Isolation and Characterization of cDNAs Corresponding to an Additional Member of the Human Histone Deacetylase Gene Family*

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Several human cDNAs encoding a histone deacetylase protein, HDAC3, have been isolated. Analysis of the predicted amino acid sequence of HDAC3 revealed an open reading frame of 428 amino acids with a predicted molecular mass of 49 kDa. The HDAC3 protein is 50% identical in DNA sequence and 55% identical in protein sequence compared with the previously cloned human HDAC1. Comparison of the HDAC3 sequence with human HDAC2 also yielded similar results, with 51% identity in DNA sequence and 52% identity in protein sequence. The expressed HDAC3 protein is functionally active because it possesses histone deacetylase activity, represses transcription when tethered to a promoter, and binds transcription factor YY1. Similar to HDAC1 and HDAC2, HDAC3 is ubiquitously expressed in many different cell types.

The organization of chromatin structure is a fundamental and significant component of transcriptional regulation in all eukaryotic cells. Transcriptionally active or repressed chromatin is determined, at least in part, by the modification of histones. For example, hyperacetylation of histones generally leads to an increase in transcription, whereas hypoacetylation of histones appears to have the opposite effect (reviewed in Refs. 1–4). Nuclear histone acetyltransferases such as transcription factors GCN5, PCAF, p300/CBP, and TAF1230/260 have been identified from different organisms (5–9).

Several yeast and mammalian histone deacetylases have been identified, and their corresponding genes have been cloned (10–12). In yeast, the HDA1 protein, which shares sequence similarity to RPD3, is a subunit of a large histone deacetylase complex HDA. RPD3 is also associated with another yeast histone deacetylase complex HDB. Using a trapoxin (an inhibitor of histone deacetylase) affinity matrix, Taunton et al. (10) purified and cloned a human 55-kDa protein related to the yeast protein RPD3. Immunoprecipitation of this 55-kDa protein, HDAC1 (also called HD1), showed that it contains histone deacetylase activity. A second human histone deacetylase protein, HDAC2, with high homology to yeast RPD3 was identified based on a yeast two-hybrid trap experiment with the YY1 transcription factor as a bait (11). YY1 negatively regulates transcription by tethering HDAC2 to DNA as a co-repressor. Both HDAC1 and HDAC2 exist in a complex with the co-repressor mSIN3 and mediate Mad transcriptional repression (13–15). In addition, HDAC1 and HDAC2 are essential components of two thyroid hormone receptor corepressors, N-CoR and SMRT (16–18). HDAC1 is also an important factor that represses transcription by progesterone receptor (19).

The yeast RPD3 protein was originally identified in genetic screens for transcriptional repressors (20). Besides human and mouse, highly homologous yeast RPD3 sequences have been identified in Drosophila (21), Caenorhabditis elegans (X78454 and 1176665), and Xenopus laevis (gi:576995). Currently, it has not yet been established whether the C. elegans or X. laevis RPD3-related proteins have histone deacetylase activities or whether they play a role in transcriptional repression. Here, we describe the identification of a third human RPD3-related protein, HDAC3, that contains histone deacetylase activity. Like HDAC1 and HDAC2, HDAC3 represses transcription and binds transcription factor YY1, suggesting that it may participate in a large complex that mediates a wide variety of repression systems in human.

MATERIALS AND METHODS

Identification of Human HDAC3 cDNAs—Expressed sequence tags (ESTs)1 were searched in the data base of ESTs with the NCBI BLAST Server. One human cDNA clone (EST200871, accession number R89879) with significant homology to HDAC1 and HDAC2 was used as a probe to screen oligo(dT)-primed human fibroblast (22) and HeLa (a gift from B. Shan at Tularik, Inc.) cDNA libraries. Briefly, the cDNA fragment was labeled with32P by random priming, and hybridization was performed with32P-labeled human HDAC1 (10); pME18S-FLAG-HDAC2, which expresses FLAG epitope-tagged HDAC2 (14); pCep4F, which contains the cytomegalovirus immediate early promoter and an ATG sequence followed by the FLAG epitope (23); pBj5-HDI-1-F; which encodes a carboxy-terminal FLAG epitope-tagged HDAC1 (10); pME18S-FLAG-HDAC2, which expresses FLAG epitope-tagged HDAC2 (14); pM2, pm3, and pSG424, which contain the Gal4 DNA-binding domain under the control of the SV40 promoter/enhancer (24, 25); pGal4-mRPD3 (pGal4-HDAC2), which expresses a Gal4-

1 The abbreviations used are: EST, expressed sequence tag; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase.
mRPD3 fusion protein (11); and pGal4tkCAT (26), which contains five Gal4-binding sites upstream of the thymidine kinase TATA box in plasmid pBLCAT2 (ptkCAT [27]).

pCMV-FLAG-HDAC3 was constructed by taking the HDAC3 cDNA and subcloning it downstream and in-frame with the FLAG sequence in pCEP4F. pCMV-HDAC3 was constructed by taking the HDAC3 cDNA and subcloning it into the EcoRI site of pcDNA3 (Invitrogen). pGal4-HDAC1 and pGal4-HDAC3A were constructed by taking HDAC1 and HDAC3A cDNA, respectively, and fusing them in-frame with the Gal4 DNA-binding domain in pM2. pGal4-HDAC3 was generated by subcloning HDAC3 cDNA in the pSG424 vector. pGal4-HDAC3DN was constructed by taking the original EST200871 clone and ligating it into the pM3 vector. To construct pGST-HDAC2, pGST-HDAC3, and pGST-HDAC3A, fragments containing the entire open reading frame of each gene were subcloned into the pGSTag (28) or pGEX-3X (Pharmacia Biotech Inc.) vectors, in-frame with the glutathione S-transferase (GST) polypeptide. All constructions were verified by dideoxy sequencing.

pGST-HDAC1 was kindly provided to us by C. Hassig and S. Schreiber (Harvard University). pT7-YY1 (a gift from Y. Shi at Harvard Medical School) contains the entire YY1 cDNA under the control of T7 promoter for in vitro transcription.

Histone Deacetylase Assays—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin/streptomycin. For transfections, 10^6 cells were seeded in a culture dish for 16 h. Twenty mg each of pCEP4F, pBJ5-HD1-F, and pME18S-FLAG-HDAC2 plasmids were introduced into cells with the calcium phosphate coprecipitation method (29). 48 h after transfections, cells were harvested, lysed with low stringency buffer, and immunoprecipitated with an anti-FLAG antibody (International Biotechnologies, Inc.) as described (14). Immunocomplexes were assayed for their ability to deacetylate chicken histone as described (30).

Western Blot Analysis—Standard protocols were followed (31). Briefly, 10^6 HeLa cells were lysed by sonication and boiling for 5 min in sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 5% β-mercaptoethanol). Two micrograms of the resulting extract were separated on a 8% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. After blocking with nonfat dried milk, the membrane was treated with 1:4000 diluted anti-FLAG antibody followed by
1:750 diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG. Subsequently, the blot was developed by 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**DNA Transfections and Chloramphenicol Acetyltransferase (CAT) Assays**—HeLa cells were grown on 60-mm tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Five mg of plasmids directing the synthesis of various effector proteins plus 5 mg of a reporter were transfected using the calcium phosphate method (29). 48 h after transfection, cells were lysed by repeated freeze/thaw cycles and extracts assayed for CAT activity by thin-layer chromatography (32) and quantified with the PhosphorImager (Molecular Dynamics).

**In Vitro Transcription/Translation and in Vitro Binding Assays**—To generate 35S-labeled YY1, pT7-YY1 was transcribed and translated with T7 RNA polymerase and [35S]methionine in a transcription/translation coupled system (Promega). Bacterially expressed GST protein and different GST fusion proteins were purified according to Frangioni and Neel (33) with modifications. Briefly, DH5α cells transformed with GST plasmids were grown to log phase and induced with isopropyl-thio-β-D-galactoside for 4 h. After sonication in STE buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol) containing 1% sarcosyl (w/v, final concentration), solubilized proteins were recovered by centrifugation, incubated with glutathione-agarose beads in the presence of 3% Triton X-100 (final concentration) for 30 min at 4 °C, and washed three to four times with ice-cold phosphate-buffered saline containing 0.2% Nonidet P-40.

For binding assays, beads were mixed with in vitro translated, 35S-labeled YY1 protein for 1 h at room temperature. Unbound proteins were washed extensively with STE buffer containing 0.2% Nonidet P-40, and bound proteins were eluted from the beads by boiling in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 0.3% 2-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol). Final products were analyzed on a 10% SDS-polyacrylamide gel and detected by autoradiography.

**Northern Blot Analyses**—Multiple human tissue Northern blots were

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### Table I

**Comparison of histone deacetylase sequences**

| Percent Similarity | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|--------------------|---|---|---|---|---|---|---|---|---|----|----|
| 1                  |   |   |   |   |   |   |   |   |   | 1  | hHDAC1 (U50079) |
| 2                  |   |   |   |   |   |   |   |   |   | 2  | hHDAC2 (U31814) |
| 3                  |   |   |   |   |   |   |   |   |   | 3  | hHDAC3 (U75697) |
| 4                  |   |   |   |   |   |   |   |   |   | 4  | mHDAC1 (X98207) |
| 5                  |   |   |   |   |   |   |   |   |   | 5  | mHDAC2 (U31758) |
| 6                  |   |   |   |   |   |   |   |   |   | 6  | dRPD3 (Y09258)  |
| 7                  |   |   |   |   |   |   |   |   |   | 7  | cRPD3-1 (1176665) |
| 8                  |   |   |   |   |   |   |   |   |   | 8  | cRPD3-2 (Z81486) |
| 9                  |   |   |   |   |   |   |   |   |   | 9  | yRPD3-1 (566438) |
| 10                 |   |   |   |   |   |   |   |   |   | 10 | yRPD3-2 (X91837) |

| Percent Divergence | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|--------------------|---|---|---|---|---|---|---|---|---|----|----|
| 1                  |   |   |   |   |   |   |   |   |   | 11 | hHDAC1 (U50079) |
| 2                  |   |   |   |   |   |   |   |   |   | 10 | hHDAC2 (U31814) |
| 3                  |   |   |   |   |   |   |   |   |   | 9  | hHDAC3 (U75697) |
| 4                  |   |   |   |   |   |   |   |   |   | 8  | mHDAC1 (X98207) |
| 5                  |   |   |   |   |   |   |   |   |   | 7  | mHDAC2 (U31758) |
| 6                  |   |   |   |   |   |   |   |   |   | 6  | dRPD3 (Y09258)  |
| 7                  |   |   |   |   |   |   |   |   |   | 5  | cRPD3-1 (1176665) |
| 8                  |   |   |   |   |   |   |   |   |   | 4  | cRPD3-2 (Z81486) |
| 9                  |   |   |   |   |   |   |   |   |   | 3  | yRPD3-1 (566438) |
| 10                 |   |   |   |   |   |   |   |   |   | 2  | yRPD3-2 (X91837) |
FIG. 2. **Functional assays of HDAC3.** A, histone deacetylase activity of recombinant HDAC1, HDAC2, and HDAC3. HeLa cells were transfected with plasmids encoding different FLAG epitope-tagged HDACs and histone deacetylase activity was assayed from immunoprecipitates using an anti-FLAG antibody (top panel). +peptide competitor indicates the addition of FLAG peptide, and +trichostatin A indicates treatment with 400 nM of trichostatin A (Wako). The amount of released [3H]acetic acid correlates with immunoprecipitated histone deacetylase activity. Each assay was performed in duplicate from two to four independent samples, and the values shown are the averages ± S.D. Expression of HDAC1, HDAC2, and HDAC3 was determined by protein immunoblot analysis using anti-FLAG antibody (bottom panel). B, transcriptional repression by the cloned human HDAC3. Top panel, schematic drawing of plasmids used in HeLa cell transfection assays. Bottom panel, transfection assays showing that Gal4-HDAC fusions repress transcription when targeted to promoters. All transfections were normalized to equal amounts of DNA with parental expression vectors. The results are the mean ± S.D. from three separate transfections. C, interaction between HDAC3 and YY1. Right panel, autoradiogram of in vitro translated YY1 protein captured by GST-HDAC fusion proteins. Bound proteins were eluted and analyzed by SDS-polyacrylamide gel as described under “Materials and Methods.” Three independent experiments yielded consistent results. Left panel, Coomasie Blue stain of the identical gel before autoradiography. The sizes of molecular mass markers are indicated on the left.
obtained from CLONTECH. HDAC1, HDAC2, and HDAC3 cDNA probes were prepared with [α-32P]dCTP using a random prime kit (Stratagene). Prehybridization, hybridization, and washing were performed under high stringency conditions before exposure to x-ray films (34). To control for the relative amount of RNA in each lane, after hybridization with each HDAC cDNA, the blots were stripped by incubation in 0.5% SDS at 95 °C and reprobed with the human β-actin cDNA.

RESULTS AND DISCUSSION

Cloning of Human cDNAs Encoding a Novel Member of the Histone Deacetylase Family—While searching the GenBank data base of ESTs for DNA and protein sequences with homology to HDAC1 and HDAC2, we identified a cDNA (EST200871, accession number R98879) from a human fetal liver library encoding a partial open reading frame with significant sequence similarity to HDAC1 and HDAC2. We refer to the product encoded by this newly identified cDNA as HDAC3. To obtain a full-length HDAC3 cDNA, a human fibroblast cDNA library was screened with a radiolabeled probe corresponding to the entire EST200871 clone. The complete DNA sequence and deduced amino acids from two newly isolated identical clones are illustrated in Fig. 1A. Analysis of the predicted amino acid sequence of HDAC3 revealed an open reading frame of 428 amino acids (1.3 kilobases) with a predicted molecular mass of 49 kDa. Analysis of HDAC3 by SDS-polyacrylamide gel electrophoresis revealed that it migrates as a 43-kDa protein (data not shown). Protein sequence homology alignment revealed that HDAC3 shares 53% identity (60% similarity) to
HDAC1 and 52% identity (58% similarity) to HDAC2 (Fig. 1B and Table I). HDAC3 is 50% identical to HDAC1 and 51% identical to HDAC2 in DNA sequences. As expected, HDAC3 is also highly homologous to RPD3-related proteins from other species (Table I).

Interestingly, an additional cDNA clone (designated HDAC3C) isolated from the fibroblast library contains a DNA sequence identical to HDAC3 from nucleotide 197 to the 3' end of the cDNA (Fig. 1A); the remaining 5' end, however, is only 43% identical. An in-frame TGA termination codon is found 42 nucleotides upstream of nucleotide 227 (Fig. 1A) in the HDAC3C clone, suggesting that the HDAC3C protein is an amino-terminal truncated form of HDAC3. Using a HeLa Actg10 library, five more partially overlapping cDNA clones were identified. Unlike HDAC3 and HDAC3C, the product encoded by these five cDNAs designated HDAC3A contains two direct repeats of amino acids at the amino terminus (Fig. 1C).

In short, we have identified and characterized cDNA clones encoding three almost identical proteins designated HDAC3, HDAC3A, and HDAC3C. Currently, we do not know whether the different forms of HDAC3 are the result of alternative splicing or other post-transcriptional modifications. Whatever the modification, the changes must be minor because we could only detect a single HDAC3 species on Northern blots (see Fig. 3). The 428-amino acid HDAC3 protein closely resembles the previously cloned HDAC1 and HDAC2 proteins (especially between amino acids 128 and 196). However, the carboxyl terminus of HDAC3 from amino acids 384 to 428 is quite different from those of HDAC1 and HDAC2.

HDAC3 Is a Member of Human Histone Deacetylases—To determine whether HDAC3 possesses histone deacetylase activity, plasmids expressing FLAG-tagged proteins were transfected into HeLa cells and low stringency immunocomplexes were assayed for their abilities to deacetylate chicken histones. As shown in Fig. 2A, low stringency anti-FLAG immunoprecipitates contained deacetylase activities in cells transfected with plasmids expressing FLAG sequence fused to HDAC1, HDAC2, or HDAC3, but not in cells expressing FLAG alone. Deacetylase activity is significantly higher in HDAC1 than in HDAC3-containing complexes. Western blot analysis indicates that this lower deacetylase activity is not a reflection of lower HDAC3 expression. Histone deacetylase activity was abolished when precipitation was performed with excess FLAG blocking peptide or when treated with the deacetylase inhibitor trichostatin A. These results demonstrate that like HDAC1 and HDAC2, HDAC3 possesses functional histone deacetylase activity in vivo.

The Cloned HDAC3 Represses Transcription—Previously, we have shown that a Gal4-HDAC2 fusion protein represses transcription when directed to promoters containing Gal4-binding sites in transient transfection experiments (11). We wondered if our newly cloned HDAC3 also possessed repression activity. A Gal4-HDAC3 expression plasmid was constructed and transfected into HeLa cells together with the target plasmid pGal4tkCAT containing five Gal4-binding sites. As shown in Fig. 2B, a 10-fold repression was seen in the presence of Gal4-HDAC3 but not HDAC3 without the Gal4 DNA-binding domain. Repression was also dependent on the presence of the Gal4-binding sites because CAT expression was not affected when ptkCAT lacking Gal4-binding sites was used as a target. A Gal4-HDAC3A fusion protein differing in 13 amino acids in the amino-terminal part of HDAC3 or a Gal4-HDAC3AN with the first 68 HDAC3 amino acids deleted did not affect repression. To compare repression activity between HDAC3 with HDAC1 and HDAC2, we also constructed pGal4-HDAC1 and similarly tested for its ability to repress transcription. Consistent with our earlier studies, a Gal4-HDAC2 protein represses transcription in a promoter containing Gal4-binding sites. Surprisingly, although HDAC1 contains higher deacetylase activity, there is no significant difference in its ability to repress transcription compared with HDAC2 and HDAC3. Nevertheless, our results suggest that like our previous finding with HDAC2 and now with HDAC1, HDAC3 also represses transcription when tethered to a promoter.

Expression of HDAC3 Transcript in Different Human Cell Lines and Tissues—To explore the cell and tissue distribution of HDAC3 mRNA, we performed Northern blots with HDAC1, HDAC2, and HDAC3 cDNA as probes using different human tumor cell lines and normal tissues. As shown in Fig. 3, all three forms of HDAC mRNA are detectable in all cell lines and tissues examined. With the exception of brain tissue, where HDAC1 and HDAC3 are expressed in lower levels, relatively equal amounts of HDAC mRNA were found in every cell line and tissue. We, therefore, conclude that HDAC1, HDAC2, and HDAC3 are ubiquitously expressed.

Summary—Based on our characterization of the primary structures and functional properties of HDAC1, HDAC2, and HDAC3, we conclude that these three proteins constitute a human HDAC family. All three proteins possess histone deacetylase activity, repress transcription when bound to a promoter, and interact with transcription factor YY1. mRNA from all three HDACs are almost uniformly expressed in all tissues examined. At this time, the major difference between HDAC3 and the other two HDACs appear to surround on sequence similarities, with HDAC1 and HDAC2 being closer to each other than to HDAC3. Also, the question concerning the existence of additional human HDAC(s) that resembles yeast RPD3 remains open. Recently, an acidic nucleolar phosphoprotein that was not homologous to yeast RPD3 was identified as a maize histone deacetylase (35). Our finding of three human HDACs closely related to yeast RPD3 therefore does not exclude the possibility that there are additional human histone deacetylases with little or no nucleotide and amino acid sequences homology to yeast RPD3.

The identification of multiple HDACs raises an important question with respect to specific individual protein functions. The fact that there exists more than one human HDAC presents the possibility that some of the HDACs could have unique functions. This is reinforced by our finding that the carboxyl terminus of HDAC3 is quite different from those of HDAC1 and HDAC2. Although our data so far suggest that all three HDACs possess similar functions, it is certainly possible that they may not be totally redundant in function. The availability of HDAC3 cDNA and a functional recombinant protein allows us now to
address this important point. Ultimately, we predict that a detailed characterization of HDAC3 will help increase our understanding of histone deacetylation, chromatin structure, and transcription regulation.

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