Identification of a transcriptional repressor related to the noncatalytic domain of histone deacetylases 4 and 5

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Histone deacetylases (HDACs) are involved in regulating transcription by modifying the core histones of the nucleosome. To date, six HDACs have been identified in mammalian cells: the yeast RPD3 homologs HDAC1, 2, and 3 and the yeast HDA1 homologs HDAC4, 5, and 6. HDAC4 and HDAC5 contain a noncatalytic N-terminal domain. Herein, we report the identification of a protein HDRP (*HD*AC-*r*elated *p*rotein) that shares 50% identity in deduced amino acid sequence to the noncatalytic N-terminal domain of HDAC4 and 5. The steady-state levels of HDRP mRNA are high in human brain, heart, and skeletal muscle and low in the several other tissues. HDRP has an apparent molecular mass of approximately 75 kDa. HDRP does not possess intrinsic HDAC activity but forms complexes with both HDAC1 and HDAC3. HDRP represses both basal and activated transcription in transient transfection assays when tethered to DNA as a Gal4-fusion protein. HDAC inhibitors do not reverse transcriptional repression mediated by Gal4–HDRP. Thus, HDRP is a transcriptional repressor and can repress transcription in the presence of HDAC inhibitors.

RNA polymerase II-mediated transcription in eukaryotic cells is regulated by both transacting activators and repressors (1–4). Classical activators contain distinct activation domains and function through interaction either directly or indirectly with components of the basal transcription machinery (2). Similarly, active repressors contain distinct domains for repression and can function either through the interference of the action of activators or by directly targeting components of the basal transcriptional machinery (3, 4). Passive repressors do not contain active repressional domains and repress transcription by several mechanisms, including competing with activators or basal transcription machinery for DNA binding, recruiting other repressors to the promoter, or modifying the environment to make it hostile for active transcription (3, 4).

Histone deacetylases (HDACs) are believed to repress transcription by removing the acetyl groups from the N-terminal tails of the core histones of the chromatin, because hypoacetylated chromatin is often associated with a transcriptional inert state (5). Five proteins in yeast (RPD3, HDA1, HOS1, HOS2, and HOS3) have been identified as HDACs (6, 7). Six HDACs have been identified in mammalian cells, the yeast RPD3 homologs HDAC1, HDAC2, and HDAC3 (8–12) and the yeast HDA1 homologs HDAC4/HDAC-A, HDAC5/mHDA1 (murine HDAC5), and HDAC6/mHDA2 (murine HDAC6; refs. 13–17). HDAC4 and HDAC5/mHDA1 contain a noncatalytic N-terminal domain (13–17).

In this study, we have identified a HDAC-related protein (HDRP) that shares 50% identity of the deduced amino acid sequence with the N termini (the noncatalytic domains) of HDAC4 and 5. HDRP mRNA is expressed in all human tissues examined and is highest in brain, heart, and skeletal muscle. HDRP forms complexes with both HDAC1 and HDAC3. HDRP represses both basal and activated transcription when tethered to DNA as a Gal4-fusion protein. Furthermore, HDRP can repress transcription in the presence of HDAC inhibitors.

Materials and Methods

Database Search and Sequence Analyses. The nonredundant peptide and nucleotide sequence database of GenBank was searched with the yeast HOS2 amino acid sequences by using the BLASTP program at the National Center for Biotechnology Information to identify human HOS2 homologs and ORFs. A previously uncharacterized human protein KIAA0288 (GenBank accession no. AB006626) was identified and found to contain intrinsic HDAC activity (X.Z., V.M.R., R.A.R., and P.A.M., unpublished data). Searching the GenBank with KIAA0288 retrieved a previously uncharacterized human protein KIAA0744 (GenBank accession no. AB018287) that shares 50% identity with the N-terminal amino acid sequence of KIAA0288. This protein is designated HDRP.

Northern Blot Analysis. The Multiple Human Tissue Northern Blot was obtained from CLONTECH. Hybridization was performed according to the manufacturer's instructions by using ExpressHyb solution (CLONTECH). The ^{32}P-random-priming-labeled coding region cDNA for human HDRP was used as probe. A ^{32}P-random-priming-labeled β-actin cDNA probe (CLONTECH) was used as a loading control. The blot was stripped according to the manufacturer's instructions between hybridizations with different probes.

Plasmids. The cDNA clone for KIAA0744 (HDRP) was kindly provided by T. Nagase (Kazusa DNA Research Institute, Chiba, Japan). The coding region of HDRP was cloned into the pFLAG-CMV-2 vector (Sigma) in-frame with a FLAG epitope at the N terminus to generate pFLAG-HDRP. pGal4-HDRP was constructed by fusing the coding regions of HDRP in-frame with the Gal4 DNA-binding domain (1–147) by using the pFA-CMV vector (Stratagene). All constructs were confirmed by DNA sequencing with an automated DNA sequencer at the New York State Center for Advanced Technology, BioResource Center, DNA Sequencing Facility of Cornell University. The 5xGal4-*c-fos-Luciferase* (Luc), 5xGal4–2xLexA-*E1B-Luc*, 5XGal4-*TK-CAT*, pCMV-Gal4 (1–147), pCMV-LexA (1–202), and pCMV-LexA (1–202)-VP16 (410–490) were generous gifts from J. Nevado and M. Ptashne (Memorial Sloan–Kettering Cancer Center). pCMV-HDAC3 and pGal4-HDAC1 were kindly provided by W.-M. Yang and E. Seto (University of South Florida, Tampa, FL). pRL-TK was from Promega.

Transfection, Immunoprecipitation (IP), Antibodies, and Western Blot Analyses. Cos7 and T24 cells (American Type Culture Collection) were cultured in DME HG medium (GIBCO/BRL) supple-

Abbreviations: HDAC, histone deacetylase; HDRP, HDAC-related protein; HA, hemagglutinin; TSA, trichostatin A; Luc, luciferase; Fluc, firefly Luc; Rluc, *Renilla* Luc; CAT, chloramphenicol acetyltransferase; kb, kilobase; IP, immunoprecipitation.

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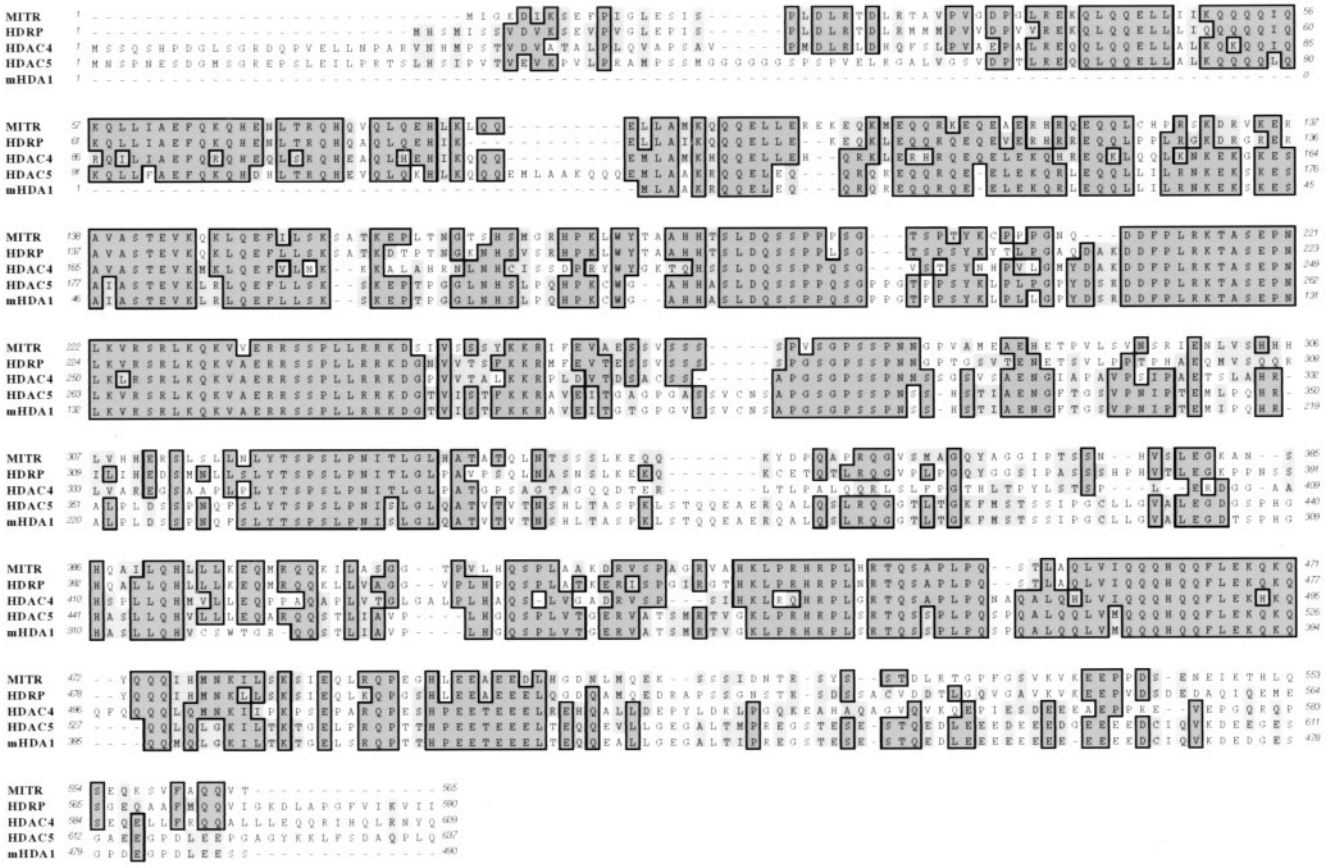

Fig. 1. Sequence alignment. Deduced amino acid sequences of HDRP (GenBank accession no. BAA34464) and MITR (GenBank accession no. CAB10167) are aligned with those of the N termini of HDAC4 (GenBank accession no NP006028) (1–609), HDAC5 (GenBank accession no. NP005465) (1–637), and mHDA1 (GenBank accession no. AAD09834) (1–490). The identical residues in all proteins are outlined and shaded. The similar residues are lightly shaded.

mented with 10% (vol/vol) FBS at 37°C in a 5% CO_2 atmosphere. Transient transfection was performed by using Lipofectamine (GIBCO/BRL) or Fugene 6 (Roche Molecular Biochemicals) according to the manufacturers' instructions. Cells were harvested 24–48 hours after transfection and lysed in IP lysis buffer (50 mM Tris·HCl, pH 7.5/120 mM NaCl/5 mM EDTA/0.5% NP-40) at 5×10^7 cells per ml. IP with anti-FLAG M2-agarose (Sigma) was performed according to the manufacturer's instructions. Immunoprecipitated proteins were released from the agarose beads by using FLAG-peptide and either used directly for HDAC enzymatic activity assays or resolved on SDS/PAGE for Western blot analyses.

Antibody against hemagglutinin (HA) epitope was purchased from Berkeley Antibody (Richmond, CA). Anti-FLAG antibody was purchased from Sigma. Antibody against HDRP was raised in rabbit by using a synthetic peptide corresponding to amino acids 587–590 (GKDLAPGFVIKVII, a region not conserved in HDAC4 and 5) of the HDRP protein conjugated to BSA and KLH (Pocono Rabbit Farm, Canadensis, PA). Antibody against human HDAC3 was raised in rabbit by using a synthetic peptide corresponding to amino acids 413–428 (FYDGDHDNDKESDVEI) of the human HDAC3 (GenBank accession no. AAC52038) conjugated to BSA and KLH (Pocono Rabbit Farm) and was kindly provided by L. Butler from our laboratory. Cos7 cell lysates containing transfected human HDAC3 and HDRP were used to confirm the validity of the HDAC3 and HDRP antibodies, respectively (data not shown). Western blot analyses were performed as described (18). A dilution of 1:2,000 was used for antisera against HDAC3 and HDRP.

Reporter Gene Assay. The reporter genes (100 ng per well) were transiently transfected into Cos7 or 293T cells (gift of S. S. Millard and A. Koff, Memorial Sloan–Kettering Cancer Center) in 12-well plates either alone or with other genes indicated. Salmon testis genomic DNA was used to adjust the DNA to an equal amount in each transfection. pRL-TK (Promega) was included as a control for transfection efficiency. Trichostatin A (TSA; 100 ng/ml) treatment was initiated 12 hours after the addition of DNA to the culture. Transient transfection assays were performed, because it has been shown that TSA has similar effects in both transient and stable transfection assays (19). The Luc activities were measured by using the Dual-Luc assay system from Promega according to the manufacturer's instructions with a TD-20e luminometer (Turner, Palo Alto, CA). Chloramphenicol acetyltransferase (CAT) enzyme assays were performed by using the CAT Enzyme Assay System from Promega according to the manufacturer's instructions with ^{14}C-chloramphenicol (58.5 mCi/mmol; NEN) as a substrate. All experiments were repeated at least three times with very similar normalized results obtained. Representative results are shown.

Results

Identification of a Protein That Has Homology to HDAC4 and HDAC5. Because no mammalian homologs have been identified for the yeast HOS family proteins of potential HDACs, HOS2 was used to screen the GenBank for mammalian homologs. One previously uncharacterized protein termed KIAA0288 was found to share 26% deduced amino acid identity to the catalytic domain

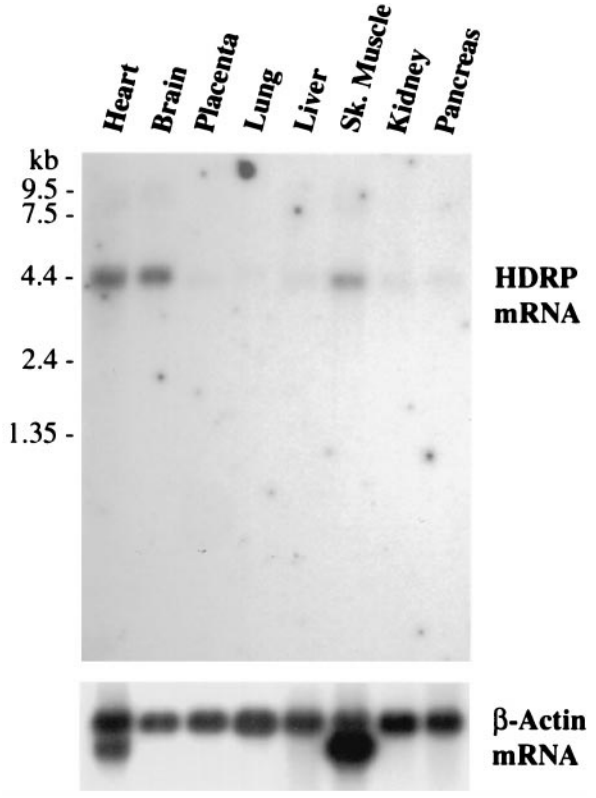

Fig. 2. HDRP mRNA is differentially expressed among human tissues. A multiple human tissue Northern blot was probed to determine mRNA expression of HDRP. The blot was stripped and reprobed with β-actin cDNA as a normalization control. The tissues examined are indicated at the top of each lane. Positions of the RNA size marker in kilobases are indicated on the left of the blot.

of HOS2. We found that KIAA0288 possesses intrinsic HDAC activity (X.Z., V.M.R., R.A.R., and P.A.M., unpublished data). KIAA0288 was also independently identified as an HDAC by other investigators and was designated HDAC-A (15) or HDAC4 (13, 16, 17).

A human protein, KIAA0744, that shares 50% amino acid

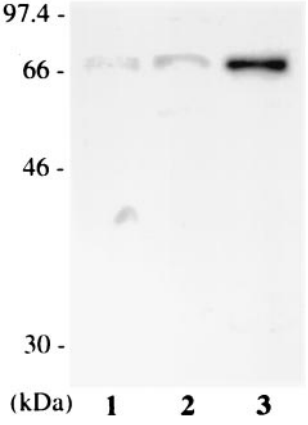

Fig. 3. HDRP protein is expressed. Whole-cell lysates (10 μg of protein) prepared from T24 cells (lane 1) or from Cos7 cells transfected with vector alone (lane 2) or with pCMV-HDRP (lane 3) were resolved on SDS/10% PAGE, and Western blot analysis was performed. Enhanced chemiluminescence was used for visualization.

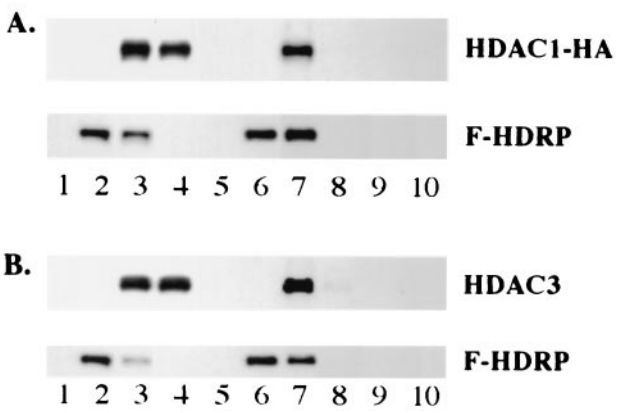

Fig. 4. HDRP interacts with both HDAC1 and HDAC3. FLAG-HDRP was immunoprecipitated from transfected Cos7 lysates by using anti-FLAG antibody, resolved by SDS/PAGE, and subjected to Western blot analyses. (*A*) Cos7 cells were transfected with vector alone (lanes 1 and 5), pFLAG-HDRP alone (lanes 2 and 6), pFLAG-HDRP with pHA-HDAC1 (lanes 3 and 7), or pHA-HDAC1 alone (lanes 4 and 8). Lanes 1–4, whole-cell lysates; lanes 5–8, anti-FLAG IP; lanes 9 and 10, anti-FLAG IP in the presence of FLAG-peptide for pFLAG-HDRP alone and for pFLAG-HDRP with pHA-HDAC1, respectively. (*Upper*) Anti-HA antibody was used for Western blotting; (*Lower*) anti-FLAG-M2 antibody was used for Western blotting. (*B*) The samples shown were described for *A*, except that pCMV-HDAC3 replaces pHA-HDAC1 in the transfection and that anti-HDAC3 antibody was used for Western blotting in *Upper*.

sequence identity with the N-terminal 600 amino acids (the noncatalytic domain) of KIAA0288 (Fig. 1) was identified through the search of GenBank and designated HDRP. HDRP is 590 amino acids in length with a theoretical pI and molecular mass of 8.86 and 65,886.5 Da, respectively. HDRP does not contain the catalytic domain of an HDAC. Sequence analysis indicates that HDRP contains a leucine-zipper motif at amino acids 488–502.

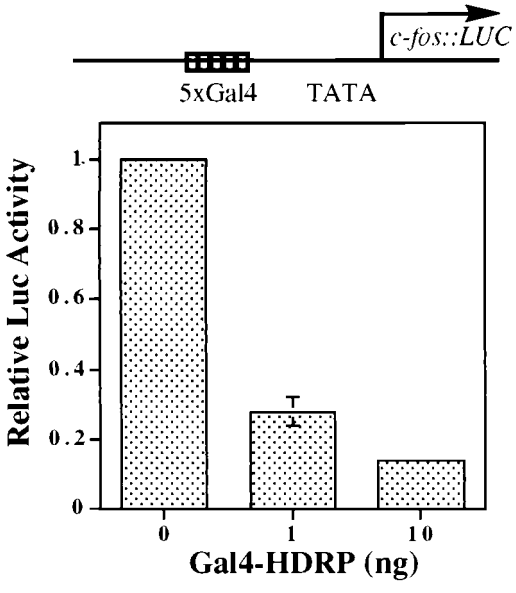

Fig. 5. Gal4-HDRP represses basal transcription. Gal4-*c-fos-Luc* was transfected into 293T cells along with the indicated amount of pGal4-HDRP. pCMV-Gal4 (1–147) (10 ng) was included in the experimental group that had no Gal4-HDRP. Results are shown as the means of three independent transfections ± standard deviation. The Fluc activity was first normalized to the cotransfected *Renilla* Luc (Rluc) activity. The basal value was set to 1.

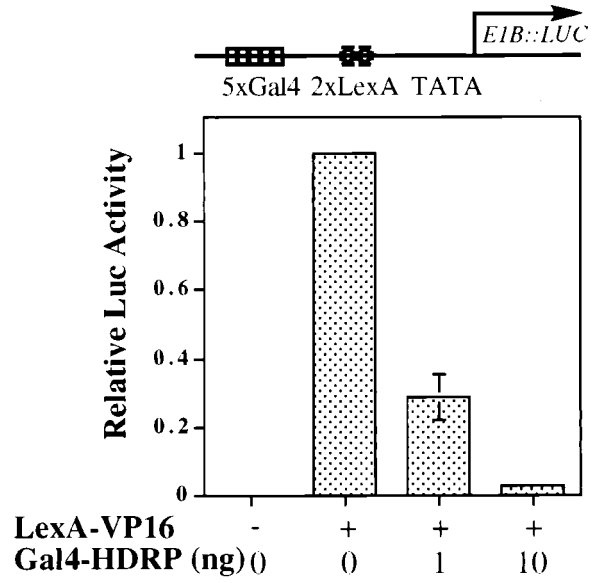

Fig. 6. Gal4-HDRP represses activated transcription. Gal4-LexA-*E1B-Luc* was transfected into Cos7 cells with 10 ng of pCMV-LexA (1–202) (−) or with 10 ng of pCMV-LexA-VP16 (+) along with the indicated amount of pGal4-HDRP. pCMV-Gal4 (1–147) was included in experimental groups with pCMV-LexA (1–202) or pCMV-LexA-VP16 alone. Results are shown as the means of three independent transfections ± standard deviation. The Fluc activity was first normalized to the cotransfected Rluc activity. The VP16 activated value was set to 1. VP16 activated transcription 238-fold compared with the basal level.

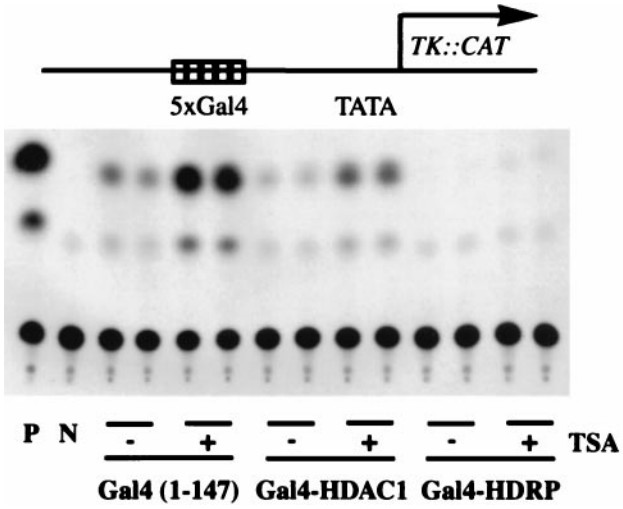

Fig. 7. Gal4-HDRP represses transcription in the presence of TSA. Gal4-*TK-CAT* was transfected into 293T cells with pCMV-Gal4 (1–147) (100 ng), pGal4-HDAC1 (100 ng), or pGal4-HDRP (20 ng). TSA (100 ng/ml) treatment was started 12 hours later, and CAT enzyme assays were performed 30 hours after the addition of TSA. Each transfection was performed in duplicate. P, positive control (CAT enzyme); N, negative control (untransfected cell lysate).

Searching the GenBank with the deduced amino acid sequence of HDRP retrieves a *Xenopus* HDRP homolog, the MILZ/MITR protein (GenBank accession no. Z97214; ref. 20) that is 71% identical to HDRP. HDRP also shares 50% identity in deduced amino acid sequence with that of the N termini of HDAC5 and mHDA1 (the murine homolog of HDAC5). The leucine zipper motif is conserved in MITR but not in the HDACs. Fig. 1 shows the amino acids sequence alignment of the full length of HDRP with the full length of MITR and the N termini of the HDACs.

By searching GenBank with the HDRP nucleotide sequence, it was found that HDRP maps to human chromosome 7p15-p21, a region that has been implicated in cancers and neuronal disorders (21–23). The entire cDNA sequences [spanning about 172 kilobases (kb)] are present in two overlapping bacterial artificial chromosome clones from human chromosome 7p15-p21, clone RG180O01 (GenBank accession no. AC002124), and clone RG317M02 (GenBank accession no. AC002433).

HDRP mRNA Is Differentially Expressed Among Human Tissues. The expression of HDRP mRNA was determined by Northern blot analysis by using the human multiple tissue Northern blot from CLONTECH. A single 4.4-kb HDRP transcript is readily detected in the heart, brain, and skeletal muscle (Fig. 2). This mRNA is also expressed in the other tissues examined (placenta, lung, liver, kidney, and pancreas), albeit at a lower level.

HDRP Is an Expressed Protein. To test whether HDRP is an expressed protein, Western blot analyses were performed on whole-cell lysates from T24 (human bladder carcinoma cell line) and Cos7 (monkey origin) cells transfected with vector alone or pCMV-HDRP by using rabbit polyclonal antibody raised against a synthetic peptide corresponding to the C-terminal 14 amino acids of HDRP. The antibody against HDRP recognizes an endogenous protein in both T24 cells and vector control Cos7 cells with an apparent molecular mass of ≈75 kDa (Fig. 3, lanes 1 and 2), the same size as the overexpressed HDRP protein (Fig. 3, lane 3). The apparent molecular mass of 75 kDa is consistent with the predicted molecular mass of 69 kDa. The antibody recognizes HDRP of human (T24 human bladder carcinoma cells and the overexpressed protein from pCMV-HDRP in Cos7 cells), monkey (Cos7 cells; Fig. 3), and mouse (C2C12 cells) origin (data not shown).

HDRP Forms Complexes with Both HDAC1 and HDAC3. HDRP shares 50% deduced amino acid sequence identity with the N-terminal noncatalytic domain of HDAC4. To rule out the possibility that HDRP may possess intrinsic HDAC activity, HDAC enzymatic activity assays were carried out with FLAG-HDRP fusion protein immunoprecipitated from transfected Cos7 cells. HDRP was found to possess no intrinsic HDAC activity (data not shown), consistent with the finding that the N-terminal domain of HDAC4 has no HDAC activity (15).

To test whether HDRP is associated with HDACs, pFLAG-HDRP was transfected into Cos7 cells with either pCMV-HDAC3 or pHA-HDAC1. Western blot analyses were performed on proteins obtained by IP with the anti-FLAG antibody on whole-cell lysates prepared from the transfected Cos7 cells. FLAG-HDRP is associated with both HDAC1 and HDAC3 as evidenced by the presence of HA-HDAC1 and HDAC3 in the anti-FLAG immunoprecipitates (Fig. 4 *A* and *B Upper*). No signals or markedly reduced signals were detected when a FLAG peptide was present during the FLAG IP or when cells were transfected with pHA-HDAC1 or pCMV-HDAC3 alone. The expression levels of FLAG-HDRP among these transfection combinations were similar (Fig. 4 *A* and *B Lower*). These results indicate that HDRP forms complexes with HDAC1 and HDAC3.

HDRP Represses Basal Transcription. It is known that HDACs repress transcription when tethered to DNA as Gal4 fusion proteins (10). We confirmed this repression effect of HDAC by

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using a 5XGal4-*TK-CAT* reporter gene and Gal4-HDAC1 (see below, Fig. 7).

We then investigated the effect of HDRP on transcription. Full-length HDRP was fused with Gal4 DNA-binding domain to tether it to DNA. A firefly Luc (Fluc) reporter gene under the control of a *c-fos* minimal promoter with five upstream Gal4-binding sites was transfected into 293T cells along with indicated genes. This promoter supports an easily detectable level of transcription in 293T cells, and the Fluc activity can be determined reliably (Fig. 5). pRL-TK (Rluc) was included as a control for transfection efficiency. pCMV-Gal4 (1–147) was included in the experimental group that had no pGal4-HDRP (Fig. 5, 0 ng of Gal4-HDRP). Gal4-HDRP represses transcription from the *c-fos* minimal promoter in a dose-dependent manner (Fig. 5). Gal4-HDRP did not repress the cotransfected TK promoter that does not contain the Gal4-binding site (pRL-TK) in the same experiment (data not shown). Likewise, FLAG-HDRP did not repress the Gal4-*fos-Luc* transcription activity (data not shown). These results indicate that HDRP is a transcriptional repressor when tethered to a promoter.

HDRP Represses Activated Transcription. To test whether HDRP can repress activated transcription, the effect of HDRP on the transcriptional activation of VP16 was determined by using a Fluc reporter gene under the control of an *E1B* minimal promoter that contains five Gal4-binding sites upstream of two LexA-binding sites. LexA-VP16 fusion protein was used to activate transcription. pCMV-Gal4 (1–147) and pCMV-LexA (1–202) were included in the experimental group that had no pCMV-LexA-VP16 and pGal4-HDRP. pCMV-Gal4 (1–147) was included in the experimental group that had pCMV-LexA-VP16 but no pGal4-HDRP. Gal4-HDRP inhibits VP16-mediated transcriptional activation in a dose-dependent manner (Fig. 6). Gal4-HDRP also inhibits SP1-mediated transactivation in the same system (data not shown). These results indicate that HDRP represses activated transcription when tethered to a promoter.

HDRP Can Repress Transcription in the Presence of HDAC Inhibitors. To test whether the transcriptional repression effect of HDRP is primarily due to its association with HDACs, the effect of HDAC inhibitors on Gal4-HDRP mediated-transcriptional repression was examined by using a 5XGal4-*TK-CAT* reporter gene in 293T cells. Gal4-HDAC1 was included as a positive control for confirmation of the effectiveness of the HDAC inhibitors used. Indeed, Gal4-HDAC1 represses transcription as indicated by the reduced CAT activity in the presence of Gal4-HDAC1, which was substantially reversed by TSA (Fig. 7). However, the transcriptional repression mediated by Gal4-HDRP was not reversed by TSA (Fig. 7). Another HDAC inhibitor, suberoylanilide hydroxamic acid (24, 25), was also unable to reverse transcriptional repression mediated by HDRP (data not shown). These results suggest that HDRP-mediated transcriptional repression is not primarily due to its association with HDACs.

Discussion

We report herein the identification and characterization of HDRP, a potent transcriptional repressor. HDRP is expressed with an apparent molecular mass of ≈75 kDa and shares 50% sequence identity with the N-terminal noncatalytic domain of HDAC4 and HDAC5. HDRP forms complexes with HDAC1 and HDAC3 but does not possess intrinsic HDAC activity.

HDRP represses both basal and activated transcription. The association of HDRP with HDAC1 and HDAC3 suggests that HDAC activity may be responsible for the transcriptional repression mediated by HDRP. However, the finding that HDRP-mediated transcriptional repression is not reversed in the presence of HDAC inhibitors does not support this hypothesis. Rather, HDRP may use an as yet unidentified mechanism to repress transcription. In support of this alternative mechanism is the finding that the N-terminal domain of HDAC4 is able to repress transcription in the presence of an HDAC inhibitor (17). The functional significance of the association of HDRP and HDAC1 and HDAC3 is unclear. However, RPD3 (the yeast HDAC1) mutants that lack deacetylase activity decrease but do not completely lose RPD3-mediated transcriptional repression (26). It is possible that HDAC1 and HDAC3 may act as corepressors by recruiting HDRP to repress transcription under certain circumstances.

HDRP may be a transcriptional repressor by itself or may recruit other transcriptional repressors. A basic domain at the N terminus of HDRP might be involved in its transcriptional repression activity, because a highly basic synthetic peptide has been found to be an effective transcriptional repressor (27).

While this manuscript was in preparation, Sparrow *et al.* (20) reported the identification of a *Xenopus* homolog of HDRP, MITR. MITR interacts with the transcription factor myocyte enhancer-binding factor 2 (MEF-2) and represses MEF-2-mediated transcription. Sparrow *et al.* (20) also showed that hMITR inhibits the transcription of a TK promoter as a Gal4-fusion protein and interacts with HDAC1. MEF-2 interacts with the basic helix–loop–helix bHLH family transcription factors MyoD (28, 29) and mammalian achaete-scute homolog 1 (MASH1; refs. 30 and 31) to induce muscle and neural-specific genes, respectively. The expression pattern of HDRP reported herein is consistent with a role for HDRP in both muscle and brain development, because levels of its mRNA are high in the skeletal muscle, the heart, and the brain. HDRP may modify the activities of the MEF2/MyoD and MEF2/MASH1 complexes during development.

The HDRP gene is located on human chromosome 7p15-p21, a region implicated in tumors and neuronal disorders (21–23). Because HDRP is a transcriptional repressor, it will be interesting to know whether mutation in HDRP is the cause of some of these diseases.

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