Two Catalytic Domains Are Required for Protein Deacetylation*

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Histone deacetylase (HDAC)-6 was recently identified as a dual substrate, 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 deacetylase activity. Taken together, our results demonstrate for the first time that the spatial arrangement of hdac domains is critical for in vivo deacetylation reaction and may provide a useful model for the development of novel HDAC inhibitors.

Protein acetylation, especially histone acetylation, is one of the most important posttranslational modifications. It is involved in the regulation of protein structure and functions and therefore has potentially important roles in most of cellular processes. In particular, the impact of histone N-terminal acetylation on chromatin organization and gene expression has been well documented. Acetylation and deacetylation of histone tails or of other proteins are catalyzed by histone acetyltransferases (HATs)2 and histone deacetylases (HDACs), respectively. In mammals, there are more than 18 HDACs that can be grouped into Class I, Class II, and Class III HDACs (1, 2, 8). In cells most, if not all, HDACs are part of large molecular weight complexes that typically contain several HDAC polypeptides and are recruited to DNA via their interactions with sequence-specific or nonspecific DNA binding proteins. Among the HDACs, HDAC-6 was recently identified as a dual substrate, and possibly multisubstrate, deacetylase that can deacetylate both histone tails and also α-tubulin Lys40 in vitro and in vivo (3, 4, 5). Interestingly, HDAC-6 is not known to be part of an obligatory higher molecular weight complex and has a unique structure with two intact hdac catalytic domains. HDAC-6 thus mimics in one molecule the presence of more than one hdac domain, as it is observed in other HDAC-containing protein complexes.

In this report, using various in vitro and in vivo assays we demonstrate that both hdac domains of HDAC-6 are essential for activity of this enzyme and propose that the presence of more than one hdac domain is a general requirement for the deacetylation reaction.

RESULTS
Tubulin Acetylation

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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-nitrotriacyclic acid and QFF columns, His6-tagged HDAC-6 proteins could be purified as single protein and equal amount of wild type and mutant proteins were used for activity assays (Fig. 1C, left panel). As shown in Fig. 1C, right panel, the HDAC and TDAC activity of HDAC-6 is intrinsic and requires both intact catalytic cores.

To confirm these in vitro results, tubulin deacetylation was also tested in vivo. For this, HDAC-6 wild type and mutant constructs were transfected into NIH3T3 cells, and the cells were subsequently immunostained for acetylated tubulin and expression of the HA epitope, which marks transfected cells. As shown in Fig. 2A, the results of these experiments agree well with the above in vitro assays; endogenous tubulin acetylation was dramatically reduced by over...
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 4 versus lane 1 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 a control for these experiments a cell clone expressing wild type HDAC-6 at an intermediate level which was similar to that of the mutant proteins (lanes 2-4, Fig. 2B, lower panel). Examination of the degree of tubulin acetylation in the different cell lines (lanes 2-4) revealed that in the two cell lines expressing mutant HDAC-6 tubulin acetylation was not reduced compared with control-transfected cells expressing GFP (lane 1). This result indicates that these two HDAC-6 mutants are inactive when tested in cells lacking endogenous HDAC-6.

The Spatial Arrangement of the hdac Domains in HDAC-6 Is Important for 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 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 insertions (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 show no deacetylase 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 de novo 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 each other 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 IGCSN/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 (Lys56), 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, in all known HDAC–containing complexes, there are functionally two HDACs (10). For example, HDAC-1 is shown 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...
are inactive in the context of the SMRT/N-CoR-HDAC-3 complex, binding between the catalytic domain of HDAC-4 and HDAC-3 via N-CoR/SMRT is crucial for the activity of the complex. *In vivo* analysis of HDAC-1 function in *Drosophila* found that flies have different phenotypes when they are either completely deficient for HDAC-1 or only have a single point mutation, which may toxify HDAC-containing complexes (13). This evidence also indirectly suggested that the mutation in one of the hdac domains in the HDAC-containing complexes could inactivate the whole complex. So far, HDAC-6 is the only HDAC that has been shown to have catalytic activity independently from other HDACs or dimerization. Our results showed that the catalytic activity of HDAC-6 is dependent on both intact hdac domains. Moreover, artificially tethering parts of HDAC-6 and HDAC-4 or -5 were found to result in *de novo* catalytic activity; this mimics the result from N-CoR/SMRT complex, where binding of parts of HDAC-3 via N-CoR/SMRT is crucial for the activity of the complex.

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