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)† 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.

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Materials and Methods

Plasmids and Mutagenesis—Mutations and deletions were generated by QuikChange kit (Strategene). For EGFP insertions, part of the EGFP was PCR-amplified from pEGFP (Clontech) and subsequently cloned into the Xbal site in the linker region of HDAC-6 cDNA. To clone the chimeric HDAC, the hdac domains from either HDAC-4 or -5 were PCR-amplified and used to replace 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 (S798A) 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 (L402A) 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-nitrotriacetic acid and QFF columns, His6-tagged HDAC-6 proteins could be purified as single protein and equal amounts of wild type and mutant HDAC-6 were purified from insect cell transformation were generated using the Bac-To-Bac Baculovirus Expression System (Invitrogen). The recombinant enzymes were purified with a nickel-nitrotriacetic acid Superflow (Qiagen) and HiTrap Q FF columns (Amersham Biosciences).

Immunofluorescence and Immunoblotting—Cells were fixed and stained with anti-HA polyclonal antibody (Santa Cruz Biotechnology) and Tu6—11 for acetylated tubulin (Sigma). The antibodies used were: Tu6—11, DM1A, FLAG M2 (Sigma), anti-HA (Santa Cruz Biotechnology), Gliu-tubulin and Tyr-tubulin (Synaptic Systems), and mHDAC-6 (6).

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 motifs; Ref. 7) (Fig. 1A). The different proteins were prepared from transiently transfected 293T cells by immunoprecipitation with an anti-HA antibody and were used for in vitro activity assays with chemically acetylated histone H4 or α-tubulin peptides (5). The expression levels of each mutant in transiently transfected cells were similar as demonstrated 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 (HD1m/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 (L402A) 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.

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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 expression of 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 vitro 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 HDAC-6 by stably transfecting the 3T3 cells with the HDAC-6 cDNA (Fig. 2B, lane 6). 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. 3C, 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). The most dramatic loss of activity was observed when the entire linker region was deleted (Δ411–478). Interestingly, the impairment of 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

FIGURE 1. Two intact HDAC domains are required for deacetylation by HDAC-6 in vitro. A, schematic representation of the HDAC-6 protein and the mutations made. The conserved hdac domains are shown as gray boxes with the catalytic cores highlighted in red. The ER motif is shown in yellow. The HDAC-6 mutants have the following structure: HD1m, Asp250 and Asp252 are replaced by Asn, and His254 and His255 are replaced by Val; HD2m, Asp648 is replaced by Asn and His650 and His651 are replaced by Val. The mutants in the ER motif have either Leu187 replaced by Ala (L180A) or Leu188 replaced by Ala (L187A). B, in vitro HDAC or TDAC activity assays with wild type and mutant HDAC-6s. 293T cells were transfected with appropriate expression vectors and cell extracts were immunoprecipitated with an anti-HA antibody. Upper panel, equal protein input was confirmed by Western blotting with HA antibody. Lower panel, for the assays, acetylated tubulin peptide or histone H4 tail peptide were used as substrates. The results presented are averaged from three independent experiments. C, recombinant HDAC-6 needs two intact hdac domains to be active. Left panel, purified wild type and mutant HDAC-6 proteins (5 µl) were analyzed by SDS-PAGE. Right panels, acetylated tubulin peptide or histone H4 tail peptide were used as substrates for deacetylation assays performed with equal amounts of purified proteins. The results presented are averaged from three independent experiments.

Y. Zhang, unpublished data.
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 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 (Lys18), 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 deacetylase activity for histones, and possibly other proteins, is found in HDAC-containing multiprotein complexes. Interestingly, in 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 5 and 6). 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, detyrosinated (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.o., knock-out.
The results presented are the average from three independent experiments. The spatial arrangement of two hdac domains in HDAC-6 is important for the selective activities on different substrates. A, schematic representation of insertion and deletion mutants of HDAC-6. Fragments from EGFP, varying from 5 amino acids to full-length (shown by green), were inserted between the two hdac domains in wild type (W.T.) HDAC-6. In the deletion mutants, various lengths were deleted from the linker region (depicted by the dotted boxes). B, 293T cells were transfected with appropriate expression vectors and cell extracts were immunoprecipitated with an anti-HA antibody. Western blotting (WB) with HA antibody was used to check the protein input used for the activity assays, as in Fig. 1B. C, extracts from B were used HDAC or TDAC activity assays. The results presented are the average from three independent experiments.

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 be made active by being tethered to HDAC-3. Based on these observations, we propose a possible general model for the deacetylation reaction, in which two hdac domains are required. As in the case for HDAC-6, HDAC-containing complexes might have a specific spatial arrangement of hdac domains, originating from two different HDAC molecules. These two hdac domains cooperate to confer the catalytic activity of the whole complex. The components of the different complexes determine the spatial arrangement of the two core hdac domains and therefore the specific activity of the complexes.

FIGURE 3. The spatial arrangement of two hdac domains in HDAC-6 is important for the selective activities on different substrates. A, schematic representation of insertion and deletion mutants of HDAC-6. Fragments from EGFP, varying from 5 amino acids to full-length (shown by green), were inserted between the two hdac domains in wild type (W.T.) HDAC-6. In the deletion mutants, various lengths were deleted from the linker region (depicted by the dotted boxes). B, 293T cells were transfected with appropriate expression vectors and cell extracts were immunoprecipitated with an anti-HA antibody. Western blotting (WB) with HA antibody was used to check the protein input used for the activity assays, as in Fig. 1B. C, extracts from B were used HDAC or TDAC activity assays. The results presented are the average from three independent experiments.

FIGURE 4. Generation of active artificial chimeric HDACs from inactive HDAC fragments. A, schematic representation of HDAC-4, -5, and -6 and chimeric HDACs thereof. The hdac domains from either wild type HDAC-4 or HDAC-5 were PCR amplified and used to replace the first and/or second hdac domains in wild type HDAC-6. B, chimeric HDACs and HDAC-6 were immunoprecipitated from transfected 293T cells and used for HDAC or TDAC assays. The results averaged from three independent experiments are presented.

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