Biochemistry

International Edition: DOI: 10.1002/anie.201508174
German Edition: DOI: 10.1002/ange.201508174

Interrogating Substrate Selectivity and Composition of Endogenous Histone Deacetylase Complexes with Chemical Probes

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Abstract: *Histone deacetylases (HDACs) regulate the function and activity of numerous cellular proteins by removing acetylation marks from regulatory lysine residues. We have developed peptide-based HDAC probes that contain hydroxamate amino acids of various lengths to replace modified lysine residues in the context of known acetylation sites. The interaction profiles of all human HDACs were studied with three sets of probes, which derived from different acetylation sites, and sequence context was found to have a strong impact on substrate recognition and composition of HDAC complexes. By investigating K382 acetylation of the tumor suppressor p53 as an example, we further demonstrate that the interaction profiles reflect the catalytic activities of respective HDACs. These results underline the utility of the newly established probes for deciphering not only activity, but also substrate selectivity and composition of endogenous HDAC complexes, which can hardly be achieved otherwise.*

Lysine acetylation is a highly abundant post-translational modification (PTM) of proteins. Global analysis of this PTM has uncovered that mammalian acetylomes comprise several thousand acetylation sites on functionally diverse proteins involved in various cellular processes including DNA packaging, transcription, signal transduction, and metabolism.[1] Acetylomes are regulated by the balanced action of lysine acetyltransferases and lysine or histone deacetylases (HDACs). The latter class of enzymes has emerged as a promising drug target, and small-molecule HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), have already been approved as clinical drugs.[2] Mammalian genomes encode 11 Zn^{2+}-dependent HDACs, which are grouped by their phylogenetic relation to the yeast homology Rpd3 and Hda1 into class I, IIa, IIb, and IV.[3] In addition, seven NAD^+-dependent deacetylases of class III, the so-called sirtuins, can also erase acetylation marks.[4] Biochemical research on Zn^{2+}-dependent HDACs (hereafter referred to as HDACs) is hampered by their assembly into multiprotein complexes, which are only poorly understood and might modulate the activity and selectivity of the deacetylases.[5]

As a consequence, the knowledge about the preferred substrate sites of HDACs and how substrate selectivity is modulated by complex formation is very limited. Current approaches to investigate the substrate sites of HDAC complexes include the determination of lysine acetylomes of cells treated with HDAC inhibitors.[1,6] In addition, activity-based probes and immobilized HDAC inhibitors represent promising tools for probing HDAC activity in cell lysates.[7]

Here we report the synthesis of peptide-based probes for interrogating the substrate selectivity and composition of endogenous mammalian HDAC complexes. These designed probes contain an HDAC inhibitor moiety grafted onto an amino acid scaffold. These HDAC inhibitor amino acids replace acetylated lysines in peptides derived from known acetylation sites. To this end, we resorted to hydroxamic acids that chelate the catalytic Zn^{2+} ion of HDACs with nanomolar affinities and serve as inhibitory units in several HDAC inhibitors.[2,7a] The spacing between the hydroxamate group and the peptide backbone appeared critical, and consequently we synthesized hydroxamate amino acid building blocks with spacers ranging from five (2-aminosuberic acid ω-hydroxamate, AsuHd), four (2-aminopimelic acid ε-hydroxamate, ApmHd), three (2-aminoadipic acid δ-hydroxamate, AadHd), to two methylene groups (glutamic acid γ-hydroxamate, GluHd) (Figure 1).[8] The synthesis of AsuHd (**1**) and ApmHd (**2**) building blocks was guided by established routes and further optimized (Figure 1a and Note S1 in the Supporting Information).[9] However, the analogous building blocks AadHd and GluHd were unstable during solid-phase peptide synthesis (SPPS) (Figure S1). Consequently, we established an alternative strategy that enabled on-resin

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201508174.

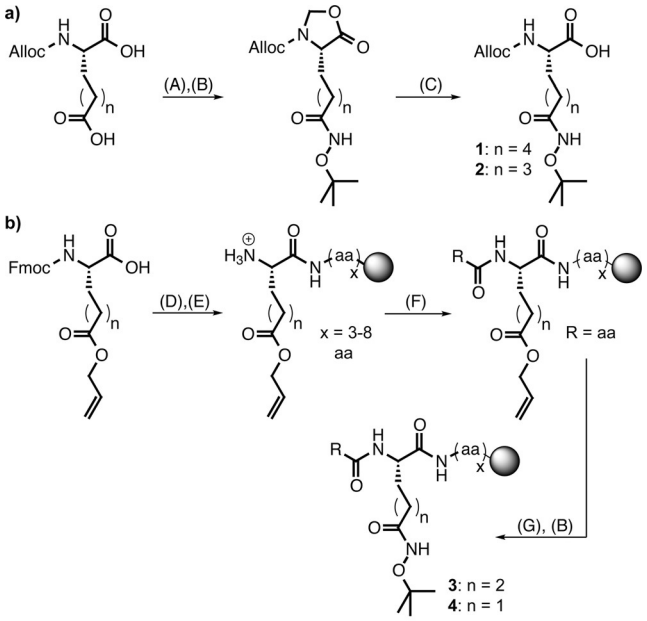

Figure 1. Synthesis of hydroxamate amino acid building blocks and HDAC probes. a) Synthesis of AsuHd and ApmHd building blocks for SPPS. b) Strategy for incorporating AadHd and GluHd into peptide probes. Reagents and conditions: A) paraformaldehyde, TsOH, in ACN, 130 °C, microwave; B) *O*-tBu hydroxylamine, EDC, DMAP, DIPEA in DCM; C) 1 M LiOH (aq) in MeOH; D) SPPS. PyOxim, NMM in DMF; E) 2% DBU/DMF, 1 min flow-wash, then 1% TFA in DMF; F) Fmoc amino acid, SPPS; G) $[Pd(PPh_3)_4]$, $BH_3{\cdot}HNMe_2$ in DCM. ACN: acetonitrile, DMAP: 4-dimethylaminopyridine, EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, DIPEA: *N*,*N*-diisopropylethylamine, DCM: dichloromethane, TFA: trifluoroacetic acid.

installation of the hydroxamates in peptides containing AadHd (**3**) and GluHd (**4**) (Figure 1b).

With these strategies and building blocks in hand, we synthesized a first set of peptide probes consisting of only two Gly or Ala residues flanking a central hydroxamate amino acid (Figure 2a). These miniprobes were immobilized on agarose resin and served as baits in subsequent pull-down experiments. Native whole-cell lysates from HeLa cells expressing all HDACs (Figure S2) were used at a fixed concentration (1 mg mL^{-1}, 0.2 mg total) as input and the pull-downs were analyzed by Western blot using specific antibodies for HDAC 1-11 (Figure 2b). All HDACs of class I and IIb were efficiently recovered on mini-AsuHd and to a lesser extent on the mini-ApmHd and mini-AadHd probes. With the exception of HDAC5, HDACs of classes IIa and IV did not bind to the miniprobes at a detectable level. These findings indicate a general utility of the HDAC probes. Importantly, the spacing between the hydroxamate moiety and backbone modulated the affinity. In the following, we confirmed the pull-down of the HDACs with mini-AsuHd in comparison to the mini-Lys probe by liquid chromatography tandem mass spectrometry (LC-MS/MS), and determined at the same time the interactome of the enriched HDACs. For this, proteins eluted from mini-AsuHd and mini-Lys probes were alkylated, trypsinized, identified, and relatively quantified by label-free LC-MS/MS analysis (Figure S3). All HDACs of classes I and IIb were significantly enriched on mini-AsuHd when compared to the control, confirming the findings of the Western blot analysis (Figure 3a). The analyses further uncovered known and potential components of HDAC complexes that were enriched together with the HDACs (Table S1). These included REST corepressors 1 and 3 (RCOR1 and RCOR3) as well as lysine demethylase LSD1 (KDM1A), which are part of the CoREST complex that also comprises class I HDACs 1 and 2 (Figure 3a).[10]

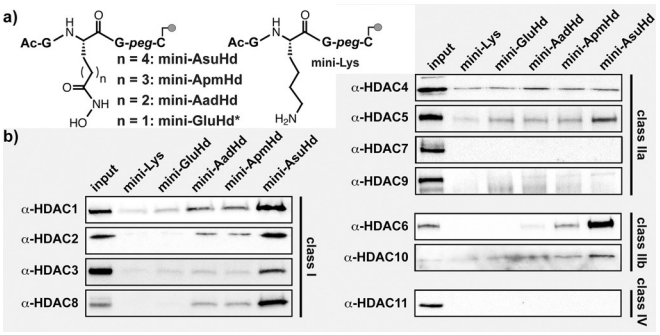

Figure 2. HDAC pull-downs with miniprobes. a) Structure of immobilized miniprobes. b) Western blot analysis of pull-down experiments. * Probe with a C-terminal Ala residue (see Note S1). peg: polyethylene glycol.

Encouraged by these findings we synthesized a new set of probes derived from the regulatory acetylation site at K382 of the tumor suppressor protein p53. The p53 probes contained either AsuHd, ApmHd, AadHd, or lysine at position 382 and were overall positively charged due to the high content of Lys and Arg residues (Figure 3b). Pull-down experiments showed that HDACs 1–5 were recovered on p53 probes (Figure 3c and Figure S4). The concentration of input HeLa extract was reduced at least fivefold ($\leq$ 0.2 mg mL^{-1}, 40 μg total), and under these conditions recovery of HDACs 1–3 on mini-AsuHd was strongly reduced. Contrary to this, HDACs 1–3 were efficiently enriched from diluted lysates on p53-AsuHd and even on p53-ApmHd and p53-AadHd which contained lower-affinity hydroxamates. HDAC6 and HDAC8 showed a different binding behavior and were enriched on p53-AsuHd and mini-AsuHd with comparable efficiency.

A further set of probes based on the acetylation site of K4 on the nuclear transport factor 2 (NTF2) showed a different interaction profile (Figure 3c and Figure S5). Acidic and hydrophobic residues govern the sequence context of NTF2 probes, which mediated only weak interactions with HDACs 1–3 when compared to p53-AsuHd (Figure 3c and Figure S6). However, HDAC8 and HDAC6 were still recovered on NTF2-AsuHd. Collectively, these observations indicate that the sequence context of the HDAC probes modulates the substrate recognition of HDACs and further support an important role of HDACs 1–3 for the deacetylation of p53-K382ac.

In the following, we explored the potential deacetylase complexes of p53-K382ac in proteomics experiments (Note S2 and Tables S2 and S3). Pull-downs of p53-AsuHd versus mini-AsuHd and p53-AadHd versus mini-AsuHd were performed using the SILAC (stable isotope labeling with

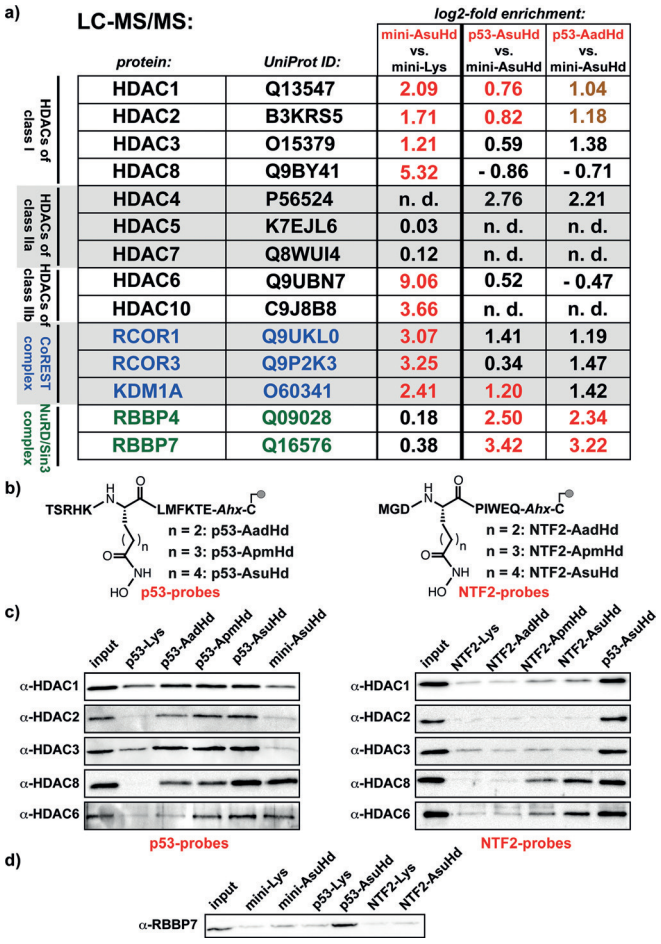

Figure 3. HDAC pull-downs with p53 and NTF2 probes. a) Selected proteins identified and quantified in pull-downs by LC-MS/MS analysis. Numbers indicate median enrichment from two (for p53-AsuHd and p53-AadHd) and three (for mini-AsuHd) independent experiments, respectively; red: significant ($p < 0.05$); brown: less significant proteins (p 0.05–0.09) with log2-fold change > 0.6; black: not significant ($p > 0.1$) or not enriched with log2-fold change > 0.6. b) Structure of p53 and NTF2 probes. c) Western blot analysis of pulled-down HDACs. d) Western blot analysis of pulled-down RBBP7.

amino acids in cell culture) approach to accurately quantify even small-fold enrichments.[11] The LC-MS/MS analyses showed that HDACs 1 and 2 were enriched on both p53 probes with statistical significance (p53-AsuHd, $p \leq 0.05$) or marginal significance (p53-AadHd, $p = 0.08$) and log2-fold changes > 0.6. Other HDACs were either not significantly ($p > 0.1$) or not enriched to the same level (Figure 3c). Potential involvement of HDACs 1 and 2 in p53-K382ac deacetylation was further supported by the enrichment of retinoblastoma binding proteins 4 and 7 (RBBP4 and RBBP7), which can be considered as signature proteins of the NuRD and Sin3A complex, on both p53 probes. These complexes contain HDACs 1 and 2, but in contrast to components of the CoREST complex, RBBP4 and RBBP7 were not significantly enriched on mini-AsuHd.

We confirmed the observed selectivity of RBBP7 for p53-AsuHd independently by Western blot analysis (Figure 3d). These observations suggest that the NuRD and Sin3A complexes steer the substrate selectivity of imbedded HDACs 1 and 2 towards the p53-K382ac site.

Reports on deacetylases of p53-K382ac are inconsistent and either include HDAC1, HDAC6, HDAC8, or sirtuin SIRT1 depending on the experimental settings.[12] In case of HDAC8, the recombinant enzyme shows a strong preference for p53-K382ac-derived substrates, but the pull-down experiments with the p53 probes do not support a major role of this HDAC in the deacetylation of K382ac. In order to validate whether the pull-down experiments with the newly established HDAC probes enable proper predictions of deacetylase substrate selectivity, we studied the catalytic properties of selected HDACs in cellular lysates. We have previously shown that global deacetylase activity in cellular lysates can be monitored with synthetic acetylated peptides by MALDI-TOF MS.[13] We synthesized a p53-derived peptide substrate with acetylated K382 that was readily deacetylated by endogenous HDACs from cell lysates (Figure S7). An isotope-labeled reference peptide was used to quantify the amount of formed deacetylated product. Initial experiments indicated that activities of HDACs 1 and 2 are redundant for erasing the acetylation mark of p53-K382ac (Figure S8).

We focused on HDAC1 and compared the deacetylase activity of this enzyme to that of HDAC6 and HDAC8. Native lysates of HEK cells overexpressing HDAC1, HDAC6, or HDAC8 were prepared and analyzed for p53-K382ac deacetylase activity (Figure 4a). Only overexpression of HDAC1 resulted in a significant twofold-increased p53-K382ac deacetylation rate (Figure 4b). Furthermore, we studied p53-K382ac deacetylation in living cells and transfected HEK cells with constructs for the expression of p53 and p300/CBP,

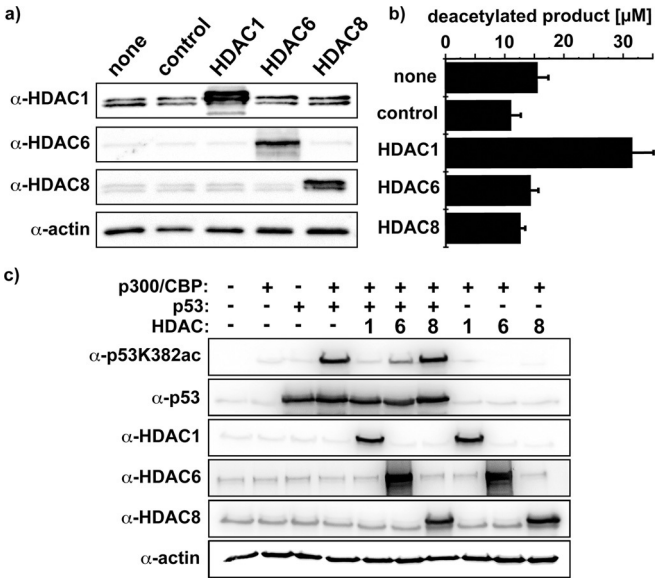

Figure 4. Deacetylation of p53-K382ac by HDACs. a) Overexpression of HDACs 1, 6, and 8 in HEK cells. b) Deacetylation of p53-K382ac in HEK cell lysates. The concentrations of deacetylated products were determined after 4 h incubation with 200 μM acetylated p53-K382ac substrate by MALDI-TOF MS. An isotope-labeled peptide derived from the product served as an internal reference for quantification. c) Western blot analysis of in vivo deacetylation of p53-K382ac. Control: transfection of an empty control vector.

an acetyl-transferase capable of acetylating p53-K382ac.[12e] Co-transfection of both constructs resulted in acetylation of p53-K382, which could be detected with a modification-specific antibody (Figure 4c). Additional expression of HDAC8 did not reduce the acetylation level of p53-K382ac, in contrast to HDAC1. Overexpression of HDAC6 also reduced the p53-K382ac signal, but not to the same extent as HDAC1. This finding could be explained by the strong overexpression of HDAC6, but it should also be noted that the peptide–hydroxamate probes might not recapitulate all aspects of HDAC substrate recognition.

In summary, we have established peptide-based HDAC probes that can be used to investigate the selectivity and redundancy of endogenous deacetylase activities in cell extracts. These probes can be further used to investigate the composition of HDAC complexes when combined with proteomic approaches. The flexibility of SPPS allows simple access to probes derived from further acetylation sites as well as investigations with probes that carry additional PTMs. Modern protein semisynthesis strategies should enable the incorporation of the hydroxamate amino acids into proteins. Based on this, the established HDAC probes appear to be very versatile tools for investigating Zn^{2+}-dependent lysine deacetylase complexes.

Acknowledgments

This work was supported by Priority Program SPP 1623 of the Deutsche Forschungsgemeinschaft (DFG) with grant SCHW 1163/4-1 and Emmy Noether grant FI 1655/1-1.

Keywords: activity-based protein profiling · chemical probes · HDAC · lysine acetylation · proteomics

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1192–1195
Angew. Chem. **2016**, *128*, 1208–1211

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Received: September 1, 2015
Published online: December 14, 2015

