Enhanced Deacetylation of p53 by the Anti-apoptotic Protein HSCO in Association with Histone Deacetylase 1

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HSCO (hepatoma subtracted-cDNA library clone one, also called ETHE1) was originally identified by its frequent overexpression in hepatocellular carcinomas. HSCO inhibits function of NF-κB by binding to RelA and accelerating its export from the nucleus. We show here that HSCO exhibits anti-apoptotic activity in cells exposed to DNA-damaging agents by suppressing transcriptional activity of p53. Induction of pro-apoptotic genes, Noxa, Perp, PIG3, and Bax were suppressed in cells overexpressing HSCO. By increasing ubiquitylation and degradation of p53, HSCO reduces p53 protein levels. HSCO specifically associates with histone deacetylase 1 (HDAC1) independently of p53, HSCO reduces p53 protein levels. HSCO specifically associates with histone deacetylase 1 (HDAC1) independently of Mdm2 and facilitates deacetylation of p53 at Lys-373/382 by HDAC1. The metallo-β-lactamase family consensus sequence in HSCO is important for its effect on p53 deacetylation. Co-immunoprecipitation and immunofluorescence studies suggested that HSCO, HDAC1, and p53 form a complex in the nucleus. Thus, HSCO is a cofactor that increases the deacetylase activity of HDAC1 toward p53, leading to suppression of apoptosis. Treatment of hepatocellular carcinomas that retain wild-type p53 and overexpress HSCO with anti-HSCO agents might re-establish the p53 response and revert chemoresistance.

The p53 tumor suppressor is mutated in ~50% of many different cancers (1) and is probably rendered inactive by a range of indirect mechanisms such as Mdm2 amplification and loss of p14ARF in the remaining 50% (2). In unstressed cells, p53 is normally maintained at low levels by continuous ubiquitylation catalyzed by E3 ubiquitin ligases such as Mdm2, COP1, PIRh2, TOPORS, and ARF-BP1/Mule, and subsequent degradation by the 26 S proteasome (3–5). In response to genotoxic stress, p53 is rapidly stabilized and activated. The activated p53 mainly functions as a sequence-specific DNA-binding transcription factor to activate or repress a large number of target genes, which mediate cell-cycle arrest, apoptosis, senescence, differentiation, DNA repair, and inhibition of angiogenesis and metastasis (6). The activity of p53 is largely controlled by the cellular p53 level, its DNA-binding activity, subcellular localization, and recruitment of transcriptional co-activators or co-repressors. Although the precise mechanisms of p53 activation are not fully elucidated, accumulating evidence indicates that post-translational modifications of p53, including phosphorylation of Ser and Thr residues and ubiquitylation, acetylation, and sumoylation of Lys residues, play important roles in regulating its stability and transcriptional activity (5, 7). Furthermore, these modifications are interrelated. For example, phosphorylation of Ser-15 or Ser-33/37 increases the affinity of p53 for p300 and promotes acetylation of p53 at Lys-373/382 (7, 8). Because the Lys residues acetylated in p53 overlap with those that are ubiquitylated, p53 acetylation has been considered to be important for p53 degradation as well as transcriptional activation (2, 5).

Histone deacetylase (HDAC) activity has been linked to diet, premalignant cell changes, aging, and development of diseases, including cancer. Eighteen potential HDACs have been identified in humans (9, 10), which are classified into three groups based on homology to yeast proteins. The enzymatic activities of HDACs in Class I (Rpd3-like) such as HDAC-1, -2, and -3, and Class II (Hda1-like) are zinc-dependent and sensitive to the inhibitor trichostatin A (TSA). Class I HDACs are ubiquitously expressed small nuclear proteins, whereas Class II HDACs are larger proteins that shuttle between the cytoplasm and the nucleus. The NAD-dependent enzymatic activities of Class III (Sirt2-like) HDACs are inhibited by nicotinamide but not by TSA (11). Histone acetylation can be reversed by HDACs. HDAC recruitment to promoter regions by p53 through interaction with mSin3A, which directly binds to HDACs, down-regulates gene expression by core histone deacetylation (6). Thus, HDACs act as p53 co-repressors. HDACs can deacetylate non-histone proteins as well (10). HDAC1 interacts with p53 possibly through mSin3A or PIP/MTA2, deacetylates p53 in vitro and in vivo, and down-regulates p53 transcriptional activity (12–14). Deacetylation of p53 is required for its effective degradation mediated by the ubiquitin ligase Mdm2, and Mdm2 can promote p53 deacetylation by recruiting a complex containing HDAC1 (15). In addition to HDAC1, a Class III HDAC, SIRT1, interacts with p53 and deacetylates it at Lys-382 (2).

Hepatocellular carcinoma (HCC) is currently the fifth most common solid tumor worldwide and the fourth leading cause of

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3 The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; HSCO, hepatoma subtracted-cDNA library clone one; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; TSA, trichostatin A; HEC, human embryonic kidney; DKO-MEF, doubly knocked-out mouse embryonic fibroblast; shRNA, short hairpin RNA; HA, hemagglutinin; CMV, cytomegalovirus; GFP, green fluorescent protein; EGFP, enhanced GFP; GST, glutathione S-transferase; TRITC, tetramethylrhodamine isothiocyanate; ActD, Actinomycin D; WB, Western blot; IP, immunoprecipitation.

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cancer-related death (16). Although screening of high risk populations by ultrasonography and measurement of the serum α-fetoprotein level has facilitated the early detection of HCC, a majority of patients present with advanced disease. Even for those patients who undergo surgical resection, the recurrence rates are as high as 50% at 2 years, and nonsurgical treatments are ineffective or minimally effective at best (16, 17). It is therefore important to identify molecules that can be used to develop novel diagnostic, preventive, or therapeutic strategies.

By constructing subtracted cDNA libraries, we have previously identified 19 genes overexpressed in HCCs, including two novel genes (18, 19). One of these genes was named HSCO (hepatoma subtracted-cDNA library clone one) (20). HSCO mRNA was overexpressed in 20 of 30 HCCs analyzed. Overexpression of HSCO inhibits caspase 9 activation and apoptosis induced by DNA-damaging agents such as adriamycin and etoposide, whereas it augments apoptosis induced by tumor necrosis factor-α. HSCO is a nuclear-cytoplasmic shuttling protein that binds to RelA and sequesters it in the cytoplasm by accelerating its export from the nucleus, resulting in inhibition of NF-κB activity. This activity of HSCO underlies the abrogation of p53-induced apoptosis in Saos-2 cells. In addition to our discoveries, Oue et al. (21) used serial analysis of gene expression and found that HSCO (also called YF13H12) was overexpressed in 52% of 46 gastric carcinomas. Tiranti et al. (22), via positional cloning, identified HSCO as the gene responsible for ethylmalonic encephalopathy. Ethylmalonic encephalopathy is characterized by neurodevelopmental delay and regression, prominent pyramidal and extrapyramidal signs, recurrent peticiae, orthostatic acrocyanosis, and chronic diarrhea, leading to death in the first decade of life. They proposed the name of the gene be changed from HSCO to ETHE1 and suggested that its product is a mitochondrial protein. Here, we show that HSCO can control the transcriptional activity of p53 by enhancing its deacetylation in association with HDAC1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Adenovirus-transformed human embryonic kidney (HEK) 293 cells (p53 wild type), SV40 large T antigen-expressing 293T cells (p53 wild type), human osteosarcoma U-2 OS cells (p53 wild type), human non-small cell lung carcinoma A549 cells (p53 wild type), and p53−/−/mdm2−/− doubly knocked-out mouse embryonic fibroblasts (DKO-MEFs, kindly provided by Dr. D. P. Lane) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Human lung adenocarcinoma H1299 cells (p53-null) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Mouse NIH/3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. All cells were grown at 37 °C in a humidified atmosphere of 5% CO2 in air. DNA transfection was performed by using the calcium phosphate method or FuGENE6 reagent (Roche Applied Science).

Induction of Apoptosis and Assays for Caspase Activity—Exponentially proliferating cells (2 × 104) were plated into a 35-mm plate and treated with actinomycin D (7.5 ng/ml), Adriamycin (1 μg/ml), etoposide (50 μg/ml), or tumor necrosis factor-α and cycloheximide (50 ng/ml and 100 μg/ml, respectively), or transfected with p53 cDNA in pcDNA3.1(+) -Neo or vector alone. Cell numbers were determined in triplicates at different time points. Viability of cells was determined by staining with trypan blue, and cell numbers were counted under a microscope. Caspase 3 activity and caspase 9 activity were determined by using the Caspase-3 Colorimetric Assay Kit and Caspase-9 Colorimetric Assay Kit (Medical & Biological Laboratories, respectively).

Reporter Gene Assays—Luciferase reporter plasmids, p53-Luc or pAP-1-Luc, containing p53- or AP-1-binding sites, respectively (Stratagene), and pRL-TK (Promega, Madison, WI) were co-transfected with wild-type or mutant (H79N or R159H) HSCO cDNA fused to HA or FLAG tag in expression vector pCMV4–3HA or pcDNA3.1(+) -Neo as described (20). 24 or 48 h later, cells were assayed for luciferase activity or treated with actinomycin D and assayed 24 h later. Luciferase activity was measured by the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. In some experiments, plasmids expressing p53 were co-transfected as well. pFC-MEK1 plasmid (Stratagene) served as a positive control for the AP-1 reporter assay.

Analyses of Gene Expression and Protein-Protein Interactions—RNA extraction, Northern blot analysis, immunoprecipitation, and Western blot analysis were performed as described (20). For immunoprecipitation, anti-p53 antibody (DO-1 and FL-393, Santa Cruz Biotechnology), anti-HA antibody (12CA5, Roche Applied Science), anti-FLAG antibody (M2, Sigma), anti-GFP antibody (Nacalai), agarose-immobilized anti-p53 antibody (DO-1, Santa Cruz Biotechnology), and agarose-immobilized anti-FLAG antibody (M2, Sigma) were used. About 5–10% (10–25 μg of protein) of total cellular lysates used for immunoprecipitation was also analyzed by Western blotting. Protein bands were visualized by using the Enhanced Chemiluminescence kit (Amersham Biosciences). Western blot analysis was performed using the antibody against p53 (DO-1, Santa Cruz Biotechnology, DO-7, BD Pharmingen), HA, FLAG, β-actin (C4, Chemicon), ubiquitin (FL-76, Santa Cruz Biotechnology), Mdm2 (SMP14, Santa Cruz Biotechnology), HDAC1 (Santa Cruz Biotechnology), HDAC2 (Santa Cruz Biotechnology), HDAC3 (Santa Cruz Biotechnology), SIRT1 (Santa Cruz Biotechnology), acetylated Lys (Cell Signaling Technology), acetylated histone H4 (Upstate Biotechnology), acetylated p53 (Lys-320, Upstate), and acetylated p53 (Lys-373/382, Upstate). In some experiments, biotinylated anti-GFP antibody (B-2, Santa Cruz Biotechnology) and anti-FLAG antibody (BioM2, Sigma) were used. Histones were isolated from cells by HCl extraction and acetone precipitation (23).

For GST pulldown assays, full-length p53, HSCO, RelA, and HDAC1 cDNAs were cloned into the expression vector pGEX-4T or pGEX-6P-1 (Amersham Biosciences) and expressed as proteins fused to GST in Escherichia coli (DH5α or BL21 strain). The fusion proteins and GST were immobilized on glutathione-Sepharose and incubated with recombinant HSCO or HDAC1 protein from which GST had been removed after cleavage with PreScission protease (Amersham Biosciences), or immunoprecipitates prepared from cell lysates. After incubation at 4 °C for 60 min, bound proteins were analyzed by SDS-PAGE and Western blotting.
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**Electrophoretic Mobility Shift Assays—**A 27-bp double-stranded oligonucleotide probe selected from the human gadd45α promoter (5′-TACAGAAACTCATTGACAT-GCTGGG-3′) was labeled with [γ-32P]ATP and purified with MicroSpin TM G25 columns (Amersham Biosciences). As a control, Sp1 binding-site probe (5′-GGATAGGGCGGGCG-GAGG-3′) was used. Nuclear extracts (10 µg) from HEK293 cells were incubated with 5 ng of labeled probe in a 20-µl reaction buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 5% glycerol) containing 10 µg of bovine serum albumin and 1 µg of poly(dl-dC):poly(dl-dC) for 20 min at room temperature. For competition tests, 50-fold excess (250 ng) of unlabeled wild-type or mutant (5′-TACA-GAATCGCTCTAAGCATGCTGGGG-3′) gadd45α probes were added to each reaction. The reaction mixture was electrophoresed in 4% polyacrylamide gel. The gels were dried and exposed to film at −80 °C with an intensifying screen.

**Analysis of p53 Stability and Ubiquitylation in Vivo—**For in vivo p53 degradation assays, U-2 OS cells stably expressing HSCO-FLAG or FLAG alone (three clones each) and H1299 cells co-transfected with plasmids expressing p53 alone or in combination with HSCO-FLAG were used and analyzed as described (24). To analyze the effects of HSCO down-regulation, U-2 OS cells transfected with HSCO-specific short hairpin RNA (shRNA) were used.

For in vivo ubiquitylation assays, H1299 cells or DKO-MEFs in 60-mm dishes were co-transfected with plasmids expressing p53 (1.0 µg), HSCO-FLAG (1.0 µg), HA-Mdm2 (1.0 µg), and His-tagged ubiquitin in various combinations. Prior to collection after 48 h, cells were treated with proteasome inhibitors, MG115 (10 µM) and MG132 (10 µM), for 6 h. Cells were then lysed in buffer (6.0 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4, 10 mM Tris-HCl, pH 8.0, 5 mM imidazole, and 10 mM 2-mercaptoethanol), and sonicated. The lysates were incubated with nickel-nitrilotriacetic acid-agarose beads. After extensive washing, bound proteins were eluted and analyzed by Western blotting.

In Vivo p53 Acetylation and Deacetylation Assays—For in vivo p53 acetylation assays, 293T cells were transfected with plasmids expressing wild-type or mutant (H79N or R159H) HSCO, Mdm2, and p300. The cells were cultured in the presence or absence of 5 mM TSA and lysed in buffer (20 mM Tris-HCl, pH 7.6, 170 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol) supplemented with protease inhibitors and TSA. To detect acetylated p53, cell lysates (250–500 µg of proteins) were incubated with 1 µg of agaroase-immobilized antibody specific to human p53 (DO-1) for 4 h at 4 °C. The p53 content in each precipitated sample was equalized and subjected to Western blotting to assess the amount of acetylated p53.

For in vivo p53 deacetylation assays, p53-null H1299 cells were co-transfected with plasmids expressing p53, wild-type or mutant (H141A) HDAC1, HSCO, and p300. In some experiments, H1299 and U-2 OS cells were co-transfected with plasmids expressing p53, p300, FLAG-tagged HSCO, and HDAC1-specific shRNA. The cells were lysed and analyzed by Western blotting as described above.

**In Vitro p53 Acetylation Assays—**The cDNA for human HSCO was cloned into pGEX6P-1 (Amersham Biosciences) and expressed as GST fusion protein in E. coli strain BL21. The GST tag was cleaved using PreScission protease (Amersham Biosciences). p300 HAT domain fused to GST (1.0 µg, Upstate) was preincubated with the purified HSCO or Mdm2 for 10 min at room temperature. Substrate (1.0 µg of GST-p53) was added, and the mixture was incubated with 50 nCi of [14C]acetyl-CoA in 20 µl of reaction buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1.0 mM dithiothreitol, 0.1 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride) for another 60 min at 37 °C. Acetylation was analyzed by SDS-PAGE followed by autoradiography.

In Vitro p53 Deacetylation Assays—The expression vector for wild-type or mutant (H141A) HDAC1 tagged with FLAG was co-transfected with HSCO cDNA in expression vector pcDNA3.1(+)−Neo or vector alone onto 293T cells. The cells were lysed in low stringency buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40) in the presence of protease inhibitors. After pre-clearing with protein A beads, the extracts were immunoprecipitated with anti-FLAG antibody in the presence of protein A beads for 2 h at 4 °C. Then, the beads were washed twice with low stringency buffer, twice with low stringency buffer containing 0.5 mM NaCl, and twice with deacetylation buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10% glycerol). The immune complexes were then incubated with acetylated p53 in 20 µl of deacetylation buffer for 2 h at 37 °C and analyzed by Western blotting. Acetylated p53 was prepared via an in vitro p53 acetylation reaction (25).

**Inhibition of Endogenous Gene Expression by shRNA—**For production of small interfering RNA in the cells, we used the pSuper vector expressing shRNA as described (24). To suppress expression of endogenous HDAC1 and p53, oligonucleotides corresponding to human HDAC1 (wt1: 5′-CTCATATGCTGTGATATCC-3′, wt2: 5′-CAGGCTGACTACATGG-3′, and mutant: 5′-CAGGTGACTTATCAAAT-3′) and p53 (5′-GACTGCGACTTCTACGAC-3′), respectively, were cloned into the pSuper plasmid. Transfection of pSuper plasmids was performed using FuGENE6 Reagent (Roche Applied Science).

**Immunofluorescence Staining—**Immunofluorescence staining was performed as described (20). FLAG-tagged HSCO was detected with anti-FLAG antibody (BioM2, Sigma) and streptavidin-allophycocyanin (BD Pharmingen). HA-tagged p53 was detected with anti-HA antibody (Sigma) and TRITC-conjugated anti-rabbit IgG (DAKO). EGFP-tagged HDAC1 was detected by GFP fluorescence. They were observed using a confocal laser microscope (Olympus). In some experiments, cells stably expressing HSCO N- or C-terminally tagged with FLAG were treated with MitoTracker (Molecular Probes). After fixation, FLAG was detected with anti-FLAG antibody (M2, Sigma), fluorescein isothiocyanate-conjugated antimouse IgG (DAKO), and MitoTracker with its fluorescence using a confocal microscope.
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FIGURE 2. Effects of HSCO on transcriptional activity of p53. A, reduced transcriptional activity of p53. U-2 OS cells were co-transfected with a p53-responsive luciferase reporter, pRL-TK, and p53-expressing plasmids (lower panel). The results were normalized to Renilla luciferase activity and represent the mean ± S.D. of triplicates. Cell lysates were also analyzed by Western blotting using the indicated antibodies (lower panel). B, p53-null H1299 cells were co-transfected and analyzed as in A except that p53-expressing plasmids were co-transfected instead of ActD treatment. C, U-2 OS cells were co-transfected and analyzed as in A except that an AP-1-responsive luciferase reporter was used instead of a p53-responsive reporter. pFA-MEK was expressed as a positive control. D, mdm2+/−/p53−/− DKO-MEFs were co-transfected and analyzed as in B. E, mdm2+/−/p53−/− DKO-MEFs were co-transfected and analyzed as in C. F, reduced induction of p53-inducible genes. U-2 OS cells were transfected with plasmids expressing HSCO-FLAG or FLAG alone (Mock). After incubation with (+) or without (−) ActD, gene expression was analyzed by Northern blotting using the indicated cDNA probes.

RESULTS

HSCO Increases Resistance to p53-dependent Apoptosis—In human U-2 OS cells, HSCO confers resistance to apoptosis induced by DNA-damaging agents but not tumor necrosis factor-α (20). DNA damage induces p53-dependent apoptosis, whereas tumor necrosis factor-α triggers apoptosis p53-independently (26). As shown in Fig. 1A, expression of HSCO increased the survival of U-2 OS cells exposed to actinomycin D. The reduced activation of caspases 3 and 9 indicated that the increased survival was due to suppression of apoptosis (Fig. 1B). The anti-apoptotic effect of HSCO was also observed in actinomycin D-treated A549 cells, a human lung carcinoma cell line expressing wild-type p53, but not in actinomycin D-treated HLE cells, a human HCC cell line expressing mutant p53 (data not shown). In p53-null human H1299 cells, apoptosis and activation of caspase 9 induced by introduction of p53 were also inhibited by overexpression of HSCO (Fig. 1A and data not shown).

When HSCO-specific shRNA was expressed in U-2 OS cells, the cytotoxic effect of actinomycin D and activation of caspase 9 were enhanced (Fig. 1, C and D). The effect of HSCO-specific shRNA on caspase-9 activity was reduced by concomitant suppression of p53 expression. Expression of shRNAs specific to HSCO, its mutant, or p53 did not affect the caspase-9 activity without actinomycin D treatment (data not shown). Taken together, these results suggest that HSCO suppresses the pro-apoptotic signaling pathway mediated by p53.

HSCO Suppresses Transcriptional Activity of p53—After genotoxic stress, p53 transactivates many genes involved in apoptotic pathways (3). To analyze the effects of HSCO on the transcriptional activity of p53, we used U-2 OS cells with a luciferase p53-cis reporter plasmid. Actinomycin D induced luciferase activity, which was suppressed dose dependently by HSCO (Fig. 2A). Interestingly, the mutant (H79N) HSCO having a missense mutation within the consensus sequence conserved throughout the metallo-β-lactamase family (20) did not

FIGURE 1. p53-sensitive anti-apoptotic activity of HSCO. A, increased survival. Three clones each of U-2 OS (upper panel) and H1299 (lower panel) cells stably expressing HSCO-FLAG (black bars) or FLAG alone (white bars) were exposed to actinomycin D (upper panel) or transfected with p53-expressing plasmids (lower panel). Cell numbers were counted at the indicated times after treatment. Data represent mean ± S.D. of triplicates. B, increased survival. Three clones each of U-2 OS cells expressing HSCO-FLAG (black bars), HSCO-mut (striped bars), or FLAG alone (white bars) were exposed to actinomycin D (ActD) as indicated. Data represent mean ± S.D. of triplicates. C, effects of HSCO down-regulation on apoptosis. U-2 OS cells were transfected with pSuper-HSCOwt1 (black bars), pSuper-HSCOmut (striped bars), or pSuper vector alone (white bars), which produce shRNAs for wild-type HSCO, its mutant, or none, respectively. Cells were treated with ActD, and the cell numbers were counted at the indicated times. Data represent mean ± S.D. of triplicates. D, effects of HSCO down-regulation on caspase activity. U-2 OS cells were transfected with pSuper-p53, which produces shRNA for p53, pSuper-HSCowt1, pSuper-HSCOmut1, or pSuper-HSCOmut and treated with ActD as indicated. pSuper-HSCOmut1 also encodes shRNAs for wild-type HSCO, but the targeted region is different from pSuper-HSCowt1. Caspase 9 activity was determined as in B. Data represent mean ± S.D. of triplicates (upper panel). Cell lysates were also analyzed by Western blotting using the indicated antibodies (lower panels).

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FIGURE 3. Effects of HSCO on p53 protein levels. A, inhibition of nuclear p53 DNA-binding activity by HSCO overexpression. Equal amounts of nuclear extracts were subjected to electrophoretic mobility shift assays with radiolabeled p53-binding site oligonucleotides. U-2 OS cells and H1299 cells were transfected with plasmids expressing FLAG-tagged HSCO or p53 and treated with actinomycin D (ActD, +−) or vehicle alone (−−) as indicated. Nuclear lysates were prepared from them and analyzed by electrophoretic mobility shift assays. For competition analysis, a 50-fold excess of unlabeled wild-type (wt) or mutant (mut) p53-site oligonucleotide DNAs were added to the reaction mixture. Comparability of the nuclear extracts was verified by electrophoretic mobility shift assays with a radiolabeled Sp1 probe. B, effects of HSCO overexpression on p53 levels. H1299 cells transiently transfected with plasmids expressing p53, HSCO-FLAG, and GFP as indicated were analyzed by Western blotting (WB, upper panels) and Northern blotting (NB, lower panels) using indicated antibodies and cDNA probes, respectively. C, enhanced p53 degradation by HSCO overexpression. Three stable clones each of U-2 OS cells expressing HSCO-FLAG (○) or FLAG alone (control, □) were treated with cycloheximide, and harvested at indicated times. Lysates were analyzed by WB, and a representative result is shown (upper panels). The intensity of the bands was quantified, and the result represents the mean ± S.D. of triplicates (lower panel). D, effects of HSCO down-regulation on p53 levels. U-2 OS cells transiently transfected with plasmids expressing shRNA specific to HSCO (○) or vector alone (control, □) were analyzed as in C. F, increased ubiquitylation of p53 by HSCO overexpression. DKO-MEFs were co-transfected with plasmids expressing p53, Mdm2, HSCO-FLAG, and His-ubiquitin and cultured in the presence of MG132 as indicated. Cell lysates and those affinity-purified using indicated antibodies and Western blotting (WB, upper panels) were analyzed as indicated.

The degradation of p53 is mainly mediated by the ubiquitin-proteasome system (5). Expression of an E3 for p53 (Mdm2, COP1, or PIRh2) decreased the exogenous p53 protein level in H1299 cells, and co-expression of HSCO further decreased it (data not shown). In HeLa cells, in which p53 is predominantly degraded through the human papillomavirus 18 E6 pathway (27), overexpression of HSCO decreased the p53 protein level (data not shown). These results suggest that the activity of HSCO is independent of the kind of E3 for p53.

We next examined the effects of HSCO on ubiquitylation of p53. As shown in Fig. 3F, expression of HSCO increased the ubiquitylation of exogenous p53 by Mdm2 in DKO-MEFs. These results suggest that HSCO accelerates degradation of p53 by increasing its ubiquitylation and that the decreased p53-cis reporter activity in the presence of HSCO is due, at least partly, to this effect.

Interaction of HSCO with p53—When exogenous p53 and HSCO were co-expressed in H1299 cells, HSCO co-immunoprecipitated with p53 (Fig. 4A). Mutant HSCO (H79N) also co-immunoprecipitated with p53 under similar conditions. As suppress the induction of luciferase. In the p53-null H1299 and Saos-2 cells, expression of HSCO suppressed the p53-cis reporter activity induced by exogenous p53 (Fig. 2B, and data not shown). The inhibition was specific to the p53 transactivation, since HSCO did not affect the luciferase AP-1-cis reporter activity (Fig. 2C). Essentially similar results were obtained using mdm2−−/−/p53−−− DKO-MEFs (Figs. 2D and 2E), indicating that the observed effect is independent of Mdm2. In U-2 OS cells exposed to actinomycin D, induction of p53-inducible genes involved in apoptosis, such as Noxa, Perp, PIG3, and Bax was suppressed by HSCO (Fig. 2F).

HSCO Reduces the Amount of p53 Bound to the p53-responsive Element—To clarify the mechanisms by which HSCO decreases the transcriptional activity of p53, we analyzed the effect of HSCO on binding of p53 to the p53-responsive element in vitro. As shown in Fig. 3A, incubation of the labeled p53-binding consensus sequences with nuclear extracts from actinomycin D-treated U-2 OS cells produced a slow migrating band in the electrophoretic mobility shift assay. Co-expression of HSCO decreased the band intensity. The band was supershifted in the presence of anti-p53 antibody (data not shown). In p53-null H1299 cells, the band appeared only when exogenous p53 was expressed, and the band intensity was decreased by co-expression of HSCO (Fig. 3A). The band disappeared in the presence of excess unlabeled wild-type, but not mutant, p53-binding consensus sequences. These results indicate that the DNA-binding activity and/or the amount of nuclear p53 are decreased by overexpression of HSCO.

HSCO Accelerates Degradation of p53 by the Ubiquitin-Proteasome System—We therefore assessed the effects of HSCO on the p53 protein level. When exogenous p53 was expressed alone or in combination with HSCO in p53-null H1299 cells, the p53 protein level was lower in the presence of HSCO, whereas the p53 mRNA levels were equivalent (Fig. 3B). In U-2 OS cells stably overexpressing HSCO, the level of endogenous p53 protein was decreased, and its half-life was shortened (Fig. 3C). Reciprocally, when endogenous HSCO protein level was down-regulated, the endogenous p53 protein level was increased (Fig. 3D), and its half-life was lengthened (Fig. 3E).
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In vitro GST-pulldown assays did not show binding of recombinant HSCO and recombinant p53 (data not shown), suggesting that the interaction between p53 and HSCO is indirect or modification of HSCO and/or p53 is necessary for the binding. We, therefore, used GST-HSCO and the lysates from U-2 OS cells treated with actinomycin D as indicated. The amount of lysates was adjusted so that each input contained an equal amount of p53 protein. As shown in Fig. 4D, only a small amount of p53 was captured by the immobilized HSCO from lysates prepared from untreated cells. Treatment of cells with actinomycin D markedly increased the amount of p53 bound to HSCO. A similar effect on the p53-HSCO binding was observed when cells were treated with Adriamycin or etoposide. These results suggest that one or more molecules induced by DNA damage and/or modification of p53 are necessary for the binding of HSCO to p53.

HSCO Decreases Acetylation of p53—The stability and activity of p53 are affected by post-translational modifications (2). Phosphorylation of multiple Ser residues of p53 has been proposed to interfere with the ability of Mdm2 to negatively regulate p53. HSCO did not affect the phosphorylation of Ser residues induced by actinomycin D (Fig. 5A).

p53 is specifically acetylated at multiple Lys residues of the C-terminal regulatory domain by p300/CREB and p300/CBP-associated factor, and the acetylation levels of p53 correlate well with its activation and stabilization induced by stress (6). As shown in Fig. 5B, HSCO as well as Mdm2 reduced the p300-induced p53 acetylation in 293T cells. The reduction at Lys-373/382 was more prominent than that at Lys-320. The effect of HSCO was reversed by an HDAC inhibitor TSA, but not nicotinamide. The missense mutations within the metallo-β-lactamase family consensus sequence (H79N or R159H) abolished the effect of HSCO on p53 acetylation (Fig. 5C and data not shown). Overexpression of HSCO did not change the global histone H4 acetylation status (data not shown). When expression of HSCO was suppressed in the presence of proteasome inhibitors in U-2 OS cells, the level of acetylated p53 was increased (Fig. 5D), demonstrating that HSCO negatively regulates p53 acetylation in vivo.

We further analyzed the effect of HSCO on p53 acetylation in vivo. In contrast to Mdm2, HSCO did not affect the acetylation of p53 by p300 (Fig. 5E). HSCO did not show deacetylase activity by itself (data not shown), suggesting that HSCO might induce p53 deacetylation by recruiting a TSA-sensitive deacetylase. In vitro deacetylase assay using acetylated p53 and the immunoprecipitates prepared from cells expressing FLAG-tagged HDACs with anti-FLAG antibody demonstrated that HSCO enhances deacetylase activity of HDAC1, but not HDAC2 nor HDAC3 (Fig. 5F). No effect on p53 deacetylation was observed when HSCO was incubated with immunoprecipitates containing mutant (H141A) HDAC1 with no enzymatic activity.

To determine whether HSCO facilitates deacetylation of p53 by HDAC1 in vivo, HSCO and/or HDAC1 were co-expressed with p53 and p300 in H1299 cells. As shown in Fig. 5G, p53 acetylation was decreased by expression of HDAC1 alone. HSCO alone also decreased it, probably by enhancing the endogenous HDAC1 activity. Co-expression of HSCO and HDAC1 further decreased the p53 acetylation. Upon co-expression with mutant (H141A) HDAC1, the effect of HSCO was lost and p53 acetylation was restored, indicating that H141A HDAC1 acts as a dominant negative mutant.

When expression of HDAC1 was down-regulated in H1299 cells, the effect of HSCO on acetylation of exogenous p53 was abrogated (Fig. 5H, left). A similar effect of HDAC1 down-regulation was additionally observed on endogenous p53 (Fig. 5H, right), suggesting that the effect of HSCO on p53 acetylation is HDAC1-dependent.

Interaction of HSCO with HDAC1—When we co-transfected U-2 OS cells with plasmids expressing FLAG-tagged HSCO and EGFP-tagged HDAC1, HDAC1 co-immunoprecipitated with HSCO from the cell lysates (Fig. 6A). This interaction was specific, because HDAC2, HDAC3, and SIRT1 did not co-immunoprecipitate with HSCO under similar conditions (data not shown). The HSCO point mutant (H79N) co-immunoprecipitated with HDAC1 (Fig. 6B), although it is defective in enhancing deacetylation of p53 (Fig. 5C). The interaction of HSCO and HDAC1 was also observed in p53-null H1299 cells (Fig. 6C), suggesting that the interaction between p53 and HSCO is indirect.
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indicating that the binding was not mediated by p53. By using a series of HSCO deletion mutants in co-immunoprecipitation and Western blot analysis, full-length HSCO was found to be necessary for the binding (data not shown). As shown in Fig. 6D, endogenous HSCO was co-immunoprecipitated with endogenous HDAC1 from A549 cell lysates. When A549 cells were treated with actinomycin D, the endogenous protein levels of HSCO and p53 increased and more endogenous HSCO and p53 were co-immunoprecipitated with HDAC1.

In U-2 OS cells HSCO C-terminally tagged with FLAG was observed mainly in the nucleus, cytoplasm, or both (22.3 ± 2.6, and 67.0 ± 2.9%, respectively, of transfected cells), and some of the cytoplasmic HSCO was localized in the mitochondria (Fig. 7D). The subcellular localization of N-terminally tagged HSCO was suggested as a mitochondrial protein responsible for ethylmalonic encephalopathy (22).

Although no direct binding between recombinant HSCO and recombinant HDAC1 was observed (Fig. 6E), a GST-pulldown assay using lysates prepared from U-2 OS cells expressing FLAG-HDAC1 and treated with actinomycin D demonstrated an interaction between GST-HSCO and HDAC1 (Fig. 6F). The binding was also observed using lysates from p53-null H1299 cells expressing FLAG-HDAC1 (Fig. 6G). These results suggest that HDAC1 indirectly interacts with HSCO and/or a modification of HDAC1 is necessary for this p53-independent interaction. As shown in Fig. 6H, HSCO co-immunoprecipitated with HDAC1 from lysates prepared from DKO-MEFs, indicating that neither Mdm2 nor p53 is necessary for the binding of HSCO to HDAC1.

HSCO co-immunoprecipitated with p53 (Fig. 4) and with HDAC1 independently of p53 (Fig. 6). We, therefore, asked whether HDAC1 affects the interaction between p53 and HSCO. From U-2 OS cell lysates, HSCO was co-immunoprecipitated with p53, the amount of which was increased by co-expression of HDAC1 (Fig. 7A). The amount of p53 co-immunoprecipitated with HSCO was also increased in the presence of HDAC1. When the expression of endogenous HDAC1 was down-regulated, the binding of HSCO and p53 was decreased (Fig. 7B).

Results with confocal microscopy were consistent with a notion that HSCO, p53, and HDAC1 form a complex in the nucleus (Fig. 7C). HSCO shuttles between nucleus and cytoplasm (20). Recently, HSCO was suggested as a mitochondrial protein responsible for ethylmalonic encephalopathy (22).
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**FIGURE 6. Interaction of HSCO with HDAC1.** A, co-immunoprecipitation (IP) of exogenous proteins. U-2 OS cells were co-transfected with plasmids expressing HSCO-FLAG and EGFP-HDAC1 as indicated. Cell lysates (10% input) and immunoprecipitates prepared by IP with indicated antibodies were analyzed by Western blotting (WB). Arrows indicate mobilities of specific bands. B, co-IP of HDAC1 and mutant HSCO. U-2 OS cells were co-transfected with plasmids expressing mutant (H79N) HSCO tagged with FLAG and EGFP-HDAC1 as indicated and analyzed as in A except that biotinylated antibodies were used in WB. C, co-IP in the absence of p53. H1299 cells were co-transfected with plasmids expressing HSCO-FLAG and HA-HDAC1 as indicated and analyzed as in A. D, co-IP of endogenous proteins. A549 cells were treated with actinomycin D for 0 or 8 h. Cell lysates (10% input) and immunoprecipitates prepared with anti-HDAC1 antibody or control IgG were analyzed by WB using indicated antibodies. E, GST-pulldown assays. GST-HDAC1 fusion protein, GST-RelA, or GST was incubated with recombinant HSCO, and bound proteins and 10% inputs were analyzed by WB using anti-HDAC antibody (upper panel). GST-HSCO or GST was incubated with recombinant HDAC1, and bound proteins and 10% inputs were analyzed by WB using anti-HDAC1 antibody (lower panel). F, GST-pulldown assay using cell lysates. GST-HSCO or GST was incubated with lysates of U-2 OS cells transfected with plasmids expressing FLAG-HDAC1. Bound proteins and 10% inputs were analyzed by WB using anti-FLAG antibody. G, binding in the absence of p53. GST-HSCO or GST was incubated with lysates of H1299 cells transfected with plasmids expressing FLAG-HDAC1 and analyzed as in F. H, co-IP in the absence of p53 and Mdm2. DKO-MEFs were co-transfected with plasmids expressing HSCO-FLAG and EGFP-HDAC1 as indicated and analyzed as in A.

HSCO was co-precipitated with HDAC1 in the absence of p53, although one or more modifications and/or cofactors of HDAC1 were necessary for the binding. Recombinant p53 did not interact with HSCO in vitro, but p53 was co-precipitated with HSCO in vivo. Co-expression of HDAC1 increased, and suppression of HDAC1 expression decreased, the p53-HSCO co-precipitation. Thus, HDAC1 probably mediates the interaction between p53 and HSCO, and binding with HSCO facilitates its binding to p53. HDAC1 does not directly interact with p53, and Mdm2, the oncogenic form of PML, and PID/MTA2 have been shown to recruit HDAC1 to exert their negative control on p53 function (14, 15, 28). Recently, the nuclear co-repressor KAP1 has been shown to inhibit p53 acetylation by interacting with Mdm2 and stimulating formation of a p53-HDAC1 complex (29). In the p53-HDAC1-HSCO complex, however, Mdm2 is not involved. The molecule mediating the binding of p53 to HDAC1 remains to be determined.

HDAC1 levels are elevated in highly proliferative tissues, embryonic stem cells, and several transformed cell lines (30). Over-expression of HDAC1 increases resistance of melanoma cells to sodium butylate-induced apoptosis by suppressing acetylation of p53 and up-regulation of Bax (31). We found that HSCO enhances the activity of HDAC1 and decreases acetylation and transcriptional activity of p53. Thus, the suppressive effect of HSCO on p53-dependent apoptosis could, at least partly, be explained by its effect on HDAC1. Interestingly, by using gain-of-function p53 acetylation mutants (Gln in place of glutamine) in combination with HDAC1.

**FIGURE 7. Complex formation of HSCO with HDAC1 and p53.** A, effects of HDAC1 overexpression on co-immunoprecipitation (IP) of HSCO and p53. U-2 OS cells were co-transfected with plasmids expressing HSCO-FLAG, HA-p53, and EGFP-HDAC1 as indicated. Cell lysates (10% input) and immunoprecipitates prepared by IP with indicated antibodies were analyzed by Western blotting (WB) using indicated antibodies. B, effects of HDAC1 down-regulation. U-2 OS cells were co-transfected with plasmids expressing HDAC-FLAG, HA-p53, and shRNA for HDAC1 as indicated and analyzed as in A. C, co-localization of HSCO, HDAC1, and p53. U-2 OS cells stably expressing HSCO-C-terminally tagged with FLAG (HSCO-FLAG) were transfected with plasmids expressing HA-p53 and EGFP-HDAC1. HSCO-FLAG was detected with anti-FLAG antibody and streptavidin-allophycocyanin and appears blue under the confocal laser microscope. HA-p53 detected with anti-HA antibody, TRITC-anti-rabbit IgG, and EGFP-HDAC1 detected by GFP fluorescence appear red and green, respectively. D, mitochondrial localization of HSCO. U-2 OS cells stably expressing HSCO-FLAG were incubated with MitoTracker. HSCO-FLAG was detected with anti-FLAG antibody and fluorescein isothiocyanate-antimouse IgG and appears green under the confocal microscope. MitoTracker appears red.
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Lys) as well as DNA-damaging agents, Knights et al. (32) have shown that acetylation of Lys-373 enhances the interaction of p53 with promoters of pro-apoptotic genes, whereas acetylation of Lys-320 promotes cell survival. Consistent with their findings, HSCO mainly reduced acetylation of Lys-373/382 rather than Lys-320 and suppressed apoptosis after DNA damage. Recently, the decision to undergo apoptosis upon DNA damage has been reported to be mediated through acetylation of p53 at Lys-120 in the DNA-binding domain (33, 34). Whether HSCO affects p53 acetylation at this site is presently unknown.

Because the C-terminal Lys residues of p53 acetylated in response to DNA damage are also ubiquitylated by Mdm2, it has been suggested that p53 acetylation stabilizes p53 (5). In support of this notion, increased levels of p53 acetylation by deacetylase inhibitors inhibit p53 degradation in vivo, and the p53 degradation requires deacetylation (2, 35). However, the physiological effect of the C-terminal modifications on p53 stabilization has been questioned by in vivo knock-in experiments (7, 36, 37). Consistent with the traditional view, we found that overexpression of HSCO decreased p53 acetylation and reduced the p53 protein level by enhancing its ubiquitylation and degradation in U-2 OS cells (Fig. 3). Reciprocally, down-regulation of HSCO increased p53 acetylation and suppressed its degradation (Fig. 5). However, exogenous p53 protein levels were not affected by HSCO in DKO-MEFs, whereas p53 acetylation was decreased and transcriptional activity of p53 was suppressed (Fig. 2D).4 This finding suggests that degradation of p53 may not be the dominant mode of action by which HSCO inhibits the function of p53. Reduction of p53 acetylation is probably more important, which could down-regulate the transcriptional activity of p53 and reduce apoptosis (2).

We found that expression of HSCO enhances p53 deacetylation, especially at Lys-373/382 and suppresses p53 transcriptional activity. Gu and Roeder (38) found that p300/CBP acetylates the C terminus of p53, specifically, Lys-370/372/373/382, and that acetylated p53 has stronger ability to bind to p53-responsive elements in vitro. Several subsequent studies also supported the idea in vitro and in vivo (8, 39). However, the role of p53 acetylation in stimulating its DNA binding is controversial (40), and several studies have shown that p53 acetylation is important for the recruitment of coactivators to p53-dependent promoters (41, 42). In the present study, although in vitro electrophoretic mobility shift assay demonstrated a reduced binding of nuclear p53 to short p53-responsive elements when HSCO was overexpressed, this could be due to a reduced amount of p53. By repressing p300-mediated p53 acetylation, HSCO probably inhibits coactivator recruitment and transcriptional activation of the p53-inducible pro-apoptotic genes.

Recently, p300-mediated acetylation has been shown to promote the cytoplasmic localization of p53 (43). Apoptotic functions of p53 include a nuclear role as a transcription factor and a non-nuclear role in the cytoplasm and mitochondria (44). In response to stress, p53 moves to the mitochondria and activates pro-apoptotic proteins such as Bid, Bak, and Bax. Thus, by enhancing deacetylation of p53, HSCO could suppress nuclear-cytoplasmic trafficking of p53 and prevent its interaction with the apoptosis machinery in mitochondria. Consistent with this possibility, HSCO expression was inducible after DNA damage and p53 co-localized with HSCO and HDAC1 in the nucleus of cells overexpressing HSCO. Mutations that replace the amino acid of HSCO conserved within the metallo-β-lactamase family members abrogated its activity to enhance deacetylation and suppress transcriptional activity of p53. That the mutant HSCO retained the ability to bind to HDAC1 and form a complex with p53 suggests a possibility that an unknown enzyme-like activity of HSCO is important for the observed enhancement of HDAC1 activity. Because mutations in the HSCO gene cause ethylmalonic encephalopathy that affects the brain, gastrointestinal tract, and peripheral vessels (22), it might be worthwhile to investigate the effects of mutations on the subcellular localization and cytoplasmic function of p53 in these tissues.

Death receptor 5 is a pro-apoptotic protein considered to be a potential target for cancer therapy, and etoposide-induced death receptor 5 expression requires cooperation between p53 and RelA (45). HSCO binds to and inhibits RelA (20). HSCO also binds to HDAC1 and inhibits p53 (this study). Thus, the anti-apoptotic effect of HSCO in etoposide-treated cells expressing wild-type p53 could, at least partly, be explained by these effects. HCCs have been regarded as tumors quite resistant to chemotherapeutic agents (16). p53 mutation is not frequent in HCCs, especially in low grade or low stage tumors, compared with other types of tumors (46). Down-regulation of HSCO sensitizes human cancer cell lines with wild-type p53 to apoptosis induced by DNA-damaging agents (Ref. 20 and this study). Considering its function and relatively frequent overexpression in HCCs, we may be able to design effective chemotherapeutic interventions by inhibiting HSCO and repairing the apoptotic p53 response in HCCs.

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