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Factors affecting the substrate specificity of histone deacetylases

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Abstract

Histone deacetylases (HDACs) catalyze the deacetylation of ε-acetyl-lysine residues within the N-terminal tail of core histones and thereby mediate changes in the chromatin structure and regulate gene expression in eukaryotic cells. So far, surprisingly little is known about the substrate specificities of different HDACs. Here, we prepared a library of fluorogenic tripeptidic substrates of the general format Ac-P_{-2}-P_{-1}-Lys(Ac)-MCA (P_{-1}, P_{-2} = all amino acids except cysteine) and measured their HDAC-dependent conversion in a standard fluorogenic HDAC assay. Different HDAC subtypes can be ranked according to their substrate selectivity: HDAH > HDAC8 > HDAC1 > HDAC3 > HDAC6. HDAC1, HDAC3, and HDAC6 exhibit a similar specificity profile, whereas both HDAC8 and HDAH have rather distinct profiles. Furthermore, it was shown that second-site modification (e.g., phosphorylation) of substrate sequences as well as corepressor binding can modulate the selectivity of enzymatic substrate conversion.

Keywords: Histone deacetylase (HDAC); Substrate specificity; Combinatorial peptide library; Fluorogenic assay; Bordetella/Alcaligenes FB188; Corepressor; Phosphorylation

Histone deacetylases (HDACs) are important players in the regulation of gene expression in eukaryotic cells affecting angiogenesis, cell-cycle arrest, apoptosis, terminal differentiation of different cell types and the pathogenesis of malignant disease [1,2]. Not surprisingly therefore, a number of HDAC inhibitors show a potency as promising antitumor agents [2]. Notably, however, HDACs and their bacterial counterparts are also active in the deacetylation of non-histone proteins as well. The HDAC superfamily has been categorized on the basis of sequence homology and functional properties [1]. RPD3-like class 1 HDACs (human HDACs 1, 2, 3, 8), HDA1-like class 2 HDACs (human HDACs 4, 5, 6, 7, 9, 10), and class 4 enzymes (human HDAC11) are structurally related zinc-dependent enzymes whereas class 3 enzymes comprise the structurally unrelated NAD-dependent sirtuins.

Relatively little is known about the substrate specificities of HDACs. It has been reported that HDACs recognize different and highly specific acetylation patterns in nucleosomes [3,4]. It was assumed that site specificity, mainly depends on cofactors that form complexes with HDACs [4]. This view was supported by a recent study, which claimed that sirtuin SirT1 does not exhibit a marked specificity towards peptidic substrates [5]. However, more recent work argues that SIRT1 does show substrate specificity [6,7]. For class 1 and class 2 HDAC subtypes, recent studies on a small number of peptidic substrates suggest that these enzymes may selectively recognize substrate peptide sequence [8–11] and the nature of the acyl leaving group [9,12]. As the use of fluorogenic peptide substrates is a well-established method for the determination of hydrolase specificity [13] we used a soluble positional library of 361 AMC (7-amino-4-methylcoumarin) peptide

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substrates of the general format Ac-P_{-2}-P_{-1}-Lys(Ac)-MCA (P_{-1}, P_{-2} = all amino acids except cysteine; MCA = 4-methylcoumarin-7-amide) to study the substrate specificity of human HDAC1, HDAC3, HDAC6, HDAC8, and bacterial FB188 HDAH, which has been shown to also accept eukaryotic acetylated histones as substrates [8]. Furthermore, the effect of additional substrate phosphorylation and the influence of corepressor binding on enzymatic deacetylation activity has also been studied.

Materials and methods

The Ac-XX-Lys(Ac)-MCA tripeptide library was synthesized using a MultiPep (Intavis AG, Cologne, Germany) according to standard Fmoc-/*t*Bu protocols of the supplier (for details see Supplementary material). His-tagged FB188 HDAH was prepared as described elsewhere [8], human recombinant HDAC8 was expressed in *Escherichia coli* and purified as described [9]. For HDAC1 and HDAC6 preparations mass cultures of HEK293 cell lines with stable expression of human full-length HDAC1 and HDAC6 were lyzed and flag-tagged proteins purified by M2-agarose affinity chromatography (Sigma Art. No. A-2220). Human HDAC3-Flag was co-expressed with the SMRT DAD domain in Sf21 insect cells and purified accordingly. Fractions from the purification were analyzed by Western blotting using antibodies specific for human HDAC1–8 and for specific activity in the biochemical enzyme assay. Human HDAC1 contains small amounts of endogenous HDAC2. In contrast, HDAC3 and 6 were not contaminated with other HDAC isoenzymes (data not shown). Stock solutions of enzymes contained 102 μg/ml (rHDAC1-Flag), 145 μg/ml (rHDAC3-Flag-DAD), 268 μg/ml (rHDAC6-Flag), 650 μg/ml (rHDAC8), and 480 μg/ml (HDAH). For comparison of free HDAC3 and HDAC3/NCoR2 complex commercially available recombinant preparations of HDAC3 (prepared in Sf9 cells) and NCoR2 (prepared in *E. coli*) were obtained from BPS Bioscience Inc. (San Diego, CA). According to the supplier no NCoR2 was traced in the HDAC3 preparation.

Fluorogenic HDAC assays were performed in black 96-well microplates essentially as described previously [10,11]. K_M and V_{max} values were determined using at least eight different substrate concentrations. The experimental data were analyzed using the GraphPad Prism software. The AMC signals were recorded against a blank with buffer and substrate. All experiments were carried out in triplicate. As controls the deacetylation of peptides of format NNK(Ac)GG was quantified using the fluorescamine assay as described ([8]; for details see Supplementary material).

Results

Synthesis of a complete diverse HDAC substrate library

A 361-member, spatially separated library of the format Ac-P_{-2}-P_{-1}-Lys(Ac)-MCA was prepared with all combinations of proteinogenic amino acids (except Cys) at the P_{-2} and P_{-1} sites. Special care was taken to purify all members of the library in order to exclude free 7-amino-4-methylcoumarin (AMC) and substrates without acetylation of the side chain of the lysine which could result in false positive signals in the HDAC assay developed previously [9–11]. The assay is based on the 2-step conversion of fluorogenic peptidic substrates comprising the HDAC-dependent deacetylation of an ε-acetylated lysyl moiety and the subsequent trypsin-dependent release of the 7-amino-4-methylcoumarin (AMC) fluorophor. Therefore, we first confirmed, that even molecules with tripeptide sequences reported to be non-optimal substrates for trypsin [14] were fully converted under conditions of the assay (see Supplementary information). Thus, differences between fluorogenic (acetylated) substrates of the library concerning the release of AMC in the 2-step assay are assumed to reflect differences in the deacetylation step of the assay, i.e., different enzymatic deacetylase activities. To confirm that the relative conversion of different substrates is not affected by the particular design of the assay substrates [15,16], i.e., the presence of the AMC fluorophor in place of peptide sequence as in acetylated proteins such as histones, we performed fluorescamine assays with selected, fluorophor-free peptides of the format NNK(Ac)GG. Indeed, peptides of the format NNK(Ac)GG show the same ranking as the corresponding NNK(Ac)MCA substrates. Not surprisingly, however, the original 2-step fluorogenic assay, proved to be better suited to resolve differences between peptides with very low conversion rates (see Supplementary material).

Specificity of histone deacetylases

The substrate library was used to analyze the specificities of human recombinant class 1 HDAC1, 3 and 8, recombinant class 2 HDAC6, as well as of class 2 histone deacetylase-like amidohydrolase (HDAH) from Bordetella/Alcaligenes FB188 (Fig. 1). For each enzyme a separate scaling was used with 100% activity for the respective best substrate. Fig. 1F shows the distribution of all 361 substrate molecules among 15 arbitrary activity classes for each enzyme tested. Determining the distance between the center of the distribution and the respective class of highest activity as a measure of selectivity, the enzymes can be ranked according to their substrate selectivity: HDAH > HDAC8 > HDAC1 > HDAC3 > HDAC6. The bacterial histone deacetylase-like amidohydrolase (HDAH) from Bordetella/Alcaligenes strain FB188 appeared to be the enzyme with the most distinct substrate specificity (Fig. 1E) efficiently converting only substrates with basic amino acids (Lys, Arg) at the P_{-2} position. Other amino acids such as methionine were much less favored. At the P_{-1} position HDAH showed an exclusive preference for serine or rather small aliphatic and relatively hydrophobic amino acids (Gly, Ala and much less pronounced Met and Thr).

Next, human HDAC8 still had a very marked substrate selectivity (Fig. 1D) that significantly differed both from that of HDAC1, 3 and 6 as well as from that of HDAH. At the P_{-2} position, HDAC8 favored Pro, Ser > Phe, Met, Lys, Arg, Gln, Asn, and Ala. However, it strongly disfavored other small aliphatic amino acids (Gly, Val), tryptophan and acidic amino acids, particularly glutamic acid. At the P_{-1} position HDAC8 displayed a preference for aromatic amino acids including histidine and to some extent also larger aliphatic amino acids (in particular Val, Ile) and methionine.

The specificities of HDAC1 (Fig. 1A), HDAC3 (Fig. 1B) and HDAC6 (Fig. 1C) were similar with HDAC1 being the