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Characterization of the two catalytic domains in histone deacetylase 6

Hua Zou, Yiqin Wu, Marc Navre, Bi-Ching Sang *

Takeda San Diego Inc., San Diego, CA 92121, USA

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Abstract

Histone deacetylase 6 (HDAC6) is the only known HDAC with two potentially functional catalytic domains, yet the role towards substrate played by these two domains remains ambiguous. Most studies report HDAC6 activities measured using either immune complexes or in vitro translated products. Here, we characterize the activity of highly purified recombinant HDAC6, mutants with active site histidine mutations in each domain (H216A and H611A), and individual catalytic domains. The deacetylase activities of these proteins, as well as their kinetic parameters, were measured using histone, α-tubulin, and fluorogenic acetylated lysine as substrates. Mutant H216A only slightly lowers the catalytic rate. However, mutant H611A decreases the catalytic rate more than 5000-fold. The first domain expressed alone is not catalytically active. In contrast, the second domain shows only a modest decrease in substrate binding and product formation rate. Our results indicate that the in vitro deacetylase activity of HDAC6 resides in the C-terminal second catalytic domain.

Keywords: Histone deacetylase 6; HDAC6; Catalytic domain; Histone; α-Tubulin

Reversible acetylation and deacetylation of histone molecules plays a pivotal role in the regulation of chromatin remodeling and affects gene expression of eukaryotic organisms [1–4]. A class of Zn binding enzymes, the histone deacetylases (HDACs), catalyzes the deacetylation of lysine residues in histones. Eleven human HDACs have been identified and grouped into two classes [5]. Class I HDACs are related to yeast transcriptional regulator RPD3 and are localized exclusively in the nucleus, whereas class II HDACs are larger proteins that are shuttled between the cytoplasm and the nucleus [6].

HDAC6, a class II HDAC, is predominantly cytoplasmic and a fraction of the protein translocates into the nucleus when cell proliferation is arrested [7]. High expression levels of HDAC6 mRNA are detected in the heart, liver, kidney, and pancreas [8]. HDAC6 is the largest member of the HDAC family with 1215 amino acid residues and the only HDAC that contains two intact catalytic domains, located at the N-terminal and the central region of the protein. Many HDAC inhibitors such as trichostatin A (TSA), trapoxin-B (TPX), and sodium butyrate have been used in HDAC functional studies [3]. The histone deacetylation activity of HDAC6 is TSA sensitive. Unlike other HDACs, however, HDAC6 is much less sensitive to inhibition by TPX and sodium butyrate [9–11]. HDAC6 has been reported to associate with microtubules and it deacetylates α-tubulin at residue lysine-40 in vitro [12,13]. This tubulin deacetylase activity has also been demonstrated in vivo as well. The tubulin deacetylase activity of HDAC6 is sensitive to TSA but not to TPX or sodium butyrate.

The presence of two tandem catalytic domains within HDAC6 has presented a challenge in understanding the contribution and interaction of these putative active sites to HDAC6 biochemistry and cellular physiology. There are conflicting results and interpretations in the recent HDAC6 literature. Work from the Schreiber group [8,14] using recombinant HDAC6 immunoprecipitated from mammalian cells, incubated with labeled histone or tubulin proteins, suggests that both domains are active as histone deacetylases, whereas only the second domain carries tubulin deacetylase (TDAC) activity. In contrast, more recent work from Guardiola and Yao [15] and Zhang et al. [13], which also utilized immunoprecipitated HDAC6 variants,

* Corresponding author. Fax: +1 858 550 0526.
E-mail address: biching.sang@takedasd.com (B.-C. Sang).

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suggests that inactivation of the first domain does not change the sensitivities of HDAC6 towards various inhibitors [15] and that neither domain is active unless the other active domain is present [13]. To better characterize the catalytic domains of HDAC6, we have used purified forms of recombinant HDAC6, mutants, and individual catalytic domains to analyze their binding affinities and catalytic activities towards different substrates and sensitivity to various inhibitors. Our results indicate that the second domain of HDAC6 is catalytically competent on its own, and moreover, is responsible for essentially all the catalytic activity of the wild-type enzyme against histone and α-tubulin substrates.

Materials and methods

Reagents. NuPAGE Novex Bis-Tris gels, LDS sample buffer, and molecular weight standards for SDS–PAGE, SDS running buffer, and PVDF membrane were from Invitrogen. Monoclonal anti-acetyl histone H3 and anti-acetyl tubulin antibodies, trypsin, HC-toxin, and TSA were purchased from Sigma. Goat anti-mouse Alexa Fluor 680 antibody was obtained from LI-COR Bioscience. The fluorogenic deacetylase substrate (*Fluor de Lys*) was from BIOMOL Research Laboratories. General molecular biology methods were used as described in Sambrook et al. [16].

Production of recombinant HDAC6, mutants, and individual domains. Full-length HDAC6 (FLWT) (residues 1–1215, GenBank Accession No. NM_006044) was cloned from a human brain cDNA library (Gibco) through PCR. Mutants and domains were made by mutagenesis using the FLWT as template. All clones, except for the second catalytic domain, were constructed into the pFastBac vector with 6× His affinity tag at their C-terminal ends. The second catalytic domain had a 6× His affinity tag at its N-terminal end with a TEV cleavage site (ENLYFQG) behind. The recombinant HDAC6 proteins were expressed in baculovirus system and purified though nickel affinity chromatography as described [17]. This was followed by a size-exclusion column purification in buffer A containing 25 mM Hepes, pH 7.1, 250 mM NaCl, and 0.125 mM TCEP. The second catalytic domain containing N-terminal 6× His tag was further cleaved by rTEV protease and passed through a nickel column, and the purified protein was then dialyzed against buffer A. The molecular weight of each purified HDAC6 protein was confirmed by mass spectrometric analysis. All the proteins were stored in −70 °C as small aliquots in buffer A containing 5% glycerol.

Preparation of histones and tubulins from HeLa cells. HeLa cells were grown to 95% confluency and then treated with 100 μM SAHA for 5 h. Cells were harvested, washed twice with PBS, and suspended in 5 volumes of lysis buffer containing 10 mM Hepes, pH 7.2, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, protease inhibitor cocktail, and TSA (100 ng/ml), and then adding HCl to a final concentration of 0.2 N. The acid-insoluble fraction was discarded by centrifugation at 11,000*g* for 10 min at 4 °C. The acid-soluble fraction included histones, tubulins as well as PCNA. The acid-soluble proteins were then dialyzed against 0.1 N acetic acid twice for 1–2 h each, followed by three consecutive dialysis steps against H_2O for 1, 3 h, and overnight, respectively. The protein was quantified and stored at −70 °C in small aliquots.

HDAC assay with fluorogenic Lys(Ac) substrate. Typically in a 50 μl reaction mixture, HDAC6 or mutants at various concentrations were mixed with *Fluor de Lys* substrate in HDAC assay buffer composed of 25 mM Hepes, pH 7.2, 100 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 0.01% Brij 35, 0.05 mM EDTA, and 0.125 mM TCEP. Reaction was performed at room temperature and stopped at the end of 60 min by mixing with 50 μl assay buffer containing 2 mg/ml trypsin. After 30 min incubation, free AMC was detected by a Microplate Spectrophotometer (Molecular Devices) at $\lambda_{excitation} = 370$ nM and $\lambda_{emission} = 450$ nm.

Tubulin and histone deacetylation assay by Western blot. A 4 μg aliquot of tubulin plus histone prep was incubated with different amount of HDAC6 or mutants in assay buffer to a final total volume of 30 or 50 μl. After incubating for 1 or 2 h, the reaction solution was subjected to 4–20% SDS–PAGE and transferred to PVDF membrane followed by probing with monoclonal anti-acetyl-histone H3 and anti-acetyl-tubulin antibodies. Washed blots were then incubated with goat anti-mouse Alexa Fluor 680 antibody and the signals were detected and quantified with an Odyssey Infrared Imaging System (LI-COR Bioscience). The same membranes were then further incubated with monoclonal anti-PCNA antibody (EMD Biosciences) and detected with the same secondary antibody.

Determination of kinetic parameters of HDAC6 and its mutants. The K_m for the fluorogenic substrate (*Fluor de Lys*) was determined for wild-type HDAC6 and each form of its mutants. The detail of the assay was described above. In each reaction, 0.5 nM of FLWT, WT or H216A was used, while the amount of CDII and H611A was 5 and 50 nM, respectively. The catalytic rate was calculated based on the amount of fluorescence generated after 1 h reaction. K_m and V_{max} were determined using a nonlinear regression method to fit the Michealis–Menten equation: $V = V_{max}[S]/([S] + K_m)$, where V is the initial catalytic rate, in nanomole of AMC/h, [S] is the concentration of the substrate in micromolar, and V_{max} is a limiting value of V at sufficiently high or saturating [S].

Results and discussions

The only HDAC that contains two potentially functional catalytic domains is HDAC6. There have been conflicting reports whether both catalytic domains are essential for enzyme activity against histone or tubulin substrates [8,13–15]. To characterize the role that the two catalytic domains play in HDAC6 activity, we expressed recombinant HDAC6 and different catalytic domain mutants in a baculovirus system as follows (Fig. 1A): full-length wild-type HDAC6 (FL-WT; amino acids 1–1215), wild-type HDAC6 (WT; amino acids 73–845), H216A wild-type (H216A/WT), H611A wild-type (H611A/WT), first catalytic domain (CDI; amino acids 73–455) and second catalytic domain (CDII; amino acids 479–845). All recombinant proteins were tagged with 6× His residues and purified through Ni–NTA beads followed by a size-exclusion column to homogeneity (Fig. 1B). Enzyme activities of the purified proteins are illustrated in Fig. 1C. We used a fluorescence-labeled deacetylase substrate which contained an acetylated lysine side chain. At the end of enzyme reaction, trypsin was used to selectively release the fluorescent molecule from deacetylated substrate [18]. The fluorescence intensity was used as the measure of HDAC6 activity. The FL-WT product exhibited the highest enzyme activity. By removing the sequences outside of the two catalytic domains, indicated as WT, the enzyme activity was lowered slightly. Because the construct WT had a much higher level of expression compared with that of the FL-WT, we used the WT backbone to generate catalytic domain mutants. The mutation at H216 in the first catalytic domain did not alter enzyme activity compared to that of WT. Under the same assay conditions, the CDII construct activity was reduced, whereas H611A/WT and CDI mutants showed no activity at up to 12.5 nM of protein.

In addition to histones, HDAC6 has been known to deacetylate several other cellular proteins such as α-tubulin and HSP90 [12,13,19]. The α-tubulin deacetylase activities

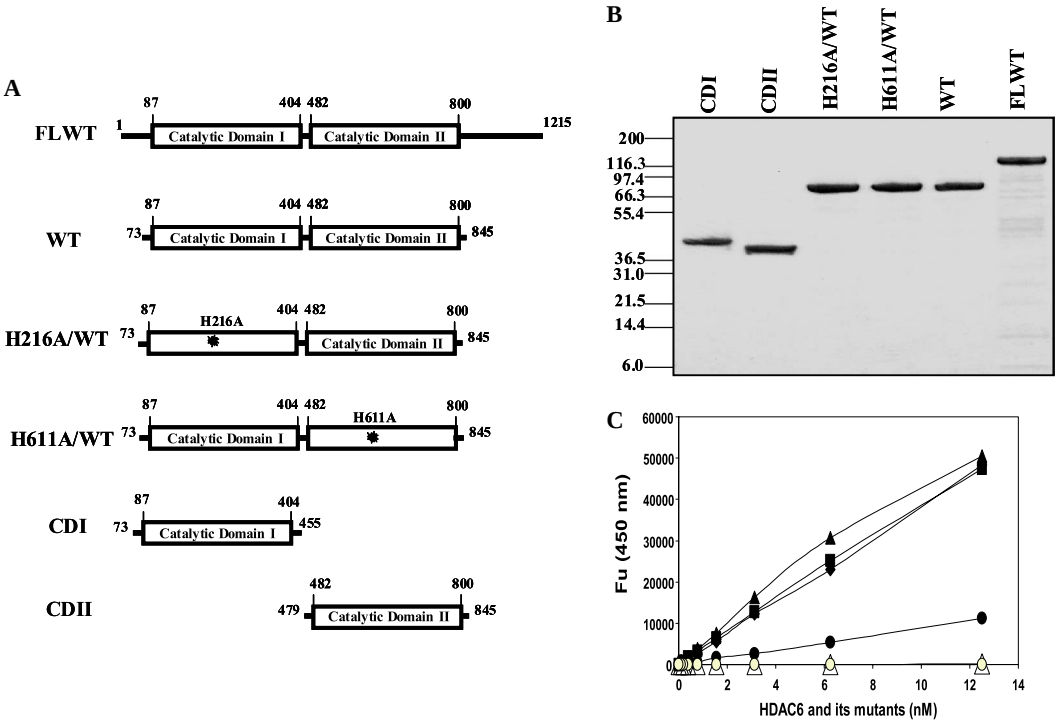

Fig. 1. Enzymatic activities of HDAC6 and its mutants. (A) Schematic diagram of wild-type HDAC6 and its mutants. (B) Recombinant full-length HDAC6 and its mutants were subjected to 4–20% SDS–PAGE and detected with Coomassie blue staining. (C) HDAC6 or its mutants were incubated with 50 μM fluorogenic substrate (*Fluar de Lys* from BIOMOL Research Laboratories) in a total of 50 μl in assay buffer. After incubation at room temperature for 1 h, the reactions were stopped by adding 50 μl of trypsin (final 0.5 mg/ml), and the enzymatic activities were detected as described under Materials and methods. FLWT (▲); WT (♦); H216A/WT (■); H611A/WT (Δ); CDI (○); CDII (●).

of HDAC6 and its mutants were measured by incubating recombinant proteins with the acid-soluble fraction of HeLa cell extracts followed by immunoblotting with anti-acetylated α-tubulin. The changes in immunoblot signals indicated the extent of α-tubulin deacetylation. In order to compare the histone and α-tubulin deacetylase activity, the same blot was simultaneously probed with anti-acetylated histone H3 antibodies. As PCNA co-purified with histone and tubulin in the acid-soluble fraction, it was used as an internal standard for the amount of sample loaded in each lane on the blot. Figs. 2A and B illustrate the results of immunoblots. Since the enzyme activities towards α-tubulin and histones by these recombinant proteins were largely different, a broad range of concentrations of recombinant proteins as well as different reaction time were applied to the acid-soluble extracts to obtain activity curves. The intensity of each immunoblotting band was measured by densitometry and the percentage of band intensity lost was plotted against enzyme concentrations as shown in Figs. 2C and D. FL-WT, WT, and H216A/WT had similar activities on both α-tubulin and histone substrates. On the other hand, neither CDI or H611A/WT exhibited α-tubulin or histone deacetylase activity under the same assay condition. CDII had an approximately 5-fold decrease in α-tubulin deacetylation activity (Fig. 2C) and its histone deacetylase activity (Fig. 2D) was reduced by more than 20-fold. Since the deacetylation of α-tubulin and histone H3 was performed in the same reaction mixture and for the same incubation time periods, the results in Fig. 2 also indicate that histone H3 deacetylation was faster than that of α-tubulin.

To determine kinetic parameters of these proteins, we used the fluorescence-labeled acetylated lysine substrate. Table 1 lists the kinetic data of HDAC6 and mutants. As was observed using other methods, FL-WT, WT, and H216A/WT had comparable K_m and k_{cat} values. Inactivation of the first catalytic domain did not affect the enzyme catalytic efficiency. H611A/WT increased its K_m to 154 μM compared to the 3.6 μM of WT. At the same time, its k_{cat} decreased to 1.9×10^{-3} s^{-1} compared to the 2.6×10^{-1} s^{-1} of WT. The second catalytic domain inactivation by mutation of H611 severely impaired the HDAC6 enzyme activity. These data are consistent with the immunoblotting. For the individual catalytic domain, CDII showed a slightly lowered K_m of 2.4 μM but a 10-fold decreased k_{cat} to 3.7×10^{-2} s^{-1}. Thus, CDII itself exhibited the same binding affinity as WT and remained catalytically competent. In contrast, no K_m or k_{cat} could be measured for CDI.

The catalytic domains of HDACs are highly homologous among different family members as well as across species [5]. The amino acid sequence alignment of the two catalytic domains of HDAC6 indicates there is 61% similarity and 46% identity. All the residues reported to be involved in catalysis are conserved between the two domains [8,20–22]. Based on the deacetylation mechanism proposed by Schultz et al. [22], the H131 residue in *Aquifex*

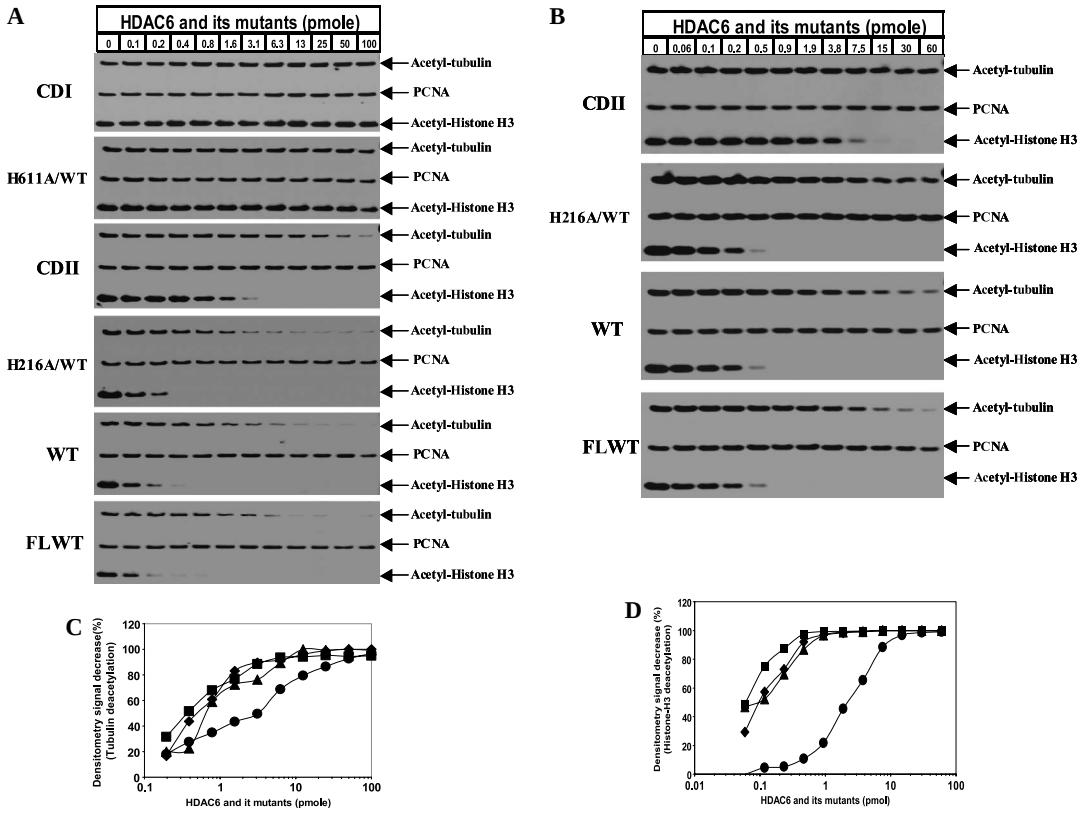

Fig. 2. Wild type HDAC6 and mutant mediated histone and α-tubulin deacetylation activities. A designated amount of wild-type HDAC6 or mutant was mixed with 5 μl of HeLa cell extract containing both histones and tubulin in a total of 50 μl (A) or 30 μl (B) in assay buffer. After incubation at room temperature for 2 h (A) or 1 h (B), the reactions were stopped by adding 3.5 μl SDS NuPAGE loading buffer and boiled. The α-tubulin and histone deacetylation activities were analyzed by Western blot using both anti-acetyl-histone H3 and anti-acetyl-tubulin antibodies simultaneously, as described under Methods and materials. The same Western blot membrane was also blotted with anti-PCNA antibody to indicate that the same amount of substrate was used in each reaction and subjected to SDS–PAGE. (C,D) Densitometry analysis of the Western blot (A or B) showing the percentage of acetyl-tubulin (C densitometry of Western blot A) or acetyl-histone H3 (D densitometry of Western blot B) signals decrease when HDAC6 enzymes were increased. FLWT (▲); H216A/WT (■); WT (♦); CDII (●). Symbols are the same for both (C,D).

Table 1
Kinetic parameters of HDAC6 and its mutants

HDAC6	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M)
FLWT	2.9×10^{-1}	3.3	8.8×10^{4}
WT	2.6×10^{-1}	3.6	7.0×10^{4}
H216A/WT	2.7×10^{-1}	3.8	6.9×10^{4}
H611A/WT	1.9×10^{-3}	1.5×10^{2}	1.2×10^{1}
CDII	3.7×10^{-2}	2.4	1.5×10^{4}

The K_m and k_{cat} for fluorogenic substrate were determined for each form of HDAC6, and each value was calculated using a non-linear regression method to fit Michaelis–Menten equation as described under Materials and methods.

aeolicus HDAC homologue protein (HDLP) is involved in deprotonating a water molecule which forms a hydroxide ion to react with the carbonyl moiety of the acetyl group. The H216 and H611 within the two catalytic domains of HDAC6 are equivalent to that of H131 in HDLP. Our studies, however, demonstrated that these histidine residues behave differently biochemically. Mutation of the histidine in the first catalytic domain did not affect deacetylation activity towards either histone or α-tubulin compared with that of the wild-type. The lack of effects on K_m and k_{cat} in response to inactivation of the first domain explained its unchanged enzyme activity. In contrast, alteration of the histidine in the second catalytic domain results in a loss of enzyme activity for both substrates, with a 42-fold increase in K_m and an over 135-fold decrease in k_{cat}. This suggests that the catalytic activity of HDAC6 is derived mainly from the second domain. For individually expressed domains, CDII was still catalytically competent, but not CDI. Of course, the improper protein folding could be the cause of the missing activity of CDI. But protein folding was not likely to explain the very different behavior in H216A/WT and H611A/WT. Sequences outside of the catalytic domains also appear to contribute to enzyme activity, since FL-WT consistently exhibited a slightly higher activity than WT.

The X-ray crystal structures of HDAC-inhibitor co-complexes reveal that various inhibitors, including TSA, interact with key catalytic residues. The X-ray data suggest that these inhibitors bind to the same site where the substrate binds [23–25]. Many reports have documented that HDAC6 exhibits different sensitivity towards well-characterized HDAC inhibitors [9–11]. We investigated the effect of these HDAC inhibitors on the recombinant HDAC6

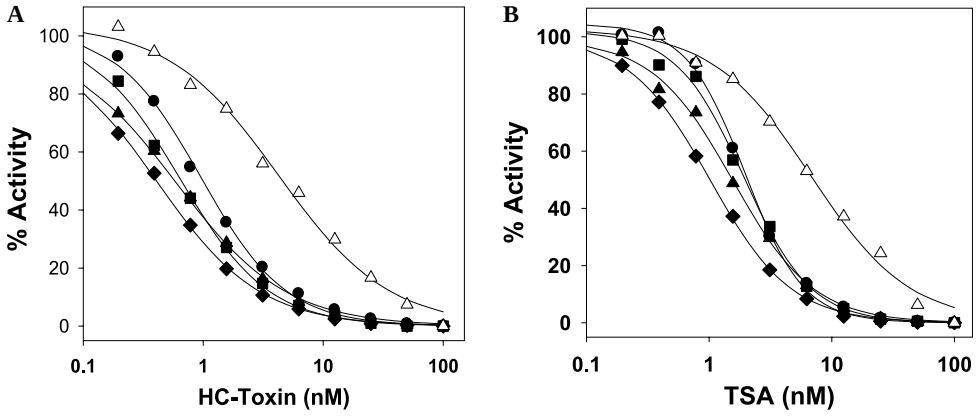

Fig. 3. HC-toxin and TSA mediated inhibition of HDAC6 and its mutants. HDAC6 or its mutant was incubated with different amount of inhibitors. The activity of the HDAC6 enzymes was determined by deacetylation of fluorescene-labeled acetylated lysyl substrate. The amount of the fluorogenic substrate in each reaction is about the same as the K_m value determined in Table 1. FLWT (▲); WT (♦); H216A/WT (■); H611A/WT (Δ); CDII (●).

proteins. Fig. 3 illustrates the inhibition of enzyme activity by HC-toxin (Fig. 3A) and TSA (Fig. 3B). The concentration of compounds needed to inhibit 50% of the enzyme activity (IC_{50}) is shown in Table 2. Our IC_{50} data for TSA and HC-toxin against these recombinant HDAC6 proteins was also in agreement with kinetic parameters measured with substrates. The IC_{50} values of HC-toxin for different recombinant proteins were higher than those of TSA, indicating the HC-toxin was a weaker inhibitor but both inhibitors demonstrated the same inhibition profiles. TSA inhibited FL-WT, WT, H216A/WT, and CDII with similar potency. It was a less effective inhibitor however on H611A/WT, where the IC_{50} changed from 1 to 14.5 nM. The first catalytic domain in H611A/WT could still bind to substrate but the K_m was largely increased which might account for the requirement of higher amounts of TSA to reach the same extent of inhibition in H611A/WT. Thus, both fluorogenic substrate and inhibitor exhibited lower affinity towards H611A/WT.

While preparing this manuscript, a report by Zhang et al. [26] was accepted for publication reporting that multiple mutations in either domain of HDAC6 result in complete loss of histone and tubulin deacetylase activities. These mutations occur at residues involved in coordinating the Zn^{2+} ion and in forming the active-site pocket wall [22–25]. This is different from our result with H216A single mutation which showed no effect on HDAC6 activity. Furthermore, we were able to demonstrate that the individual second domain alone was catalytically active. Since the mutants described in this work differ largely from those made by Zhang et al. [26], the exact cause of these discrepancies is unclear.

Table 2
IC_{50} (nM) of HC-toxin or TSA mediated inhibition of HDAC6 and its mutants

Inhibitors	FLWT	WT	H216A/WT	H611A/WT	CDII
HC-toxin	6.2×10^{2}	4.2×10^{2}	6.4×10^{2}	7.5×10^{3}	9.4×10^{2}
TSA	1.6	1.0	1.9	1.5×10^{1}	1.9

HC-toxin or TSA mediated inhibition of HDAC6 and its mutants was measured in Fig. 3. The IC_{50} of the inhibition was calculated by fitting the equation, $\%I = [\mathrm{I}]\max^{*}(1 - ([\mathrm{I}]^{n}/(\mathrm{IC}_{50}^{n} + [\mathrm{I}]^{n})))$.

We have presented a detailed study of the catalytic activity of numerous variants of human HDAC6 protein purified to homogeneity. Using synthetic and physiological substrates, we have demonstrated unambiguously that the second catalytic domain is the major functional domain of HDAC6. Additionally, the first domain is essentially devoid of catalytic activity whether expressed in the context of the intact HDAC6 protein or expressed as an isolated subdomain. Moreover, we have demonstrated that the inhibition of intact HDAC6 by small molecule inhibitors can be attributed solely to the simple interaction of these compounds with the second catalytic domain and is not impacted by domain–domain interactions. We believe the results of these studies with pure proteins resolve inconsistencies of the results on HDAC6 with immunoprecipitated proteins and can lead to improved models of the role HDAC6 plays in cellular regulation.

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