Assembly of the SMRT–histone deacetylase 3 repression complex requires the TCP-1 ring complex

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Nucleosomes, the basic building block of chromatin, are composed of histone octamers. Posttranslational modification of histone tails by kinases, acetyltransferases, deacetylases, and methylases contributes to remodeling of chromatin structure that regulates gene expression. The “histone code” hypothesis holds that specific modification patterns serve as markers for transcriptional regulatory complexes with different transcriptional consequences [Jenuwein and Allis 2001]. Lysine residues are modified by both acetyltransferases and methylases. Acetylation of lysines is associated with transcriptional activation, whereas lysine methylation can be associated with activation or repression (Jenuwein and Allis 2001). The acetylation of histone tails is a primary determinant of gene activity. Histone deacetylase 3 (HDAC3) requires the nuclear receptor corepressor SMRT for HDAC enzyme activity. Here we report that HDAC3 interacts with SMRT only after priming by cellular chaperones including the TCP-1 ring complex (TRiC), which is required for proper folding of HDAC3 in an ATP-dependent process. SMRT displaces TRiC from HDAC3, yielding an active HDAC enzyme. The SMRT–HDAC3 repression complex thus joins the VHL–elongin BC tumor suppression complex and the cyclin E–Cdk2 cell cycle regulation complex as critical cellular machines requiring TRiC for proper assembly and function. The strict control of HDAC3 activity underscores the cellular imperative that histone deacetylation occur only in targeted regions of the genome.

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Nucleosomes, the basic building block of chromatin, are composed of histone octamers. Posttranslational modification of histone tails by kinases, acetyltransferases, deacetylases, and methylases contributes to remodeling of chromatin structure that regulates gene expression. The “histone code” hypothesis holds that specific modification patterns serve as markers for transcriptional regulatory complexes with different transcriptional consequences [Jenuwein and Allis 2001]. Lysine residues are modified by both acetyltransferases and methylases. Acetylation of lysines is associated with transcriptional activation, whereas lysine methylation can be associated with activation or repression [Jenuwein and Allis 2001]. Thus the balance among unmodified, acetylated, and methylated lysine is a critical determinant of gene expression. Histone deacetylases [HDACs] reverse the activated state characterized by acetylation, and are associated with transcriptional repression [Hassig and Schreiber 1997]. HDACs contribute to the dynamic nature of chromatin remodeling, because they convert acetylated lysine to an unmodified state that is subject to methylation in addition to reacetylation. HDACs also impose direct control on nonchromatin targets such as the p53, E2F, and NF-kB transcription factors [Martinez-Balbas et al. 2000; Chen et al. 2001; Luo et al. 2001; Vaziri et al. 2001] and can influence microtubule-dependent cell motility by deacetylation of α-tubulin [Hubbert et al. 2002]. Consistent with their important role in various cellular processes, HDACs function in multiprotein repression complexes, and their activities are highly regulated. Class II HDACs, such as HDAC4, are retained in the cytoplasm following direct phosphorylation, thus preventing their function in the nucleus [McKinsey et al. 2000]. Enzymatic activity of HDAC4 may require complex formation with HDAC3 [Fischle et al. 2002]. HDAC3 is inactive on its own but is activated by forming a stable complex with the nuclear hormone receptor corepressor SMRT (or N-CoR, Wen et al. 2000; Guenther et al. 2001), which functions as a subunit in an active HDAC3–SMRT enzyme [Guenther et al. 2001]. The deacetylase activating domain (DAD) of SMRT/N-CoR, containing an essential SANT domain, is sufficient for HDAC3 interaction and activation [Guenther et al. 2001; Zhang et al. 2002].

While investigating the role of SMRT in activating the HDAC3 enzyme, we discovered the requirement for a preceding energy-requiring step, involving the TRiC multiprotein chaperone complex. This stringent regulation of HDAC3 activity provides a mechanism to ensure that histone deacetylation occurs specifically in targeted regions of chromatin.

Results and Discussion

An energy-dependent priming step is required for HDAC3 activation by SMRT

HDAC3 produced in rabbit reticulocyte lysate (RLR) is enzymatically inactive but can be activated by addition of SMRT [Guenther et al. 2001]. HDAC3 purified from insect cells was also inactive [Fig. 1a]. However, addition of SMRT was insufficient to activate this preparation of HDAC3 unless the purified HDAC3 was first preincubated or “primed” with unprogrammed RRL [Fig. 1a]. This priming step was inhibited by nonhydrolyzable ATP [Fig. 1b], indicating that ATP was required. Interaction of HDAC3 with SMRT also required priming by RRL [Fig. 1a,b]. The effect of RRL was dose-dependent [Fig. 1c], suggesting that priming might require a stoichiometric rather than a catalytic interaction with one or more components of the RRL.

HDAC3 enzyme activation by SMRT requires prior binding to the ATP-dependent TRiC protein folding machine

To determine the mechanism of HDAC3 priming, purified Flag–HDAC3 was incubated with RRL and immunopurified using Flag-agarose. Silver staining of the bound material revealed a band corresponding to HDAC3, as well as several other bands whose intensity suggested a stoichiometric complex [Fig. 2a]. Direct sequencing re-
revealed one of these proteins to be Hsc70, which has been previously shown to interact with HDAC3 (Johnson et al. 2002). The role of Hsc70 in HDAC3 priming was supported by the effects of deoxyspergualin (DSG), an inhibitor of Hsc70 function (Nadler et al. 1992) that blocked HDAC3 activation and interaction with SMRT (data not shown). Remarkably, all of the other proteins were components of the TCP-1 ring complex (TRiC; also called chaperonin-containing TCP-1, or CCT). TRiC is an ~800-kD, ATP-dependent protein folding machine comprised of eight orthologous subunits that are arranged similarly to bacterial GroEL (Dunn et al. 2001; Hartl and Hayer-Hartl 2002). The association of HDAC3 with TRiC was confirmed by immunoprecipitation of HDAC3 followed by immunoblotting for TRiC constituents (Fig. 2b). Thus, HDAC3 forms a stable and stoichiometric association with the TRiC (Fig. 2b).

**TRiC dissociates from the active SMRT–HDAC3 complex**

We investigated whether TRiC remains bound to HDAC3 after formation of the active SMRT–HDAC3 complex. Excess Flag–HDAC3 was primed by reticulocyte lysate prior to addition of SMRT. Immunoprecipitated SMRT was associated with HDAC3, but not with TRiC subunits, whereas HDAC3 not associated with SMRT was associated with TRiC (Fig. 2d). Similar results were obtained after overexpression of HDAC3 in 293T cells, where cellular TRiC was found to associate with Flag–HDAC3 alone but not in the context of the SMRT–HDAC3 complex (Fig. 2e).

**Geldanamycin inhibits TRiC–HDAC3 complex formation**

Interestingly, the protein folding inhibitor geldanamycin (GA) prevented the formation of the HDAC3–TRiC complex (Fig. 3a). GA is a relatively specific inhibitor of heat shock protein 90 (Hsp90; Scheibel and Buchner 1998). However, we were unable to demonstrate a stable interaction between HDAC3 and Hsp90 in reticulocyte lysate (data not shown). The Hsp90 homolog GRP94 is also GA-sensitive (Blagosklonny 2002), but GRP94 could not be demonstrated to interact with HDAC3 (data not shown) and is located in the endoplasmic reticulum, making it an unlikely chaperone for HDAC3. Thus, the TRiC–HDAC3 interaction is dependent upon Hsp90 (or another GA-sensitive chaperone). As predicted from its prevention of HDAC3 priming by TRiC, GA also prevented formation of the HDAC3–SMRT complex and activation of HDAC3 by SMRT (Fig. 3b). This effect was specific for HDAC3, as SMRT interaction with TBL1 was unaffected (Fig. 3b). However, when GA was added after formation of the TRiC–HDAC3 complex, it was in-
effective at preventing formation of the enzymatically active HDAC3–SMRT complex (Fig. 3c). Thus the effect of GA on priming of HDAC3 for SMRT interaction occurs proximal to the formation of the TRiC–HDAC3 complex.

Prevention of HDAC3 priming blocks nuclear localization of HDAC3

Because GA blocks priming of HDAC3 with TRiC and, consequently, interaction with SMRT/N-CoR, it was used as a tool to determine the effects of priming on the subcellular localization of HDAC3. In agreement with others [Yang et al. 2002], we found that HDAC3 was distributed between the cell nucleus and cytoplasm [Fig. 3d]. GA completely prevented nuclear localization of HDAC3 [Fig. 3d]. N-CoR was constitutively nuclear in the presence or absence of GA [Fig. 3d, iv], indicating that GA did not generally prevent nuclear import of proteins and, furthermore, that nuclear localization of N-CoR does not require HDAC3. These data suggest that nuclear localization of HDAC3 requires priming by TRiC and, potentially, interaction with N-CoR or SMRT.

Conclusion

Based on our findings, we propose the following model of HDAC3 activation. After translation, unfolded HDAC3 is likely bound to Hsp90, explaining the GA sensitivity. The HDAC3–Hsp90 association is transient, and Hsp90 is rapidly replaced by the Hsc70–TRiC (Fig. 4). This step is ATP-dependent [Melki et al. 1997], as is the putative Hsp90 interaction. Interestingly, although the HDAC3–TRiC complex is stable in reticulocyte lysate and in cells, Flag–HDAC3 purified from insect cells is not associated with TRiC [Fig. 2a] and also cannot be activated by SMRT [Fig. 1a]. It is possible that the amount of HDAC3 produced overwhelms the insect cell TRiC machinery, or that the TRiC complex is lost upon stringent purification of the recombinant HDAC3. Coexpression of HDAC3 and the SMRT DAD in insect cells does lead to formation of a stable, active complex [Guenther et al. 2001]. We suggest that this is due either to transient association of HDAC3 with insect TRiC, or to a direct ability of coexpressed SMRT to stabilize newly folded HDAC3. In mammalian systems, HDAC3 is only primed for interaction with SMRT when bound to TRiC. Upon SMRT binding, TRiC dissociates from HDAC3 which together with SMRT now constitutes a stable, enzymatically active deacetylase complex.

The requirement of TRiC for formation of enzymatically active HDAC3–SMRT complex has important implications. TRiC is also critical for the formation and activity of the VHL–elongin BC elongase complex [Feldman et al. 1999; Hansen et al. 2002] and the cyclin E–cdk2 protein kinase complex [Won et al. 1998]. Yet most protein–protein interactions do not require priming by TRiC [Hartl and Hayer-Hartl 2002]. Thus, TRiC plays
a special, thematic role in the formation of enzymatically active multiprotein complexes that mediate critical cellular functions such as translation (VHL-BC), cell cycle regulation (Cyclin E-cdk2), and transcription (SMRT-HDAC3). Moreover, corepressors have been implicated in the pathophysiology of acute myelogenous leukemias [Minucci et al. 2001]. HDAC inhibitors have been used to treat these diseases, but current HDAC inhibitors target multiple HDACs and have numerous side effects [He et al. 2001; Marks et al. 2001]. The search for specific HDAC inhibitors requires in vitro reconstitution of active HDACs, and the present work shows that proper folding will be critical. In addition, the chaperonin complexes required for formation of the HDAC3–SMRT repression complex represent novel and potentially HDAC-type-specific therapeutic targets. It is interesting in this context that GA is a chemotherapeutic agent for cancer [Neckers et al. 1999]. Finally, the multilevel regulation of HDAC3 deacetylase activity is of critical importance to normal cell function. The control of HDAC3 activity by TRiC as well as a SANT-containing SMRT ortholog is likely to extend to yeast [Pijnappel et al. 2001]. Conservation of this strict control of HDAC activity is undoubtedly related to the deleterious consequences of unregulated nuclear HDAC activity. Proper folding of HDAC3 by chaperone machines coupled with the formation of the active repression complex with SMRT/N-CoR thus regulates both HDAC3 localization and enzyme activity. This is in keeping with the recent suggestion that coupling of multicomponent cellular machines coordinates the activity, efficiency, and specificity of each step in gene expression [Maniatis and Reed 2002].

Materials and methods

**Plasmids**

Flag-HDAC3 was a gift from Drs. Eric Verdin and Wolfgang Fischle. Myc–HDAC3 was produced by cloning HDAC3 cDNA (encoding amino acids 1–428) into pcDNA3.1myc/his (Invitrogen). Myc–TBL1 was produced by cloning TBL1 cDNA (encoding amino acids 1–577) into pcDNA3.1myc/his (Invitrogen). Gal4–mSMRT plasmid containing Gal4–DBD fusion to mSMRT amino acids 1–763 was described previously [Guenther et al. 2001].

Production of active HDAC3 complexes in vitro

Myc–HDAC3, Gal4–SMRT 1–763, and Myc–TBL1 were translated individually with a TNT T7 Quick Coupled Transcription/Translation Kit (Promega) according to the manufacturer’s instructions; 100 µL of translated material was used as a source for each protein unless otherwise noted. After translation, the desired proteins were mixed and added to two volumes of buffer D-300 (300 mM KC), 20 mM HEPES at pH 7.9, 0.25
Figure 4. Model depicting the production of an active HDAC3 enzyme. Newly translated, inactive HDAC3 associates with the multiprotein TRiC complex and Hsc70, an energy-dependent step that allows SMRT to interact with and activate HDAC3. TRiC association is prevented by the Hsp90 inhibitor geldanamycin, suggesting a role for Hsp90, although the interaction of HSP90 with HDAC3 has not been directly shown. The TRiC/Hsc70 complex dissociates from the enzymatically active SMRT-HDAC3 deacetylase complex, which functions as a corepressor for nuclear receptors (NR).
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