Two Catalytic Domains Are Required for Protein Deacetylation*

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Histone deacetylase (HDAC)-6 was recently identified as a dual substate, possibly multisubstrate, deacetylase that can act both on acetylated histone tails and on α-tubulin acetylated on Lys40. HDAC-6 is unique among deacetylases in having two hdac domains, and we have used this enzyme as a useful model to dissect the structural requirements for the deacetylation reaction. In this report, we show that both hdac domains are required for the intact deacetylase activity of HDAC-6 in vitro and in vivo. The spatial arrangement of these two domains in HDAC-6 is essential and alteration of the linker region between the two domains severely affects the catalytic activity. Artificial chimeric HDACs, made by replacing the hdac domains in HDAC-6 with corresponding domains from other class II HDACs, show de novo deacetylation activity. Taken together, our results demonstrate for the first time that the spatial arrangement of hdac domain is critical for in vivo deacetylation reaction and may provide a useful model for the development of novel HDAC inhibitors.

RESULTS

Two Hdac Domains Are Required for Deacetylation by HDAC-6—To investigate whether the two hdac domains in HDAC-6 might have different roles in the deacetylation reaction, we prepared several mutant constructs, with mutations in the catalytic cores or in the presumed substrate recognition regions (so-called ER motif; Ref. 7) (Fig. 1A). The different proteins were purified and tested from transiently transfected 293T cells by immunoprecipitation with an anti-HA antibody and were used for in vitro assays with chemically acetylated histone H4 or α-tubulin peptides (5). The expression levels of each mutant were determined by Western blot analysis (Fig. 1B, upper panel). As shown in Fig. 1B, lower panel, point mutations in the catalytic core of both hdac domains (HD1/2m) completely abolished the HDAC and the TDAC activities of HDAC-6. Surprisingly, mutating either of the hdac catalytic cores (HD1m or HD2m) also destroyed the whole activity on both substrates. This suggests that the cooperation between the two hdac catalytic cores is critical for the deacetylation reaction mediated by HDAC-6. Interestingly, the mutations in the presumed substrate recognition region (7) led to somewhat different effects on enzymatic activities. The mutation in the second substrate recognition site (L798A) completely inactivated the catalytic activity on both peptides, whereas the mutation of the corresponding region in the first hdac domain (L802A) retained partial activity. Interestingly, the latter mutation had a stronger effect on TDAC activity than on HDAC activity. This result suggests that in the deacetylation reaction the two different peptide substrates might interact differently with the N-terminal catalytic domain of HDAC-6.

To rule out the possible interference of co-immunoprecipitated proteins with HDAC-6, we purified recombinant wild type or mutant HDAC-6 from a baculovirus expression system. After nickel-nitrioltriacetic acid and QFF columns, HDAC-6, we purified recombinant wild type or mutant HDAC-6 from a baculovirus expression system. After nickel-nitrioltriacetic acid and QFF columns, HDAC-6, we purified recombinant wild type or mutant HDAC-6 from a baculovirus expression system. After nickel-nitrioltriacetic acid and QFF columns, HDAC-6.
expression of wild type HDAC-6 but not by the different HDAC-6 catalytic core mutants.

To rule out any possible effect from endogenous HDAC-6 on these assays, we also made use of HDAC-6-deficient 3T3 cell lines. In the complete absence of HDAC-6, 3T3 cells showed dramatically increased tubulin acetylation (lane 1 versus lane 4 in Fig. 2B, upper panel), in agreement with the notion that tubulin is the physiological substrate of HDAC-6. Stable re-introduction of HDAC-6 into the knock-out cells at an expression level comparable with the wild type efficiently reduced tubulin acetylation (Fig. 2B, lane 6). Clones expressing lower levels of HDAC-6 also showed a decrease in tubulin acetylation, albeit less pronounced (lane 5 in Fig. 2B). Interestingly, whereas the tubulin acetylation increased dramatically in the absence of HDAC-6, other tubulin modifications, such as tyrosinated (Tyr) or detyrosinated (Glu) tubulin did not change. Next, we measured the capacity of HDAC-6 mutants to deacetylate tubulin in vivo by stably re-introducing them into HDAC-6-deficient cells. Specifically, we tested HDAC-6 constructs with mutations in the first or in the second hdac catalytic core. Because it was difficult to obtain high expression of these mutants, we used as a control for these experiments a cell clone expressing the Selective Activities on Different Substrates—To examine whether the spatial arrangement of the two catalytic domains is important for the activity of HDAC-6, we prepared a series of constructs in which the distance between the two hdac domains was modulated by insertions or deletions. Fragments derived from the EGFP protein, ranging from 5 amino acids to full-length of EGFP (239 amino acids), were inserted between the two hdac domains (Fig. 3A). To shorten the distance between the two hdac domains, the linker region was deleted by 5, 25, or 68 amino acids, respectively. As shown in Fig. 3B, all constructs were equally expressed in transiently transfected 293T cells and subsequently used for immunoprecipitation and activity assays. Surprisingly, even slight modulation of the linker length, by addition or removal of only 5 amino acids, dramatically affected the catalytic activity (Fig. 3C). Generally, both HDAC and TDAC activities decreased along with increasing length of the inserts (Fig. 3C). The most dramatic loss of activity was observed when the entire linker region was deleted (Δ411–478). Interestingly, the impairment of the activity clearly showed substrate preference and TDAC activity was found to be more sensitive to spatial changes than HDAC activity.

Generation of Active Artificial Chimeric HDACs from Inactive HDAC Fragments—Finally, we tested whether artificial HDACs, made from combinations of different class II HDACs, might be selectively active. To create chimeric HDACs, we replaced the first or the second hdac domain of HDAC-6 by the hdac domains from either HDAC-4 or HDAC-5 (Fig. 4A). Since the distance between two hdac domains is important for activity (Fig. 3), in the
chimeric HDACs we used the linker region from HDAC-6 to keep the distance between two catalytic cores as it is in the wild type HDAC-6 protein. After transfection into 293T cells, the extracts were subsequently used for immunoprecipitation and activity assays. The specific activities were normalized to the protein expression levels determined by Western blot. As shown in Fig. 4B, replacement of the second hdac domain of HDAC-6 by domains from either HDAC-4 or HDAC-5 resulted in a chimeric protein with almost no activity on either tubulin or histone substrates. On their own, full-length HDAC-4 and -5 displayed catalytic activity (Refs. 5 and 12 and results not shown). On the other hand, chimeric proteins with the first hdac domain derived from either HDAC-4 or HDAC-5 and the second domain from HDAC-6 showed activity on both histone and tubulin substrates. Here again activity was greater on the histone peptide than on the tubulin substrate, which might be due to selective recognition and/or enzymatic activity of HDAC-4 or -5 on the histone but not on the tubulin peptide. These experiments showed that artificial combination of the hdac domains from HDAC-4 or -5 and the second HDAC-6 domain, either of which are inactive by themselves, led to new activity.

**DISCUSSION**

HDAC-6 contains two intact hdac catalytic domains which might mimic native HDAC-containing complexes. Since the two hdac domains in HDAC-6 are well conserved, the first question was whether they are both important and/or functionally different. Here we demonstrate that mutating a single hdac core is sufficient to inactivate HDAC-6 on both histone and tubulin substrates, both in vitro or in vivo. In addition, changing the spatial arrangement between these two domains by insertions or deletions has a significant impact on the activity. This confirms that both hdac domains are necessary for the activity and also suggests that their precise arrangement relative to another is important. We have shown previously that each hdac domain in HDAC-6 is sufficient to bind on its own β-tubulin, and this interaction is maintained even when the catalytic core is mutated (5). Within the hdac domain, in addition to the conserved catalytic core, a region of homology between HATS (such as Esa1) and HDACs (such as Rpd3) has been identified. This motif, termed ER (Esa1-Rpd3) motif (7), is located near the active center in the tertiary structure of Esa1. Recent structure analysis of the IGCNS/CoA/H3 complex (9) showed that this motif might be involved in the interaction and recognition with histone tails. Mutation analysis revealed that the ER motif regions of Esa1 or Rpd3 are required for HAT activity of Esa1 and HDAC activity of Rpd3, respectively (7). By mutating these putative substrate recognition motifs in HDAC-6, we find that the second ER motif might be more important for interaction with the substrate(s). This is the first evidence to show a functional difference between the two hdac domains in HDAC-6. The results obtained with chimeric HDACs further support this hypothesis (Fig. 4). Moreover, we observed that the mutation in the first ER motif had different effects on deacetylation of the tubulin or histone peptide: TDAC activity is more sensitive to this mutation than HDAC activity. This suggests that the two hdac domains selectively interact with and recognize different substrates. Interestingly, modulation of the distance between the two hdac domains in HDAC-6 also has a stronger effect on tubulin peptide deacetylation than on histone peptide deacetylation. We think that this might be also partially due to selective substrate recognition. While the tubulin substrate only has one acetylated lysine (Lys40), histone tails usually have several acetylated lysine residues. In vitro experiments have demonstrated that class I and II HDACs could deacetylate all acetylated lysines on core histone substrates, albeit with slightly different efficiencies (2). This suggests that there might be a dynamic sliding between HDACs and histone tails to allow deacetylation of all residues. Because of this, the recognition of the histone substrates might be inherently more flexible than that of tubulin. In the deletion and insertion constructs of HDAC-6, the fact that the effect was weaker on histone than on tubulin substrates also suggests that tubulin deacetylation needs a more tightly controlled conformation of HDAC-6.

It is generally assumed that most of the in vivo deacetylation activity for histones, and possibly other proteins, is found in HDAC-containing multiprotein complexes. Interestingly, all known HDAC-containing complexes, there are normally two HDACs (10). For example, HDAC-1 is commonly found to work together with HDAC-2. Moreover, HDAC-1 itself can homo-oligomerize through its N-terminal domain; the same domain is necessary for interaction in vitro with HDAC-2 or -3 and also for catalytic activity (11). This raises the question whether the in vivo deacetylation reactions also need two HDAC molecules together. Previous results demonstrated that class II HDACs regulate transcription by bridging the enzymatically active SMRT/N-CoR-HDAC-3 complex and select transcription factors, independently of any intrinsic class II HDAC activity (12). While HDAC-4 and other class II HDACs

**FIGURE 2.** Two intact HDAC domains are required for deacetylation by HDAC-6 in vivo. A, in vivo tubulin deacetylation assays with wild type and mutants HDAC-6. Expression vectors encoding wild type or mutant HDAC-6 proteins were transiently transfected into NIH3T3 cells, as indicated. Immunofluorescence stainings for HA and acetyltubulin were performed after 2 days. B, in vivo tubulin deacetylation in 3T3 cells. Upper panel, retroviruses encoding wild type (w.t.) HDAC-6 were used to infect control (lanes 2 and 3) or HDAC-6-deficient 3T3 cells (lanes 1 and 4). For the wild type 3T3s the parental clone is presented (609, lane 1) as well as two derivatives overexpressing HDAC-6 at intermediate (9F5, lane 2) or high level (9F8, lane 3). For the HDAC-6 deficient 3T3s, the parental clone is presented (615, lane 4) as well as two derivatives expressing HDAC-6 at either low (lane 5) or high level (lane 6). Western blot analysis is shown for expression of HDAC-6, α-tubulin, as well as for the level of acetylated, deacetylated (Glu antibody) or tyrosinated (Tyr antibody) tubulin. Genotyping of the different cells is shown. Lower panel, retroviruses encoding wild type (w.t.) or mutant HDAC-6 (HD1m or HD2m, as in Fig. 1A) were used to infect HDAC-6-deficient 3T3s (615, lanes 1–4). In lane 1 an empty expression vector expressing only GFP was used as a control. In lane 2 a clone expressing WT HDAC-6 at intermediate level, similar to the expression level of the mutants (lanes 3 and 4), is presented. Western blot analysis is shown for expression of α-tubulin and for the level of tubulin acetylation. k.a., knock-out.
The results presented are the average from three independent experiments.

**REFERENCES**
