Molecular Mechanisms of Transactivation and Doxorubicin-mediated Repression of survivin Gene in Cancer Cells

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Human maintenance DNA cytosine methyltransferase (DNMT1) regulates gene expression in a methylation-dependent and -independent manner. Anti-apoptotic survivin gene down-regulation is mediated by p53 recruitment of DNMT1 to its promoter. Survivin inhibits programmed cell death, regulates cell division, and is expressed in cancer cells. The survivin gene promoter is CG-rich containing several Sp1 canonical, Sp1-like, cell cycle-dependent element/cell cycle gene homology region, and p53-binding sites. Here we demonstrate that Sp1 transcription factor(s) play a role in transcriptional activation of the survivin promoter in Drosophila and human cells. Sp1 inhibition in vivo by mithramycin A leads to down-regulation of a luciferase reporter driven by the human survivin promoter in transfected cells. Mithramycin A or Sp1-specific short interfering RNA down-regulated the endogenous survivin gene expression, confirming Sp1 as the primary determinant for transcriptional activation. Furthermore, immobilized DNMT1 ligand bound to seven consensus amino acids corresponding to the N-terminal region of the Sp class of transcription factors in a phage display analysis. In the immunoprecipitation assay, the endogenous Sp1 or Sp3 pulled down DNMT1 and methyltransferase activity. Similarly, a glutathione S-transferase pull-down assay between DNMT1 and Sp1 demonstrates a direct interaction between the two proteins. Fluorescent fusions of DNMT1 and Sp1 co-localized in the mammalian nucleus, thus supporting binary complex formation between both the proteins. The kinetics of survivin promoter occupancy via chromatin immunoprecipitation following doxorubicin treatment show the presence of Sp1 and gradual accumulation of transcriptional repressors p53, DNMT1, histone methyltransferase G9a, and HDAC1 onto the promoter along with histone H3K9me2. These data suggest that the Sp1 transcription factor acts as a platform for recruitment of transcriptional repressors.

In cancer cells de-regulation of genetic control of cell death and cell survival is common. Members of the inhibitor of apoptosis gene families have emerged as unique regulators of cell death (1) and are expressed robustly in cancer cells. One such member is Survivin, expressed exclusively in fetal and cancer cells but not in normal adult cells. Survivin binds to the mitotic spindle during mitosis and is thought to be responsible for anti-apoptotic activity in cancer cells via aberrant interaction with the mitotic spindle. There is also speculation that the Survivin protein can bind caspase-3 (an enzyme required for apoptosis) and inhibit its function. Architecturally the survivin gene promoter contains several Sp1 canonical, Sp1-like, and p53-binding elements, suggesting participation of the Sp1 transcription factor and/or p53 in gene regulation. Furthermore, survivin transcription is down-regulated by the DNA-damaging agent doxorubicin, which mediates p53 induction in acute lymphoblastic leukemia (2). In the same study, binding of p53 to the survivin promoter is shown using immunoprecipitation with p53 antibodies, and overexpression of p53 led to down-regulation of Survivin. However, deletion and mutation analysis of p53-binding sites in the survivin promoter suggested that neither p53-binding site is required for survivin gene repression, although overexpression of wild type p53 led to repression of the survivin promoter in various cell types. It was also suggested that modification of chromatin in the survivin promoter might play a significant role in silencing of survivin gene transcription by p53 (3).

The tumor suppressor p53 gene product is a key transcriptional activator/repressor. Its function is to eliminate and inhibit the proliferation of abnormal cells, thereby preventing neoplastic development. The p53 signaling pathway is in standby mode in normal cells and may be activated by cellular stress such as DNA damage or strand breaks, and in turn may activate programmed cell death. When the p53 gene is mutated, cells cannot respond correctly to various signals and are predisposed to neoplastic development. Indeed, in many cancers p53 gene mutation is common. p53 is shown to be associated with several protein factors that help transcriptional activation or repression of p53-responsive genes. The mechanism of p53-mediated repression is facilitated by histone deacetylases (HDACs) in association with the core repressor protein Sin3a. The p53-Sin3a complex targets HDACs to p53-repressive promoters to create a chromatin environment nonconducive for transcription (4). Tumor suppressor p53 is also shown to interact with Sp1 (5, 6), which is also believed to be a key element in regulation of important biological processes controlled by p53 via p21 gene activation such as DNA repair, cell growth, differentiation, and apoptosis.

Sp1 was one of the first mammalian transcription factors to be cloned (7). It is a member of the family of transcription fac-
itors with a zinc finger-type DNA binding domain that binds GC-rich sequences, including GC and GT boxes (8, 9). Sp1 transcription factor is essential for mammalian development since Sp1 knock-out mice die approximately at day 11.5 of gastrulation (10). Sp1 is also required for terminal cell differentiation. This transcription factor undergoes post-translational modifications such as phosphorylation and glycosylation (11, 12), indicating secondary modifications may regulate its biological function. Earlier studies indicate that Sp1 is responsible for recruitment of TATA-binding proteins to the promoter and fixing the transcription start site of TATA-less genes (13–15). Most housekeeping genes are TATA-less and contain CpG islands (16). Often embedded within the CpG islands are the Sp1-binding sites. Binding of Sp1 transcription factors on the CpG-rich regions protects it from de novo methylation (17). Sp1 transcription factors contain regions that are crucial for transactivation, DNA binding, and oligomerization and were identified using Drosophila SL2 cell lines that are naturally deficient in the Sp class of transcription factors and their related activities. The N-terminal glutamine, serine/threonine-rich domains of Sp1 are essential for transcriptional activation (18). The C-terminal domain is shown to be involved in interaction with other transcription factors (19). Furthermore, Sp1 interacts physically and cooperates functionally with several sequence-specific activators such as NF-kB, GATA, YY1, E2F1, pRb, SREBP1, and p53 (20). Sp1 is also shown to bind human maintenance DNA (cytosine-5-)methyltransferase (DNMT1) in vivo via immunoprecipitation of HeLa cell nuclear extract (21).

Like Sp1, human DNMT1 is known to be associated with several different proteins during DNA replication and to participate in transcriptional regulation (22). DNMT1 has two domains. The N terminus and the C terminus are known as regulatory and catalytic regions, respectively. The N terminus is shown to recruit transcriptional repressors such as histone deacetylases (23), Sin3a (24), and retinoblastoma (25, 26) to specific classes of promoters for transcriptional gene silencing. For example, recruitment of HDACs alters the acetylation status of the histones on chromatin, thus making the promoter less accessible to transcription factors. Alternatively, DNMT1 may be recruited by specific transcription factors such as p53 (27) or PML-RAR (28, 29) for hypermethylation of responsive promoters. The above phenomenon of methylation-independent and -dependent gene repression by DNMT1 makes it an ideal transcriptional repressor. Furthermore, DNMT1 can also recruit de novo methyltransferases such as DNMT3A and DNMT3B for DNA methylation (30). In colorectal cancer cells, DNMT1 and DNMT3B are essential for maintenance of genomic methylation (31). Although de novo methyltransferases participate in maintenance of DNA methylation, they may recruit other transcriptional repressors such as RP58 and HDACs for methylation-independent gene repression (32). Furthermore, homozygous deletion of DNMT1 or DNMT3A and DNMT3B leads to embryonic lethality (33, 34) like that of Sp1 null mice (10) suggesting involvement of these proteins in mammalian development. In cancer cells, aberrant methylation of the tumor suppressor gene promoters is a hallmark (35, 36), and overexpression of DNMT1 has been speculated (37).

In mammalian cells gene expression is coordinated with precision. Therefore, aberrant expression of a key gene often leads to failure of cellular function, ultimately challenging the very survival of a cell. In human cells the balancing act between transcriptional activators and repressors brings harmony to transcription of a gene. Generally the proximal part of a human gene contains DNA sequences that can bind transcription factor(s) and other transcriptional co-activator(s). In our previous work in colorectal carcinoma cells (27), we have demonstrated that survivin gene regulation is mediated by both epigenetic factors in conjunction with p53. After DNA damage, the survivin promoter was found to be hypermethylated, and chromatin immunoprecipitation shows the presence of p53-, DNMT1-, and K9-methylated H3s (27). These observations gave a snapshot of the repressed promoter, but it did not address the transition between an actively expressed to a repressed survivin promoter. In this study we have worked to identify transcriptional activators/repressors of the survivin promoter and their dynamics during transcriptional activation and repression upon DNA damage.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Constructs**—All the following cell lines were obtained from the ATCC: Jurkat (human T cell lymphoblast), HEK293 (human embryonic kidney), and COS-7 (SV40 transformed fibroblast). Cells were grown as per ATCC recommendations. Cultures of Drosophila melanogaster Schneider cell line 2 (SL2) were maintained in Schneider’s Drosophila medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomyacin at 27 °C. Parental HCT116 and p53 knock-out cell lines p53+/− (HCT116 p53+/−) were grown as described previously (38). Doxorubicin was dissolved in Me2SO and treated with different concentrations of mithramycin A for an hour before transfections. All GST-DNMT, DNMT1fl-InCDB, and DNMT1Δ580-InCBd constructs have been described previously (30). The GFP2 fusion construct of DNMT1 was made by PCR representing amino acids 1–1409. DsRed-Sp1 and pPACDNMT1/p53 constructs were made by PCR cloning. SpII, Cdc25C (pcdc25C-luc), Cdc2 (pdc2-cdc2-luc), pPAC-Sp1, and -Sp3 plasmids are described elsewhere (2, 39, 40).

**Immunoprecipitation, GST Pulldown, and Western Blot Analysis**—Antibodies for p53, p21, and Survivin were from Cell Signaling Technology. Anti-DNMT1 antibody was from New England Biolabs. Antibodies for PCNA, Sp1, Sp3, and HDAC1 were from Upstate Biotechnology, Inc. Anti-GFP antibody was purchased from Roche Applied Science. Nuclear extracts were made as described previously (41). All the pulldowns were per-

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2 The abbreviations used are: GFP, green fluorescent protein; siRNA, short interfering RNA; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; PCNA, proliferating cell nuclear antigen; HDAC, histone deacetylases; RNAi, RNA interference.
formed as described previously (30). Densitometric analysis (NIH image 1.59) was done on two independent blots.

For peptide competition assay, GST fusion DNMT1 (DNMT1-(788–1109)) was incubated with competitor peptide (TLPSPLLTVT) or a nonspecific (KFPSPLRPGGNIIYS-PLKSP) peptide for 30 min. The interaction between DNMT1 and Sp1 was probed by Western blot analysis.

**DNA (Cytosine-5-)-Methyltransferase Assay—**Methyltransferase assays were carried out at 37 °C for 30–60 min in duplicate with a total volume of 25 μl of reaction mix. Reaction conditions were as described previously (42).

**Transient Transfections, Luciferase Assay, and Immunocytochemistry—**For transient transfections, COS-7 and HEK293 cells were incubated with a mixture of DNA and FuGENE 6 (Roche Applied Science) at a ratio of 1 μg/3 μl. The Cellfectin reagent (Invitrogen) was used to transfect DNA into SL2 cells. As an internal control, a constant amount of pCMVβ (Clontech), a vector containing the β-galactosidase gene, was also co-transfected. Forty eight hours post-transfection, the cells were harvested, and luciferase activity was measured and normalized with β-galactosidase activity. For immunocytochemistry, COS-7 cells were transfected with plasmid and visualized using a confocal microscope with a 63 x 1.4 oil Zeiss objective lens at 488 nm for GFP-DNMT1 and 583 nm for DsRed-Sp1 fusions using Apotome device.

**Chromatin Immunoprecipitation Assays—**HEK293 and HCT116 cells were grown on 150-mm dishes and were treated with 100 μM mithramycin A and/or 1 μM doxorubicin. After 24 h of treatment, 1% formaldehyde was added for 10 min at 37 °C to cross-link proteins to DNA. Cells were washed two times with ice-cold 1× phosphate-buffered saline, scraped, and lysed with SDS lysis buffer (Upstate Biotechnology, Inc.) in the presence of a mixture of protease inhibitors (Sigma). The lysates were sonicated to shear DNA to lengths between 200 and 1000 bp. After 10-fold dilution of the sonicated cell supernatants in ChIP dilution buffer (Upstate Biotechnology, Inc.) supplemented with protease inhibitors, immunoprecipitations were carried out overnight at 4 °C with rotation by using 4 μg of anti-Sp1, anti-DNMT1, anti-p53, anti-G9a (Upstate Biotechnology, Inc.), anti-dimethylated H3K9 (Upstate Biotechnology, Inc.), and anti-HDAC1 polyclonal antibodies for 2–4 μg of protein. Monoclonal GFP antibody (Roche Applied Science) and anti-dimethylated H3K9 (Upstate Biotechnology, Inc.), anti-dimethylated H3K9 (Upstate Biotechnology, Inc.), and anti-HDAC1 polyclonal antibodies for 2–4 μg of protein. Monoclonal GFP antibody (Roche Applied Science) was used as a control. 40 μl of protein G coupled to magnetic beads pre-adsorbed with salmon sperm DNA were added to the chromatin-antibody complexes for 1 h at 4 °C with rotation. The beads were then isolated and washed according to the manufacturer’s recommendation (Upstate Biotechnology, Inc.). The protein-DNA complexes were eluted with a buffer containing 1% SDS and 0.1 M NaHCO3. The protein DNA cross-links were reversed by incubating the eluates with NaCl (5 μl) for 6 h at 65 °C. A small portion of samples was tested first for their content in DNMT1, Sp1, or p53 by Western blot. To the rest of the samples, proteinase K (New England Biolabs) was added for 1 h at 45 °C, and the DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Immunoprecipitated DNA was analyzed for the presence of survivin gene (GenBank™ accession number U75285) promoter sequence by PCR with proximal (GACCA-CGGGCAGAGCCACGCGCG and GCGCCCTGGGCGCAACCGTCTCCACC) and distal (TCCTGGAACCTCGTGTGTTTGAG and ACCACTTTGGGGCCAGAGTG) primer sets. PCR was performed using the following amplification parameters. After a hot start, the cycling parameters were 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for a total of 30 cycles. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide and were observed under UV light.

**Phage Display Assay—**Human DNMT1 with fused intein chitin binding domain (full-length DNMT1, DNMT1fl-InCBD; N-terminal 580 deletion DNMT1, DNMT1Δ580-inCBD) was expressed in Sf9 cells using the baculovirus expression system, and these fusions were incubated with chitin magnetic beads. Enzymes bound to the beads were isolated as depicted in Fig. 3A and incubated with a Ph.D.-12 phage display peptide library (New England Biolabs). After several washes, phages specifically bound to DNMT1 were isolated by incubating phage DNMT1fl-InCBD in 50 mM dithiothreitol. This process cleaves the DNMT1 from the InCBD fusion tag resulting in isolation of DNMT1 and the bound phages, whereas the In-CBD tag remains on the magnetic matrix. The phages were amplified for another round of bio-panning. The bio-panning procedure was repeated two more times to enrich specific DNMT1-binding phages.

**Sp1 Knockdown Assay—**HCT116 cells were transfected using Lipofectamine™ 2000 reagent (Invitrogen) with two different Sp1 Validated Stealth™ RNAi duplex sequences (Invitrogen) according to the manufacturer’s recommendations. For control transfection, a Stealth™ RNAi negative control duplex was used (Invitrogen). 48 h after transfection, Western blots were performed.

**RESULTS**

**Sp1 Transcription Factor Is Crucial for Transcriptional Activation of the survivin Promoter—**Sequence analysis of the survivin promoter in both human (43) and mouse (44) genes reveals the absence of a TATA box. A CpG island lies upstream of the transcription start site with at least seven putative Sp1 transcription factor-binding sites. Deletion analysis of the survivin promoter correlated promoter activity in the presence of Sp1–binding sites in a transfection assay (44). Also prominent in the promoter region are two putative p53-binding sites (Fig. 1A). To examine the functional association between Sp1-like transcription factors and the p53 tumor suppressor, we transfected SpII, a luciferase reporter plasmid construct under human survivin promoter regulation (2), in combination with Sp1 or Sp1 and p53 into COS-7 cells, and we then measured luciferase activity. Overexpression of Sp1 in the COS-7 cells leads to ~8-fold transcriptional activation of the survivin promoter, and this transactivation was strongly repressed in the presence of p53 (Fig. 1B) suggesting that the presence or absence of p53 directly affects survivin expression. We further deleted the p53–binding sites of the SpII plasmid to create SpIIΔp53, a plasmid also lacking two Sp1–binding sites, and used it as a negative control (Fig. 1A) for luciferase activity assay in the presence of exogenous p53 and/or Sp1 in COS-7 cells. Luciferase activity was not strongly affected in the presence of exogenous p53 in the SpIIΔp53 transfection assay confirming that p53–binding sites are crucial for survivin promoter repres-
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**FIGURE 1.** The survivin gene is repressed by tumor suppressor p53 and activated by transcription factor Sp1. A schematic diagram of the proximal region of the human survivin gene. Binding motifs for different proteins are shown as follows: Sp1 (empty box), p53 (filled box), and cell cycle-dependent element and cell cycle gene homology region (shaded box). Transcription start region is shown with an arrow. Fusion constructs of the survivin promoter fused to luciferase gene (SpI) and the survivin promoter without p53-binding site (SpIIΔp53) are shown. B, luciferase reporter assay demonstrating survivin promoter activation by Sp1 and repression by p53 in COS-7 cells. Deletion of p53-binding region on the survivin promoter (SpIIΔp53) impairs p53 repressive activity. The transfected plasmids are shown below. The transfection was performed in COS-7 cells with 100 ng of p53 expression plasmid. C, both Sp1 and Sp3 transcription factors can transactivate survivin promoter, and p53 can repress either Sp1- or Sp3-mediated transactivation in SL2 cells. D, transfected Sp1 in HCT116 cells shows up-regulation of Survivin. Western blot of the transfected cell extracts are probed for Sp1, Survivin, and control PCNA using specific antibodies as shown below each blot. B and C, transfected plasmid(s) are shown below with a plus symbol. Relative luciferase activity is shown at left.

**Mithramycin A Down-regulates the Wild Type survivin Promoter via Effective Blocking of Sp1 on the Promoter**—To further investigate the role of the Sp1 class of transcription factors on survivin gene transcription, we transfected SpI plasmid into COS-7 and HEK293 cells and treated these cells with an increased concentration of mithramycin A, a drug that binds to the minor grooves of the GC-rich regions of the DNA and hence blocks Sp1 binding (45). Luciferase expression by the survivin promoter in these cells would be dependent on the availability of an endogenous Sp1 class of transcription factors. Thus blocking endogenous Sp1 binding to the target survivin promoter will reduce luciferase transactivation. Indeed, both cell lines, COS-7 and HEK293, showed mithramycin A dose-dependent reduction of luciferase expression (Fig. 2, A and B), confirming survivin gene expression indeed is dependent on endogenous Sp1 transcription factor binding to the survivin promoter.

Hoffman et al. (2) have demonstrated that the survivin promoter is repressed by stabilization of p53 tumor suppressor protein following DNA damage mediated by doxorubicin. Our previous report (27) suggests that endogenous survivin repression is dependent on both p53 and DNMT1 in conjunction with K9-methylated histone H3 after DNA damage. To dissect out the contribution of endogenous p53 on survivin promoter repression, we transfected SpII plasmid into wild type HCT116 with wild type p53 and HCT116 p53 null cells and treated them with mithramycin A in an identical way as that of COS-7 and derived cell line SL2 for our studies. These cells are devoid of the Sp1 class of transcription factors or Sp1-associated activities. Thus they represent a unique biological system to study Sp1-dependent transcriptional mechanisms. Both Sp1 and Sp3 expression constructs were transfected either in combination with SpII or SpI and p53. As in COS-7 cells (Fig. 1B), Sp1 and Sp3 transcription factors were able to transactivate the survivin promoter between 12- and 37-fold, and this transactivation was significantly reduced in the presence of p53 in SL2 cells, suggesting that p53 is capable of transcriptional repression despite the presence of Sp1 or Sp3 (Fig. 1C). To unequivocally confirm that the Sp1 class of transcription factors is indeed involved in survivin gene activation in human cells, we transfected HCT116 cells with a construct containing full-length Sp1, and we performed Western blot analysis on the total cell extract 48 h post-transfection to assess the changes in the level of Sp1 and survivin (Fig. 1D). Indeed, the Survivin level was up-regulated in a dose-dependent manner despite the presence of the endogenous Sp1 correlating with the amount of the transfected plasmid. The level of control PCNA remained similar (Fig. 1D). To confirm the role of Sp1 in transactivation, we co-transfected the Sp1 expression plasmid with two other positive controls, Cdc2 and Cdc25C, and compared the promoter activity to SpII wild type and SpIIΔp53 in SL2 cells. Cdc25C, Cdc2, and survivin (SpII) promoters were up-regulated, but SpIIΔp53 displayed ~5-fold reduced transactivation, suggesting Sp1-binding sites are crucial for transactivation (supplemental Fig. 1). These experiments clearly demonstrate the transcriptional activation of the survivin promoter is dependent on Sp1 and Sp3, and p53 is a potent survivin down-regulator in the mammalian and insect cell system.

**Discussion**

Introduction of Sp1 alone or with p53 in the SpIIΔp53 transfection assay did not affect the promoter activity, despite the presence of five more Sp1-binding sites on the deletion survivin promoter (Fig. 1B). These data suggest Sp1 can be recruited onto the survivin promoter for transactivation of the gene, and p53 can act as a negative modulator of the survivin promoter.

In the above experiments (Fig. 1B), mammalian cells such as COS-7 contain the endogenous Sp1 class of transcription factors, which may influence the gene expression pattern. Thus, to avoid influence of the endogenous Sp1 class of transcription factors on transgene activation, we chose Drosophila embryo-
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FIGURE 2. Mithramycin A represses the survivin promoter-mediated gene expression. Transfected plasmid SpII is shown below with an increased concentration of mithramycin A (50, 100, 200, and 500 nM). Relative luciferase activity is shown at left. Cell lines used for transfection assay are as follows: A, COS-7; B, HEK293; C, HCT116, with wild type p53; and D, HCT116 p53 null. Mithramycin A represses endogenous survivin expression in wild type and p53 null HCT116 cells. Western blots of the cell extracts are shown with antibodies as indicated below each panel. Note doxorubicin-mediated Survivin repression is exclusive to wild type HCT116 cells.

E, mithramycin A blocks synergistic transactivation of p21. Western blot analysis of p21, p53, and loading control PCNA is shown along with a densitometric scan of the level of p21 at the bottom panel. G, siRNA-mediated knockdown of Sp1 reduces Survivin expression in human cells. The knockdown status of the extracts is indicated at the top. Sp1 RNAi-I and Sp1 RNAi-II are two different but specific siRNAs for Sp1. The control RNAi-ve is indicated. The level of Survivin and Sp1 expression by densitometric scan is shown below. H, chromatin immunoprecipitation of survivin promoter from HCT116 cells with (+) or without (−) mithramycin A (100 nM). Antibodies used for ChIP are indicated at the top. For both G and H panels, the lane numbers are indicated at the bottom.
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HEK293. Luciferase expression was down-regulated in both cell types (Fig. 2, C and D), suggesting Sp1 is unable to transactivate the promoter possibly because of mithramycin A-mediated blocking. A control experiment with Sp1Δp53 activity in the presence or absence of mithramycin A did not yield any changes (data not shown). In order to elucidate the mechanism of mithramycin A on endogenous survivin gene expression, both HCT116 and HCT116 p53 null cells were treated either with mithramycin A or doxorubicin for p53 induction. The cell extracts were Western-blotted and quantitated for p53, Survivin, Sp1, and PCNA. PCNA was used as a loading control because its expression is independent of p53 presence in HCT116 cell line (46). Both mithramycin A and doxorubicin transactivated p53 in HCT116 cells suggesting p53 stabilization is also mediated by mithramycin A (Fig. 2E, lane 1 versus lane 3 and lane 7 versus lane 9). Endogenous Survivin level in mithramycin A-treated cells were down-regulated irrespective of p53 status in the cells indicating factors other than p53 may be involved in mithramycin A-mediated down-regulation (Fig. 2E, lane 1 versus lane 3 and lane 4 versus lane 6). It is also plausible that Sp1-mediated transactivation of the survivin promoter is compromised in the presence of mithramycin A because of its inability to bind to the promoter. Control experiments in doxorubicin-treated cells showed a robust repression of survivin in HCT116 wild type (Fig. 2E, lane 7 versus lane 9) but not in HCT116 p53 null cells (lane 10 versus lane 12), although the control PCNA level remained the same. Similarly, Sp1 levels remained similar in all the concentrations of mithramycin and doxorubicin (Fig. 2E). These results demonstrate that neither mithramycin A nor doxorubicin has any influence in Sp1 expression, although an Sp1-transactivated gene, such as survivin, shows down-regulation in the presence of the Sp1 inhibitor mithramycin A.

In order to validate the mithramycin- and doxorubicin-mediated gene response, we Western-blotted and probed both mithramycin A- and doxorubicin-treated extracts for the level of cyclin-dependent kinase inhibitor p21 gene product. Both Sp1 and p53 transcription factors synergistically transactivate p21 (47); thus mithramycin A-mediated functional disruption of Sp1 activity and p53 binding to target site DNA will result in a failed transactivation process. Indeed, p53 induction by mithramycin A treatment in the presence of Sp1 failed to transactivate p21 in the HCT116 wild type cells (Fig. 2E, lanes 2 and 3). However, doxorubicin-mediated p53 induction in the presence of endogenous Sp1 led to severalfold activation of p21 (Fig. 2E, lanes 8 and 9). HCT116 p53 null cells either with doxorubicin or mithramycin A did not show p21 induction. These results suggested that neither Sp1 nor p53 could bind to the target promoter, such as p21, in the presence of mithramycin A for synergistic transactivation. To confirm this observation, HCT116 wild type cells were treated with either doxorubicin or mithramycin A or both doxorubicin and mithramycin A, Western-blotted, and probed for the level of p21 and p53. Indeed, in the presence of both doxorubicin and mithramycin A, p21 level decreased below the basal level (Fig. 2F, lane 1 versus lane 4), and severalfold less than doxorubicin-mediated transactivation (Fig. 2F, lane 3 versus lane 4).

If Sp1 is the transactivating factor for survivin gene activation, then the knockdown of the endogenous Sp1 will lead to reduced level of survivin expression. The control siRNA had no effect on either Sp1 or Survivin level. However, two different Sp1-specific siRNAs reduced Sp1 protein expression correlating with Survivin reduction (Fig. 2G, lane 1 versus lanes 2 and 3). The loading control PCNA level remained similar between lanes.

To further investigate if mithramycin A treatment on the cells interferes in endogenous transcription factor Sp1 recruitment to the survivin promoter, we used ChIP. HCT116 colorectal carcinoma cells were treated with mithramycin A, and total cell extracts were incubated in the presence of anti-Sp1 and control anti-GFP antibodies. DNA protein complexes captured by the antibodies were PCR-amplified to detect the presence of the survivin promoter. Sp1 antibodies will be able to pick up the survivin promoter if Sp1 alone or in a complex is bound to the survivin promoter. In the event of mithramycin A blocking the Sp1-binding sites in vivo, Sp1 will either demonstrate a reduced or zero occupancy on the promoter. Data presented in Fig. 2H demonstrate that in absence of mithramycin A the survivin promoter indeed contained Sp1. After mithramycin A treatment of the cells, Sp1 was unable to bind to the survivin promoter (Fig. 2H, lane 3 versus lane 4).

DNMT1 Binds Directly to the Sp1 Class of Transcription Factors—Earlier we have shown that DNMT1 can associate with p53 and mediate repression of the p53 repressive survivin promoter, and this repression was relieved by DNMT1-specific siRNA or the demethylating drug 5-aza-2′-deoxycytidine (27). To determine what role DNMT1 and Sp1 might be playing in survivin gene regulation, a phage display library was screened to find peptides that bind specifically to DNMT1. These binding peptides facilitate identification of interacting protein partners and binding motifs via homology search. A diagram describing the assay is shown in Fig. 3B. Purified DNAs from the phages were sequenced, and the displayed peptide sequences of the phages were analyzed. Consensus peptide sequence represented a motif of 12 amino acids out of which 7 middle amino acids (PSPLALL) had a perfect match with the Sp1 class of transcription factors, namely Sp1, Sp3, and Sp4 (Fig. 3C). Another DNMT1 fusion with the intein chitin binding domain lacking the first 580 amino acids (PSPLALL) had a perfect match with the Sp1 class of transcription factors and zinc and bromo-binding region of DNMT1 (Fig. 3D).

To demonstrate that the DNMT1 and Sp1 bind to each other, several GST fusion fragments of DNMT1 covering the whole protein were expressed in Escherichia coli and used in a GST pulldown assay with baculovirus-expressed Sp1. Two DNMT1 fusion fragments representing amino acids 432–836 and 778–1109 bound strongly to full-length Sp1 (Fig. 3D). These binding data support and confirm the phage display observations demonstrating direct interaction between both DNMT1 and Sp1 involving the repressor domain (R region) of the Sp1 class of transcription factors and zinc and bromo-binding region of DNMT1 (Fig. 3E).

To confirm that PSPLALL sequence of Sp1 binds to DNMT1, an in vitro binding assay of GST-DNMT1–(788–1109) and Sp1 was performed in the presence of a competitor or nonspecific
peptide. The competitor peptide reduced the interaction between GST-DNMT1-(788–1109) and Sp1 as compared with the nonspecific peptide (data not shown).

DNMT1 and Sp1 Class of Transcription Factors Are Associated in Vivo in the Presence or Absence of p53

To determine whether DNMT1 and Sp1 class of transcription factors are associated in vivo, immunoprecipitation experiments were performed with nuclear extracts from HEK293 and Jurkat cells. Whereas both cell lines express DNMT1 well, Sp1 expression is robust in HEK293, and Sp3 expresses well in Jurkat cells. Both anti-DNMT1 and anti-Sp1 antibodies were able to pull down each other in HEK293 nuclear extracts (Fig. 4A, left panel). A similar observation was made in Jurkat cells with respect to Sp3 and DNMT1 interactions. In both the cell lines, the control anti-GFP monoclonal antibodies were unable to pull down either DNMT1 or Sp1, demonstrating these complexes are specific (Fig. 4A, right panel). Thus, DNMT1 binds strongly to either Sp1 or Sp3 in vivo. A previous report also suggests that DNMT1 binds to HDAC1 for methylation-independent gene repression (23). To determine that DNMT1, Sp1, and HDAC1 are in a complex in HEK293, nuclear extracts were immuno-

FIGURE 3. Phage display with DNMT1 ligand. A, schematic diagram of the two DNMT1 ligands used for bio-panning with a phage display library. Various domains of the DNMT1 are shown, and the intein-chitin binding domain fusion is at the C terminus of the DNMT1. B, schematic diagram of the bio-panning protocol. DNMT1fl-inCBD indicates full-length DNMT1 fused to intein chitin binding domain, and DNMT1S80-inCBD indicates 80-amino acid deleted DNMT1 fusion. ChitMgBeads indicates chitin magnetic beads, and the phages used are from the phage display kit. C, the consensus display motif (display peptide) from 15 independent bound phage particles is shown at the top. The homologous region of the transcription factors Sp1, Sp3, and Sp4 are shown below the display motif, and the consensus amino acids are shown at the bottom. D, GST pulldown assay with DNMT1 fusions and Sp1. The overlapping fusions are shown at the top with amino acids in parentheses. The antibody used for Western blot is shown below. E, schematic diagrams of the interacting regions between DNMT1, Sp1, Sp3, Sp4, and p53 are shown. R and A motifs indicate repression and activation regions, respectively. Functional regions of p53 are indicated as follows: TA, transactivation; PR, proline-rich; DBD, DNA binding domain; TD, tetramerization; NR, negative regulation. DNMT1 binding (DNMT1BD), Sp1 binding (Sp1BD), and p53 binding (p53BD) regions are shown with amino acids numbers.

FIGURE 4. In vivo interaction between DNMT1 and Sp class of transcription factors. A, endogenous co-immunoprecipitations of DNMT1 and Sp1 in HEK293 cell extract (left panel) and DNMT1 and Sp3 in Jurkat cell extract (right panel) are shown. IP, immunoprecipitation. B, repressor protein HDAC1 co-immunoprecipitates with Sp1 transcription factor in HEK293 cell extracts. Antibodies (Ab) used for immunoprecipitation are indicated at the top, and the antibodies used for Western blot analysis are shown at the bottom of A and B. C, DNMT1 activity co-immunoprecipitates with Sp1 transcriptional activator. DNMT1 activity measured in counts/min is shown at left, and the antibodies used for co-immunoprecipitation are shown at the bottom. Anti-GFP antibody was used as a control. Each reported activity was measured in duplicate assays. D, co-localization between DNMT1 and Sp1 in the mammalian cell nucleus. GFP-DNMT1 fusion is visualized as green, and DsRed-Sp1 fusion is in red. Merge pattern of DNMT1 and Sp1 is shown in yellow.
precipitated with anti-HDAC1, -DNMT1, and -Sp1 antibodies. Western blot of the immunoprecipitate revealed that DNMT1, Sp1, and HDAC1 are indeed associated with each other (Fig. 4B). We also examined if DNA methyltransferase activity is associated with such complexes. Anti-DNMT1 as well as Sp1 antibodies were able to pull down DNA methyltransferase activity demonstrating catalytically competent complex formation (Fig. 4C). To confirm DNMT1-Sp1 interaction visually, we co-transfected GFP-DNMT1 and DsRed-Sp1 fusion constructs into COS-7 cells, followed by cytochemical localization using a fluorescent microscope. GFP-DNMT1 fusion proteins were observed only in the nucleus as bright green spots, and DsRed-Sp1 fusions were observed as red nuclear spots. Superimposition of both GFP-DNMT1 and DsRed-Sp1 yielded bright yellow nuclear spots confirming DNMT1 and Sp1 association exclusively in the nucleus (Fig. 4D).

DNMT1 and p53 Synergistically Repress the survivin Promoter in Conjunction with Sp3—Because Sp3 shares the identical motif with that of Sp1 for DNMT1 interaction (Fig. 3C), and Sp1 and Sp3 can bind to the same DNA sequence, the conventional hypothesis would be that Sp3 will act in a manner similar to that of Sp1. We examined the influence of DNMT1 in the presence or absence of Sp3 and p53 for transcriptional repression of the survivin promoter. We transfected SL2 cells with SpII and various combinations of p53, Sp3fl, and DNMT1. Sp3fl like Sp1 up-regulated survivin promoter expression, and this activation was repressed up to ~70–80% in the presence of DNMT1 or p53 alone. The repression was almost to the basal level in the presence of both p53 and Sp1, suggesting synergy in gene repression (Fig. 5). A similar experiment was performed with a full-length Sp1, and similar results were obtained, confirming the above observation (data not shown).

Doxorubicin-mediated DNA Damage Recruits p53, DNMT1, G9a, and HDAC1 Repressors to Previously Sp1 Occupied survivin Promoter—In HCT116 cells the anti-apoptotic survivin gene is expressed robustly. To dissect the cooperative role of DNMT1, Sp1, and p53 in p53-mediated gene repression, cells were treated with the DNA-damaging agent doxorubicin that triggers and stabilizes cellular p53. The control and doxorubicin-treated cells were fixed with formaldehyde to cross-link protein complexes assembled on the DNA. These DNA-protein complexes were captured with anti-DNMT1, anti-p53, or anti-Sp1 antibodies and reverse cross-linked with sodium chloride. The whole reverse cross-linked protein fractions were separated on a gel to identify protein-protein interacting partners by Western blot analysis. In doxorubicin-treated cells anti-DNMT1 antibodies were able to pull down p53 and Sp1, and the other two antibodies, anti-p53 and anti-Sp1, were able to pull down DNMT1 and Sp1 or p53 and Sp1, respectively. In the control antibody (anti-GFP) pulldown experiment, none of the complexes were pulled down. These data confirm ternary transcriptional repressor complex formation between DNMT1, Sp1, and p53 in the presence of DNA-damaging agents that stabilize p53 (Fig. 6A).

In order to elucidate the sequence of recruitment of repressor proteins onto the survivin promoter, we performed chromatin immunoprecipitation assay of the survivin promoter after doxorubicin treatment using a set of proximal and a set of distal primers. The proximal primer PCR will include the Sp1- and p53-binding sites, and the distal primer is about 2.3 kbp downstream of the proximal primer site without any p53- or Sp1-binding sites (Fig. 6B). Cells were cross-linked and harvested at a fixed timed interval (0, 9, and 15 h), and the cross-linked DNA protein complexes from the cell extracts were probed with control antibody (anti-GFP), anti-DNMT1, anti-p53, anti-Sp1, anti-G9a, anti-HDAC1, and anti-H3K9me2. The captured DNAs were PCR-amplified for detection of the survivin gene. After 9 h of doxorubicin treatment, p53 and Sp1 were found on the promoter. The recruitment of p53 onto the promoter had a small effect on protein expression level (data not shown). At the 15-h time point, DNMT1, p53, Sp1, HDAC1, G9a, and H3K9me2 were found (Fig. 6C) correlating with down-regulation of Survivin (data not shown). Our results did not show displacement of Sp1 after doxorubicin treatment until 15 h post-treatment. Thus it appears that Sp1, the transcription factor alone occupied the promoter suggesting that Sp1 transcription factor is indeed involved in in vivo transactivation of the survivin gene. After 9 h of doxorubicin treatment, p53 and Sp1 were found on the promoter. The recruitment of p53 onto the promoter had a small effect on protein expression level (data not shown). At the 15-h time point, DNMT1, p53, Sp1, HDAC1, G9a, and H3K9me2 were found (Fig. 6C) correlating with down-regulation of Survivin (data not shown). Our results did not show displacement of Sp1 after doxorubicin treatment until 15 h post-treatment. Thus it appears that Sp1, the activator of the survivin promoter, remained bound with the promoter during gene activation and perhaps acted as an anchor to recruit DNMT1 and p53 to repress the survivin gene (Fig. 6D). To validate if Sp1 participates as a recruiting platform, HCT116 cells were treated with either doxorubicin or mithramycin A or a combination of doxorubicin and mithramycin A, and the factor occupancy on the survivin promoter was examined by ChIP.
survivin gene expression is essential and evolutionarily conserved in late-stage cell division especially in cytokinesis, potentially involving cleavage furrow formation. A dramatic increase in survivin expression has been demonstrated in tumor and transformed cells hypothesizing its global deregulation in cancer cells, driving overexpression of the protein at all cell cycle stages. Molecular lesions and epigenetic changes are also associated with survivin expression. These include amplification of the survivin locus 17q25 in neuroblastoma (48) and demethylation of survivin in exon 1 in clinical ovarian cancer samples (49). Another molecular abnormality in human cancer that is suspected of driving survivin expression is deregulation of Wnt/TCF signaling (50). This may have particular relevance during colorectal cancer (51) and is consistent with an embryonic like survivin expression pattern, potentially exploited during transformation (50). Furthermore, down-regulation of Survivin by Wnt-2 antibody and siRNA in human non-small cell lung cancer cells suggested that inhibition of Wnt-2-mediated signaling induces apoptosis through inactivation of Survivin (52). Several groups have independently demonstrated that survivin repression is p53-dependent (2, 3). In those studies it was not clear if the p53-binding sequence of the survivin promoter is involved in its repression in response to p53 activation and stabilization or the changes in the chromatin structure of the promoter. Furthermore, survivin expression also antagonized p53-induced apoptosis (2). Using molecular antagonists such as antisense, ribozyme, siRNA, or a dominant negative survivin mutant to interfere in survivin expression or function resulted in suppression of tumor cell growth in various cancer models alone or in combination with other anticancer strategies (53–55).

In this study we have conclusively demonstrated that it is indeed the Sp1 transcription factor that plays a vital role in transcriptional activation of the survivin gene. In an in vitro experiment, using a HeLa nuclear extract, a DNA-Sp1 complex formed that could be disrupted by excess of cold canonical Sp1 probe, but an Sp1 mutant probe did not. Furthermore, mutation of Sp1-binding sites led to reduction of promoter activity (44), suggesting Sp1 may be involved in survivin repression. Expression of an additional Sp1 transcription factor led to an increased level (1.7 times), and RNAi-mediated knockdown of Sp1 led to a 2-fold decrease of endogenous Survivin expression. Thus how did a transcriptionally active gene get repressed in response to certain stimuli such as doxorubicin treatment? An obvious hypothesis would be the release of the transcription factor(s) from the promoter followed by recruitment of repressor complex. That means the Sp1 transcription factor(s) from the promoter must be released from the survivin promoter. Indeed transcription factor Sp1 never leaves the promoter region (44), suggesting Sp1 may be involved in survivin promoter repression based on ChIP analysis. Transcription OFF DNA methylation HDAC1 green gene. In a fully repressed promoter, DNMT1 (blue), p53 (red), G9a (yellow), HDAC1 (green) along with methylated DNA and histones are present.

As expected, Sp1 was found on the promoter with or without doxorubicin treatment, and DNMT1 accumulated in response to doxorubicin treatment (supplemental Fig. 2, upper panel). Cells treated with mithramycin A, or a combination of doxorubicin and mithramycin A, lost Sp1 binding to the promoter concurrent with the loss of DNMT1 occupancy (supplemental Fig. 2, lower panel), supporting Sp1 as the primary recruitment factor. However, at 24 h Sp1 transcription factor was removed from the promoter and p53 and HDAC1 remained (data not shown). This may be due to severe apoptotic events on the cells. This cascade of events suggests a well coordinated effort between Sp1, p53, DNMT1, G9a, and HDAC1 in survivin gene repression.

**DISCUSSION**

The transcription mechanism is a local phenomenon, and every cell nucleus coordinates expression of genes for survival.
events occur leading to recruitment of DNMT1, p53, G9a onto the promoter and subsequent methylation of histone H3 and DNA, enforcing transcriptional silencing of the gene. This hypothesis is strengthened by an observation by Koutsodontis et al. (47), demonstrating a strong binding affinity between Sp1 and p53 in a GST pulldown assay. They have also observed endogenous association between Sp1 and p53 in COS-7 cells in a co-immunoprecipitation assay. The p53-binding site spans between 610 and 702 amino acids of the Sp1 transcription factor. We have also observed a similar binding between both Sp1 and p53 (data not shown). Therefore, transcription factor Sp1 can recruit DNMT1 at its extreme N-terminal region and p53 to the midregion creating a heterotrimeric repressor protein complex (Fig. 3E). Because DNMT1 and p53 can form binary complex, it is conceivable that the survivin promoter can recruit this binary complex via Sp1 or directly through binding to p53-binding sites. Recruitment of DNMT1 may facilitate another repressor complex HDAC1 onto the promoter because HDAC1 is shown to bind strongly to DNMT1 and is present on the repressed survivin promoter (Fig. 6D).

All of the above observations were documented with a time course chromatin immunoprecipitation assay using DNMT1, p53, G9a, HDAC1, H3K9me2, and Sp1 antibodies. The data obtained allowed us to analyze promoter occupancy at specific time intervals. At zero time, the survivin gene promoter contained Sp1 transcription factor that acted as a transcriptional activator. During this time Survivin protein expression remained robust. However, at 9 h post-doxorubicin treatment, p53 appeared on the promoter without the loss of Sp1. This perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene via perhaps is an example of Sp1 mediating dual function of both transcription activator and repressor, on the same gene.
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