Molecular Characterization of Propionyllysines in Non-histone Proteins*

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Lysine propionylation and butyrylation are protein modifications that were recently identified in histones. The molecular components involved in the two protein modification pathways are unknown, hindering further functional studies. Here we report identification of the first three in vivo non-histone protein substrates of lysine propionylation in eukaryotic cells: p53, p300, and CREB-binding protein. We used mass spectrometry to map lysine propionylation sites within these three proteins. We also identified the first two in vivo eukaryotic lysine propionyltransferases, p300 and CREB-binding protein, and the first eukaryotic depropionylase, Sirt1. p300 was able to perform autopropionylation on lysine residues in cells. Our results suggest that lysine propionylation, like lysine acetylation, is a dynamic and regulatory post-translational modification. Based on these observations, it appears that some enzymes are common to the lysine propionylation and lysine acetylation regulatory pathways. Our studies therefore identified first several important players in lysine propionylation pathway. Molecular & Cellular Proteomics 8:45–52, 2009.

Acetylation of the ε-amino group of lysine residues, or lysine acetylation (Lys\textsuperscript{ac}), is one of several abundant post-translational modifications of the lysine side chain, and it has important roles in cellular physiology. Lysine acetylation was first identified in the 1960s in histones (1). Discovery of enzymes responsible for adding and removing acetyl groups (histone acetyltransferases (HATs))\textsuperscript{1} and histone deacetylases (HDACs) as well as non-histone substrate proteins (e.g. p53) in the mid-1990s marked a turning point in the field of lysine acetylation biology (2–4). Extensive studies over the past decade have established that lysine acetylation has diverse cellular functions and plays an important role in multiple diseases (5–10).

The high abundance of lysine acetylation in mammalian cells, as demonstrated in a proteomics screen (11), led us to hypothesize that the ε-amino group of lysine residues undergoes the structurally similar modifications of propionylation and butyrylation (Lys\textsuperscript{prop} and Lys\textsuperscript{buty}, respectively) (12). We confirmed our hypothesis in human histones and verified the discovery by 1) comparing the tandem mass spectra of the modified histone peptides with spectra obtained from synthetic peptides and 2) identifying the first two in vitro propionyl- and butyryltransferases, p300 and CBP (12). We also demonstrated that these two enzymes, which are also acetyltransferases, can carry out in vitro autopropionylation and autobutyrylation on lysine residues. In unpublished work\textsuperscript{2} we have also found lysine propionylation and butyrylation of histones from Saccharomyces cerevisiae. Lysine propionylation was subsequently reported in the Salmonella enterica propionyl-CoA synthetase enzyme PrpE (13). Elegant enzymological studies by Smith and Denu (14) suggest that some HDACs have measurable activity toward peptides containing propionyllysine and butyryllysine residues. Given the unique metabolic roles of acetyl-CoA, propionyl-CoA, and butyryl-CoA, which are the co-substrates for the modification reactions, as well as subtle structural differences among the modifications, we propose that lysine propionylation and lysine butyrylation have different biological functions than lysine acetylation. However, in vivo regulatory enzymes and non-histone substrates in eukaryotic cells remain to be characterized, hindering biological studies of the two modification pathways.

p53 is a short-lived protein whose activity is maintained at low levels in normal cells. Tight regulation of p53 is essential for its effect on tumorigenesis as well as maintaining normal cell growth. The cellular functions of p53 are rapidly activated in response to stress. Although the mechanisms of p53 activation are not fully understood, they are generally thought to entail post-translational modifications of p53, such as ubiquitination, phosphorylation, and acetylation (15–17). In fact,
p53 was the first non-histone protein found to be acetylated on lysine residues (3). Lysine acetylation regulates the protein’s stability (by competing with ubiquitination for modification of specific sites), its interactions with binding partners (e.g. Mdm2 and Mdmx), and its DNA-binding activity (18). Lysine acetylation status modulates p53-regulated effects in both cell cycle arrest and apoptosis. We recently demonstrated that p53 can also be lysine propionylated and butyrylated in vivo, catalyzed by p300 and CBP. Nevertheless, it remains unclear whether p53 is also propionylated and butyrylated by the same enzymes in human cells. Research into any biological pathway at the molecular level is dependent on the identification and isolation of the key players, which remain unknown for lysine propionylation pathway. The present work was undertaken to identify in vivo non-histone substrates of lysine propionylation in eukaryotic cells and the enzymes responsible for adding and removing the modification.

MATERIALS AND METHODS

Materials: Plasmids, Antibodies, and Other Reagents—Plasmids used in this study were described previously (12, 22). p300 HAT-dead mutant, p300DY, was generated by mutation of aspartic acid in 1399 to tyrosine; CBP HAT-dead mutant, CBP-LD, was generated by mutation of both leucine in 1345 and aspartic acid in 1346 to alanine; SirT1, enzymatic defective mutant was generated by replacing histidine in 363 by tyrosine. Generation of anti-propionyllysine antibody was described in Supplemental Fig. S1; anti-acetyllysine antibody was from (ImmunoChem Pharmaceuticals Inc. (Burnaby, British Columbia, Canada); anti-HA antibody was from Roche Diagnostics; anti-Sirt1 antibody was purchased from Upstate (Upstate, Charlotteville, VA); anti-FLAG M2 antibody, FLAG-M2 beads, and HA-agarose beads were purchased from Sigma-Aldrich. Modified porcine trypsin was purchased from Promega Inc. (Madison, WI). HPLC-grade acetonitrile, water, and ethanol were purchased from EMD Chemicals Inc. (Gibbstown, NJ); and acetic acid was purchased from Sigma.

Cell Culture, Cell Transfection, and Treatment—H1299 or 293T cells were maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO2. Cell transfection was carried out with Lipofectamine 2000 as described by the manufacturer (Invitrogen). For H1299 cells, cells were treated with 5 mM sodium butyrate; for 293T cells, cells were treated with HDAC inhibitor mixture (2 μM trichostatin A, 30 μM nicotinamide, and 50 mM sodium butyrate) for six hours to inhibit the activity of endogenous HDACs before harvesting the cells.

Detection of Lysine Propionylation of p53, p300, and CBP in Cells—Twenty-four hours after transfection with various plasmids, as indicated, transfected cells were treated with described HDAC inhibitors for 6 h, harvested, and lysed in FLAG-lys buffer (50 mM Tris-HCl, pH 7.9, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 10% glycerol, and fresh protease inhibitor mixture (Sigma)) plus 10 mM sodium butyrate. Propionylation of p53 in the total cell extracts or in material immunoprecipitated with M2-agarose beads was detected by Western blotting analysis using the anti-propionyllysine antibody described above. Propionylation of p300 or CBP in material immunoprecipitated with M2 or HA-agarose beads was detected by Western blot analysis using the anti-propionyllysine antibody.

Purification of p53, p300, and CBP Proteins—Transfected cells were lysed by NETN buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing described HDAC inhibitors and protease inhibitor mixture. Cell lysates were clarified by centrifugation at 100,000 × g for 20 min at 4 °C and incubated with either FLAG-M2 beads or HA beads (Sigma-Aldrich) at 4 °C for 1 h. Antibody beads were washed five times with NETN buffer. The bound proteins were eluted with SDS-PAGE sample buffer.

In-gel and In-solution Digestion with Trypsin—The proteins of interest purified above were separated by SDS-PAGE and visualized by staining with colloidal Coomassie Blue. The protein bands were excised, in-gel digested, and extracted as described previously (19). The extracted peptides were pooled, dried in a SpeedVac, and desalted using a μC18 ZipTip (Millipore, Billerica, MA) prior to HPLC/MS/MS analysis.

HPLC/MS/MS Analysis and Database Searching—The dried peptide sample was dissolved in 3 μl of HPLC buffer A (acetonitrile:water:acetic acid = 2:97.9:0.1, v/v/v) and loaded onto a home-made capillary column (10 cm length × 75 μm ID) packed with Jupiter C12 resin (4 μm particle size, 90 Å pore diameter; Phenomenex, St. Torrance, CA) connected to an Agilent 1100 nanoflow HPLC system (Agilent, Palo Alto, CA). Peptides were eluted with a gradient of 10% to 90% HPLC buffer B (acetonitrile:water:acetic acid = 90:9.9:0.1, v/v/v). Peptides were ionized and introduced into an LTQ mass spectrometer using a nano-spray source. The mass spectrometric data were acquired in data-dependant mode. Tandem mass spectra were analyzed using PTMap software, an algorithm we recently developed to identify all possible post-translational modifications in a substrate protein (20). The specified parameters for protein sequence database searching included Met oxidation as a variable modification, trypsin as the enzyme, three allowed missing cleavages, and mass errors of 4 Da for precursor ions and 0.6 Da for fragment ions. All identified peptides bearing modifications were manually inspected according to a procedure we described previously (21).

RESULTS

p300 and CBP Are Propionyltransferases for p53 in Vivo—Our previous experiments (12) demonstrated that p300 and CBP can catalyze in vitro lysine propionylation of p53. Given that p300 and CBP can catalyze both acetylation and propionylation reactions in vitro and that they can acetylate p53 in vivo, we reasoned that the enzymes are likely able to perform propionylation of p53 in vivo. To test this hypothesis, we used co-transfection experiments in H1299 cells followed by Western blotting analysis using a pan-propionyllysine antibody. As expected, lysine propionylation of p53 was dramatically increased when cells were co-transfected with either p300 or CBP (Fig. 1A), but not mutated, HAT-dead forms of these enzymes (B). These data in combination with our earlier in vitro results showing p53 lysine propionylation by p300 and CBP (12) suggest that these two enzymes can catalyze p53 propionylation in vivo.

We tested the ability of other acetyltransferases to propionylate p53 in vivo. For example, Tip60 is an acetyltransferase that can acetylate p53 at lysine-120. This modification site is critical for p53-dependent apoptosis, but not p53-mediated growth arrest (22). In contrast to p300 and CBP, little lysine propionylation activity was detected for Tip60 either in vitro (12) or in vivo (Fig. 1B). Co-transfection experiments using
four HATs (MOF, PCAF, Tip60, and HBO1) suggest that these proteins are not major enzymes responsible for propionylating lysine residues in p53 (Fig. 1B).

Sirt1 Can Catalyze Lysine Depropionylation in p53—Sirt1 is a known lysine deacetylase for p53 (23). To test if Sirt1 have enzymatic activity toward lysine propionylation in p53, we detected lysine propionylation levels in 293T cell by Western blotting analysis using a pan-propionyllysine antibody following co-transfection of p53 with wild type Sirt1 or its HDAC-defected mutant. As expected, treatment of cells with HDAC inhibitors induced lysine propionylation (Fig. 1C). Lysine propionylation level was decreased when co-transected with wild type Sirt1 but not its HDAC-defected mutant. Our results suggest that Sirt1 is likely a lysine depropionylation enzyme for p53.

Mapping Lysine Propionylation Sites in p53 by Mass Spectrometry—Although we previously identified the lysine residues within p53 that were propionylated by p300 and CBP in vitro (12), the in vivo modification sites remain unknown. To identify these sites, we co-transfected p53 into H1299 cells with either p300 or CBP, then isolated p53 by immunoprecipitation (Fig. 2A). The isolated proteins were subjected to SDS-PAGE, in-gel digestion, and HPLC/MS/MS analysis to map sites of lysine acetylation and propionylation. As expected, our analysis identified 11 lysine acetylation sites in p53 (Supplemental Fig. S2). We also identified one lysine propionylation site and three lysine butyrylation sites (Fig. 2, B and C). The propionyllysine sites were identified in p53 from cells co-transfected with p300/CBP, but not from cells without p300/CBP co-transfection (Fig. 2B), further suggesting that p53 is an in vivo propionyltransferase substrate for p300/CBP.

As expected, we found that several lysine residues (Lys372, Lys373, and Lys382) at the C terminus can be butyrylated, supporting the notion that multiple protein modifications of the C terminus including ubiquitination, acetylation, and methylation are involved in functional regulation of p53. Interestingly, we found that Lys292 is propionylated in cells, suggesting that modification of Lys292 by propinylation may mediate specific functions in vivo. Moreover, tumor-associated mutations of Lys292 have been observed in several different types of human tumors. Thus, it is important to understand the precise functional consequence of this modification in p53-mediated tumor suppression, which warrants future investigations.

p300 and CBP Are Lysine Propionylated in Vivo—We previously found that p300 and CBP can perform auto-lysine propionylation in vitro (12). To determine whether these proteins are propionylated in vivo, we transfected 293T cells with p300 or CBP, immunoprecipitated the proteins, and analyzed
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A

B In vivo lysine propionylated peptides and sites identified in p53

| Protein | K<sup>Prop</sup>/K<sup>Buty</sup> Sites | Peptide Sequence | Co-expression with |
|---------|--------------------------------------|------------------|-------------------|
| p53     |                                      |                  | control | p300        | p300 + Sirt1 | CBP |
| K292    | K<sup>Prop</sup>GEPHELPPGSTK<sup>R</sup> |                  | -       | +           | -            | +   |
| K372    | SK<sup>Buty</sup>K<sup>Prop</sup>GQSTSR |                  | -       | -           | -            | +   |
| K373    | SK<sup>Buty</sup>K<sup>Prop</sup>GQSTSR |                  | -       | -           | -            | +   |
| K382    | HK<sup>Buty</sup>LMFK                 |                  | -       | +           | +            | +   |

C

![MS spectrum of propionylated p53 peptides](image)

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for propionyllysine by Western blotting. Lysine propionylation of p300 was detected in these cells (Fig. 3A). In a subsequent attempt to increase lysine propionylation levels, we treated the cells with HDAC inhibitors for six hours before they were harvested. This treatment increased the amount of lysine propionylation detected (Fig. 3A), suggesting that deacetylases contribute to regulate the propionyllysine status. Likewise, in vivo propionylation of CBP was also detected under this experimental condition (Fig. 3B). To test if Sirt1, a class III HDAC, can remove propionyl group from p300, we co-transfected p300 into 293T cells along with Sirt1 or its HDAC-dead version of Sirt1. Lysine propionylation was significantly decreased when p300 was co-transfected with wild type Sirt1, but not the mutated form (Fig. 3C), implying that Sirt1 can catalyze in vivo lysine depropionylation of p300.

Next, we carried out mass spectrometric analysis of endogenous p300 and CBP immunoprecipitated directly from H1299 cells. To boost lysine propionylation levels, the cells were treated with HDAC inhibitors for six hours before the cells were harvested. HPLC/mass spectrometric analysis in combination with protein sequence database searching identified 27 lysine acetylation sites (Supplemental Fig. S3), 4 propionylation sites, and 4 lysine butyrylation sites in p300 (Fig. 4, A and C). Likewise, we identified 31 acetylation sites (Supplemental Fig. S4), and 2 lysine butyrylation sites (Fig. 4B) in CBP. We were not able to identify propionylation sites in CBP with mass spectrometry possibly because of low stoichiometry of the modification. Nevertheless, together with Western blotting analysis, these results demonstrate that (i) p300 is in vivo lysine propionylation substrate; (ii)
A) Lysine propionylated and butyrylated peptides identified in p300

| Protein | \( K^{\text{Prop}} / K^{\text{Buty}} \) Site | Peptide Sequence |
|---------|-----------------------------------|-----------------|
| p300    | K1554                             | NNK^{\text{Prop}}_{1554}TSK^{\text{Prop}}_{1554}NK^{\text{Ac}}_{1554}SSLSR |
|         | K1555                             | NNK^{\text{Prop}}_{1555}TSK^{\text{Prop}}_{1555}NK^{\text{Ac}}_{1555}SSLSR |
|         | K1558                             | NNK^{\text{Prop}}_{1558}TSK^{\text{Prop}}_{1558}NK^{\text{Ac}}_{1558}SSLSR |
|         | K1560                             | NNK^{\text{Prop}}_{1560}TSK^{\text{Prop}}_{1560}NK^{\text{Ac}}_{1560}SSLSR |
|         | K1554                             | NNK^{\text{Buty}}_{1554}TSK^{\text{Buty}}_{1554}NK^{\text{Ac}}_{1554}SSLSR |
|         | K1555                             | NNK^{\text{Buty}}_{1555}TSK^{\text{Buty}}_{1555}NK^{\text{Ac}}_{1555}SSLSR |
|         | K1558                             | NNK^{\text{Buty}}_{1558}TSK^{\text{Buty}}_{1558}NK^{\text{Ac}}_{1558}SSLSR |
|         | K1560                             | NNK^{\text{Buty}}_{1560}TSK^{\text{Buty}}_{1560}NK^{\text{Ac}}_{1560}SSLSR |

B) Lysine propionylated and butyrylated peptides identified in CBP

| Protein | Modification Sites | Peptide Sequence |
|---------|--------------------|-----------------|
| CBP     | K1595              | NNK^{\text{Buty}}_{1595}NKSSISR |
|         | K1597              | NNK^{\text{Buty}}_{1597}SSISR |

C) MS/MS spectrum of “NNKAcKPropTSKAcNKAcSSLSR” that identified Lys^{1555} as in vivo lysine propionylation site in p300.

Fig. 4. Mapping lysine propionylation sites in p300 by mass spectrometry. In Vivo lysine propionylated and butyrylated peptides and sites identified in p300 (A) and in CBP (B). C, MS/MS spectrum of “NNKAcKPropTSKAcNKAcSSLSR” that identified Lys^{1555} as in vivo lysine propionylation site in p300.
p300 and CBP are in vivo lysine butyrylation substrates; and (iii) SirT1 can catalyze p300 in vivo lysine depropionylation reaction.

DISCUSSION

Lysine propionylation is originally identified in histones (12), but whether it, like lysine acetylation, is present in non-histone proteins remains unknown, as do the identities of the enzymes responsible for regulating the modification. Here, we show that (1) lysine propionylation is present in three non-histone proteins (p53, p300, and CBP); (2) p300 and CBP can catalyze lysine propionylation of p53; (3) p300 and CBP have auto-lysine propionylation activities; and (4) SirT1 is a depropionylase that removes lysine propionylation from p53 and p300. These results indicate that lysine acetylation and lysine propionylation pathways are likely to share many substrates and regulatory enzymes in common. Although three regulatory enzymes for lysine acetylation (SirT1, p300, and CBP) also affect lysine propionylation, Tip60 and the other three HATs tested seem to have much higher selectivity and are likely to restrict their enzymatic activity to lysine acetylation or have much lower activity to lysine propionylation, at least for the substrate p53. Our analysis indicates that stoichiometry for lysine acetylation is much higher than that for lysine propionylation and lysine butyrylation. For example, the mass spectrometric signal for propionylated lysine (Lys\textsuperscript{prop}) is 25 times less that that of acetylated lysine (data not shown). Nevertheless, the stoichiometric ratios for the three modifications are not sequentially decreased with the lengths of the short acyl chains. This is evidenced by the fact that two lysine butyrylation sites (Lys\textsuperscript{1595} and Lys\textsuperscript{1597}) were identified in CBP and three lysine butyrylation sites (Lys\textsuperscript{372}, Lys\textsuperscript{373}, and Lys\textsuperscript{380}) in p53, but not their propionyllysine counterparts. This result suggests that there might be the existence of enzymes that prefer lysine butyrylation to lysine propionylation. Lysine propionylation status can be enhanced moderately by HDAC inhibitors among three substrate proteins, raising the possibilities of other depropionylation enzymes in cells the activities of which cannot be blocked by HDAC inhibitors. It should also be pointed that we are likely to fail to identify lower stoichiometric propionyllysines and butyryllysines sites among the three substrate proteins as only 0.5–1 pmol of p300/CBP and ~3 pmol of p53 were used in our experiment.

Our studies raise a few interesting questions. How are the relative levels of acetylation and propionylation at a particular lysine residue regulated by the same enzyme? In the case of the transferase reaction, Lys\textsuperscript{Ac} and Lys\textsuperscript{Prop} status may be regulated by the intrinsic activity of the enzyme toward the substrate compounds (acytyl-CoA and propionyl-CoA) and also by the relative concentrations of the two acetyl-CoA molecules. Propionyl-CoA is generated by the catabolism of short-chain lipids and certain amino acids. Therefore, diet may affect the concentrations of the acyl-CoAs, which in turn modulate the lysine modification status. It is also possible that some transferases prefer one acyl-CoA to the other as a substrate. If this is so, cells will contain Lys\textsuperscript{Ac} or Lys\textsuperscript{Prop}-specific transferases. The question of how specificity is achieved also arises for the depropionylases. Because no modification-specific cofactors are involved in the demodification reaction, this reaction is mainly dependent on intrinsic enzymatic activity. Finally, what are functional differences between Lys\textsuperscript{Ac} and Lys\textsuperscript{Prop} in a given substrate protein? Although acetyllysine and propionyllysine are structurally similar, they may cause differences in protein-protein interactions. For example, acetylated and propionylated proteins may associate differently with bromo-domain proteins.

Because Lys\textsuperscript{Prop} has been identified in several proteins known to be substrates for lysine acetylation (histones, p53, p300, and CBP), we anticipate that a large number of other Lys\textsuperscript{Ac} substrates may also be lysine propionylated and lysine butyrylated. Similarly, additional regulatory enzymes for Lys\textsuperscript{Prop} and Lys\textsuperscript{Buty} may be found among enzymes known to be acetyltransferases or deacetylases.
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