Histone Deacetylases Specifically Down-regulate p53-dependent Gene Activation*

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p53, the most commonly mutated gene in cancer cells, directs cell cycle arrest or induces programmed cell death (apoptosis) in response to stress. It has been demonstrated that p53 activity is up-regulated in part by posttranslational acetylation. In agreement with these observations, here we show that mammalian histone deacetylase (HDAC)-1, -2, and -3 are all capable of down-regulating p53 function. Down-regulation of p53 activity by HDACs is HDAC dosage-dependent, requires the deacetylase activity of HDACs, and depends on the region of p53 that is acetylated by p300/CREB-binding protein (CBP). These results suggest that interactions of p53 and HDACs likely result in p53 deacetylation, thereby reducing its transcriptional activity. In support of this idea, GST pull-down and immunoprecipitation assays show that p53 interacts with HDAC1 both in vitro and in vivo. Furthermore, a pre-acetylated p53 peptide was significantly deacetylated by immunoprecipitated wild type HDAC1 but not deacetylase mutant. Also, co-expression of HDAC1 greatly reduced the in vivo acetylation level of p53. Finally, we report that the activation potential of p53 on the BAX promoter, a natural p53-responsive system, is reduced in the presence of HDACs. Taken together, our findings indicate that deacetylation of p53 by histone deacytases is likely to be part of the mechanisms that control the physiological activity of p53.

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human HDAC2 was identified based on a yeast two-hybrid screening using YY1 transcription factor as bait (22). YY1 negatively regulates transcription by tethering HDAC2 to DNA as a corepressor. Both HDAC1 and HDAC2 associate stably with mSin3A in mediating transcriptional repression. This HDAC-mSin3 complex can be recruited to specific promoters via interactions with a growing number of sequence-specific transcription factors (19–20). These include unliganded nuclear hormone receptors (e.g., RAR and TR), Mad/Max and Mxi/Max heterodimer (23), MeCP2 (24), and p53 (25). As well as being associated with mSin3A, HDAC1/2 are components of the NuRD (nucleosome-remodeling histone deacetylase) complex which has been implicated a role in transcriptional repression by DNA methylation (26–27). Mammalian HDAC3 was cloned by the searches of EST data bases (28–29) and found to repress YY1-mediated transcriptional repression via direct interactions with YY1 (28). Searches of EST data bases led to the discovery of three more histone deacetylases HDAC4, HDAC5, and HDAC6 (30–33). Sequence alignment analysis reveals that the mammalian HDACs identified so far fall into two groups, the yeast RPD3 protein-like (HDAC1, -2, and -3) and the yeast HDAC protein-like (HDAC4, -5, and -6) (21, 30, 33). It is becoming evident that each group of HDACs is utilized by distinct sets of transcriptional repressors (20).

Whether HDACs play a role in regulating p53 function remains unaddressed. In the discovery of HIV Tat acetylation (16), treatment of cells with histone deacetylase inhibitor TSA (34) was found to synergistically work with Tat in activating HIV-1 promoter, presumably through inhibiting the deacetylation of Tat. Furthermore, it was found that the retinoblastoma (Rb)-associated histone deacetylase could deacetylate E2F1 (18). These studies raise the possibility that, similar to Tat and E2F1, modification of p53 by HDACs might play a direct role in regulating p53 function. In support of this hypothesis, in the course of studying the transcriptional repression function of p53 in apoptosis, Murphy et al. (25) found p53 recruited HDACs, via interactions with mSin3A, to repress two genes, Map4 and statomin. The underlying mechanism at least involves core histone deacetylation. However, the possibility of p53 deacetylation has not been examined. In the present study, we report that mammalian HDAC1, -2, and -3 specifically down-regulate the transactivation activity of p53. The inhibition is HDAC dosage-dependent and requires the deacetylase activity of HDACs. Most importantly, the down-regulation of p53 function by HDACs relies largely on the C-terminal 30 residues of p53, the region containing the basic lysines (Lys-373 and Lys-382) that have been shown to be acetylated by p300/CBP in vivo (4, 8, 9). These results of functional assays are further supported by the fact that HDACs form a complex with p53 and significantly deacetylate p53 both in vitro and in vivo. Finally, we show that HDACs inhibit the activity of BAX promoter, a nature system responsive to p53, in a p53-dependent manner. Our findings strongly suggest that HDAC1, -2, and -3 participate in p53-mediated gene regulation, at least in part by directly deacetylating p53.

MATERIALS AND METHODS

Plasmids—The following plasmids have been described previously: pSVp53V143A and pSVp53V143ACD30, both of which express temperature-sensitive derivatives of p53V143A (35), the p300 promoter construct p3PRE-CAT (35); pME18S-FLAG-HDAC2, which expresses FLAG epitope-tagged HDAC2 (36); pCMV-FLAG-HDAC3, which expresses FLAG epitope-tagged HDAC3 (28); pFLAG-HDAC1(H141A), both of which express FLAG epitope-tagged mutant HDAC1 defective in deacetylase activity (37); pGV5VP, which expresses GAL4VP16, and pG5E1BCAT (38); pGST-HDAC1, pGST-HDAC2, and pGST-HDAC3 (28); pBAX-Luc (39).

To construct plasmid pcDNA3-HDAC1-FLAG, which encodes a C-terminal FLAG epitope-tagged HDAC1, the BamHI fragment from pBJS-HD1-F (21) was subcloned into the BamHI site of pcDNA3 (Invitrogen). Plasmid pME18S-FLAG-HDAC2(1–372), which encodes FLAG epitope-tagged mutant HDAC2 defective in deacetylase activity, was created by digestion of plasmid pGEM7z-mRDP3 (22) with SnaBI and BamHI, followed by fill-in and self-ligation. A further digestion of the FLAG epitope-tagged mutant HDAC2 defective in deacetylase activity, was constructed by digestion of the plasmid pBS-SK-HDAC3(1–428) with HindIII. The cut out fragment was subsequently cloned into pME18S-FLAG vector (40) between the corresponding XhoI and BamHI sites. pGEM3-p53 was created by subcloning of the cDNA corresponding to p53 sequence into pGEM3 vector (Promega) between HindIII and BamHI sites.

Cell Culture, Transfection, Chloramphenicol Acetyltransferase (CAT) Assay, and Luciferase Assay—The human lung carcinoma cells H1299 and the human osteosarcoma cells Saos-2 were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Approximately 2.5 × 105 cells (H1299) or 7.5 × 105 cells (Saos-2) were seeded in each 60-mm culture dish 18–24 h before transfection. Calcium phosphate-mediated DNA transfection was performed as described previously with some modifications (41). Typically, transfection lasted 18 h. CAT activity was measured 72 h after transfection and quantified as described previously (41). For temperature shift assays, the incubation temperature was reduced to 30 °C 4 h after incubation at 37 °C. For luciferase assays, cell culture and DNA transfection were done as described above except that 0.5 µg of pBAX-Luc, 0.1 µg of pRL-TK, 0.5 µg of pSVp53V143A, and 2.5 µg of plasmids encoding HDACs were introduced into cells. After harvesting the cells, the luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega).

In Vitro Translation of Proteins—In vitro transcription/translation was performed with the TNT system (Promega) according to the manufacturer’s instructions. The template was pGEM3-p53.

GST Pull-Down Assays—GST, GST-p53, GST-HDAC1, GST-HDAC2, and GST-HDAC3 were expressed in and purified from Escherichia coli BL21DE3pLysS strain according to standard protocols (42–44). The ligand concentrations, using bovine serum albumin as a standard, were 1 mg/ml GST beads and 0.3 mg/ml each of GST-HDAC1, GST-HDAC2, and GST-HDAC3 beads. Aliquots (50 µl, ~15 µg of proteins on beads) of the GST, GST-HDAC1, GST-HDAC2, and GST-HDAC3 beads washed with 2.5% NP-40 containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, and 0.5% sodium deoxycholate were translated, [35S]methionine-labeled p53. After being washed with buffer D (45), bound proteins were eluted from beads with buffer D containing 0.1 M glutathione and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel.

Immunoprecipitation—Approximately 5 × 105 cells were seeded in each 100-mm culture dish 18–24 h before transfection. Cell cultures and DNA transfections were performed as described above. Briefly, 20 µg each of the corresponding plasmids was transfected into H1299 cells. Cell extracts were prepared by lysis in Nonidet P-40 buffer (20 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1% sodium deoxycholate, 0.1% Nonidet P-40, 1 mM EDTA) containing 1% protease inhibitor mixture (Complete™, Roche Molecular Biochemicals). Equal amounts of lysates were incubated with anti-p53 (Ab-6, Oncogene) and FLAG epitope (Sigma), respectively, using the EZ1™ system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Western Immunoblotting—An equal amount (approximately 10 µg) of proteins from extracts of transfected cells was boiled in a sample buffer (125 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 20% glycerol, 0.005% bromphenol blue) for 5 min and then loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon membrane (Millipore). p53, FLAG-HDAC derivatives were detected with antibodies directed against p53 (Ab-6, Calbiochem) and the human osteosarcoma cells Saos-2 were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Approximately 2.5 × 105 cells (H1299) or 7.5 × 105 cells (Saos-2) were seeded in each 60-mm culture dish 18–24 h before transfection. Calcium phosphate-mediated DNA transfection was performed as described previously with some modifications (41). Typically, transfection lasted 18 h. CAT activity was measured 72 h after transfection and quantified as described previously (41). For temperature shift assays, the incubation temperature was reduced to 30 °C 4 h after incubation at 37 °C. For luciferase assays, cell culture and DNA transfection were done as described above except that 0.5 µg of pBAX-Luc, 0.1 µg of pRL-TK, 0.5 µg of pSVp53V143A, and 2.5 µg of plasmids encoding HDACs were introduced into cells. After harvesting the cells, the luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega).

Chemical Acetylation of Peptide—Peptides corresponding to the C-terminal 26 amino acids of p53 (residues 364–389, 5′-AHS(5′H)-SKGGKSGTIRHHKLMPKTEG-3′) or the N-terminal fragment of histone H4 (5′-SRGKGGKGGKLOGKKHRKRVRKL-3′) were synthesized by the core facility center of the Institute of Zoology, Academia Sinica,
and purified to 95% purity by high pressure liquid chromatography. To acetylate the peptides, 0.4 mg of peptides were incubated with 0.5 ml of [3H]CH3COONa (NEN Life Science Products, catalog number NET-003H, 5 mCi (185 M bq), 2–5 Ci (74.0–185 Gbq/mmol in ethanol) and 10 µl of BOP (BOP solution is always prepared freshly. For 100 µl of BOP solution, take 0.01 g of BOP (Aldrich catalog number 22,608-4) into 97 µl of acetonitrile (Aldrich catalog number 27,071-7) at room temperature overnight with stirring. After mixing well to dissolve, 3 µl of triethylamine (Aldrich catalog number 13,206-3) was added and mixed thoroughly. The final concentration for BOP and triethylamine were 0.24 m and 0.2 m, respectively. This labeling was processed in the chemical hood. To purify the radiolabeled peptides, the Microcon-SCX (Millipore catalog number 42460) was prewashed with 500 µl of 10 mM HCl in methanol once and then with 500 µl of 10 mM HCl, 10 mM methanol in double distilled water. An aliquot (250 µl) of the labeling mixture was loaded onto the prewashed Microcon-SCX column and then spun at 1200 × g for 1 min. The column was then washed twice with 500 µl of 10 mM HCl in 10% methanol, followed by centrifugation at 1200 × g for 1 min. To elute the sample, 50 µl of 3 N HCl in 50% isopropyl alcohol was added to the reversed column, followed by centrifugation at 14,000 × g for 15 s. Finally, the eluate was dried out and dissolved into 500 µl of double distilled water and stored at −80 °C. To evaluate the successful labeling, 1 µl of the purified peptide was taken into 100 µl of water and extracted with 400 µl of ethyl acetate for scintillation counting. The counts were adjusted to 20,000 cpm for each peptide deacetylase assay.

Peptide Deacetylase Assay—H1299 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. For transfection, 5 × 10⁶ cells were seeded in a 100-mm culture dish for 16 h. Twenty µg of pcDNA3-HDAC1-FLAG was introduced into cells with the calcium phosphate coprecipitation method as described (41). 72 h after transfections, cells were harvested, lysed in Jurkat lysis buffer (37). Immunoprecipitation of the HDAC1 immunocomplex with FLAG antibodies (Sigma) was performed as described (37). The precipitated HDAC1 complex was stored in 150 µl of ice-cold HD buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol) and subsequently used in the deacetylase reactions as described with some modifications (28). Briefly, 20,000 cpm of purified peptides were incubated with immunoprecipitates in 150 µl of ice-cold HD buffer at room temperature overnight with mild shaking. To stop the reactions, 50 µl of STOP solution (0.16 mM acetic acid, 1.0 M HCl) was added into each reaction and mixed well by vortexing. The released [3H]acetate was extracted by adding 600 µl of ethyl acetate. 250 µl of upper layer was mixed with 5 ml of scintillation mixture to detect the HD activity.

RESULTS AND DISCUSSION

Dosage-dependent Inhibition of p53 Function by HDAC1,-2, and -3—Regulation of p53 function by posttranslational acetylation has been demonstrated by several laboratories (4, 8, 9). A recent finding by Murphy et al. (25) further indicates that p53 utilizes HDACs, basically through histone deacetylation, to repress the transcription of two genes, Map4 and statmin. However, question as to whether deacetylation of p53 itself plays a role has not yet been addressed. To assess the possibility of HDACs in regulating p53 function, a CAT reporter construct p3PREcCAT (35) containing three p53-responsive elements (PRE) was transiently transfected into cells, in the absence or presence of expression plasmids encoding p53 or HDACs. Since p35 has been shown to inhibit the activities of promoters lacking PREs, we took advantage of the temperature-sensitive p53 mutant p53V143A (35) to ensure optimal expression of HDACs. In this way, HDACs were expressed first, and the function of wild type p53 was then induced by a shift of the assaying temperature from 37 to 30 °C. Two p53-deficient cell lines were used throughout the study, the human lung carcinoma cells H1299 and the human osteosarcoma cells Saos-2 (data not shown). Similar results were obtained. As shown in Fig. 1, p53 alone strongly activated the p3PREcCAT reporter (lanes 2, 10, and 18). In the presence of increasing amounts of HDAC1 (upper panel, lanes 3–8), HDAC2 (middle panel, lanes 11–16), or HDAC3 (lower panel, lanes 19–24), the CAT activity driven by p53 was decreased in a HDAC dosage-dependent manner, suggesting a direct inhibition of p53 function by HDACs.

Recruitment of HDACs by sequence-specific DNA-binding proteins has been reported to repress transcriptional activity of many promoters in vivo (19). To eliminate the possibility that overexpression of HDACs affects the protein level of p53, thus resulting in the apparent loss of p53 function, similar experiments were applied, followed by a Western blot probed with antibodies directed against p53. As shown in Fig. 2A, co-expression of HDACs led to a dramatic decrease in the CAT activity driven by p53 (upper panel, compare lanes 3–5 to lane 2), consistent with the previous observation described in Fig. 1. However, co-expression of HDACs did not affect the protein level of p53 to a significant extent (lower panel, compare lanes 3–5 to lane 2). Since acetylation of p53 by p300 greatly enhances its DNA-binding ability (4), and hence the transactivation activity (47), it is very likely that the observed loss of p53 transactivation activity is due to direct deacetylation of p53 by HDACs. This possibility was explored in the following experiments.

HDAC Down-regulation of p53 Function Requires the Deacetylase Activity of HDACs—The specificity of HDAC inhi-
Hbibetion of p53-dependent transactivation was further examined. First, we asked whether the deacetylase activity of HDACs was required to repress p53 function. Several residues within the central regions of the HDACs have been found to be essential for its deacetylase activity (37, 48). To demonstrate that p53 down-regulation by HDACs depends on the deacetylation event, wild type p53 was induced in the absence or presence of FLAG epitope-tagged mutant HDACs (FLAG-mHDACs) defec-

**FIG. 2. Specific inhibition of p53 activity by HDACs.** A, HDAC1, -2, and -3 down-regulate p53 function in transient transfection assays. Upper panel, H1299 cells were transfected with 2.5 μg of p53 reporter construct p3PRE-cAT, 0.5 μg of p53V143A expression plasmid, and 2.5 μg of plasmids expressing FLAG-HDACs, as indicated. The relative CAT activity (RCA) is indicated above each track of the autoradiogram. RCA is the mean fold inhibition of transcription compared with the transactivation activity of p53 in the absence of HDACs. Experiments were repeated three times. The standard errors were 0, ±0.09, ±0.07, and ±0.13 for lanes 2–5, respectively. Lower panel, protein levels of p53V143A. Transient transfections were performed as in the upper panel. Proteins of H1299 cells transfected with the vector alone (lane 1), with p53V143A (lane 2), or with p53V143A and HDACs (lanes 3–5) were fractionated on a 12% SDS-polyacrylamide gel, followed by immunoblotting with antibodies against p53. The positions of molecular mass markers in kilodaltons are shown on the left. B, the deacetylase activity of HDACs is required for the repression of p53 function. C, down-regulation of p53 function by HDACs is largely attributed to the C-terminal 30 amino acids of p53. D, GAL4VP16-dependent transactivation is not inhibited by HDACs. The CAT reporter construct pG5E1BCAT containing five GAL4-binding sites upstream of the E1B TATA box was illustrated above the autoradiogram. B–D, transfections and Western blots were performed as described in A, except that effectors and antibodies used were as indicated in each figure. The standard errors of the RCAs were 0, ±0.05, ±0.18, and ±0.09 for lanes 2–5 in B; 0, ±0.11, ±0.07, and ±0.09 for lanes 2–5 in C; and 0, ±0.1, ±0.26, and ±0.21 for lanes 2–5 in D, respectively.
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The deacetylase activity of these mutants were constructed by either site-directed mutagenesis (H199F) in HDAC1 (37) or deletion of the conserved deacetylase motif in the cases of HDAC2 and HDAC3 (see "Materials and Methods"). As shown in Fig. 2B, repression of p53 activity was greatly alleviated in the presence of mutant HDACs (compare lanes 2–5 in Fig. 2B, upper panel, to lanes 3–5 in Fig. 2A, upper panel). Both wild type and mutant HDACs expressed to similar levels, as detected in the Western blot probed with FLAG antibodies (Fig. 2B, lower panel and data not shown). It should be noted that mutant HDAC1 (H199F) fails to interact with the mSin3A protein (37), mSin3A is a transcriptional corepressor that bridges HDAC function to sequence-specific DNA-binding proteins (23). To exclude the possibility that the restoration of p53 function in the presence of the deacetylase mutant HDAC1(H199F) was due to the loss of the mediator protein, but not the loss of deacetylase activity, we examined the effect of another deacetylase mutant HDAC1(H141A) (37), which still associates with mSin3A, on repressing p53 activity. Similar results were obtained (data not shown). These experiments indicate that the deacetylase activity is indeed essential for HDACs to repress p53 function.

Down-regulation of p53 Function by HDACs Is Dependent on the C-terminal Domain of p53, Which Contains the Acetylation Sites for s300/CBP.—We next determined the p53 domain(s) involved in the down-regulation by HDACs. As shown in Fig. 2C, deletion of the C-terminal 30 residues of p53 (p53V143ACD30) did not affect its transactivation activity (lane 2). However, the HDAC-mediated inhibition of p53 activity was greatly abolished (compare lanes 3–5 in Fig. 2C, upper panel, to lanes 3–5 in Fig. 2A, upper panel). The observed loss of HDAC-mediated inhibition was not likely due to protein instability since protein level of mutant p53 remained unchanged in the absence or presence of HDACs (Fig. 2C, lower panel). These data are of particular interest in that the C-terminal 30 residues of p53 contain the acetylation sites for p300/CBP (4). The acetylation of lysines 373 and 382 of p53 has been clearly demonstrated by several laboratories (4, 8, 9). It is reasonable to speculate that HDACs directly deacetylate p53 at its C-terminal domain and thus alleviate its ability to activate gene. It should be noted that deletion of C-terminal 30 residues of p53 (wild type, 393 amino acids) did not totally release the repression by HDACs. This can be explained in part by the fact that additional lysine (Lys-320) is acetylated by PCAF in vivo (8–9). Deleting the C-terminal 30 amino acids from p53 only removed the acetylation sites for p300/CBP (Lys-373 and Lys-382). The residual activity observed may be due to Lys-320 acetylation. Alternatively, it may be contributed by the general phenomenon of core histone deacetylation on local promoter.

GAL4VP16-dependent Transactivation Is Not Affected by HDACs.—To test if p53 is a specific target for HDACs, the effect of HDACs on GAL4VP16-mediated gene activation was assayed. The expression plasmid pSGVP that encodes GAL4VP16, was transfected into cells in the absence or presence of HDACs. A CAT reporter construct containing five GAL4-binding sites upstream of the E1B TATA was used. As shown in Fig. 2D, overexpression of HDACs exhibited little effect on GAL4VP16-dependent transactivation (compare lanes 3–5 to lane 2, upper panel), demonstrating the specificity of HDACs on the repression of p53 activity. This finding clearly indicates that HDACs only regulate a subset of transcription factors. It is possible that GAL4VP16 does not physically interact with HDACs and thus fails to recruit HDACs to the specific promoter. In contrast, p53, presumably its C-terminal domain, is likely to be one of the direct sites for the binding of HDACs.

Direct Interactions of p53 and HDACs—The results described are consistent with the notion that HDACs might directly deacetylate p53, reduce its DNA-binding ability, and therefore render it less active in stimulating gene transcription. If deacetylation of p53 is relevant for its transcriptional activity in vivo, then associations between p53 and HDACs should be determined. To examine whether HDACs directly interact with p53, the ability of GST-HDAC fusion proteins to interact with in vitro translated 35S-labeled p53 was investigated by a protein pull-down assay (Fig. 3A). Remarkably, we found that HDAC1, -2, and -3 (linked to GST) retained 35S-labeled p53 to similar intensity (lanes 3–5). In contrast, GST alone showed little affinity toward p53 (lane 2). These data indicate that p53 might directly interact with HDAC1, -2, and -3 in vitro. Our results conflict with the conclusions made by Murphy et al. (25). In their study, p53 inhibited downstream genes by indirect association with HDACs, basically mediated by the corepressor mSin3A. No direct interactions between p53 and HDACs were observed in their in vitro GST pull-down assays. This discrepancy can be argued by the fact that GST-p53 recombinantly purified from bacteria, instead of the in vitro translated 35S-labeled p53, was used in their pull-down experiments. It is of interest to note that we did not observe significant association between p53 and HDACs as well, when GST-p53 expressed in bacteria was used (data not shown). Only p53 translated in vitro in the rabbit reticular lysates was evident to interact with HDACs (Fig. 3A). Given that HDAC1, -2, and -3 directly interact with the DNA-binding protein YY1 (22, 28), we believe p53 might as well directly associate with HDACs independently of mSin3A. This hypothesis is further strengthened by the fact that, indeed, mSin3A does not exist in the rabbit reticular lysates, as determined by a Western blotting using an antibody directed against mSin3A (data not shown). In addition, unless the protein level of the endogenous mSin3A in H1299 cells and Saos-2 cells is high, the observed loss of p53 activity may be contributed by the general phenomenon of core histone deacetylation.
activity in the presence of overexpressed wild type HDACs shown in Fig. 1 is unlikely due to indirect mediation by mSin3A. It is likely that certain modification(s) absent in the prokaryotic system renders p53 a better substrate for HDACs. Nevertheless, we cannot exclude the possibility that some proteins, which are present in the rabbit reticular lysates, mediate the interaction.

To confirm further that the FLAG-tagged HDACs are associated with p53 in cells, p53V143A was therefore transfected into H1299 cells along with either wild type HDAC1 (FLAG-tagged) (Fig. 3, lane 3) or with the deacetylase mutant (H199F) (lane 4). The anti-p53 antibody (FL-393, goat polyclonal, Santa Cruz Biotechnology) was then used to precipitate p53 and p53-associated proteins, which were subsequently detected by Western blot analysis. As shown in Fig. 3B, we found that both wild type and mutant FLAG-HDAC1 could be co-precipitated with p53 (compare lanes 3, 4 to lane 2), as revealed by the Western blotting using anti-FLAG antibodies. These data are consistent with previous observations made by Murphy et al. (25) in which endogenous p53 associates with HDAC1. Furthermore, our results strongly suggest that p53 may form a complex with HDACs via direct binding (see Fig. 3A). In addition, the association of p53 and deacetylase mutant HDAC1 (H199F) further supports the idea that deacetylase activity is indeed required for HDACs to fully repress p53 function (Fig. 2B).

p53 Deacetylation by HDAC1 in Vitro and in Vivo—Because direct associations of p53 and HDACs were demonstrated (Fig. 3), and because the down-regulation of p53 function by HDACs was found to target the region in p53 containing acetylation lysines for p300/CBP (see Fig. 2C), we asked further if p53 served as a direct substrate for HDACs. To test this hypothesis, a peptide corresponding to the C-terminal 26 residues of p53 (p53 peptide: AHSSHLKSGKQSTSRHKLMFKTE) was used as a substrate for HDAC1. The HD activity was determined by the dpm of the released [3H]acetate. The amino acid sequence of the p53 peptide is shown. Lysines 373 and 382 are indicated by arrows. The experiments were repeated three times, and the data shown represent the average ± S.E. of three independent experiments.
was synthesized and chemically acetylated in vitro (see “Materials and Methods”). Wild type or deacetylase mutant HDAC1 (FLAG-tagged) was expressed in the cells, and the HDAC1 immunocomplex was precipitated by FLAG antibodies to test its ability to deacetylate the p53 peptides that were [3H]acetyl-labeled in vitro. As shown in Fig. 4A, in the presence of the immunoprecipitated wild type HDAC1 (wt HDAC1), we found that the chemically acetylated p53 peptides were significantly deacetylated, as measured by the released dpm of [3H]Acetyl. By contrast, the immunoprecipitated deacetylase mutant HDAC1(H199F) only deacetylated the p53 peptides to a background level (mt HDAC1). As a control, a similar experiment using acetylated histone H4 peptides, a putative substrate for HDACs, as substrates was done in parallel. Under the same conditions, we found that deacetylation of the p53 peptide by HDAC1 was comparative to H4 deacetylation, suggesting p53 could be as an equally good substrate as histone H4 for HDACs.

We next sought to establish whether p53 could be deacetylated by HDACs in vivo. To this end, p53V143A was introduced into H1299 cells in the absence or presence of FLAG-HDAC1. Immunoprecipitation of the acetylated fractions of p53 was carried out by an antibody that specifically recognizes p53 acetylated at lysines 373 and 382, followed by a Western blot using p53-specific antisera Ab-6 (Calbiochem). As depicted in Fig. 4B, the acetylation level of p53 was significantly reduced when HDAC1 was introduced into cells (upper panel, compare lane 4 to lane 2). Again, the p53 protein level remains unchanged in the presence of ectopically expressed HDAC1 (lower panel, compare lane 4 to lane 2). Similar results were obtained when immunoprecipitation with p53 antibody (FL-393) was followed by Western blotting for acetylated p53 (Fig. 4C, compare lane 2 to lane 1). Furthermore, the observed loss of p53 acetylation in the presence of HDAC1 was partly restored when deacetylase mutant HDAC1(H199) was introduced instead (Fig. 4C, compare lane 3 to lane 2). These results strongly suggest that p53 is a physiological substrate for histone deacetylases.

**HDACs Reduce the Ability of p53 to Activate BAX Promoter—**
Finally, we assessed whether HDACs affect a natural p53-responsive system, the BAX promoter (39). Cells were transfected with a luciferase reporter gene driven by the BAX promoter and the expression plasmid of p53V143A, in the absence or presence of HDACs. As shown in Fig. 5, in the absence of p53, HDACs exhibited little effect on the BAX promoter (–p53). Expression of p53 alone activated the BAX promoter approximately 3–4-fold, as measured by the luciferase activity (+p53, open bar). However, in the presence of wild type, but not deacetylase mutant, HDAC1, -2, or -3, the p53-dependent transcriptional activation of BAX promoter was reduced approximately 50% (+p53) (Fig. 5 and data not shown). Thus, the ability of HDACs to down-regulate p53 function was not only evident on an artificial construct containing p53-binding sites (p3PRECAT, the data presented above) but was also observed with the BAX promoter, a natural system responsive to p53. These data further support the idea that HDACs might be a physiologically important regulator of p53.

**Conclusions—**Our results indicate that HDAC1, -2, and -3 were able to down-regulate p53-dependent transcription. These results were specific, because they were HDAC dosage-dependent, required the deacetylase activity of HDACs, and were not observed with GAL4VP16-dependent transcription. Interestingly, these HDAC-mediated repressions of p53 activity were largely attributed to the C-terminal 30 amino acids of p53, which encompass the acetylation sites for p300/CBP. Unlike histone acetyltransferases that often have particular sub-

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