Human HDAC7 Histone Deacetylase Activity Is Associated with HDAC3 *in Vivo**

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   Histone deacetylases (HDACs) are part of transcriptional corepressor complexes and play key roles in regulating chromatin structure. Three different classes of human HDACs have been defined based on their homology to HDACs found in *Saccharomyces cerevisiae*: RPD3 (class I), HDA1 (class II), and SIR2 (class III). Here we describe the identification and functional characterization of HDAC7, a new member of the human class II HDAC family. Although HDAC7 is localized mostly to the cell nucleus, it is also found in the cytoplasm, suggesting nucleocytoplasmic shuttling. The HDAC activity of HDAC7 maps to a carboxyl-terminal domain and is dependent on the interaction with the class I HDAC, HDAC3, in the cell nucleus. Cytoplasmic HDAC7 that is not bound to HDAC3 is enzymatically inactive. We provide evidence that the transcriptional corepressors SMRT and N-CoR could serve as critical mediators of HDAC7 activity by binding class II HDACs and HDAC3 by two distinct repressor domains. Different class II HDACs reside in the cell nucleus in stable and autonomous complexes with enzymatic activity, but the enzymatic activities associated with HDAC7 and HDAC4 rely on shared cofactors, including HDAC3 and SMRT/N-CoR.

The packaging of DNA into chromatin restricts the accessibility of DNA to factors involved in fundamental cellular processes such as DNA replication and transcription. The repeating organizing unit of chromatin is the nucleosome (1). Each nucleosome consists of a core built of two copies of histones H2A, H2B, H3, and H4, around which the DNA is tightly wrapped and bound by electrostatic interactions (2). Consistent with the repressive effects of chromatin on gene expression, gene activation is often accompanied by nucleosomal rearrangements (3, 4). Such local or extended structural changes in chromatin are achieved by ATP-driven chromatin remodeling complexes and

by posttranslational acetylation, methylation, or phosphorylation of histones (5–7).

   The most abundant and best studied posttranslational modification of chromatin is the reversible acetylation of lysines in the amino-terminal tails of the four core histones (1, 8, 9). Transcriptionally silenced regions, such as heterochromatin and the inactivated mammalian X chromosome, are associated with hypoacetylated histones (10). In contrast, transcriptionally active domains in euchromatin are often associated with histone hyperacetylation (11). Localized changes in histone acetylation levels near the transcriptional start site of certain genes are linked to gene activation or repression (12–15). The causal link between histone acetylation and transcriptional regulation is dramatically illustrated by the identification and characterization of transcriptional regulators containing histone acetyltransferase or deacetylase (HDAC)[1] activities (16–19).

   HDAC proteins are classified in three distinct families (19–21). Class I HDACs (HDAC1, 2, 3, and 8) are derived from the yeast transcriptional regulator RPD3 (22, 23). Class II HDACs (HDAC4, 5, 6, and 7) are similar to HDA1, another deacetylase in yeast (23, 24). Class III HDACs are related to the yeast silencing protein SIR2 and are dependent on NAD for enzymatic activity (25).

   In contrast to the relative compact proteins of class I, class II HDACs (HDAC4, 5, and 7) possess two distinct domains (26–30). The carboxyl-terminal domain of HDAC4 has catalytic activity *in vivo*, whereas the amino-terminal domain exerts autonomous repressor activity by an unknown mechanism (27, 30–32). Class I HDACs are expressed in most cell types; class II HDACs are expressed in a tissue-specific manner and have been implicated in the regulation of muscle differentiation (26, 30, 33–35). HDACs 4 and 5 bind to transcription factors of the MEF2 family via their amino-terminal domain (27, 36). This binding is regulated by calcium through calmodulin and calmodulin-dependent kinases (33, 34, 37, 38). Both HDAC4 and HDAC5 shuttle in and out of the cell nucleus in a regulated manner controlled by phosphorylation and interaction with 14-3-3 proteins (34, 39, 40). Class II HDACs 4, 5, and 7 interact with SMRT, N-CoR, and BCoR, an additional corepressor that mediates repression by BCL-6 (35, 41, 42).

   HDACs are part of high molecular mass corepressor complexes (19, 43). These complexes are recruited to specific promoters via their interactions with sequence-specific DNA-binding proteins, including the nuclear hormone receptors, the E box-binding factors, and the methylcytosine-binding protein

   [1] The abbreviations used are: HDAC, histone deacetylase; aa, amino acids; GST glutathione *S*-transferase; kb, kilobases; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; TSA, trichostatin A; HIV, human immunodeficiency virus.

MeCP2 (19, 20). Class I HDAC1 and HDAC2 are found in SIN3 and NURD·Mi2 complexes (19, 43). In contrast, HDAC3 is associated with the corepressors SMRT and N-CoR in a complex that mediates transcriptional repression by the thyroid hormone receptor and the oncoprotein v-ErbA (44–48).

Mutational analysis of RPD3 and HDAC1 indicates that enzymatic activity is essential for the transcriptional regulatory functions of these proteins *in vivo* (49, 50). However, with few exceptions, it has been remarkably difficult to obtain recombinant HDAC proteins demonstrating enzymatic activity *in vitro*. Immunoprecipitation experiments have been required to study and characterize the enzymatic activity of most HDACs (22, 30, 49–52). In addition, biochemical fractionation of mammalian cell extracts has indicated that several additional proteins beside HDAC1 and HDAC2 might be necessary to build an enzymatically active HDAC1·HDAC2 core complex. The limitations observed in the expression of recombinant HDAC activity could therefore be the consequence of missing essential cofactors (53).

This report describes the identification and characterization of a new member of the class II HDAC family, human HDAC7. *In vivo*, HDAC7 is associated with enzymatic activity when it is in the cell nucleus but not when it is in the cytoplasm. This enzymatic activity is dependent on the association of the carboxyl-terminal region of HDAC7 with the class I HDAC HDAC3. We provide evidence that the transcriptional corepressors SMRT and N-CoR could serve as mediators that simultaneously bind class II HDACs and HDAC3 by two distinct repressor domains.

EXPERIMENTAL PROCEDURES

Cell Culture—All cell lines were obtained from the American Type Culture Collection and grown in RPMI 10% fetal bovine serum at 37 °C in a humidified incubator. The following cell lines were used in these experiments: HISM, human smooth muscle (ATCC CRL-1692); SKN, human neuroblastoma (ATCC HTB-11); 10T1/2, mouse embryo (ATCC CCL-226); Cos7, African green monkey, SV40-transformed kidney cells (ATCC CRL-1651); HeLa, human cervical epitheloid carcinoma (ATCC CCL-2.1); and 293, transformed human kidney (ATCC CRL-1573).

Plasmids and Recombinant DNA—Computer algorithms for predicting exon-intron boundaries (Genscan (54); GenFinder (55)) and sequence similarity of HDAC7 to HDAC4 and HDAC5 (26, 30) were used to deduce a putative ORF derived from a genomic sequence located at chromosome 12q31 (GenBank™ accession number AC0004466). Overlapping human cDNA fragments were amplified from a HeLa cell cDNA library (CLONTECH) using the following primer pairs: 5′-CGGAATTC-CAGCCCATGGACCTGCGGGTG-3′ and 5′-CGGAATTCGACCGAGT-CATAGATCAGCC-3′; and 5′- CGGAATTCACCATGGCCCCGCTGCT-GACTGTGCC-3′ and 5′- CGGAATTCGAGATTCATAGGTTCTTCCTC-3′. The full-length HDAC7 ORF was generated using a unique *Bcl*I site in these fragments, and the resulting cDNA was extended in the 5′ and 3′ directions by anchored polymerase chain reaction strategies (rapid amplification of cDNA ends). Nucleotide sequencing on both strands was performed using the dideoxy sequencing method (ABI bioprism). FLAG-tagged expression vectors for different HDACs were generated as described previously (30). HDAC5 cDNA was obtained as an expressed sequence tag clone (Id 1142916) from the IMAGE Consortium, and the sequence corresponding to nucleotides 394–3369 of the HDAC5 ORF was used to construct the fusion protein (26). HDAC6 was cloned from a human cDNA library (CLONTECH) with primers corresponding to the sequence from GenBank™ accession number AJ011972 (defined as a transcription map in Xp11). The sequence of this ORF is identical to that of HDAC6 (26). Deletion constructs were generated by standard procedures (56, 57). *Eco*RI fragments from the pcDNA3.1 expression constructs were subcloned into the *Eco*RI site of pGEX4T1 (Amersham Pharmacia Biotech) to generate amino-terminal GST fusion proteins.

Northern Blot Analysis—A multiple human tissue Northern blot was obtained from CLONTECH. ^{32}P-Labeled probes corresponding to hHDAC7 and hGAPDH cDNAs were prepared with the Multiprime DNA labeling system (Amersham Pharmacia Biotech). The blots were prehybridized and hybridized with ExpressHyb hybridization solution (CLONTECH) and washed under high stringency conditions (58). Autoradiographs were analyzed with a FUJIX BAS1000 phosphorus im-

aging system (Fuji, Tokyo, Japan).

Generation of Antibodies—cDNA fragments corresponding to aa 118–662 of hHDAC4 and aa 1–487 of hHDAC7 were cloned into the pGEX4T1 vector (Amersham Pharmacia Biotech) to generate hybrid proteins fused to GST. Fusion proteins were expressed in BL21 RIP (Stratagene), purified according to standard procedures (Amersham Pharmacia Biotech), and separated from copurified contaminating bacterial proteins by large scale preparative SDS-polyacrylamide gel electrophoresis (PAGE). Antisera against the recombinant proteins were raised in New Zealand White rabbits according to standard procedures (Harlan Bioproducts). To eliminate antibody species with affinity for GST and to reduce putative crossreactivity, antisera were depleted on GST and ortholog GST-HDAC columns (*e.g.* HDAC7 antiserum was depleted on GST-HDAC4 columns) followed by purification of the IgG species according to standard protocols (59).

Immunoprecipitation—Immunoprecipitation was performed as described previously (30). For FLAG-tagged proteins, M2-agarose (Sigma) was used at 15 µl/ml. For precipitation of endogenous proteins, the polyclonal anti-HDAC4 and anti-HDAC7 antisera were used at ~10 µg/ml in combination with 20 µl/ml of a preblocked (10 mg/ml bovine serum albumin) 50% protein G-Sepharose slurry (Amersham Pharmacia Biotech).

SDS-PAGE and Western Blotting—SDS-PAGE and Western blot analysis were performed according to standard procedures (57). Western blots were developed with ECL (Amersham Pharmacia Biotech). Polyclonal anti-HDAC1 and anti-HDAC3 antisera have been described previously (60). Polyclonal anti-IKKα, anti-SIN3, anti-HDAC2, and anti-FLAG antisera used were from Santa Cruz Biotechnology (Santa Cruz, CA). SMRT and N-CoR antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY) and Affinity BioReagents (Golden, CO).

Histone Deacetylase Activity Assays—HDAC activity was measured with a chemically [^{3}H]acetylated peptide corresponding to the amino-terminal tail of histone H4 as described previously (30). Complexes immobilized on beads either from immunoprecipitations or GST pull-down assays were resuspended in 30 µl of HD buffer containing ~50,000 counts/min of the substrate.

Preparation of Nuclear and Cytoplasmic Extracts—The cell nuclei were isolated and lysed according to Osborn *et al.* (61) with slight modifications.

Immunofluorescence and Confocal Microscopy—The cells were plated onto glass coverslips and transfected the following day with LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer's instructions. After incubation overnight, the transfected cells were fixed with 1% paraformaldehyde, permeabilized with phosphate-buffered saline, pH 7.2, and 0.5% Triton X-100 and then incubated for 60 min at room temperature with the appropriate antibodies at dilutions typically between 1:100 and 1:1000. The cells were mounted onto glass slides in phosphate-buffered saline with 90% glycerol containing 1 mM paraphenylenediamine as an antifade reagent. Confocal microscopy was performed with a Zeiss LSM510 laser scanning confocal microscope at 488 nm (Alexa 488-conjugated secondary antibodies) and 543 nm (Cy3-conjugated secondary antibodies). The pinhole aperture was set to 1.0 airy unit for each channel. Band pass filters and sequential scanning of individual fluorescent probes were used to eliminate cross-talk between channels. The images were collected as 12-bit images, and each channel was independently rescaled from 0 to 255 (8-bit) for final presentation.

GST Fusion Proteins: Expression and Pull-down Assays—Fusion proteins were expressed in DH5α (Life Technologies, Inc.) or BL21 RIP (Stratagene) and purified according to standard protocols (Amersham Pharmacia Biotech). Purity, as well as protein amount, was checked on SDS-PAGE gels stained with Coomassie Blue. For pull-down reactions, extracts from 293, HeLa, A301, or SupTI cells were used (30, 62). After incubation for 3 h at 4 °C with rocking, reactions were washed three times in 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, three times in the same buffer but at 1 M NaCl, and two times in HD buffer (30). Pull-down reactions were split and either processed for histone deacetylase activity assays or for Western blotting. For pull-down reactions with ^{35}S-labeled proteins, cDNAs were transcribed and translated with the TNT reticulocyte system (Promega). The labeling reaction (5 µl) was incubated with GST fusion proteins in a total volume of 200 µl of GST buffer (20 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM dithiothreitol, 2 mM EDTA, 0.5% nonfat dry milk, 0.1% Nonidet P-40) for 1 h at 4 °C with rocking. The reactions were washed five times in the same buffer, and bound proteins were resolved on denaturing SDS-PAGE gels for analysis by autoradiography.
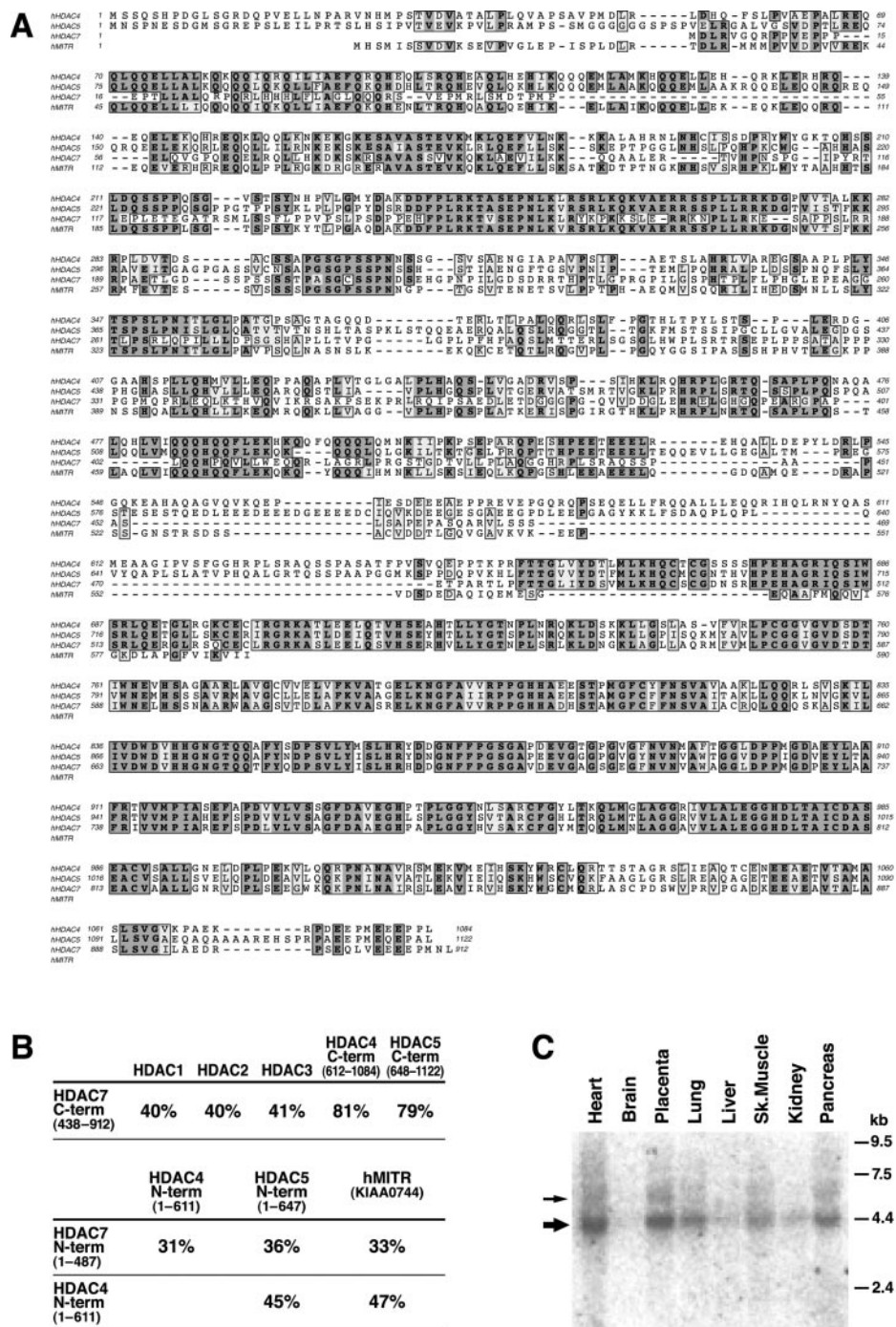
HDAC7 Functionally Depends on HDAC3

FIG. 1. **hHDAC7 is a human class II HDAC.** *A*, an alignment of the predicted amino acid sequences of human HDAC4, HDAC5, HDAC7, and MITR was created with the ClustalW routine of the MacVector program. Identical amino acids are *shaded*; related amino acids are *boxed*. *B*, degree of similarity between different HDACs. The percentages refer to the homology of amino acid sequences in the amino-terminal domains (*N-term*) or the carboxyl-terminal HDA1-like regions (*C-term*) and were calculated based on the alignment shown in *A*. *C*, expression profile of human HDAC7 mRNA. A multiple human tissue Northern blot was probed with the HDAC7 cDNA. The *arrows* indicate the major mRNA species of ~4.4 kb and a putative alternatively spliced species of ~6.0 kb. Molecular size markers are indicated on the *right*.

RESULTS

HDAC7, a New Member of the Human Class II HDAC Family—We identified previously a new human genomic sequence, located on chromosome 12q31 (GenBank™ accession number AC0004466) that potentially encoded a new human class II HDAC (30). Two different computer algorithms for the prediction of exon-intron boundaries (Genscan (54); GenFinder (55)) and the sequence similarity to HDAC4 and HDAC5 were used to deduce a putative cDNA encoded by this locus. Based on this information we devised a polymerase chain reaction strategy in combination with 5′ and 3′ rapid amplification of cDNA ends

reactions to clone several cDNAs sharing an ORF of 2739 base pairs but different 5′ extremities. The amplified cDNAs contained a single putative start codon embedded in a sequence environment favorable for the initiation of translation (63) and in-frame with an upstream stop codon (data not shown). The corresponding ORF translated to a putative polypeptide of 912 aa (Fig. 1*A*). The same polypeptide originating from the 12q31 locus was independently predicted by the Human Genome Project (GenBank™ accession number XP-007047). Interestingly, the amino terminus of human HDAC7 is 22 aa shorter than that of murine HDAC7, despite >95% conservation of the rest
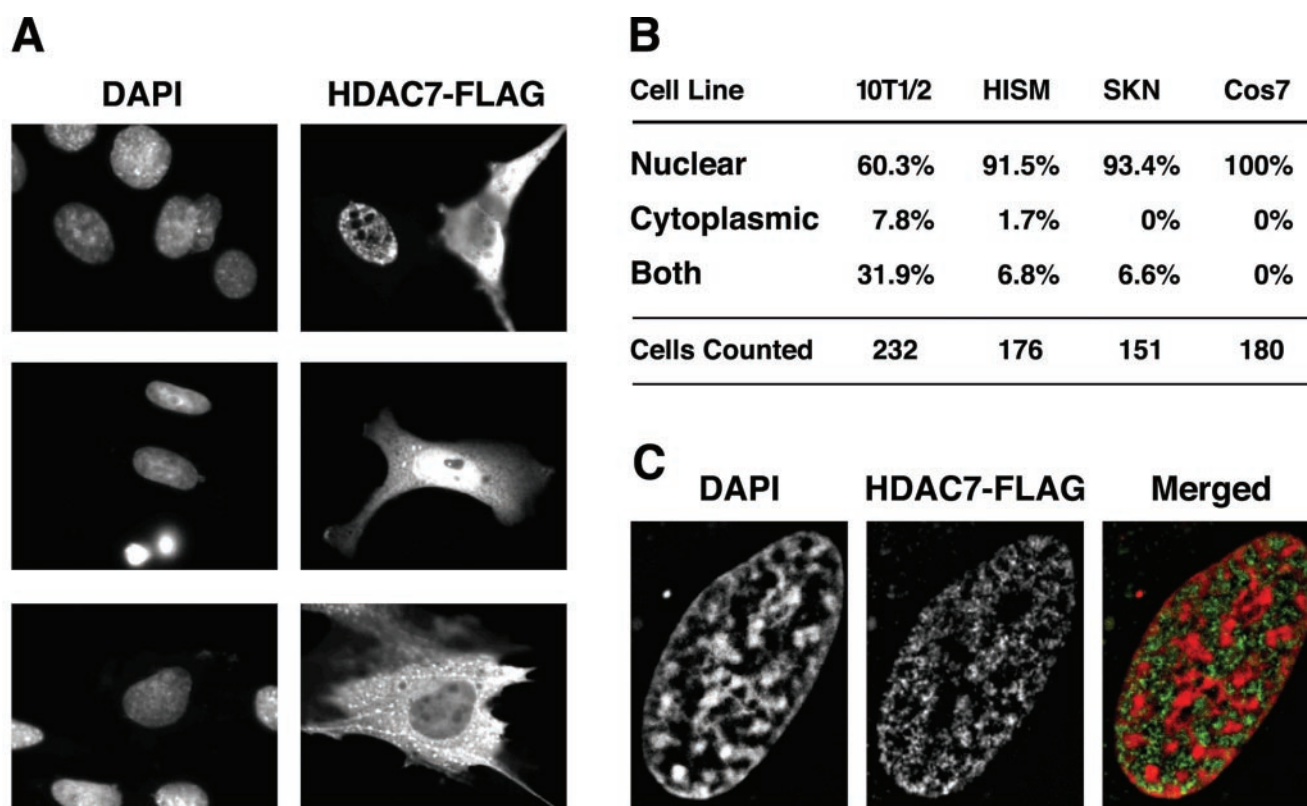
FIG. 2. **HDAC7 is a predominantly nuclear protein that localizes to discrete foci in the cell nucleus.** *A*, HDAC7-FLAG was transiently transfected into HISM cells and analyzed by immunofluorescence confocal microscopy. Each *row* represents a distinct cellular distribution of HDAC7. *Top row*, nucleus; *middle row*, nucleocytoplasmic; *bottom row*, cytoplasm. The *left panels* show the cell nucleus as reflected by the DNA stained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI), and the *right panels* show the corresponding anti-FLAG staining. *B*, the subcellular localization of HDAC7-FLAG was analyzed after transfection into 10T1/2, HISM, SKN, and Cos7 cells. The fraction of cells with HDAC7 present in the nucleus, in the cytoplasm, and in both locations is given for each cell line. *C*, HDAC7-FLAG accumulates in discrete foci within the interchromatin space. The nucleus of a HeLa cell transiently expressing HDAC7-FLAG was imaged by collecting serial z sections at 0.4-μm intervals. A single optical section near the center of the cell nucleus is shown. The *left panel* shows the DNA stained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI), and the *right panel* shows the corresponding anti-FLAG staining.

of the protein (35). A five-amino acid motif, PXDLR (64), conserved in HDAC4, HDAC5, and hMITR and implicated in the interaction with the transcriptional corepressor CtBP is missing in the human HDAC7 protein.

As previously reported for other class II HDACs, HDAC7 contains two distinct domains. The carboxyl-terminal domain (aa 438–912) shows high homology to known HDACs, in particular class II HDACs. The amino-terminal domain is related to similar regions in HDAC4, HDAC5, and hMITR (Fig. 1, *A* and *B*).

The expression of HDAC7 in different tissues was examined by Northern blot (Fig. 1*C*). A major transcript of 4.4 kb was detected mainly in heart and placenta but also in pancreas, lung, and skeletal muscle. A weaker band corresponding to 6.0 kb might represent a differentially spliced isoform of the HDAC7 mRNA. The size of 4.4 kb is in good agreement with the lengths of several cDNAs cloned (data not shown).

HDAC7 Is a Predominantly Nuclear Protein and Localizes to Discrete Foci in the Cell Nucleus—To examine the subcellular localization of the HDAC7 protein, we transfected an HDAC7-FLAG fusion construct into HISM (SKN, HeLa, 10T1/2, 293, and Cos7) cells and performed indirect immunofluorescence with an anti-FLAG M2 antibody (Ref. 65 and Fig. 2*A*). In most cells examined, the HDAC7 protein was found predominantly in the cell nucleus (Fig. 2*A*, *top row*, cell to the *left*). However, in a significant fraction of the transfected cells, HDAC7 was present in both the cell nucleus and cytoplasm (Fig. 2*A*, *top* and *middle rows*). In some cells, HDAC7 was present in the cytoplasm only (Fig. 2*A*, *bottom row*). The relative proportion of

cells containing HDAC7 expressed only in the nucleus, only in the cytoplasm, or in both the nucleus and cytoplasm varied depending on the cell line examined (Fig. 2*B*). For example, HDAC7 was exclusively nuclear in Cos7 cells, whereas only 60% of 10T1/2 cells contained HDAC7 exclusively localized in the nucleus. The relative expression levels of HDAC7 in these cell lines (10T1/2 > HISM > SKN > Cos7) suggest that higher levels of expression coincide with cytoplasmic localization of HDAC7 (Fig. 2*B*). The presence of HDAC7 in both the nucleus and cytoplasm indicates that it might shuttle in and out of the nucleus. Nucleocytoplasmic shuttling has been observed for other class II HDACs (27, 34, 39, 40) and reflects a putatively important regulatory mechanism for these factors (66). Treatment of HDAC7-transfected cells with leptomycin B, a fungal toxin that inhibits CRM1-mediated nuclear export, resulted in nuclear localization of HDAC7-FLAG in most transfected cells examined (data not shown) (67). These results are in agreement with a dynamic translocation of HDAC7 in and out of the cell nucleus. In the interphase cell nucleus, HDAC7 is excluded from the nucleoli and accumulates in discrete foci devoid of DNA, as shown by 4′,6-diamidino-2-phenylindole hydrochloride staining (Fig. 2*C*). Such chromatin-depleted foci have been reported for other HDACs (30, 65), transcription factors, and nascent mRNA species (65). HDAC7 was also depleted near the periphery of the cell nucleus and excluded from heterochromatic territories.

The Carboxyl-terminal Domain of HDAC7 Associates with HDAC Activity in Vivo—We developed a polyclonal antiserum specific for HDAC7 by immunizing rabbits with a fusion pro-
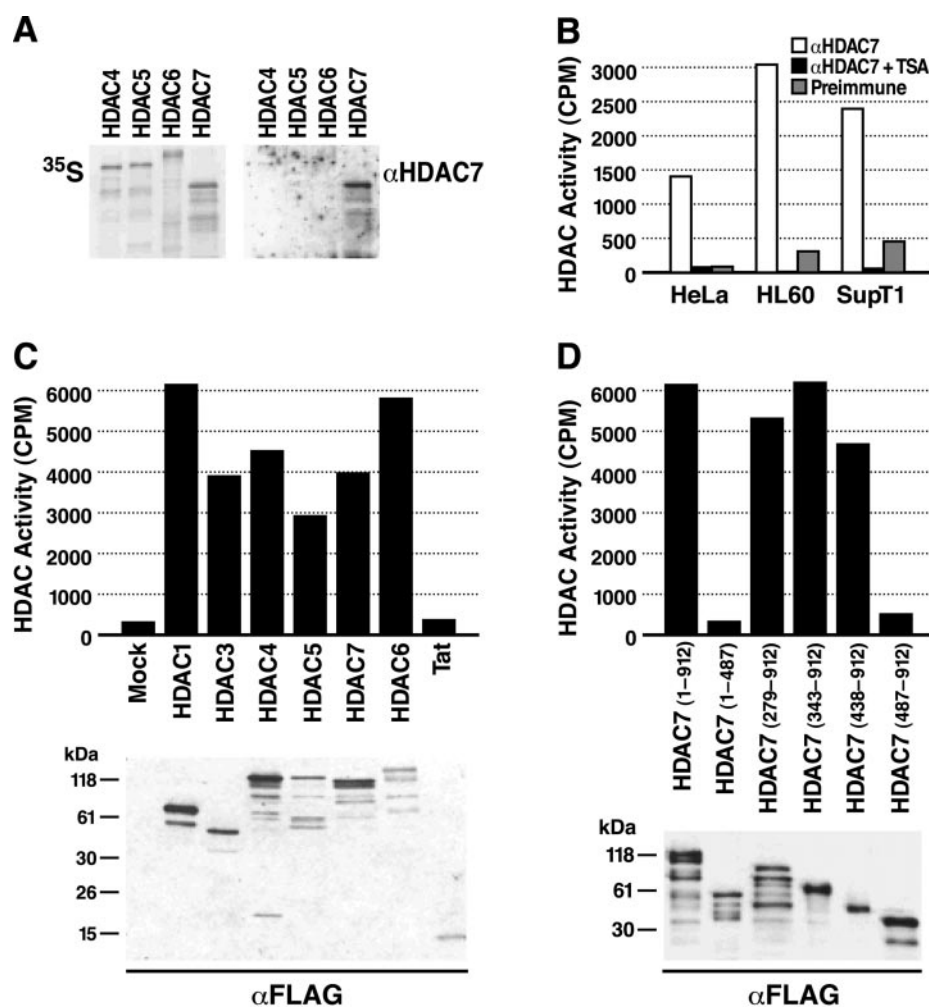
FIG. 3. **Mapping human HDAC7-associated HDAC activity to a carboxyl-terminal domain.** *A*, characterization of a polyclonal anti-HDAC7 antiserum. cDNAs for HDAC4, 5, 6, and 7 were translated *in vitro* using [35S]methionine. The corresponding recombinant proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The *left panel* shows the equal loading of all class II HDACs on the membrane by autoradiography (35S). The *right panel* shows Western blot analysis of the same membrane with our polyclonal anti-HDAC7 antiserum (αHDAC7). *B*, cellular extracts (10 mg each) from the indicated cell lines were immunoprecipitated with either HDAC7 antiserum (*αHDAC7*) or the corresponding preimmune serum. The HDAC activity of immunoprecipitates was measured with a radiolabeled histone H4 peptide in the absence or presence of 400 nM TSA. The enzymatic activity represents the average of three independent experiments. *C*, vectors encoding HDAC1, 3, 4, 5, 6, and 7 and the HIV Tat protein (all proteins tagged with the FLAG epitope) or the empty vector as control were transiently transfected into 293 cells. Fusion proteins were immunoprecipitated with anti-FLAG antiserum and tested for HDAC activity. The activity is presented as the average of three independent experiments. To control for the expression level and the efficiency of the immunoprecipitation, the immunoprecipitated material was analyzed by Western blotting with specific antisera against the FLAG epitope (*αFLAG*). *D*, the indicated FLAG-tagged constructs corresponding to full-length HDAC7 or to amino- or carboxyl-terminal deletions of HDAC7 were transiently transfected into 293 cells. Material immunoprecipitated with the anti-FLAG antiserum was tested for HDAC activity. Western blotting of the immunoprecipitated material with a specific anti-FLAG antiserum was performed to control for the expression level of the transfected constructs (*αFLAG*).

tein between GST and the first 487 aa of HDAC7 (HDAC7 (1–487)). When tested on *in vitro* translated proteins, the purified antiserum recognized only HDAC7 and did not cross-react with HDAC4, 5, or 6 (Fig. 3*A*). Western blot analysis of lysates from several cell lines showed a 105-kDa band that migrated with the same apparent molecular mass as *in vitro* translated HDAC7 (data not shown).

To test whether endogenous HDAC7 had HDAC activity, we used the anti-HDAC7 antiserum to immunoprecipitate HDAC7 from HeLa, HL60, and SupT1 cell lysates. The immunoprecipitated material was incubated with a peptide corresponding to the amino terminus of histone H4 that had been acetylated chemically, and the released acetate was quantified (Fig. 3*B*). Specific HDAC activity was associated with the HDAC7 antiserum but not with the preimmune serum in each cell line (Fig. 3*B*). This activity was inhibited by trichostatin A (TSA) (400 nM), a fungal toxin that inhibits class I and II HDACs (67).

To further study the enzymatic activity of HDAC7, we transfected FLAG-tagged HDAC7 and similar constructs encoding other class I and class II HDACs into 293 cells. HDAC assays performed after anti-FLAG immunoprecipitation showed that the activity of exogenous HDAC7 is comparable with that of HDAC1, 3, 4, 5, and 6. Western blot analysis verified that all epitope-tagged proteins were expressed and immunoprecipitated efficiently (Fig. 3*C*). To verify that the carboxyl-terminal domain of HDAC7 is sufficient for enzymatic activity, we used a series of amino- and carboxyl-terminal deletion mutants (Fig. 3*D*). All deletion mutants were tagged with a carboxyl-terminal FLAG sequence and transfected into 293 cells. A domain of 474 aa corresponding to aa 438–912 of HDAC7 was necessary and sufficient for HDAC activity. Deletion of a further 49 aa at the amino terminus of this domain abolished enzymatic activity (Fig. 3*D*). No activity was found associated with the amino-terminal region of HDAC7. These experiments
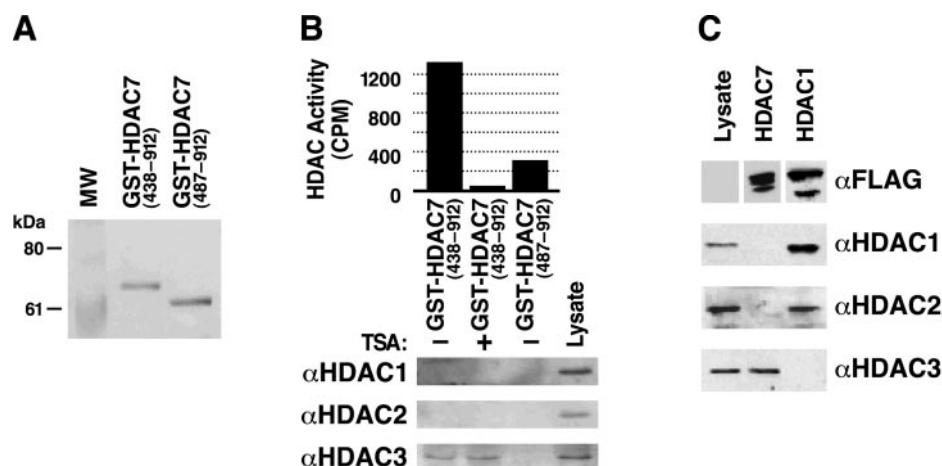
FIG. 4. **Activity associated with HDAC7 correlates with HDAC3 binding.** *A*, two carboxyl-terminal fragments of HDAC7, aa 438–912 and 487–912, were expressed as GST fusion proteins and immobilized on glutathione-agarose beads. Equal amounts of purified recombinant protein were loaded onto a SDS-PAGE gel, which was stained with Coomassie Blue. *MW*, molecular weight. *B*, the immobilized GST-HDAC7 fusion proteins were incubated with cellular extract from SupT1 cells. After intensive washing, HDAC activity associated with the bound material was analyzed in the absence or presence of TSA (400 nM). The proteins bound to the GST-HDAC7 columns were examined by Western blot with antisera specific for HDAC1, HDAC2, and HDAC3. *C*, vectors encoding HDAC7-FLAG and HDAC1-FLAG were transiently transfected into 293 cells. Material immunoprecipitated by the anti-FLAG antiserum was analyzed by Western blotting with the indicated antisera.

demonstrate that both endogenous and exogenous HDAC7 associate with HDAC activity *in vivo* and map the HDAC domain to the carboxyl-terminal region homologous to HDA1.

Enzymatic Activity Associated with HDAC7 Is Dependent on Cellular Factors, Including HDAC3—To further analyze the enzymatic activity of HDAC7, we expressed the carboxyl-terminal fragments (aa 438–912) and (aa 487–912) as GST fusion proteins in *Escherichia coli* (Fig. 4*A*). No HDAC activity was detected in the recombinant protein preparations despite numerous attempts with different bacterial strains and purification protocols (data not shown). The finding that the carboxyl-terminal domain of HDAC7 was associated with enzymatic activity when transfected into cells but not when expressed in bacteria suggested that HDAC7 activity requires one or more cellular cofactors. To test this hypothesis, we bound recombinant GST-HDAC7 proteins to glutathione-agarose beads and incubated them with extracts from SupT1 cells. After extensive washing, the bound material was tested for HDAC activity (Fig. 4*B*). GST-HDAC7 (aa 438–912) was associated with HDAC activity, whereas only minimal activity was associated with GST-HDAC7 (aa 487–912), which is inactive *in vivo*. HDAC activity associated with GST-HDAC7 (aa 438–912) was completely inhibited by TSA (400 nM). Because the class II HDACs HDAC4 and HDAC5 coimmunoprecipitate with endogenous HDAC3 (26), we tested the material associated with GST-HDAC7 (aa 487–912) for the presence of HDAC3. Western blot analysis revealed that HDAC3 was pulled out of the extracts by the active GST-HDAC7 (aa 438–912) but not the inactive GST-HDAC7 (aa 487–912) (Fig. 4*B*). This interaction was specific because neither of the highly homologous class I HDACs HDAC1 or HDAC2 did bind to GST-HDAC7. Binding was not dependent on the enzymatic activity of the proteins because it was insensitive to TSA (Fig. 4*B*).

To confirm the interaction between HDAC7 and HDAC3 *in vivo*, we analyzed material immunoprecipitated with HDAC7-FLAG by Western blotting (Fig. 4*C*). Endogenous HDAC3 coimmunoprecipitated with transfected HDAC7-FLAG but not with HDAC1-FLAG. No association of HDAC7 with HDAC1 or HDAC2 was detected (Fig. 4*C*). In contrast, HDAC1 coimmunoprecipitated with HDAC2 as predicted by the existence of complexes containing both proteins (53).

HDAC7 and HDAC3 Colocalize in Discrete Structures in the Cell Nucleus—HDAC3 is an exclusively nuclear protein (60).

We predicted that if HDAC7 and HDAC3 form a functional complex *in vivo*, they should colocalize within the cell nucleus. The subcellular localization of HDAC7-FLAG and of endogenous HDAC3 was examined in HeLa cells using confocal fluorescence microscopy (Fig. 5*A*). At low expression levels of HDAC7, a micropunctuate pattern reminiscent of the nuclear patterning of endogenous HDAC1 and HDAC3 was observed.[2] Merged images showed that HDAC7-FLAG and endogenous HDAC3 were localized in the same loci (Fig. 5*A*, *top row*). At higher expression levels of HDAC7, larger nuclear foci were detected, similar to those observed for overexpressed HDAC4 and HDAC5 (27, 40). In this case, merged images showed almost complete overlap between HDAC7-FLAG and endogenous HDAC3, suggesting recruitment of endogenous HDAC3 to such focal structures (Fig. 5*A*, *bottom row*).

Nuclear Localization of HDAC7 Is Necessary for Its HDAC Activity—The observation that HDAC7 accumulates both in the nucleus and in the cytoplasm suggested that HDAC7 could exist in two forms: in the cell nucleus complexed with HDAC3 and in the cytoplasm not associated with HDAC3. To assess the enzymatic activities of these two forms, we fractionated extracts from 293 cells transiently transfected with HDAC7-FLAG into nuclear and cytoplasmic fractions. HDAC7-FLAG was immunoprecipitated from both fractions in similar amounts (Fig. 5*B*). However, HDAC activity was 4.5 times higher (14762 *versus* 3240 counts/min) in the nuclear fraction, where HDAC7 was associated with HDAC3 (Fig. 5, *B* and *C*). Western blot analysis of the nuclear and cytoplasmic fractions with an antiserum specific for HDAC1 (exclusively nuclear) and IKKα (exclusively cytoplasmic) showed that each fraction was around 80% pure (Fig. 5*B*). We conclude that only nuclear HDAC7 interacts with HDAC3 and is associated with enzymatic activity. The results are consistent with a direct involvement of HDAC3 or HDAC3 containing complexes in the enzymatic activity of HDAC7.

HDAC7 Interacts with SMRT/N-CoR in Vivo and in Vitro—To further define the role of HDAC3 in the enzymatic activity of HDAC7, we attempted to reconstitute enzymatic active complexes *in vitro* by using recombinant proteins. After all attempts to detect direct binding between HDAC3 and

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[2] M. J. Hendzel, unpublished observations.

FIG. 5. **Nuclear HDAC7 bound to HDAC3 is associated with enzymatic activity, whereas free cytoplasmic HDAC7 is inactive.** *A,* HeLa cells were transiently transfected with an expression vector encoding HDAC7-FLAG. Endogenous HDAC3 was detected with affinity-purified anti-HDAC3 antibodies combined with a Cy3-labeled secondary antibody (*red*). FLAG-tagged HDAC7 was visualized with the M2 antibody in combination with an Alexa 488-tagged secondary antibody (*green*). For better visualization of the expression intensities of the individual HDACs, *black* and *white* images are shown. The *yellow dots* in the merged image indicate colocalization of both factors. Two different cells representing different expression levels and nuclear distribution profiles are shown. *B,* nuclear (*lanes N*) and cytoplasmic (*lanes C*) extracts were prepared from 293 cells transiently transfected with HDAC7-FLAG. Both extracts were immunoprecipitated (*IP*) with anti-FLAG antiserum, and bound material was analyzed by Western blotting with antisera against the FLAG epitope (*αFLAG*) and HDAC3 (*αHDAC3*). Extracts were also directly analyzed using Western blotting with antisera against the cytoplasmic protein IKKα (*αIKKα*) and the nuclear protein HDAC1 (*αHDAC1*). *C,* activity associated with HDAC7 from nuclear extracts (*N*) and cytoplasmic extracts (*C*) after immunoprecipitation of HDAC7-FLAG.
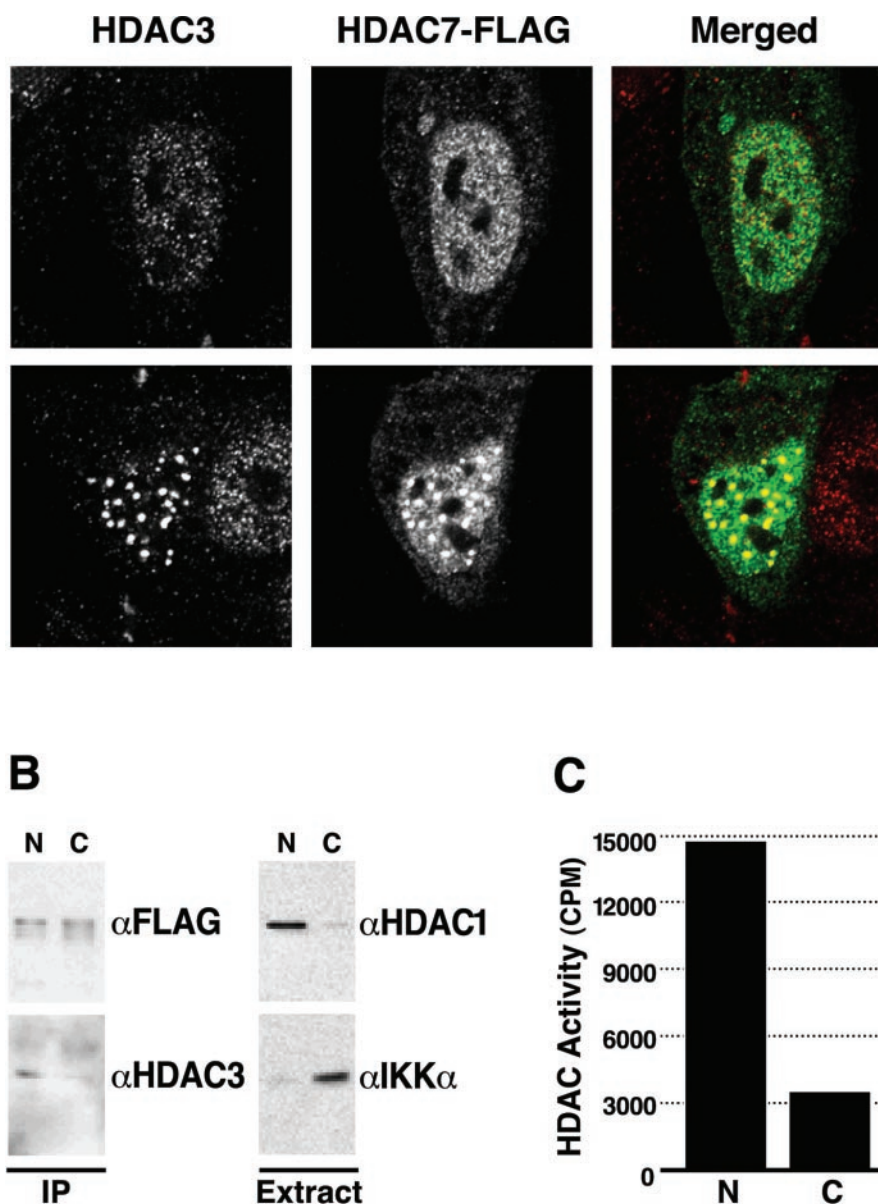
HDAC7 failed, we reasoned that additional factors might be involved in mediating the interaction. Recently, it has been reported that murine HDAC7 can directly interact with the corepressors SMRT and N-CoR (35, 68). In addition, we and others found HDAC3 in multiprotein complexes containing SMRT and N-CoR (44–48). Based on these observations we asked whether SMRT or N-CoR could be the missing link in the interaction between HDAC7 and HDAC3. Western blot analysis of material that coimmunoprecipitated with HDAC7 under the same conditions used to analyze its enzymatic activity (Fig. 2*C*) indeed demonstrated binding to N-CoR and SMRT (Fig. 6*A*). In the same experiment material immunoprecipitated with HDAC1 contained SIN3, but not N-CoR or SMRT, demonstrating specificity of the detected interaction. Importantly, deletion analysis showed that the domain of HDAC7 corresponding to aa 438–912 that was necessary for the interaction with HDAC3 (Fig. 4*B*), was at the same time mediating the interaction with SMRT and N-CoR (Fig. 6*B*). Similar results were obtained with the GST-HDAC7 pull-down assay (data not shown). These experiments indicate a strict correlation between the enzymatic activity of HDAC7 and derived constructs and their ability to simultaneously interact with HDAC3 and SMRT/N-CoR.

N-CoR contains three autonomous repression domains (RD1, RD2, and RD3) that repress transcription when tethered to sequence-specific DNA-binding domains (42, 69). To determine which domains of N-CoR are necessary for binding HDAC3 and HDAC7, we used GST fusion proteins with either RD1, RD2, or RD3 in pull-down experiments (Fig. 6*C*). Full-length HDAC7 and a deletion mutant containing the active carboxyl-terminal domain (aa 438–912) bound to both GST-RD1 and GST-RD3 but not to GST-RD2 (Fig. 6*D*). No interaction of the amino-terminal domain of HDAC7 (aa 1–487) or the inactive carboxyl-terminal fragment (aa 487–912) with RD1, RD2, or RD3 was observed. In contrast, HDAC3 was bound to GST-RD2 (Fig. 6*D*). These results suggest that HDAC7 and HDAC3 could simultaneously bind to N-CoR by interacting with separate domains.

Enzymatic Activities Associated with HDAC7 and HDAC4 Are Independent but Rely on the Same Cofactors—Different members of the class II HDACs share a high degree of homology in their carboxyl-terminal domain (Fig. 1). Because HDAC4 and HDAC5 have also been reported to bind to HDAC3 (26), we tested whether different class II HDACs rely on the same cofactors for enzymatic activity. First, we examined the possibility that GST-HDAC fusion proteins recruit common factors
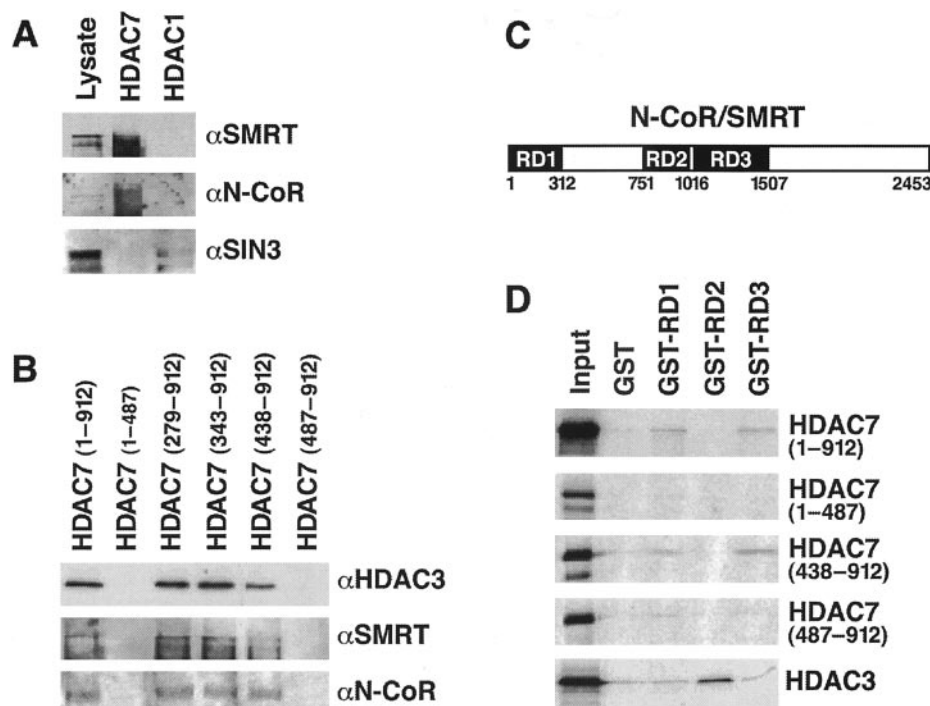
FIG. 6. **SMRT and N-CoR can mediate the interaction of HDAC7 and HDAC3.** *A*, vectors encoding HDAC7-FLAG and HDAC1-FLAG were transiently transfected into 293 cells. Material immunoprecipitated with the anti-FLAG antiserum was analyzed by Western blotting with antisera specific for SMRT, N-CoR, and SIN3. *B*, full-length HDAC7 or amino- or carboxyl-terminal deletions of HDAC7 (all FLAG tagged at the carboxyl terminus) were transiently transfected into 293 cells. Material immunoprecipitated with the anti-FLAG antiserum was analyzed by Western blotting with antibodies specific for HDAC3, SMRT, and N-CoR. *C*, schematic representation of the N-CoR protein indicating previously mapped repression domains (*RD*). *D*, binding of full-length HDAC7 and deletion mutants of HDAC7 or HDAC3 to recombinant GST-RD1, GST-RD2, and GST-RD3 of N-CoR was analyzed in pull-down assays with *in vitro* translated ³⁵S-labeled proteins. Input equaled 10% of the material used for the pull-down.

from cellular extracts. 293 or SupTl (data not shown) cellular extracts were repeatedly incubated with a recombinant fusion protein corresponding to the catalytically active domain of HDAC4 (aa 612–1084) (30) or with recombinant GST as a control. Western blot analysis demonstrated that GST-HDAC4 specifically depleted endogenous HDAC3 but not HDAC1 (Fig. 7*A*). Pull-down experiments using GST-HDAC4 (aa 612–1084) or GST-HDAC7 (aa 438–912) from the GST-HDAC4-depleted lysate showed an approximately 3-fold reduction in associated HDAC activity when compared with pull-downs from the GST-depleted lysates (Fig. 7*A*). This reduction in enzymatic activity was in good agreement with the degree of HDAC3 depletion achieved (Fig. 7*A*). The experiment demonstrates that the enzymatic activities associated with GST-HDAC4 and GST-HDAC7 depend on common factors, including HDAC3. Next, we tested whether stable autonomous complexes of HDAC7 and HDAC4 activity exist *in vivo*. Cellular extract from SupT1 cells were subjected to repeated immunoprecipitations with HDAC7 antiserum. When this HDAC7-depleted extract was immunoprecipitated with a HDAC4-specific antiserum, significant enzymatic activity was found, indicating that the enzymatic activities associated with HDAC4 and HDAC7 are present in distinct complexes *in vivo* (Fig. 7*B*, *left panel*). Similar results were obtained when the order of immunoprecipitations was reverted (Fig. 7*B*, *right panel*). These experiments indicate that although the enzymatic activities associated with HDAC4 and HDAC7 rely on a common set of nuclear proteins, the HDAC4 and HDAC7 proteins stably bind to their respective partners in the cell nucleus and do not readily exchange their cofactors.

DISCUSSION

We have identified and characterized a new human class II HDAC, HDAC7. Sequence analysis of HDAC7 indicates that its

amino-terminal region is more distantly related to HDAC4 and HDAC5 than these two factors are related to each other. Using coimmunoprecipitation experiments and *in vitro* pull-down assays, we found that the enzymatic activity associated with HDAC7 is dependent on its interaction with HDAC3, a class I HDAC. HDAC7 does not bind directly to HDAC3 but the two proteins are corecruited by the corepressors SMRT and N-CoR. Our findings are consistent with two distinct models. According to the first model, HDAC7 has lost it enzymatic potential during evolution. The function of HDAC7 would be to recruit enzymatically active HDAC3·SMRT·N-CoR complexes to distinct transcription factors. According to this model, HDAC7 would function as a corepressor, and HDAC3 alone or in conjunction with other factors would be the sole enzymatic activity of HDAC7·HDAC3 complexes. According to the second model, HDAC7 participates directly in building an enzymatically active complex but relies on HDAC3 and SMRT·N-CoR for activating its enzymatic potential. Conformational changes (allosteric regulation) or posttranslational modifications could be necessary to activate HDAC7 in the context of an HDAC7·HDAC3 complex. Because we can coimmunoprecipitate HDAC7 and HDAC3 *in vitro* (using the GST-HDAC7 pull-down) or *in vivo* (by immunoprecipitation) in the presence of TSA, the enzymatic activity of HDAC7 *per se* is not necessary for the interaction with HDAC3. Although our data do not allow us to chose in a definite manner between these models, we note that the primary sequence of the carboxyl-terminal domain of HDAC7 is highly conserved, not only with other class II HDACs and with yeast HDA1 but also when compared with class I HDACs and their ortholog in *Saccharomyces cerevisiae*, RPD3. In addition, the primary sequence of HDAC7 can be modeled onto the solved structure of a histone deacetylase-like protein with all structural requirements for putative functional HDACs fulfilled (70). These facts are more

A

FIG. 7. **The enzymatic activities associated with HDAC7 and HDAC4 rely on shared cofactors, including HDAC3, present in stable and autonomous complexes.** *A*, cellular extracts from 293 cells were repeatedly incubated either with GST or with a GST fusion protein corresponding to aa 612–1084 of HDAC4. The depleted lysates were analyzed by Western blotting with antibodies specific for HDAC1 and HDAC3. HDAC activities of pull-downs with GST-HDAC4 (aa 612–1084) or with GST-HDAC7 (aa 438–912) from the GST- and GST-HDAC4-depleted lysates are shown as the averages of two independent experiments. *B*, HDAC4 and HDAC7 were sequentially immunoprecipitated from SupT1 cellular extracts. HDAC activity of the immunocomplexes was analyzed by using acetylated histone H4 peptide as substrate. The *left panel* shows the enzymatic activity associated with three sequential immunoprecipitations with anti-HDAC7 antibodies followed by immunoprecipitation with anti-HDAC4 antibodies. The *right panel* shows the enzymatic activity associated with three sequential immunoprecipitations with anti-HDAC4 antibodies followed by immunoprecipitation with anti-HDAC7 antibodies.
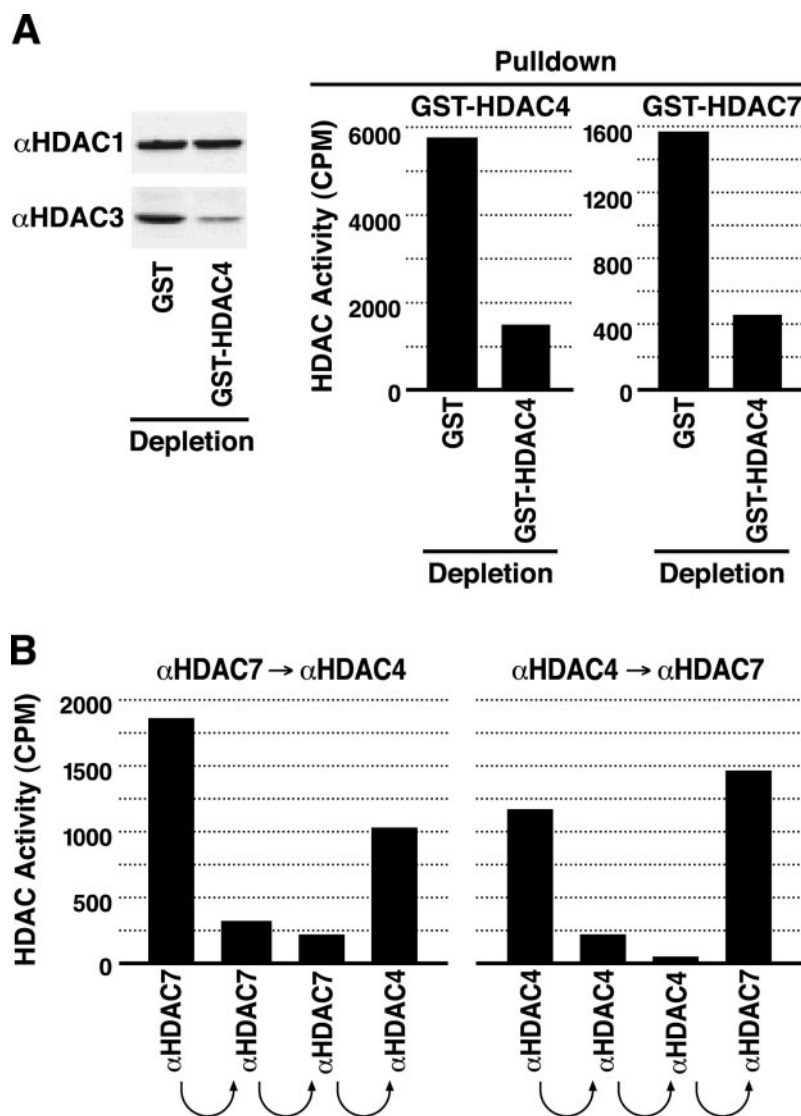
consistent with the second model and could suggest that HDAC7 is a *bona fide* HDAC that becomes only activated after interaction with the HDAC3·SMRT·N-CoR complex.

High molecular mass complexes containing the SMRT·N-CoR corepressors and HDAC3 have recently been described (44–48). In fact it has been suggested that the majority of the cellular SMRT and N-CoR proteins are constitutively associated with HDAC3 (47). *In vitro*, all class II HDACs, HDAC4, 5, and 7, display high affinity for the RD3 domain of SMRT and N-CoR (35, 42), and binding of the class II HDACs to these corepressors *in vivo* has been demonstrated (this study and Refs. 35 and 42). Our data combine these independent sets of observations and suggest that the combination of a class I (HDAC3) and a class II HDAC (either HDAC4, 5, or 7) could lead to the formation of an enzymatically active complex.

The observation that the SMRT·N-CoR·HDAC3 complex can be purified out of nuclear extracts using a GST-HDAC7 affinity matrix as reported here suggests that preassembled complexes containing SMRT·N-CoR and HDAC3 and no class II HDAC preexist in the cell. Similarly, because only a small fraction of cellular HDAC4 is bound to N-CoR under standard conditions (2–4%) (42), we predict that class II HDAC proteins exist in cells predominantly in a latent enzymatically inactive form that can be activated upon binding to the HDAC3·SMRT·N-CoR complexes. In contrast, we observed that the enzymatic activities of HDAC4 and HDAC7 were independent of each other

after immunoprecipitation (Fig. 7*B*), indicating that a fraction of both HDAC4 and HDAC7 exists in distinct stable complexes associated with the HDAC3·SMRT·N-CoR proteins in the cell nucleus.

Both HDAC4 and HDAC5 shuttle in and out of the cell nucleus (27, 34, 39, 40), and our data suggest that HDAC7 could be regulated in a similar manner. We found that only nuclear HDAC7 is associated with enzymatic activity, whereas cytoplasmic HDAC7 is enzymatically inactive. Our data indicate that cytoplasmic sequestration of class II HDACs could play an important role in regulating the enzymatic activity associated with these factors. An additional level of complexity could be added by posttranslational modification of the corepressors and/or HDACs controlling the association of enzymatically active complexes. Our observations suggest that novel regulatory mechanisms could modulate the recruitment of unique histone deacetylases to specific genes. Further work will be necessary to define fully the relationship between the HDAC3·SMRT·N-CoR complex and different class II HDACs, especially in the context of their tissue-restricted expression. It will be important to identify the specific gene targets of distinct complexes and to understand the nature of the signal transduction pathways that might modulate these interactions.

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