Transcriptional Repression of the Anti-apoptotic survivin Gene by Wild Type p53

Survivin is a member of the inhibitor of apoptosis family. This apoptosis inhibitor also has an evolutionarily conserved role as a mitotic spindle checkpoint protein. Previous studies on p53-repressed genes have implicated several genes involved in the G$_d$/M transition of the cell cycle as targets of negative regulation by p53. However, few targets of p53 repression that are anti-apoptotic have been identified. This study identifies the anti-apoptotic survivin gene as a p53-repressed gene. Notably, Survivin repression by p53 is shown to be distinct from p53-dependent growth arrest. Chromatin immunoprecipitations indicate that p53 binds the survivin promoter in vivo; immunobinding studies indicate that this site overlaps with a binding site for E2F transcription factors and is subtly distinct from a canonical p53-transactivating element. The survivin-binding site contains a 3-nucleotide spacer between the two decamer “half-sites” of the p53 consensus element; deletion of this spacer is sufficient to convert the survivin site into a transactivating element. Finally, we show that overexpression of Survivin in cells sensitive to p53-dependent cell death markedly inhibits apoptosis induced by ultraviolet light. The identification of survivin as a p53 repressed gene should aid in the elucidation of the contribution of transcriptional repression to p53-dependent apoptosis.

**survivin** was first identified as a gene with a coding region complementary to the effector cell protease receptor, EPR-1. Although these genes share homology in their coding regions, they are transcribed in a reverse orientation and appear to share no regulatory or promoter regions (1). Sequence analysis of the survivin coding region revealed a conserved motif present at the amino terminus that identified it as a member of the inhibitor of apoptosis (IAP)$^1$ family. This motif, or baculovirus death effector domain, has been shown to mediate the interaction with, and inhibition of, the caspase family of proteolytic enzymes, which are the penultimate mediators of apoptosis (2, 3). Consistent with its predicted anti-apoptotic function, expression of antisense RNA for Survivin is sufficient to induce apoptosis in a number of human tumor cell lines (4–6). Elevated expression of Survivin would be predicted to promote tumorigenesis, and in fact Survivin is highly expressed in a number of tumor types, including neuroblastoma, colorectal carcinoma, and gastric carcinoma; in these tumors, Survivin overexpression is correlated with poor prognosis (7–9). Additionally, analysis of the differences in gene expression between normal and tumor cells has revealed that survivin is one of the genes most consistently overexpressed in tumor cells relative to normal tissue (10).

Survivin is expressed widely in fetal tissues, but becomes restricted during development, and appears to be negligibly expressed in the majority of adult tissues (1, 11). The expression of Survivin is also cell cycle-regulated. This gene is repressed in the G$_d$ phase of the cell cycle and is highly expressed in G$_d$/M (12). This cell cycle regulation appears to rely on the presence of two CDE elements (cell cycle-dependent elements) that are downstream of the transcriptional start site (12), although a proximal CHR element (cyclin homology region) may also play a role. During mitosis, Survivin protein binds the mitotic spindle, and there is evidence that via this interaction this protein monitors mitotic spindle integrity; it is hypothesized that Survivin controls the elimination by apoptosis of those cells with aberrantly formed mitotic spindles (5, 13). This role for Survivin is evolutionarily conserved, as it is shared among homologues in yeast (14) and Caenorhabditis elegans (15). Although deregulation of survivin gene expression appears to be a common and significant event in tumorigenesis, little is known regarding the important regulators of the expression of this gene in normal and tumor cells.

Like Survivin, the p53 tumor suppressor protein is also a critical mediator of apoptosis and tumorigenesis. p53 is a nuclear transcription factor that is latent in normal cells but becomes activated by a variety of cellular stresses such as DNA damage, hypoxia (insufficient oxygen), and the presence of activated oncogenes (for review see Ref. 16). Following induction of p53 by these stresses, p53 up-regulates a set of genes that can promote cell death and growth arrest, such as p21$^{waf1}$, bax, fas, and KILLER/DR5 (for review see Ref. 17). p53 also negatively regulates the expression of a separate set of genes; in some cases this negative regulation has been shown to be important for the induction of apoptosis (18–20). The exact nature of the binding site for p53 in repressed promoters, and how this site differs from that in transactivated promoters, has only begun to be elucidated. The mechanism of repression by p53 is also becoming more clearly elucidated; specifically, at least one mechanism whereby p53 negatively regulates gene expression involves an association between p53 and histone deacetylases (HDACs). This p53-HDAC interaction is mediated by binding of p53 to the co-repressor protein Sin3. The p53-Sin3 interaction targets HDACs to the promoters of p53-repressed genes, where HDACs serve to deacetylate histones and...
create a chromatin environment that is unfavorable for transcription (21).

In several studies the transcriptional repression activity of p53 has been implicated in p53-dependent apoptosis; notable in these studies is the finding that deletion of the proline-rich domain of p53 renders this protein competent as a transactivator but unable to induce apoptosis or to repress transcription (22–24). Additionally, tumor-derived mutant forms of p53 that are impaired for apoptosis induction are likewise unable to repress transcription, yet retain the ability to activate transcription (25). In support of the positive association between p53-mediated repression and apoptosis, we recently mapped the Sin3-binding domain of p53 to the proline-rich domain, which is critical for apoptosis induction by p53 (26). These and other studies raise the possibility that p53 may transcriptionally repress genes with anti-apoptotic activity. In a search for genes that are negatively regulated by p53, we and others identified several genes with roles in the control of the G2/M phase of the cell cycle that are repressed following induction of wild type p53. These genes include stathmin, Map4, cyclin B1, cdc2, and cdc25c (27–30). Repression of these genes following DNA damage has been shown to require wild type p53 and is hypothesized to constitute a DNA damage-induced G2/M checkpoint (31, 32). These studies prompted us to test the possibility that Survivin, which binds to the mitotic spindle and exhibits anti-apoptotic activity, might likewise be subject to negative regulation by p53. Our studies have identified survivin as a gene that is potently repressed, at both the RNA and protein levels, following p53 induction in cells with both endogenous and inducible p53. The identification of survivin as a p53-repressed gene should aid in the elucidation of the mechanism of transcriptional repression by p53 and in the estimation of the contribution of this activity to p53-dependent programmed cell death.

EXPERIMENTAL PROCEDURES

Cell Culture, p53 Induction—MCF-7 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin. The human melanoma cell line CaMc and the human colon cancer cell line HT29 were grown in Dulbecco’s Modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin. Murine Val5 cells, containing the temperature-sensitive Mad21 allele of p53, human Val138 cells containing the valine 138 to alanine mutation, and murine Val5 cells containing the temperature-sensitive cdc25c allele of p53, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, and 50 units/ml of streptomycin. Murine Val5 cells, containing the temperature-sensitive cdc25c allele of p53, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 units/ml of streptomycin. The human osteosarcoma cell lines Saos2 and U2OS and the mouse osteosarcoma cell line MC3T3-E1 were grown in Eagle’s minimal essential medium containing 10% fetal bovine serum, 50 units/ml of penicillin, and 50 units/ml of streptomycin. The human breast adenocarcinoma cell line MCF-7 and the human lung cancer cell line A549 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, and 50 units/ml of streptomycin. The human lung adenocarcinoma cell line H1299 were cultured as described. The human lung adenocarcinoma cell line H1299 were cultured as described.

For transfection experiments, cells were plated into 10-cm plates at 1 × 10^6 cells/plate and allowed to settle overnight. The next morning cells were transfected with 4 μg of firefly luciferase reporter construct and 2–4 μg of Renilla luciferase construct (pRL-CMV or pRL-tk, Promega) or SV40 β-galactosidase plasmid (pRL-CMV) using Fugene 6 (Roche Molecular Biochemicals). After 24 h, the cells were harvested and lysed, and dual luciferase assays were performed as per the protocol derived from the manufacturer (Promega) on a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Luciferase activity was normalized to total protein levels, as well as to Renilla luciferase activity or β-galactosidase activity; β-galactosidase assays were performed according to protocols derived from the manufacturer (Promega).

Kinase Assays, Flow Cytometry—Cyclin-dependent kinase assays were performed essentially as described (35), using 250 μg of total protein extract immunoprecipitated with 0.5 μg of polyclonal antiserum to Cdk2 (Santa Cruz Biotechnology), cyclin E, and cyclin A. Following immunoprecipitation and washing, 1 μg of histone H1 (Sigma) was used as a substrate in kinase reaction buffer (20 mM Tris, pH 7.4, 7.5 mM MgCl_2, 1 mM dithiothreitol) supplemented with 5 mM sodium fluoride, 1 mM sodium 32 °C orthovanadate, and 125 ng/μl eAMP-dependent protein kinase inhibitor (Sigma). 0.5 μl of [γ-32P]ATP (PerkinElmer Life Sciences, Boston, MA) was added to each reaction, and 0.5 μg of normal rabbit IgG (Sigma) was used as a separate control. Reactions were incubated at 37 °C for 20 min, reactions were boiled in Laemmli sample buffer and loaded onto 10% SDS-PAGE. Cells for flow cytometry were fixed in ethanol and stained with propidium iodide as described (19) and analyzed on a Becton Dickinson FacScan. G_1 (synthesis), and G_2/M populations were calculated using the program CellQuest.
sitions—Immunoselection of the p53-binding site in the survivin promoter was performed essentially as described (33, 36). Briefly, 100–200 μg of whole cell extract from 10.1 (p53 null) or Val5 cells grown at (wt p53) was incubated with 300,000 cpm of end-labeled probe for the survivin promoter in McKay binding buffer (10% glycerol, 5 mM EDTA, 20 mM Tris, pH 7.2, 100 mM NaCl, 0.1% Nonidet P-40) supplemented with 1.25 μg/μl poly(dI/dC) (Amersham Biosciences) and 1 μg each 421 and 1620 monoclonal antibody. Following incubation for 1 h at 4 °C, immune complexes were collected with protein A-Sepharose and washed extensively in McKay washing buffer (2% glycerol, 5 mM EDTA, 20 mM Tris, pH 7.4, 1 mM EDTA, pH 8, phenol/chloroform extracted, precipitated, and resolved on 4% non-denaturing acrylamide gels. For competition experiments, a 20 5-fold molar excess of unlabeled DNA was preincubated with extract for 10 min prior to the binding reaction.

RESULTS

Survivin Is Negatively Regulated in a p53-dependent Manner—Previous studies using DNA micro-array technology to identify candidate p53-repressed genes revealed that a number of genes with roles in G2/M progression, including several genes that encode microtubule components, are subject to negative regulation by p53 (30). We sought to test the hypothesis that, like these genes, the microtubule-associated protein Survivin might likewise be negatively regulated by p53. For these studies the human lung adenocarcinoma cell line Val138 was utilized; these cells are derived from p53-null H1299 cells and are stably transfected with a temperature-sensitive p53 (Val138, wt p53 at 32 °C). In contrast, doxorubicin treatment results in increased expression of the p53-induced gene p21waf1, whereas the RNA levels of GAPDH remain unchanged. C, Survivin down-regulation requires the presence of wt p53. Western analysis of the human osteosarcoma cell lines U2OS (wt p53) and Saos2 (p53-null) following doxorubicin treatment results in decreased Survivin protein levels only in cells with wt p53 (U2OS). A β-actin control is included to verify equal protein loading in the lanes. D, the human papillomavirus E6 protein, which targets p53 for degradation, inhibits doxorubicin-mediated down-regulation of survivin by p53. Western analysis of the human melanoma cell line CaCl (wt p53) and a CaCl/E6 line following doxorubicin treatment results in decreased Survivin protein levels only in cells with wt p53 (CaCl/E6). A β-actin control is included to verify equal protein loading in the lanes.

such decreased levels did not occur following identical treatment of Saos2 human osteosarcoma cells, which are null for p53 (Fig. 1C, lane 4). To extend these studies, we performed Western analysis of p53 and Survivin in matched human tumor cell lines that differ in p53 status, following treatment with doxorubicin. CaCl is a human melanoma cell line with wt p53; CaCl/E6 is a clonal derivative of this cell line that stably expresses the human papillomavirus E6 protein, which targets p53 for degradation (27). As shown in Fig. 1D, the CaCl/E6 line has significantly reduced p53 levels compared with parental CaCl cells, and doxorubicin treatment failed to up-regulate the p53-induced protein p21waf1. Similarly, doxorubicin treatment failed to cause down-regulation of Survivin in CaCl/E6 cells, indicating a requirement for wt p53 for this process. Flow cytometric analyses indicated that both cell lines undergo G2/M arrest following doxorubicin treatment, indicating that Survivin down-regulation was unlikely to be an indirect effect of p53-induced G1 arrest in these cells (see below).
p53-dependent Repression of survivin Occurs Independent of G1 Arrest by p53—Because the survivin gene is known to be transcriptionally repressed during the G1 phase of the cell cycle, it became important to eliminate the possibility that repression of Survivin by p53 was a side effect of the induction of G1 growth arrest by this protein. Immunoblots of MCF-7 cells treated with doxorubicin indicated that, as expected, p53 levels were induced, and Survivin levels were markedly decreased following treatment (Fig. 2A). Flow cytometric analysis of these cells indicated that they were growth-arrested in the G2/M phase of the cell cycle following doxorubicin treatment (Fig. 2B); such a failure of human tumor cell lines with wt p53 to growth arrest in G1 following doxorubicin and gamma irradiation treatment has been noted by others (37, 38). In an effort to confirm that these doxorubicin-treated cells were in fact arrested in G2/M, we performed cyclin-dependent kinase assays in MCF-7 cells before and after drug treatment. These assays indicated that the activity of the G1-specific cyclin E-associated kinase, as well as the Cdk2 kinase, were dramatically reduced in MCF-7 cells following doxorubicin treatment (Fig. 2C, lanes 3 and 9). Additionally, the kinase activity associated with cyclin A, the S phase cyclin, was also markedly reduced (Fig. 2C, lane 6), as would be expected for cells in G2/M. Because the cyclin B1 and cdc2 genes are subject to transcriptional repression by p53, the activity of these enzymes was not analyzed; however, the combined data support the premise that doxorubicin-treated MCF7 cells are arrested in G2/M.

We extended these studies in efforts to better separate the ability of p53 to down-regulate Survivin from its ability to induce G1 arrest. We (27) and others (39) have noted that treatment of cells with low doses of UV radiation, between 2 and 4 J/m², is sufficient to induce p53 protein and transcriptional activity but is insufficient to induce G1 growth arrest. Treatment of MCF-7 breast carcinoma cells with ultraviolet irradiation led to dose-dependent decreases of Survivin protein 24 h after treatment, whereas flow cytometry indicated that these cells continue to proliferate following treatment (Fig. 3A and data not shown). We also analyzed murine embryo fibroblasts from the genetically engineered p21<sup>waf1<sup>−/−</sup> knock-out mouse; these cells are markedly impaired for growth arrest in response to p53 (40). p21-null mouse embryo fibroblasts demonstrated normal p53 induction and decreased Survivin levels, following treatment with UV radiation (Fig. 3B), but there was no evidence for G1 arrest by flow cytometry (Fig. 3C). These data solidify the notion that the ability of p53 to down-regulate Survivin is distinct from its ability to induce G1 arrest or to induce p21<sup>waf1<sup>−/−</sup>.

It became of interest to test the ability of the p53 homologue p73 to down-regulate the survivin gene; although p73 is fully capable of inducing p21<sup>waf1<sup>−/−</sup> and G1 arrest, it has been reported to be incapable of repressing the p53-repressed gene cdc25c (41). For this analysis we took advantage of p53-null H1299 cell lines that have been stably transfected with temperature-sensitive versions of p53 and p73 protein (34). As indicated previously, these proteins exist in mutant (inactive) conformation at 39 °C and become wild type conformation (and activity) at 32 °C. As depicted in Fig. 4, only temperature-sensitive p53, and not p73, was capable of down-regulating Survivin at the permissive temperature (32 °C, wt p53, lane 4). In contrast, both proteins were able to transactivate p21<sup>waf1<sup>−/−</sup> to identical levels (Fig. 4A), and both were indistinguishable in their ability to growth-arrest cells (Fig. 4B). The combined data indicate that induction of growth arrest and transactivation of p21<sup>waf1<sup>−/−</sup> are not sufficient to cause down-regulation of Survivin.

The survivin Promoter Is Sufficient to Confer Negative Regulation to a Heterologous Gene by p53, Even in Stably Transfected Cells—The survivin promoter has been characterized previously (5, 12). We cloned a 1-kb fragment of this promoter, and we analyzed it for the ability to confer negative regulation to a reporter gene by p53.

**FIG. 2.** p53 induction in MCF-7 cells leads to survivin down-regulation and G2/M arrest. A, Western analysis of p53 induction in human MCF7 breast carcinoma cells, following 24 h treatment with doxorubicin which creates DNA damage and induces p53. Although p53 levels are 4-fold induced, protein levels of Survivin are 3–4-fold decreased. A, β-actin control is included to verify equal protein loading in the lanes. B, flow cytometry profiles of MCF-7 cells treated with doxorubicin (+ DOX) for 24 h. Propidium iodide staining indicates that MCF-7 cells arrest with a 4N DNA content; the percentage of cells in each cell cycle stage are indicated. The data depicted are representative of three independent experiments. C, immunoprecipitation–kinase assays of catalytically active Cdk2 (lanes 2 and 3), cyclin A (lanes 5 and 6), and cyclin E (lanes 8 and 9) using histone H1 as the substrate. As a negative control, normal rabbit IgG is used instead of polyclonal antisera for the immunoprecipitations and kinase assays (No Ab, lanes 1, 4, and 7). Notably, whereas these kinase activities are high in asynchronously growing MCF7 cells (Asyn, lanes 2, 5, and 8), all activities are low in cells treated with doxorubicin (lanes 3, 6, and 9). This finding is consistent with flow cytometry data, which indicate that doxorubicin-treated cells are arrested with a G2/M content of DNA.
Fig. 3. Down-regulation of survivin by p53 does not require induction of G1 arrest or p21waf1. A, dose-dependent decreases in survivin gene expression in MCF7 breast carcinoma cells following ultraviolet irradiation. Cells were treated with the indicated dose of radiation, harvested 24 h later, and subjected to Western analysis for p53 and Survivin. That there is less p53 in the 10 J/m² sample (lane 4) reflects the fact p53 has already peaked in level in these cells and that levels of p53 are returning to normal. A β-actin control is included to verify equal protein loading in the lanes. B, Western analysis of murine embryo fibroblasts from the p21<sup><small>−/−</small></sup> knock-out mouse reveals dose-dependent decreases in Survivin levels in these cells following ultraviolet irradiation. A β-actin control is included to verify equal protein loading in the lanes. C, flow cytometric analyses of p21<sup><small>−/−</small></sup>-null mouse embryo fibroblasts that are treated with ultraviolet radiation (4 J/m²) or untreated. The calculated numbers indicate there is no evidence for G1 arrest following p53 induction in this cell line; the data depicted are representative from two independent experiments read in duplicate.

Fig. 4. Inducible p53, but not p73β, negatively regulates survivin gene expression. A, Northern analysis of Survivin levels in H1299 cells (p53-null human lung adenocarcinoma, lanes 1 and 2), and in H1299 cells containing stably transfected alleles for temperature-sensitive p53 (ts p53 Val138 cells, lanes 3 and 4), and in p73β (lanes 5 and 6). Cells were grown at 39 °C (mutant conformation, lanes 3 and 5) or temperature-shifted to 32 °C for 24 h (wild type p53/p73 induction) as indicated. Whereas both proteins are capable of inducing the p53-response gene p21<sup><small>−/−</small></sup> to comparable levels at 32 °C (lanes 4 and 6), only wild type p53, and not p73β, was capable of down-regulating survivin expression (lane 4). Like p73β, p73α was likewise incapable of repressing survivin expression, despite being capable of inducing growth arrest in temperature-shifted cells (not shown). A GAPDH control is included to verify equal loading and integrity of RNA. The data depicted are representative of three independent experiments. B, flow cytometric analysis of temperature-shifted, propidium iodide-stained cells containing temperature-sensitive p53 (ts p53 Val138 cells) or p73β. Both proteins are capable of inducing a G1 and G2/M arrest following 24-h temperature shift to 32 °C, whereas only ts p53 can repress survivin (A). The data presented are the average from two independent experiments, and the same plates of cells were used for the Northern analyses in A.

p53 Binds in a Sequence-specific Manner to the survivin Promoter; the p53-binding Site Is Necessary for Transcriptional Repression of Survivin in Vivo—To test whether p53 could physically associate with the survivin promoter, the assay of McKay was utilized; this assay allows for the analysis of DNA-protein interactions using large fragments of DNA, up to several kilobases in length (36). In this assay, the survivin promoter was digested with restriction endonucleases, radio-labeled with [32P]dCTP using Klenow polymerase, and incubated with whole cell extract from cells that are null for p53 (10.1 murine embryo fibroblasts) or that contain wild type p53 (Val5 cells shifted to 32 °C). Following incubation of the radio-labeled survivin promoter with cell extract, samples were immunoprecipitated with p53 antisera and protein A-Sepharose. These immunoprecipitates were washed and phenol/chloroform extracted, and bound DNA fragments were resolved on non-denaturing polyacrylamide gels. McKay assays on the full-length survivin promoter (SpII, not shown), and on the smaller SpV fragment of this promoter (Fig. 6A), indicated that this

moter, but was repressed nearly as well by wt p53 (Fig. 5A). Similar results were obtained in the human Saos2 osteosarcoma cell line (Fig. 5B); this cell line is null for both p53 and the retinoblastoma protein pRB and thus is impaired for p53-dependent growth arrest. These data also support the conclusion that p53 can down-regulate Survivin independent of p53-dependent growth arrest.

Repression of Survivin by p53 was recapitulated in stably transfected cells, where the full-length survivin promoter-luciferase construct was stably introduced into cells containing temperature-sensitive p53 (Val5-ValSP II cells). In two independently derived cell lines containing temperature-sensitive p53, the stably integrated luciferase gene driven by the survivin promoter was markedly down-regulated by p53 induction (Fig. 5C). Similarly, we found that the luciferase construct containing a truncated version of the survivin promoter (SpVI) was also sufficient to confer negative regulation by p53 in stably transfected cells (data not shown). In contrast, temperature shift alone had no effect on luciferase levels in parental 10.1 mouse embryo fibroblasts (data not shown). To our knowledge, the survivin promoter is the first promoter demonstrated to confer p53-dependent repression to a heterologous gene in both transiently and stably transfected cells.
DNA fragment could be specifically immunoprecipitated with p53 antisera, only in cells containing wild type p53 (Fig. 6A, lane 3) and not in parental p53-null cells (lane 2). As an internal negative control, vector sequences were negligibly bound to p53 in this assay (Fig. 6A). The survivin promoter was capable of binding to p53 to a level roughly comparable with the Mdm2 promoter, which was labeled to identical specific activity (Fig. 5A).

To assess the significance of the p53-binding site on transcriptional repression of survivin by p53, the survivin promoter/luciferase constructs (SpV and SpVΔp53BS) were stably transfected into Val5 cells that contain a temperature-sensitive p53 (Val5 cells) in 5-fold excess with the drug selectable plasmid pGK-hygro, which confers hygromycin resistance. Hygromycin-resistant colonies were pooled and analyzed. Temperature shift to 32 °C induces wt p53 protein and down-regulation of luciferase mRNA, as depicted in this Northern analysis. Expression of GAPDH is shown as a control for RNA loading and integrity. The results shown were recapitulated in three independent experiments, in two independent sets of pooled, stably transfected cells.
p53-dependent Repression of Survivin

p53-repressed genes like co-repressor Sin3, which recruits HDACs to the promoters of”, “whereby p53 can repress gene expression is via binding to the Survivin promoter in vivo; Deletion of the p53-binding site in the survivin promoter abrogates transcriptional repression by p53 in vivo.

**Fig. 6.** p53 binds in a sequence-specific manner to a fragment of the survivin promoter. A, McKay immunobinding assays indicate that the radiolabeled survivin promoter (SpV), but not the vector internal control, is immunoprecipitated by p53 antisera in cells with wt p53 (Val5 32 °C, lane 3) but not in cells that are null for p53 (parental 10.1 cells, p53 −/−, lane 2). 20% of the radiolabeled vector/insert is loaded in the input lane. B, sequence of the survivin promoter in the minimal p53-binding region. The arrow denotes the start site of survivin transcription; just upstream of the transcriptional start site is depicted the p53-binding site (boxed) and an overlapping consensus for E2F transcription factors (underlined). Downstream elements responsible for preferential G2/M transcription, the CDE/CHR, are underlined. C, immunobinding (McKay) assays using the radiolabeled promoter constructs listed, incubated with 100 μg of whole cell extract from cells with wt p53 and monoclonal antisera specific for p53. Following binding reactions, bound protein-DNA complexes are immunoprecipitated, washed, phenol/chloroform extracted, and resolved on a non-denaturing polyacrylamide gel. The radiolabeled vector fragment serves as an internal negative control and the Mdm2 promoter serves as a positive control (lanes 7–9). The SpV construct encodes an ~500-bp fragment of the survivin promoter; the SpV–Ap53BS encodes the same fragment, with the p53-binding site deleted using the restriction endonuclease SacI. As a control for sequence-specific binding, a 25-fold molar excess of each construct was preincubated with the extract for 10 min prior to the binding reaction (+ competitor). 10% of the radiolabeled vector/insert is loaded in the input lane. The results depicted are representative of several independent experiments. D, Northern analysis of cells with temperature-sensitive p53 (Val5 cells, murine p53-null cells transfected with the valine 135 temperature-sensitive p53 gene) that are stably transfected with the SpV-survivin promoter/luciferase construct or the SpV construct in which the p53-binding site is deleted (SpVΔp53BS). Northern hybridization to a probe specific for the luciferase gene indicates that p53 is capable of down-regulating the SpV construct but not the construct in which the p53-binding site is deleted (SpVΔp53BS). The data depicted are from pooled, stably transfected clones and are representative of three independent experiments on two independently derived sets of cell lines for each construct.

p21CIP1, as p53-mediated repression of the Sp-min construct was also evident in p21−/− cells (Fig. 7B), albeit to a slightly reduced magnitude. The combined data raise the possibility that p53 may repress survivin transcription in two ways as follows: one may involve inhibition of E2F by induction of p21CIP1, and subsequent binding of hypophosphorylated pRB to E2F to create a transcriptional repressor. Additionally, however, p53 appears to interact directly with the survivin promoter and may interfere with the ability of E2F proteins from activating transcription from an overlapping E2F site.

p53 Interacts with the survivin Promoter in Vivo; Deletion of the Sin3 Binding Domain of p53 Impairs Its Ability to Repress Survivin—We have shown previously that one mechanism whereby p53 can repress gene expression is via binding to the co-repressor Sin3, which recruits HDACs to the promoters of p53-repressed genes like Map4 (21). That p53 binds to the Map4 promoter in vivo, and recruits HDACs, was demonstrated using the technique of chromatin immunoprecipitations. Chromatin immunoprecipitations were performed on the survivin promoter in cells containing wild type (Val5–SpII cells at 32 °C) and mutant (cells at 39 °C) p53. These data indicated that wt but not mutant p53 could be found complexed to the survivin promoter in vivo (Fig. 8A); this binding was comparable with that for the Map4 promoter in these cells (Ref. 21 and data not shown). Additionally, in cells with wt p53 (cells at 32 °C), but not mutant p53 (39 °C), the survivin promoter was preferentially associated with deacetylated histone H3, consistent with the action of HDACs (Fig. 8A, lanes 5 and 6). Identical reactions performed in the absence of antibody, with irrelevant antibody, or in wash buffers alone, failed to reveal a PCR product for the survivin promoter (Fig. 8A, lanes 1–3). These data were consistent in three independent experiments, and they support our data from the McKay assay, which indicate that wild type p53 can interact with a consensus p53-binding site within the survivin promoter.

We recently mapped the interaction domain between Sin3 and p53, and we have found that amino acids 61–75 of wild type p53 are necessary for interaction with Sin3 (26). Therefore, it became of interest to determine whether a deletion mutant of p53 lacking the Sin3-binding domain (Δ61–75) was impaired for repression of the survivin promoter. p53-null H1299 cells were transfected with the luciferase construct SpVI, in the presence of 10 μg of either wt p53 or the Δ61–75 mutant of p53. Significantly, whereas wt p53 was capable of
protein (20 subclones of CaCl cells with greatly overexpressed Survivin transfection of the T34E variant of Survivin yielded several Survivin is the anti-apoptotic form of this protein (43). Stable data indicate that the threonine 34-phosphorylated form of some toxicity in cells, we used for these studies a Survivin construct that was mutated to mimic constitutive phosphorylation at the threonine residue at amino acid 34. The available construct that was mutated to mimic constitutive phosphorylation (Bio-Rad) and to the activity of equal micrograms of co-transfected β-galactosidase (Promega). Transfections were performed in H1299 cells (A) and in mouse embryo fibroblasts from the p21 knock-out mouse (p21 /− /−, B). The results depicted are the average of three independent experiments; error bars mark S.D.

**DISCUSSION**

Although originally identified as an IAP (1) and shown to inhibit caspase activity in vitro (2), that Survivin is a bona fide caspase inhibitor has been the subject of considerable debate. Nonetheless, several groups (4–6) have shown that antisense down-regulation of Survivin is sufficient to induce apoptosis in human tumor cell lines. Interestingly, we found that expression of wt Survivin in human tumor cells was associated with considerable toxicity and that only by mutating this protein at amino acid 34, to mimic constitutive phosphorylation at the threonine residue at amino acid 34. The available data indicate that the threonine 34-phosphorylated form of Survivin is the anti-apoptotic form of this protein (43). Stable transfection of the T34E variant of Survivin yielded several subclones of CaCl cells with greatly overexpressed Survivin protein (20–30-fold overexpression, see Fig. 9). CaCl-Survivin clones, as well as vector-transfected control, were treated with increasing doses of ultraviolet light to induce apoptosis, and the extent of apoptosis after 24 h was monitored by immunopositivity for the caspase-cleaved p85 fragment of poly(ADP) ribose polymerase (PARP). As depicted in Fig. 9, Western analysis of CMV- and Survivin-transfected cells treated with increasing doses of ultraviolet radiation led to significant p53 induction in both cell lines (Fig. 9). However, only in CMV-transfected cells did this p53 induction lead to dose-dependent increases of p85 PARP, a marker for caspase activation and apoptosis. Similar results were obtained in another independently generated Survivin-transfected clone (CaCl-T34E-Survivin clone 1, data not shown). We also noted reduced annexin V staining in UV-treated cells that overexpress Survivin, relative to vector-transfected control (data not show). These data support the hypothesis that overexpression of Survivin can inhibit UV-induced apoptosis and that repression of Survivin by p53 would be predicted to either directly induce, or sensitize cells to, apoptosis.

**Fig. 7. Increasing concentrations of p53 reverse E2F-mediated transactivation of the survivin promoter in p53 null (H1299) and p21-null cells.** Transient transfection/luciferase assays of 4 μg of the minimal p53-repressible survivin promoter (Sp-min, contains the p53-binding site and the overlapping E2F site, in the luciferase construct pGL3-E1B-TATA) were incubated with increasing concentrations of p53 (0, 0.01, 0.1, and 0.25 μg of p53) in the presence or absence of 0.5 μg of pCMV-E2F1. Values were normalized to protein levels using the Bio-Rad Dc assay (Bio-Rad) and to the activity of equal micrograms of co-transfected β-galactosidase (Promega). Transfections were performed in H1299 cells (A) and in mouse embryo fibroblasts from the p21 knock-out mouse (p21 /− /−, B). The results depicted are the average of three independent experiments; error bars mark S.D.
In this study we have identified the survivin gene as a member of a growing class of genes with a role in the G2/M transition of the cell cycle that are also negatively regulated by p53; these genes include cdc2, cdc25c, and cyclin B1 (28, 29, 41). The promoters of these genes, like survivin, each contain a bipartite element near the start site of transcription, termed a CDE/CHR (cell cycle-dependent element/cyclin homology region). This element interacts with an as yet uncloned binding protein termed CDF-1 in the G1 phase of the cell cycle; this binding is believed to lead to transcriptional repression of these genes in G1 and enhanced expression in G2/M (44, 45). Whereas this element is clearly important for the cell cycle-regulated expression of these genes, our data indicate that the CDE/CHR element is not required for the repression of survivin promoter constructs by p53 in transient assays. Additionally, we have found that G1 arrest alone, induced by the p53-homologue p73, is not sufficient to cause repression of survivin.

Two mechanisms for the p53-mediated repression of genes like cdc2, cdc25c, and cyclin B1 have been proposed. Repression of these genes by p53 has been proposed to involve inhibition by p53 of the NF-Y transcription factor, which binds to CCAAT boxes in these promoters and normally transactivates these genes (39, 46, 47). As the survivin promoter does not contain an obvious CCAAT box-binding site for NF-Y, it is unlikely that this mechanism plays a role in p53-dependent repression of survivin. It has also been proposed that p53-dependent repression of cdc2 by p53 relies on up-regulation of p21WAF1/CIP1. This leads to hypophosphorylation of pRB family proteins and transcriptional repression via E2F family members (29). Our data indicate, however, that this may be one of two overlapping, redundant mechanisms for repression of survivin.

In this study we show that p53 represses survivin in p21-null cells, which would argue against such a mechanism; however, we have consistently noted that the repression of survivin by p53 shows decreased magnitude in cells lacking p21 or pRB. Therefore, it is possible that p53 represses survivin in part by transactivation of p21WAF1/CIP1 and subsequent conversion of E2F complexes to E2F-RB repressor complexes. Our data indicate, however, that this may be one of two overlapping, redundant mechanisms for the repression of survivin.
ment with acetylated histones; this would be consistent with the action of the p53-Sin3-HDAC complex. We propose a model whereby direct binding of p53 to the survivin promoter confers repression by p53, via the p53-Sin3-HDAC complex. Interestingly, we have found that, when placed alone upstream of a minimal promoter, the p53-binding site of the survivin promoter is not sufficient to confer repression to a heterologous gene. Rather, the overlapping E2F-site is required along with the p53-binding site. Therefore, we favor the hypothesis that the p53-Sin3-HDAC complex binds to the survivin promoter and modifies the chromatin conformation such that E2F-binding and/or transactivation is impaired or abrogated. Such a mechanism of repression by p53 has been seen for other promoters, such as the a-fetoprotein promoter, on which p53 binds and competes for binding with the HNF-3 transcription factor.

It is formally possible that p53 and E2F proteins occupy the survivin promoter at the same time and together create a transcriptional repressor. Indeed, p53 and E2F-1 have been reported to interact, and association with p53 has been shown to reduce the ability of E2F to transactivate from this site; interestingly, increasing the space to 10 nucleotides restores transactivation (40). We propose that increasing the space changes the orientation of the p53 dimers to each other and that transactivation can occur only if the dimers are on the same face of the DNA helix. Otherwise, this site functions passively as a p53-binding site and is inactive for transactivation; instead p53 bound to this site may interfere with the binding or activity of other transcription factors, such as E2F-1.

The DNA-binding domain of p53 is able to accommodate such spacing changes in the DNA-binding site. We propose a model where direct binding of p53 to the survivin promoter is supported by the fact that a flexible linker region connects the DNA-binding domain to the oligomerization domain (51). Similar subtle changes in the p53 consensus element have been shown to influence the p53 transcriptional response (52, 53). Interestingly, two p53-binding sites, both with 3-nucleotide spacers, are present in the promoter of the p202 gene, which has recently been identified as a p53-repressed gene (54). The contribution of this type of binding site to the repression of the p202, and other p53-repressed, promoters remains the subject of future study.

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