

## Inhibiting S100B Restores p53 Levels in Primary Malignant Melanoma Cancer Cells\*

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**S100 calcium-binding proteins such as S100B are elevated in primary malignant melanoma and are used as markers for this and numerous other cancers. Wild-type p53 protein levels are relatively low in these cancer cells (i.e. when compared with cells without S100B) but are elevated when RNA antisense to S100B is introduced. This result implicates S100B in the down-regulation of p53 and is consistent with the large decreases in p53 protein levels observed previously in transient cotransfections of p53 and S100B (Lin, J., Blake, M., Tang, C., Zimmer, D., Rustandi, R. R., Weber, D. J., and Carrier, F. (2001) *J. Biol. Chem.* 276, 35037–35041). Down-regulation of p53 in primary malignant melanoma cells is likely the result of a direct interaction with S100B, which was observed by co-immunoprecipitation experiments. Furthermore, p53 binds regions of the S100B promoter, one of which matches the 20-nucleotide p53-binding consensus DNA sequence perfectly. Therefore, when p53 levels increase, it contributes to its own demise by up-regulating the transcription of S100B as part of a negative feedback loop. This is analogous to what is found for another protein that down-regulates p53, namely hdm2 (human double mutant 2).**

The tumor suppressor p53 is a transcription activator that signals for cell cycle arrest and apoptosis and plays a pivotal role in the maintenance and regulation of normal cellular functions (1, 2). Its inactivation affects cell cycle checkpoints, apoptosis, gene amplification, centrosome duplication, and ploidy (2–9). If p53 is inactivated by mutation, as is found in >50% of human cancers, the cell cycle proceeds unregulated, and cell growth proliferates. Likewise, apoptosis pathways are not induced, and proliferating cells transform into cancerous ones (3, 10). On the other hand, if p53 levels are too high, then phenotypes associated with aging, particularly those associated with problems in skin and bone, are observed (11). For these reasons, it is important that p53 protein levels are highly regulated, which is achieved by post-

translational modifications and interactions with other proteins inside the cell (5, 12–15). The interactions of p53 with members of the S100 family of calcium-binding proteins (i.e. S100B) are particularly intriguing because they link p53 biology to calcium-mediated signal transduction pathways.

There are now >20 members of the S100 family of EF-hand Ca<sup>2+</sup>-binding proteins, and they are widely distributed in human tissue (16–18). S100 proteins were given this name because they are soluble in 100% saturated ammonium sulfate (19). One member, S100B, is a 21.5-kDa symmetric homodimer that is highly conserved (>95%) among mammals (16, 19). In a manner similar to that of calmodulin, a Ca<sup>2+</sup>-dependent conformational change is required for S100B to bind target proteins such as p53 (20, 21). Although it is not completely clear how S100 proteins affect cell growth, S100B and several other S100 proteins (i.e. S100A1, mts1) interact with the tumor suppressor protein p53, resulting in significantly reduced p53 levels, and p53-dependent transcription activation of target genes is inhibited (22–24). In primary malignant melanoma cancer cells, we report here that S100B interacts directly with p53 and that inhibiting S100B with siRNA restores the functional p53 protein. These data support the notion that higher than normal levels of S100B, as found in malignant melanoma, are likely contributing to tumor progression by excessive down-regulation of the p53 tumor suppressor protein.

### EXPERIMENTAL PROCEDURES

**Cells and Cell Treatments**—Primary human melanoma C8146A cells were obtained from Dr. Frank L. Meyskens (University of California, Irvine, CA). The cells were grown in F-10 media (Invitrogen) containing 10% fetal bovine serum. The human glioblastoma U118 MG cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's media (Invitrogen) containing 10% fetal bovine serum. All of the other melanoma cell lines were purchased from the Division of Cancer Treatment and Diagnosis, NCI-Frederick, National Institutes of Health, Frederick, MD, and grown in RPMI 1640 (Invitrogen) containing 10% fetal calf serum. The human large cell lung carcinoma H1299 cells were grown in RPMI 1640 containing 10% fetal bovine serum. The human colorectal carcinoma cell line RKO was provided by Al Fornace, Jr. (NCI, National Institutes of Health, Bethesda, MD), and the cells were grown in RPMI 1640 containing 10% fetal bovine serum. The UV source was a Philips 30-watt germicidal lamp, and the intensities of the UV lamp were determined with a UVX Radiometer (UVP Inc., Upland, CA).

**Chloramphenicol Acetyltransferase (CAT) Assays**—A fragment of the S100B promoter (nucleotides (nt)<sup>1</sup> 1–3080; GenBank™ numbering) containing seven consensus p53 binding sites (prS100B-1 to

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<sup>1</sup> The abbreviations used are: nt, nucleotide(s); CAT, chloramphenicol acetyltransferase; hdm2, human double mutant 2; siRNA, small interference RNA.

prS100B-7)<sup>2</sup> and three shorter fragments (nt 1435–3080, containing prS100B-2 to prS100B-7; nt 1679–3080, containing prS100B-3 to prS100B-7; and nt 1744–3080, containing prS100B-4 to prS100B-7) of the human S100B promoter (GenBank™ accession number M59486) were amplified by PCR from the pGBS-4437 plasmid (Linda Van Eldik, Northwestern University, Chicago, IL) containing the full-length human S100B promoter (25). The four PCR products were each inserted into the KpnI and XhoI sites of the pCAT3-basic vector (Promega, Madison, WI) and used for transient transfection. The CAT reporter gene construct containing five *GADD45* p53-binding sites (p53REX) was provided by Al Fornace Jr. (NCI, National Institutes of Health). The expression vectors used in this study for p53 and *GADD153* have been described previously (23). The plasmids were transiently transfected into H1299 cells, and the CAT assay was performed as described previously (23) with the exception that 50 µg (S100B promoter) or 0.5 µg (*GADD45* p53 binding site) of cellular extracts were used for the assay. For transcription activation of the S100B promoter by endogenous p53 without or with UV treatment, the CAT assay was performed as above with the exception that no p53 plasmid was co-transfected, and 25 µg (S100B promoter) or 2.5 µg (*GADD45* p53 binding site) of cellular extracts were used for the assay. The levels of CAT activity were evaluated by a PhosphorImager (Amersham Biosciences) using the ImageQuant software, and the percentage of relative errors was <15% for each experiment done in quadruplicate.

**Small Interference RNA (siRNA)**—The 2-nt double-stranded RNA was synthesized by Ambion (Austin, TX) with standard purification. The sequence (5'-GGAAUUAUGGCCUUUGUU-3') corresponds to the S100B C-terminal end (nt 216–234) plus a two dT 3'-overhang. This region of S100B was chosen because it is a region of sequence that is not homologous with other S100 proteins and, thus, is specific for S100B. Scramble siRNA was bought from Ambion (Silencer negative control number 1 siRNA, catalog number 4611). Different concentrations of siRNA (2 and 20 nM) were transfected into C8146A melanoma cells using Ambion siPORT lipid transfection agent according to the manufacturer's recommendation. The cells were harvested 48 h later and analyzed by Western blots (see below).

**Western Blots**—The Western blot analyses were performed, as described previously (23), on 100 µg of C8146A protein extracts or on 100 µg of U118MG protein extracts. The cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% non-ionic detergent P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, and 5 mM dithiothreitol). The proteins were run on a 12% polyacrylamide gel, transferred onto nitrocellulose, and reacted with either p53 mouse monoclonal antibody (DO-1, Oncogene Research Products, Boston, MA) at 1:1000 dilution, S100B rabbit polyclonal antibody (Research Diagnostics Inc., Flanders, NJ) at 1:1000 dilution, p21 rabbit monoclonal antibody (Zymed Laboratories Inc.) at 1:100, Bcl-2 mouse monoclonal antibody (Oncogene Research Products) at 1:100 dilution, MDM2 mouse monoclonal antibody (Oncogene Research Products) at 1:100 dilution, or actin mouse monoclonal antibody (Oncogene Research Products) at 1:5000 dilution to control for even protein loading. The blots were then reacted with their respective secondary antibodies conjugated to horseradish peroxidase and reacted with a chemiluminescence substrate

(ECL, Amersham Biosciences) as recommended by the manufacturer. Recombinant S100B protein was produced and purified to homogeneity as described previously (23, 26).

**Co-immunoprecipitation**—The C8146A primary malignant melanoma cells were harvested and washed twice with ice-cold PBS. The cell pellets were lysed for 2 h in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, and 0.5% Nonidet P-40) and centrifuged at 15,000 rpm. The supernatants (1 mg) were incubated with either p53 antibody (DO-1, cross-linked to agarose beads; Oncogene Research Products) or S100B antibody for 2 h at 4 °C. Myc mouse monoclonal antibody (cross-linked to agarose bead; Oncogene Research Products) and His rabbit polyclonal antibody (Santa Cruz Biotechnology) were used as a negative controls (*i.e.* as non-related antibodies). Protein A-agarose beads (50 µl; Oncogene Research Products) were then added to the antibody-cell extract mixture, and all reactions were mixed gently overnight at 4 °C. The beads were spun down and washed six times with lysis buffer without detergent and loaded on a 12% polyacrylamide gel for SDS-PAGE. The samples were transferred to a nitrocellulose membrane and incubated with control antibody, S100B antibody, or p53 antibody as described above. A peroxidase-conjugated secondary antibody that does not cross-react with the IgG heavy chain (Jackson ImmunoResearch, West Grove, PA) was used to detect p53 co-immunoprecipitated with the S100B antibody.

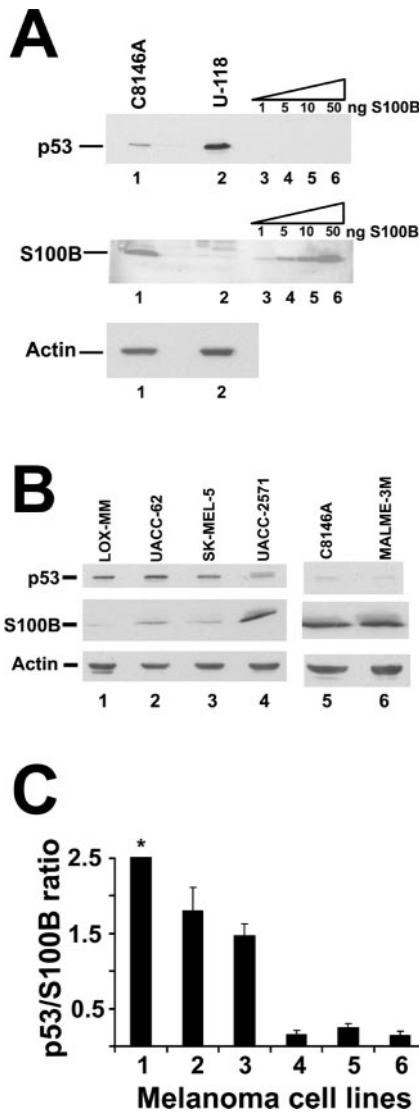
**Mobility Shift Assay**—The electrophoretic mobility shift assay was performed essentially as described previously (27) with the exception that salmon sperm DNA (1 µg) and purified recombinant p53 were used. The baculovirus-expressed p53 was obtained from Proteomics Inc. (Baltimore, MD). Baculovirus-expressed p53 was used (0.6 and 3 µg) for binding to the *GADD45* and the S100B oligonucleotides, respectively. The probes were purified by reverse phase high pressure liquid chromatography (Vydac C-4) and labeled with T4 polynucleotide kinase (New England Biolabs) as described previously (27). Oligonucleotide sequences from the S100B promoter matching 20 (termed prS100B-3), 17 (termed prS100B-1), and 16 (termed prS100B-2) nucleotides of the 20 nucleotide consensus p53 binding site (see footnote 2) were sense 5'-GCC TGG GCA AGC TCT GTG CTT CAC AGA GCA AGC CTG TGT-3', sense 5'-GTT CTG GGA CTT TCA CTA AAC TTC TCC TAC CAT-3', and sense 5'-CAG AGG GCA GGC CCG GCT GGG CCC TCC TGC TGA-3'.

## RESULTS

**Wild-type S100B and p53 Protein Levels in Primary Malignant Melanoma Cells**—Primary malignant C8146A melanoma cells have relatively high levels of S100B, but wild-type p53 levels are markedly lower in these cancer cells as compared with U118 cells with little or no S100B (Fig. 1A). This observation is consistent with what is found in transient co-transfections of S100B and p53, where levels of the tumor suppressor protein were significantly reduced by the addition of S100B (>100-fold) (23). Using increasing amounts of purified recombinant S100B protein (Fig. 1A; lanes 3–6), we estimated that the levels of S100B in the melanoma C8146A cells are at least 50 times higher than in the U118 cells (Fig. 1A). To verify whether the S100B-p53 correlation was specific to C8146A cells or could be found in other melanoma cells, we performed Western blots analysis in five other melanoma cell lines known to have a p53 wild-type genotype (28). The data shown in Fig. 1B indicate that cells having relatively low S100B levels (*i.e.* LOX-IM VI, UACC-62, and SK-MEL-5) have higher levels of p53, whereas in UACC-2571, C8146A, and Malme 3M cells the S100B levels are relatively high and the p53 levels are lower as compared with the other melanoma lines. This indicates that the S100B-p53 correlation (*i.e.* high S100B/low p53 levels) is consistent in at least six melanoma cell lines.

To determine whether S100B contributes directly to the lowering of p53 levels in the melanoma cancer cells, siRNA corresponding to the C terminus of S100B (siRNA<sup>S100B</sup>) was introduced into primary C8146A melanoma cells and compared with results from scrambled siRNA that does not interfere with S100B production (Fig. 2). The addition of the S100B antisense RNA lowered S100B levels by  $2.4 \pm 0.3$ -fold and correspondingly, increases in p53 levels ( $2.5 \pm 0.3$ -fold) were observed

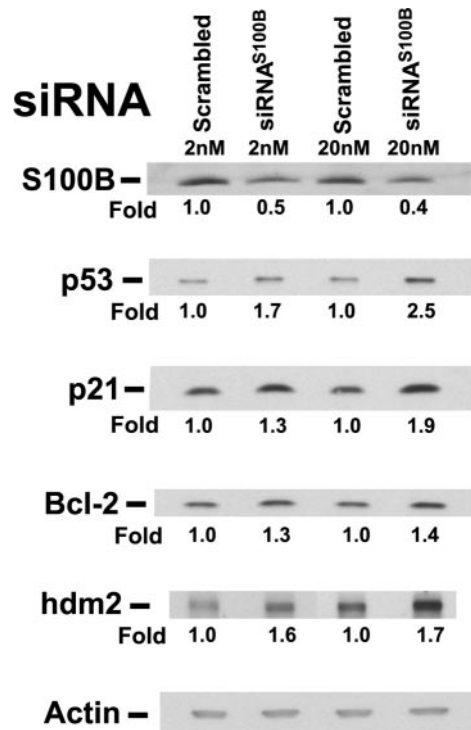
<sup>2</sup> Regions of the S100B promoter that are similar to or match the p53 binding consensus sequence (5'-RRRC(A/T)(T/A)GYYY-3' 0–13 nt 5'-RRRC(A/T)(T/A)GYYY-3') are listed below. The nucleotides 1705–1735 in the S100B promoter (termed prS100B-3; 5'-GGGCAAGCTC-3' 11 nt 5'-GAGCAAGCCT-3') match perfectly (20/20) the consensus nucleotide sequence for p53 binding. Six additional p53 binding sites occur in the S100B promoter, including the following: nucleotides 149–169 (prS100B-1; 5'-GGACTTtCaC-3' 1nt 5'-AAACTTtCC-3') and 2172–2292 (prS100B-6; 5'-GGACACATcTCT-3' 1nt 5'-AGGCATcTc-3') that match 17 nucleotides in the p53 consensus sequence (17/20); the nucleotides 1455–1478 (prS100B-2; 5'-GGGCAgGCC-3' 4nt GGGGcctCCT-3') and 2795–2820 (prS100B-7; 5'-cAGCAAGTCC-3' 6nt 5'-ctGCTTG-gTC-3') that match 16 nucleotides of the p53 consensus sequence (16/20); and the nucleotides 1995–2021 (prS100B-4; 5'-AAACTgGtTT-3' 7nt 5'-GGACgTgATg-3') and 2115–2146 (prS100B-5; 5'-AttCTTGTga-3' 12nt AAACtGGCC-3'), which match 15 sites (15/20). The p53 binding regions in the third intron of *GADD45* (19/20 match; 5'-GAACAT-GTCT-3' 0nt 5'-AAGCATGCTg-3') and the hdm2 promoter *RE1* (18/20 match; 5'-GGtCAAGTTC-3' 0nt 5'-AGACAcGTTTC-3') and *RE2* (17/20 match; 5'-AGttAAGTCC-3' 0nt 5'-tGACTTGCT-3') are also listed for comparison. Nucleotides shown in lower case do not match the consensus sequence.



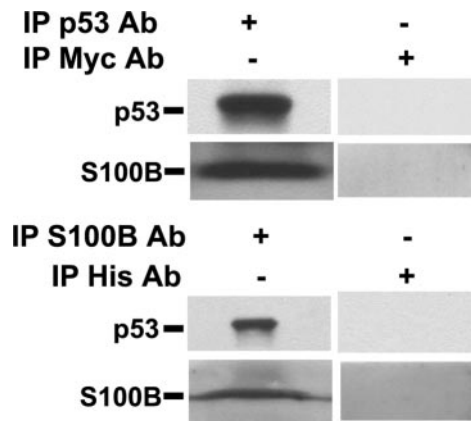
**FIG. 1. S100B and wild-type p53 levels in several cancer cell lines.** A, Western blots of p53 and S100B in human primary malignant melanoma cells (C8146A; lane 1) and control cells with little or no S100B (glioblastoma U118; lane 2). Actin levels are shown as loading controls. Shown also are blots of purified recombinant S100B used to estimate the amount of S100B in the cancer cells (lanes 3–6). B, Western blots of p53 and S100B in six melanoma cell lines having a wild-type p53 genotype (28); actin levels are shown as loading controls. C, the ratio of p53 to S100B in the six cell lines is shown corresponding to the lane numbers (1–6) from panel B. The bar labeled with the asterisk (\*) for the LOX-MM cell line is off-scale and has a p53 to S100B ratio of  $11.8 \pm 2.8$ . Together, these data illustrate that skin cancer cells with higher S100B protein levels have diminished levels of wild-type p53.

(Fig. 2; 20 nM siRNA<sup>S100B</sup>). Likewise, levels of the p21 protein, Bcl-2, and hdm2 (*i.e.* genes activated by p53) were also up-regulated by comparable amounts with the addition of the siRNA of S100B when p53 levels are elevated (Fig. 2). These results with antisense RNA indicate that S100B contributes directly to the down-regulation of functional p53 inside primary malignant melanoma cancer cells and that inhibiting S100B protein production with siRNA<sup>S100B</sup> restores active p53.

**S100B Interacts with p53 in Malignant Melanoma**—Numerous studies have characterized the high affinity Ca<sup>2+</sup>-dependent interaction between S100B and p53 *in vitro* (20, 21, 29–31). However, it is critically important to determine whether S100B and p53 interact directly inside cells. To do this, co-immunoprecipitation of the S100B-p53 complex was monitored in C8416 melanoma cells (Fig. 3). The data in Fig. 3 indicate that,



**FIG. 2. Small interfering RNA directed toward S100B (siRNA<sup>S100B</sup>) restores functional p53.** Western blot analyses of S100B, p53, p21, Bcl-2, hdm2, and actin (controls) after the transfection of melanoma with scrambled siRNA or siRNA<sup>S100B</sup>. Densitometry was used to determine the intensities of the blots, and the fold changes in protein levels were calculated by comparing blots with siRNA<sup>S100B</sup> (after normalization to actin) to corresponding blots with the same concentration of scrambled siRNA. The cells were harvested 48 h after transfection.



**FIG. 3. Interaction of S100B with p53 in human primary malignant melanoma cancer cells as detected by co-immunoprecipitation (IP) experiments.** The p53 antibody co-immunoprecipitates S100B (*top*), and a reciprocal experiment with the S100B antibody indicates that the S100B antibody co-immunoprecipitates p53 and S100B (*bottom*). The Myc mouse monoclonal antibody and the His rabbit polyclonal antibody were used as negative controls for p53 and for S100B, respectively.

in addition to the immunoprecipitation of S100B, the S100B antibody can co-immunoprecipitate p53 (lane 1). The reciprocal immunoprecipitation experiment performed with p53 antibody (lane 2) confirmed the formation of a complex between p53 and S100B in primary malignant melanoma (C8416 cells). As a control, it was shown that nonspecific antibodies of the same isotype could not immunoprecipitate p53 (lane 3) and S100B (lane 4). Therefore, it is likely that a direct interaction between the S100B and p53 proteins contributes to decreased levels of

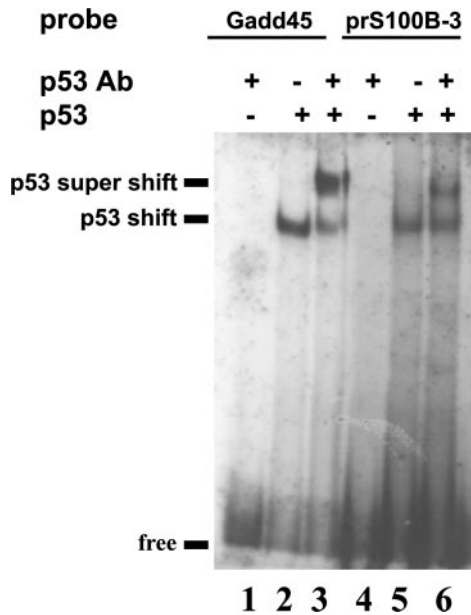


FIG. 4. Mobility shift assays of purified p53 bound to DNA derived from the third intron of *GADD45* (lanes 1–3) and a region of the S100B promoter (prS100B-3, 20/20 match) (lanes 4–6). The free probe, the p53-shifted, and the p53 antibody (p53Ab) super-shifted bands are indicated. Baculovirus-expressed p53 was used (0.6 and 3  $\mu$ g) for binding to the *GADD45* and the S100B oligonucleotides, respectively.

the tumor suppressor, p53, in melanoma with elevated S100B.

**Regions of the S100B Promoter Bind p53**—When p53 protein levels rise, the transcription of a number of genes involved in the cell cycle and apoptosis are activated. As part of a feedback loop, p53 also up-regulates the transcription of hdm2 (mdm2 in mice), a protein involved in the ubiquitin-dependent degradation of p53 itself (7, 32, 33). In a situation analogous to that of hdm2, the promoter for S100B has seven regions in its promoter (named prS100B-1 to prS100B-7) that correspond to the consensus sequence for p53 binding.<sup>2</sup> In fact, one DNA sequence, prS100B-3, matches perfectly the 20-nucleotide p53-binding consensus sequence (20/20 nucleotide match).<sup>2</sup>

Mobility shift assays (Fig. 4) were performed with purified p53 to determine whether sequences in the promoter of S100B (prS100B-1, prS100B-2, and prS100B-3) were *bona fide* p53 binding sites, using the p53 regulatory sequence from *GADD45*, a p53 downstream effector gene (34), as a positive control. The data shown in Fig. 4 indicate that a complex of slower mobility was generated with both *GADD45* and the prS100B-3 probes (Fig. 4, lanes 2 and 5, respectively). Moreover, both bands were super-shifted by the p53 antibody (Fig. 4, lanes 3 and 6, respectively) and, thus, represent genuine p53 complexes. However, despite the fact that the prS100B-3 sequence matches the consensus sequence perfectly, five times more protein and DNA were necessary to generate the protein-DNA complex with the prS100B-3 promoter sequence when compared with data from the *GADD45* sequence (Fig. 4). In a separate experiment, when equal concentrations of protein and DNA were used in such band shift assays, the intensity of the super-shifted DNA band, as measured by densitometry, was  $6.5 \pm 0.8$ -fold lower for the prS100B-3 promoter sequence than for the *GADD45* DNA. This may be due to the fact that two of the AT variants in the consensus sequence are A in the *GADD45* sequence, which is preferred, but are T in the prS100B-3 sequence (Fig. 4). Another difference between these p53 binding sites is the length of the intervening DNA sequence that links the two 10-nucleotide stretches of DNA that interact with p53 (11 nt in prS100B-3 versus 0 nt in *GADD45*;

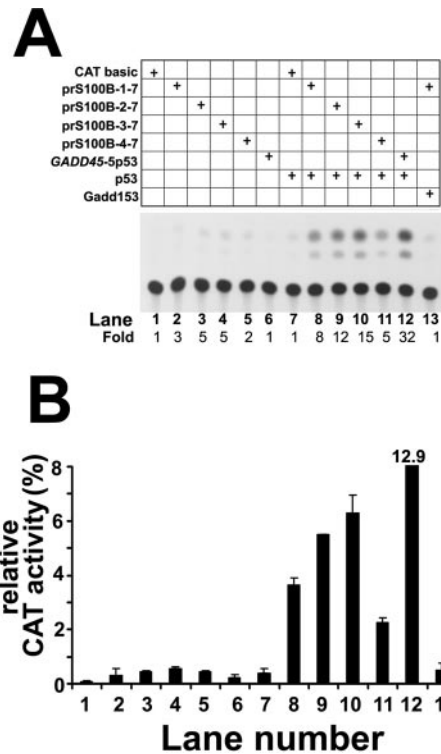


FIG. 5. Transcription activation of the S100B promoter in cells (H1299) in which the p53 gene is eliminated (p53<sup>-/-</sup>). **A**, lanes 1–6, controls for the S100B DNA constructs/transfections in the absence of p53. Lane 7, control transfection of p53 in the absence of an S100B promoter construct. Lane 8, co-transfection of p53 and the full-length S100B promoter. Transcription activation by p53 is measured by CAT activity for the full-length S100B promoter, S100B 7p53, that includes all seven p53 binding sites (nt 1–3080; prS100B-1 to prS100B-7). Lane 9, p53 transcription activation of a promoter construct of S100B with six of seven of the p53 binding sites, S100B 6p53 (nt 1435–3080; prS100B-2 to prS100B-7). Lane 10, p53 transcription activation of an S100B promoter construct that has five of seven of the p53-binding sites (nt 1679–3080; prS100B-3 to prS100B-7); this construct still retains the prS100B-3 sequence that has a 20 of the 20 nucleotides matching the p53 consensus sequence. Lane 11, p53 transcription activation by remainder of the S100B promoter, S100B 4p53 (nt 1744–3080; prS100B-4 to prS100B-7), which has four of the seven consensus p53 binding sites. Lane 12, positive control showing the p53 transcription activation of a well characterized p53-regulated gene, *GADD45*. Lane 13, negative control for CAT activation with an expression vector for an unrelated protein, Gadd153. The S100B CAT assays were performed with 50  $\mu$ g of cell extract, whereas the control CAT assay for the *GADD45* construct was performed with 0.5  $\mu$ g of cell extract. **B**, bar graph illustrating the relative CAT activity for each construct (lanes 1–13) from panel A. The bar labeled 12.9 (lane 12) is off-scale and has a CAT activity of  $12.9 \pm 1.2$  relative percentage conversion.

Fig. 4). Similar data were obtained with the two other p53 consensus sites in the S100B promoter (nt 149–169, prS100B-1; nt 1455–1478, prS100B-2); however, these sites had lower affinity for p53 than did the 20/20 sequence, prS100B-3 (data not shown). Together, these data suggest that the regulation of S100B transcription by p53 would occur only after genes with higher affinity p53 sites in their promoters are occupied.

**Transcription Is Activated When p53 Binds Regions of the S100B Promoter**—The full-length S100B promoter contains seven p53 binding sites (nt 1–3080; prS100B-1 to prS100B-7).<sup>2</sup> Three shorter constructs containing only six sites (nt 1435–3080; prS100B-2 to prS100B-7), five sites (nt 1679–3080; prS100B-3 to prS100B-7), or four consensus p53 binding sites (nt 1744–3080; prS100B-4 to prS100B-7), respectively, were also cloned into a CAT reporter gene construct to determine whether p53 affects S100B transcription. Transient transfection

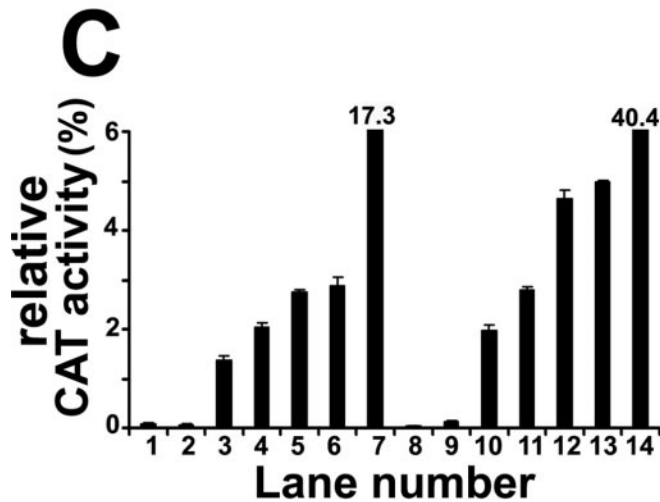
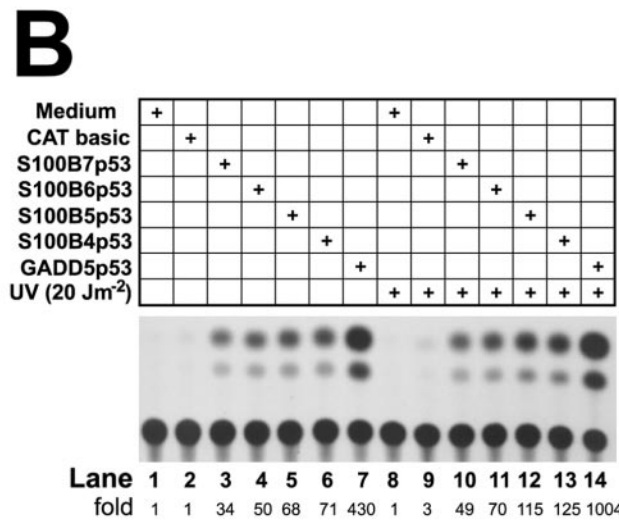
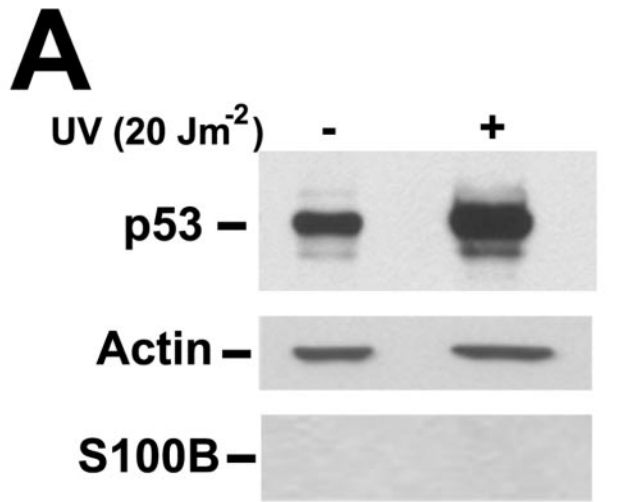


FIG. 6. Transcription activation of the S100B promoter by endogenous wild-type tumor suppressor protein p53 (in RKO cells) without or with UV treatment. A, Western blots of p53 and S100B in the human colorectal carcinoma cell line RKO before and after UV treatment. Actin was used as loading control. B, lanes 1-7, controls that are not treated with UV radiation include the following: RKO cell without any transfected construct (lane 1); control in the absence of the S100B promoter construct (lane 2); transcription activation by endogenous p53 as measured by CAT activity for the full-length S100B promoter, S100B 7p53, that includes all seven p53 binding sites (nt 1-3080; prS100B-1 to prS100B-7) (lane 3); p53 transcription activation

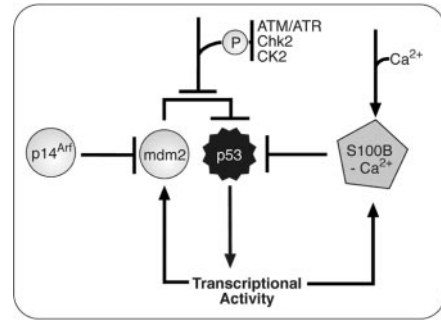


FIG. 7. Scheme for the down-regulation of wild-type p53. p53 is activated upon DNA damage or under stress and up-regulates the transcription of genes involved in apoptosis (i.e.  $\uparrow$  Bax, etc.) and cell cycle-dependent growth arrest (i.e.  $\uparrow$  p21 etc.). As part of a feedback control mechanism, p53 also up-regulates the transcription of genes involved in its own inactivation (i.e. hdm2 and S100B). As part of a cell growth response ( $\uparrow$  Ca<sup>2+</sup>), the Ca<sup>2+</sup>-dependent interaction between S100B and p53 induces a conformational change in p53 and tetramer dissociation of the tumor suppressor (29), which likely contributes to its degradation (i.e. perhaps involving hdm2/ubiquitin- and/or protease-dependent pathways). Thus, down-regulation of p53 by S100B and hdm2 ultimately facilitates cell growth. ATM, ataxia telangiectasia mutated kinase; ATR, ATM- and Rad-3-related kinase; p14Arf, alternative reading frame (human).

tions performed in the p53 null cells H1299 (p53<sup>-/-</sup>) indicated that each S100B promoter construct was activated in a p53-dependent manner (Fig. 5; lanes 8-11). Likewise, it was found that endogenous wild-type p53 in RKO cells (34) (p53<sup>+/+</sup>) could also activate the S100B promoter constructs and that UV treatment amplified this transcription activation activity as found with other genes activated by p53 (Fig. 6). However, it is likely that negative regulatory elements exist in the S100B promoter that affect the p53-dependent transcription activation of S100B, because cutting the S100B promoter back to make smaller constructs was found to increase the amount of p53-dependent transcription activation in both cell lines (Figs. 5 and 6). These results are not too surprising, however, because previous studies of this S100B promoter indicate that the overall regulation of this S100 gene is complex, often cell-type specific, and involves multiple positive and negatively regulatory elements (25).

As with the band shift assay (Fig. 4), it was found that a reporter gene construct containing p53 binding sites from the GADD45 gene sequence was more efficiently activated than the S100B promoter. Even when 10 times less protein extract was used, the CAT activity generated by the GADD45 reporter gene construct was higher than the levels obtained with the highest S100B activity (Fig. 6). Taken together, the DNA-binding and transcription activation activity assays are consistent and suggest that p53 can only activate the transcription of the S100B gene when the tumor suppressor protein levels are relatively

of a promoter construct of S100B with six of the p53 binding sites, S100B 6p53 (nt 1435-3080; prS100B-2 to prS100B-7) (lane 4); p53 transcription activation of a promoter construct from S100B that has five p53-binding sites (nt 1679-3080; prS100B-3 to prS100B-7) (lane 5); p53 transcription activation by an S100B promoter construct (nt 1744-3080; prS100B-4 to prS100B-7) containing four of the seven consensus p53 binding sites (lane 6); and positive control showing the p53 transcription activation of a well characterized p53-regulated gene, GADD45 (lane 7). Lanes 8-14 are the same as those in lanes 1-7, except that UV treatment was also applied as described under "Experimental Procedures." The S100B CAT assays were performed with 25  $\mu$ g of cell extract, whereas the GADD45 assay was performed with 2.5  $\mu$ g of cell extract. C, bar graph of the relative CAT activity for each construct (lanes 1-14) from panel A. The bars labeled with the numbers 17.3 and 40.4 (lanes 7 and 14) are off-scale and have CAT activities of 17.3  $\pm$  1.1 and 40.4  $\pm$  2.1 relative percentage conversions, respectively.

high (as compared with the p53 levels needed to activate other genes such as *GADD45*).

#### DISCUSSION

In its active form, each subunit of tetrameric p53 binds  $Zn^{2+}$  and contains a sandwich of two antiparallel  $\beta$ -sheets that serve as a scaffold for a loop-sheet-helix DNA binding domain (35). When cells are under stress (*i.e.* UV radiation, DNA damage, etc.), elevated levels of p53 bind specific DNA sequences and activate the transcription of downstream targets, including a cyclin-dependent kinase inhibitor (p21<sup>WAF/CIP1</sup>), cell cycle control proteins (cyclin G, *GADD45*), genes involved in apoptosis (*i.e.* *Bcl2*), and, eventually, proteins such as S100B and hdm2, which, in turn, negatively regulate p53 protein levels inside the cell via feedback control (1, 2, 32). In Fig. 7, a scheme is presented for how p53 is down-regulated by hdm2 and S100B as a part of feedback control to ensure that p53 levels do not get elevated to excessive levels.

For S100B, the affinity of p53 for its promoter regions are relatively low (Figs. 4–6), which suggests that regulation of S100B by p53 would occur only after higher affinity promoter sites are occupied (*i.e.* for *GADD45*). This idea is further supported by p53 transcription activation assays performed with shorter constructs of the S100B promoter (Figs. 5 and 6), which indicates that additional negative regulatory elements exist in the full-length promoter that reduce p53-dependent transcription activation of the S100B gene. Specifically, at the levels of the p53 protein necessary to activate the transcription of S100B, numerous other genes (*i.e.* *GADD45*, *p21*, etc.) will have already been transcribed. This, of course, is a scenario that one might expect for the regulation of a gene such as *S100B*, which is involved in a feedback loop that ultimately leads to lowering the levels of functional p53.

Interestingly, p53 is also known to up-regulate the transcription of another S100 protein, S100A2 (36) by binding the p53-binding consensus sequences found in its promoter.<sup>3</sup> Furthermore, several other S100 proteins (*i.e.* S100A1, S100A4, and S100A9) have consensus p53 binding sequences in their respective promoters,<sup>3</sup> so it is likely that several other S100 proteins are also regulated by p53. However, more work needs to be done before general conclusions can be made about the functional significance of regulatory pathways involving p53 and other S100 family members.

The lowering of p53 protein levels inside cells by S100B that was observed here (Fig. 2) and previously (23) is most likely related to the ability of S100B to bind directly to p53 (Fig. 3), dissociate the p53 tetramer (29), and/or cause conformational changes in the extreme C terminus and tetramerization domains of the tumor suppressor (30). Such effects by S100B may work synergistically with the hdm2 protein/pathways, which are involved in the ubiquitin-dependent degradation of p53 (37), although this concept needs to be rigorously established.

<sup>3</sup> A region of the S100A2 promoter (–2146 to –2126; GenBank<sup>TM</sup> accession number Y07755) nearly matches the p53-binding consensus sequence (18/20), binds p53, and activates p53-dependent transcription of a reporter gene (36). Other S100 proteins that have DNA sequences in their promoters that nearly match the consensus p53-binding sequence(s) include the following: S100A1 (15/20, –1011 to –984; GenBank<sup>TM</sup> accession number U26356), S100A4 (15/20  $\times$  3, –129 to –107, –453 to –426, and –680 to –658; 16/20, –145 to –119; 17/20, –1466 to –1437; GenBank<sup>TM</sup> accession number U94663), and S100A9 (16/20, –2488 to –2456; 17/20, 327 to 322; 18/20  $\times$  3, –204 to –178, –1092 to –1068, and –3171 to –3150; GenBank<sup>TM</sup> accession number AJ250496). S100A7 (GenBank<sup>TM</sup> accession number AF050167) does not have a DNA sequence in its promoter that closely matches the p53-binding consensus sequence, and the promoters for other S100 protein family members are not yet available in the GenBank<sup>TM</sup> nucleotide sequence database.

Nonetheless, an important distinction between these two negative regulatory proteins (hdm2 versus S100B) is that the S100B interaction with the C terminus of p53 is  $Ca^{2+}$ -dependent (21) and links p53 biology to calcium-mediated signaling pathways and extracellular growth responses, whereas, hdm2 binds the N terminus of p53 in a calcium-independent manner and is regulated by other proteins (*i.e.* p14<sup>ARF</sup>)/signaling pathways and the phosphorylation state of p53 (33, 37, 38) (Fig. 7).

It is important to understand how cancer cells, such as those in malignant melanoma, can proliferate even though they have wild-type p53. One possible explanation for this paradigm is that elevated levels of the proteins that negatively regulate p53, such as S100B, may be a culprit. Specifically, the overall effect of elevated S100B levels on wild-type p53 could mimic cellular aberrations such as gene amplification and mutations that are encountered with non-functional mutants of p53. Specifically, several melanomas have wild-type p53 and higher than normal levels of S100B, and the prognosis for patients such as these is generally poorer than for those with lower S100B levels (39–41). For primary skin cancer cells, we show that there is a direct interaction between p53 and S100B and that functional wild-type p53 levels can be restored when S100B levels are lowered by siRNA (Fig. 2). This result directly implicates S100B in the down-regulation of wild-type p53 and is consistent with the large decreases in p53 protein levels observed previously in transient co-transfections of p53 and S100B (23). With these results in mind, perhaps a small molecule inhibitor that binds S100B, blocks the S100B-p53 interaction, and restores wild-type p53 levels (*i.e.* an siRNA<sup>S100B</sup> mimic) could be useful in therapeutic strategies for the treatment of cancers with elevated S100B, such as some forms of malignant melanoma and glioma.

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