses of amino acid sequences, we found some general rules for the selection of the peptide sequence for the production of high quality site-specific lysine acetylation antibodies. Using these antibodies, we discovered that upon kinase inhibition, acetylation on different lysine residues on histone 3 was differentially regulated in a signaling event. This work may provide some guidelines in the selection of epitopes for successful generation of antibodies recognizing other PTMs at specific sites and demonstrates their potential use in the study of this modification in cell signaling events.

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Compliance with Ethical Standards

Conflicts of Interest The author, Shi Chen, has a potential conflict of interest given that he may be benefited financially from the sales of the acetyl lysine antibodies because of his current employment at a company that produces antibodies. The remaining authors declare that they have no conflicts of interest.

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