Sp1 Plays a Critical Role in the Transcriptional Activation of the Human Cyclin-dependent Kinase Inhibitor p21WAF1/Cip1 Gene by the p53 Tumor Suppressor Protein*

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In the present study we present evidence for the critical role of Sp1 in the mechanism of transactivation of the human cell cycle inhibitor p21WAF1/Cip1 (p21) gene promoter by the tumor suppressor p53 protein. We found that the distal p53-binding site of the p21 promoter acts as an enhancer on the homologous or heterologous promoters in hepatoma HepG2 cells. In transfection experiments, p53 transactivated the p21 promoter in HaCaT cells that express Sp1 but have a mutated p53 form. In contrast, p53 could not transactivate the p21 promoter in the Drosophila embryo-derived Schneider’s SL2 cells that lack endogenous Sp1 or related factors. Cotransfection of SL2 cells with p53 and Sp1 resulted in a synergistic transactivation of the p21 promoter. Synergistic transactivation was greatly decreased in SL2 cells and HaCaT cells by mutations in either the p53-binding site or in the −82 to −77 Sp1-binding site indicating functional cooperation between Sp1 and p53 in the transactivation of the p21 promoter. Synergistic transactivation was also decreased by mutations in the transactivation domain of p53. Physical interactions between Sp1 and p53 proteins were established by glutathione S-transferase pull-down and coimmunoprecipitation assays. By using deletion mutants we found that the DNA binding domain of Sp1 is required for its physical interaction with p53. In conclusion, Sp1 must play a critical role in regulating important biological processes controlled by p53 via p21 gene activation such as DNA repair, cell growth, differentiation, and apoptosis.

p21WAF1/Cip1/S11 (p21) is a ubiquitously expressed protein that is involved in the regulation of cell cycle progression in mammalian cells (1, 2). In the cells, p21 is found in quaternary complexes that consist of cyclins, cyclin-dependent kinases (CDKs),1 and proliferating cell nuclear antigen, a subunit of DNA polymerase δ (3). The formation of these complexes is essential for cell cycle progression (4–6). However, changes in p21 stoichiometry that could be controlled at the transcriptional level result in suppression of CDK activity, allowing the accumulation of hypophosphorylated Rb and cell cycle arrest in G1 (7, 2). By interacting directly with proliferating cell nuclear antigen, p21 prevents DNA synthesis and regulates DNA methylation (8). Thus, p21 plays important roles in DNA repair and in the control of cell senescence, apoptosis, and differentiation (9–11).

p21 gene expression is controlled by both p53-dependent and p53-independent mechanisms. A key regulator of p21 is the product of the tumor suppressor gene p53, which plays a central role in a protein network that controls cell cycle progression, differentiation, DNA repair, and apoptosis (12). Accumulation of wild type p53 in the cells causes an enhancement in the rate of transcription of several target genes (12–21).

The function of p53 as a tumor suppressor is supported by the finding that mice in which the p53 gene was inactivated by homologous recombination spontaneously developed tumors at a young age (22). Over 10,000 human tumor-associated mutations of p53 have been discovered to date (12). Most mutations are localized in the DNA binding domain and the C-terminal regulatory domain of the protein (12).

The p53 protein can be structurally and functionally divided into the following four domains: an N-terminal acidic transcription activation domain between amino acids 1 and 42 that interacts with factors of the basal transcription machinery (TAFl70 and TAFI31) as well as the negative regulator of p53 activity MDM2 protein; a DNA binding domain between amino acids 102 and 292; an oligomerization domain between amino acids 324 and 355; and a C-terminal regulatory domain between amino acids 367 and 393 that may affect the DNA binding function of p53 protein by steric or allosteric interactions (23).

In addition to p53, other inducers of p21 gene expression include factors that control differentiation of diverse cell types such as steroid hormones (24, 25), nerve growth factor (26, 27), platelet-derived growth factor (28), tumor necrosis factor-α (29), phorbol esters or phosphatase inhibitors (30), interferon γ...
(31), progesterone (32), transforming growth factor β (TGF-β), activin A (33–35), and their signaling effectors, Smad proteins (36, 37).

The majority of the regulatory modulators of p21 including TGF-β1, progesterone, phorbol esters, and phosphatase inhibitors affect p21 gene expression via Sp1 proteins (Sp1 and Sp3) bound to the proximal promotor region between nucleotides −120 and −50 relative to the transcription initiation site (38).

Sp1 is a member of a family of transcription factors with zinc finger-type DNA binding domains that binds to GC-rich or GT-rich DNA sequences (39, 40). Mice in which the Sp1 gene was inactivated by homologous recombination die at approximately day 11.5 of gestation indicating that Sp1 plays a pivotal role in development (41). Sp1 does not seem to be required for the regulation of CpG island methylation but rather is required for the maintenance of terminal cell differentiation by regulating the expression of the MeCP2 gene (42). The Sp1 protein is phosphorylated in a cell cycle-dependent manner (42) and is heavily O-glycosylated, a modification that seems to confer resistance to proteasome-dependent degradation (43). Domains in Sp1 protein that are crucial for its transcriptional activity, binding, and oligomerization functions were identified using the *Drosophila* embryo-derived Schneider’s SL2 cells (44). This cell line lacks endogenous Sp1 or Sp1-related activities, and thus it is a very useful model for studies of Sp1-dependent mechanisms of transcriptional activation of eukaryotic genes (45). The N-terminal glutamine- and serine/threonine-rich domains are essential for transcriptional activation (45). The C-terminal domain of Sp1 is involved in interaction with other transcription factors (46). Sp1 has been shown to interact directly with proteins of the basal transcription machinery such as TFIIID components (47). On the other hand, Sp1 interacts physically and cooperates functionally with several sequence-specific activators including NF-κB, GATA, YY1, E2F1, pRb, and SREBP-1 (48–51). Thus, although Sp1 has been traditionally considered as an ubiquitous factor closely associated with core promoter activities, it has been recently shown that it participates in several cases of regulated gene transcription triggered by multiple signaling pathways and metabolic or differentiation conditions.

We have shown recently that Smad3 and Smad4 proteins, which are key effectors in the TGF-β signaling pathway, and Jun members, which are activated by the c-Jun N-terminal kinase/stress-activated protein kinase in response to a variety of extracellular stimuli, transactivate the p21 promoter by interacting physically and functionally with Sp1 bound to the proximal −120/−50 region (37, 52, 53). In both cases, no direct binding of Smad or Jun proteins to DNA occurred but rather an enhanced affinity of Sp1 for its cognate sites on the p21 promoter was observed in the presence of the activators (52, 53).

In the present study we present evidence that Sp1 bound to the proximal p21 promoter is essential for the transactivation of this promoter by p53. p53 cannot transactivate the p21 promoter in *Drosophila* SL2 cells which lack endogenous Sp1 activities. Coexpression of p53 and Sp1 in SL2 cells resulted in a strong synergistic transactivation of the wild type p21 promoter but not of the promoter mutated in either the distal p53 or the proximal −82/−77 Sp1-binding site. GST pull-down experiments and *in vitro* coimmunoprecipitation assays indicated that the functional synergism between Sp1 and p53 is the result of physical interactions between the two proteins. Our findings suggest that Sp1 plays a critical role in regulating important biological processes controlled by p53 via p21 gene activation including DNA repair, cell growth, differentiation, and apoptosis.

**MATERIALS AND METHODS**

**Plasmid Constructions**—The p21 promoter plasmids −2,325/+8 luc, −215/+8 luc, −143/+8 luc, −2,325/+8 −122/−60 p21 luc, and −1,890/+8 p21 CAT have been described previously (37, 52, 53). The −2,325/+8 p21 luc reporter plasmids containing point mutations in Sp1 sites 1−6 were generated using the Gene Editor in *vitro* site-directed mutagenesis system (Promega Co). The construction of the mutants will be described elsewhere. The reporter plasmids −2,325/+8 1,890 p21 CAT and −1,890−325 p21 tk CAT were constructed as follows. The HindIII/SacI p21 promoter fragment (−2,325/−1,890) was excised from plasmid −2,325/+8 p21 CAT (37) and subcloned into the *Sma*I site of pig β-globin luciferase expression vector pGL3Basic. The reporter plasmids −2,325/−2,260 p21 tk CAT and −2,260/−1,890 p21 tk CAT were excised by cleaving the −2,325/−2,260 (HindIII/SacI) and −2,260/−1,890 (SacI/SalI) promoter fragments from plasmid −2,325/+8 p21 CAT, blunting them with Klenow DNA polymerase, and subcloning them into the *Sma*I site of vector −85 tk CAT. The −2,265/−2,260 p21 fragment was then cloned into the *Sac*I site of plasmids −215/+8 p21 luc and pG5Bluc to create plasmids (−2,325/−2,260)−215/+8 p21 luc and (−2,325/−2,260) pG5Bluc. The expression vectors pRo/CMV p53 and pRo/CMV p53 (residues 22 and 23) were the generous gift of Dr. Arnold Levine, Rockefeller University, New York (54). The bacterial expression vectors pGEX-Sp1 (83–778), pGEX-Sp1 516C, pGEX-Sp1 N619, pGEX-Sp1 Aint 349 were the generous gift of Dr. E. Flavey and have been described elsewhere (52). The original Sp1 mutants and the *Drosophila* expression vector pPac-Sp1 were the generous gift of Dr. R. Tjian, University of California, Berkeley. The his-tag expression vector used for normalization of transfections in *Drosophila* SL2 cells was the generous gift of Dr. C. Delidakis, University of Crete and the Institute of Molecular Biology and Biotechnology, Heraklion, Greece.

**Cell Cultures, Transient Transfections, CAT, Luciferase, and β-Galactosidase Assays**—Human hematoma HepG2 cells, human osteosarcoma Saos-2 cells, and human HαCaT keratinocytes were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM phenylmethylsulfonyl fluoride, 0.4% bovine serum albumin, 2 mM glutathione, 0.4% Nonidet P-40, 25 μM α- laurylsarcosine) for 10 min, at 4 °C, with 250 μM isopropyl-β-D-thiogalactopyranoside for 4 h at 37 °C. Bacteria were then harvested, resuspended in 1/10 of the original culture volume of phosphate-buffered saline (PBS), sonicated for 1 min in PBS on ice, lysed by the addition of Triton X-100 to a final concentration of 1%, and cleared by centrifugation at 8,000 rpm, at 4 °C for 10 min. The pellets were redissolved in solubilization buffer (1 mM EDTA, 25 mM triethanolamine, 1.5% N-lauroylsarcosine) for 10 min, at 4 °C, with gentle agitation. Triton X-100 to a final concentration of 2% and CaCl2 to a final concentration of 1 mM were added, and the lysates were cleared by centrifugation at 10,000 rpm, at 4 °C, for 10 min. The luciferase assay was performed using the luciferase assay kit from Promega Corp. according to the manufacturer’s instructions. Expression of Proteins in Vitro—Expression of proteins in vitro was performed using the coupled *in vitro* transcription/translation system (TNT) of Promega Corp. according to the manufacturer’s instructions. Labeling of *in vitro* expressed proteins was done by the inclusion of 20 μCi of [35S]methionine in the TNT reaction mixture. Bacterial Expression of Proteins—The GST fusion proteins were expressed in *Escherichia coli* strain DH-10β. Bacteria were grown overnight, diluted 1:25, and, after reaching an *A*<sub>600</sub> of 0.7, were stimulated with 250 μM isopropyl-β-D-thiogalactopyranoside for 4 h at 37 °C. Bacteria were then harvested, resuspended in 1/10 of the original culture volume of phosphate-buffered saline (PBS), sonicated for 1 min in PBS on ice, lysed by the addition of Triton X-100 to a final concentration of 1%, and cleared by centrifugation at 8,000 rpm, at 4 °C for 10 min. The pellets were redissolved in solubilization buffer (1 mM EDTA, 25 mM triethanolamine, 1.5% N-lauroylsarcosine) for 10 min, at 4 °C, with gentle agitation. Triton X-100 to a final concentration of 2% and CaCl<sub>2</sub> to a final concentration of 1 mM were added, and the lysates were cleared by centrifugation at 10,000 rpm, at 4 °C, for 10 min. The bacterial expression of the expressed proteins was monitored by SDS-PAGE and Coomassie Blue staining.

**GST Protein Interaction Assay**—Glutathione-Sepharose 4B beads were equilibrated in PBS and mixed with 1 volume of bacterially expressed GST fusion proteins on a rotary shaker for 2 h at 4 °C. The beads were washed one time with an equal volume of 2× interaction buffer (40 mM Hepes, pH 7.9, 10 mM MgCl<sub>2</sub>, 0.4% Nonidet P-40, 15% glycerol, 2 mM bovine serum albumin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin). Coupling efficiency was monitored by SDS-PAGE and Coomassie Blue staining.

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For the binding reaction, 50 µl of 1:1 bead slurry in 200 µl of 1× interaction buffer was combined with 5 µl of a [32P]-labeled radioactive DNA and a final volume of 400 µl on a rotatory shaker for 90 min at 4 °C. The beads were then washed 3 times with 20 volumes of washing buffer (20 mM Hepes, pH 7.9, 100 mM KCl, 5 mM MgCl2, 0.2% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 1 mM EDTA) and the bound proteins were eluted by boiling in Laemmli SDS-PAGE loading buffer and subjected to SDS-PAGE. Bound proteins were visualized by autoradiography.

Coimmunoprecipitation, Western Blotting Assays—For coimmunoprecipitation experiments, COS-7 cells were washed twice with ice-cold PBS and collected in PBS. Cells were solubilized by centrifugation at 10,000 rpm for 10 min at 4 °C and resuspended in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton X-100) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 13 µg/ml aprotinin, and 6.7 µg/ml leupeptin). Lysates were collected by centrifugation at 10,000 rpm for 10 min at 4 °C and then pre-cleared by incubation with 30 µl of protein A-Sepharose Fast Flow pre-equilibrated with lysis buffer on a rotating platform for 3 h at 4 °C, followed by 30 min of centrifugation and collection of the supernatant. Supernatants were incubated with 8 µg/ml of polyclonal anti-Sp1 antibody (PEP-2, Santa Cruz Biotechnology) on a rotating platform overnight at 4 °C, followed by incubation with 30 µl of protein G-Sepharose Fast Flow beads pre-equilibrated in lysis buffer for 3 h. Beads were washed three times in lysis buffer, precipitated, and resuspended in equal volume of 2× SDS loading buffer. Immunoprecipitated proteins were analyzed by 8.5% SDS-polyacrylamide gels followed by autoradiography. Western blotting analysis was performed with 22 ng/ml of polyclonal anti-Sp1 antibody (DO-1, Santa Cruz Biotechnology) followed by incubation with horseradish peroxidase-conjugated antimouse antibody and visualization by treatment with enhanced chemiluminescence substrate.

Gel Electrophoresis Mobility Shift Assay—Gel electrophoresis mobility shift assays were performed as described previously (55). Sense and antisense oligonucleotides were annealed to generate the double-stranded oligonucleotide probe and labeled with Klenow fragment of DNA polymerase I and [32P]dCTP as described (55). The sequence of the sense oligonucleotide used as probe is as follows: p21 (−2,325/−2,281): 5′-ATC AGG AAC ATG TCC CAA CAT GTT GAG CTC T-3′.

RESULTS

The Distal −2,325/−2,260 Region of the p21 Promoter, Which Contains a p53-responsive Element, Acts as a Transcriptional Enhancer of the Proximal Promoter That Contains Multiple Sp1-binding Sites—We have shown previously that the activity of the promoter of the human CDK inhibitor p21 gene in human hepatoma HepG2 cells is controlled by two distinct regions as follows: one distal region between nucleotides −2,325 and −1,890 that seems to act as a transcriptional enhancer, and one proximal region defined by nucleotides −120 to −50 that contains six binding sites for the ubiquitous transcription factor Sp1 (37). Deletion of the proximal or the distal region resulted in 95 and 70% reduction in p21 promoter activity in HepG2 cells, respectively (Fig. 1A and Ref. 37). These findings suggest a functional cooperation between factors bound to the distal enhancer region and the proximal region. To characterize further the distal p21 promoter and identify regions important for its enhancer activity, a series of reporter plasmids was constructed in which different segments of the −2,325/−1,890 region were placed upstream of the heterologous herpes simplex virus-thymidine kinase promoter −85/−1 that contains one Sp1-binding site (Fig. 1B). These p21/tk-CAT reporter constructs were transiently transfected into HepG2 cells, and their activity was measured by CAT assay. It was found that the −2,325/−1,890 p21 promoter region caused a 43.5- and 51.5-fold transactivation relative to the orientation of the tk promoter and that mutations in the Sp1 site 3 of the proximal promoter altered this activity. The sequence of the p53-responsive element, which contains one Sp1-binding site (Fig. 1B), was found that this region, which contains the p53-responsive element and the −2,260/−1,890 region, which lacks the p53-binding site. Both regions were placed upstream of the tk promoter and the CAT gene. Transfections of HepG2 cells and CAT analysis showed that full enhancer activity was retained by the −2,325/−1,890 region that contains the p53-responsive element and that this activity was lost when the −2,260/−1,890 region was used (Fig. 1B).

To test the ability of the −2,325/−2,260 p21 region to act as a transcriptional enhancer on its homologous promoter, this region was placed immediately upstream of the −215/−8 tk promoter. Transfection experiments in HepG2 cells showed that the −2,325/−2,260 p21 promoter region transactivated strongly (28-fold) the −215/−8 proximal p21 promoter (Fig. 1C). The combined data of Fig. 1A, C, and D indicate that the −2,325/−2,260 region of the p21 promoter, which contains a p53-responsive element, acts as a transcriptional enhancer of the proximal promoter that contains multiple Sp1-binding sites suggesting a functional cooperation between p53 and Sp1 in the transactivation of the p21 promoter.

The p53-mediated Transactivation of the p21 Promoter Is Achieved by the Functional Cooperation between p53 and Sp1 Bound to Sp1 Site 3 (−82/−77)—To study further the role of the proximal and distal p21 promoter regions in the p53-mediated transactivation, we performed transient transfection experiments in the human HaCaT keratinocyte cells. These cells contain two mutant alleles of p53, which are unable to activate transcription from the p21 promoter when overexpressed (56). Overexpression of a functional p53 protein in these cells increased p21 promoter activity by 7-fold (Fig. 2A). Transactivation was abolished by deletion of either the proximal −122/−60 or the distal −2,325/−144 p21 promoter regions (Fig. 2A). To characterize in detail the importance of the Sp1 sites present in the proximal p21 promoter, the p21 region between nucleotides −120 and −50 was mutagenized to prevent binding of Sp1 factors to these sites. The −120/−50 region contains six sequences with homology to the consensus recognition sequence: 5′-GGCGGG-3′. The mutations introduced are shown in Fig. 2B and are designated mut 1, 2, 3, 4, and 5. The wild type as well as the mutated −2,325/−82 p21 promoter-luciferase reporter plasmids were transiently transfected into HaCaT cells, and their promoter activity in the absence or the presence of wild type p53 was analyzed by luciferase assays. It was found that mutations in the Sp1 site 3 (mut 3, −82/−77) reduced the basal p21 promoter activity (18% relative to the wt promoter) as well as the p53-mediated transactivation (4.2-fold transactivation for the mutated promoter versus 7-fold transactivation for the wild type promoter) (Fig. 2C). In contrast, mutations in the other Sp1 sites (sites 1–6) reduced by 40–60% the p21 promoter activity and either did not affect or increased the p53-mediated transactivation (Fig. 2C).

These findings indicate that the p53-mediated transactivation of the p21 promoter requires the functional cooperation between p53 and Sp1 bound to site 3 of the proximal promoter. Sp1 molecules bound to other sites in the proximal region may modulate the p53-mediated transactivation of the p21 promoter.

Binding of p53 to DNA Is Crucial for Its Functional Cooperation with Sp1—To study further the mechanism of transacti-
activation of the p21 promoter by p53, a series of transient transfection experiments were performed in the Drosophila embryo-derived cell line SL2. These cells lack endogenous Sp1 or Sp1-related activities and thus they represent a very useful model to study Sp1-dependent transcriptional mechanisms.

The activity of the human p21 promoter in SL2 cells is extremely weak compared with its activity in cells expressing Sp1 such as HepG2, COS-7, or HeLa (data not shown). Overexpression of Sp1 in SL2 cells resulted in a drastic increase in p21 promoter activity (81-fold) in a dose-dependent manner (not shown) (Fig. 3A), whereas overexpression of p53 alone had no effect. Cotransfection of SL2 cells with Sp1 along with p53 and the p21 promoter resulted in an ~7-fold enhancement in transactivation compared with the sum of transactivation achieved separately by Sp1 and p53. This finding suggested strongly that Sp1 or Sp1-related factors, which are missing from SL2 cells, are essential for the p53-mediated transactivation of the p21 promoter. Mutagenesis of Sp1 site 3 reduced to 2.7-fold the enhancement in the transactivation of the p21 promoter by Sp1 and p53 compared with the transactivation achieved by Sp1 and p53 on the wt p21 promoter. The transactivation of the p21 Mut 3 promoter by Sp1 alone was not affected. Consistently with the findings of Fig. 2, the findings of Fig. 3 suggest a functional cooperation between p53 and Sp1 in SL-2 cells, which leads to a synergistic transactivation of the p21 promoter. Deletion of the proximal p21 promoter region between nucleotides 122 and 60 that contains 4 out of 6 Sp1-binding sites (sites 1–4) abolished the transactivation of the p21 promoter by either Sp1 or Sp1 and p53 (Fig. 3A), p53 was not able to transactivate a reporter construct (~215/8

Fig. 1. The −2,325/−2,260 region of the human p21 promoter that contains a binding site for the tumor suppressor p53 protein acts as a transcriptional enhancer in human hepatoma HepG2 cells. A–C, HepG2 cells were transfected with the indicated p21 promoter or p21 promoter/HSV tk minimal promoter-CAT constructs (2 μg) along with the CMV β-galactosidase plasmid (1 μg) that was used for normalization. CAT activity was determined as described under “Materials and Methods.” The normalized, relative CAT activity of at least two independent experiments performed in duplicate is shown on the right. In all panels, human p21 promoter fragments are shown as gray bars. The distal p53-binding site is shown with a black oval, whereas the multiple proximal Sp1-binding sites are shown with white ovals numbered 1–6. Numbers on the promoter refer to positions relative to the transcription start site of the human p21 gene (+1). The black bars in B represent the minimal promoter of the herpes simplex virus-thymidine kinase gene (−85/+1), which also contains an Sp1-binding site. Dotted lines indicate deletions in the promoter. D, the p53 protein is expressed in HepG2 cells. Total extracts from HepG2 cells (35-mm dish) were subjected to SDS-PAGE and Western blotting using the monoclonal a-p53 antibody DO1. The p53 band is shown with an arrow. M, molecular mass protein markers in kilodaltons. E, binding of p53 expressed in HepG2 cells to the distal region of the p21 promoter. A synthetic double-stranded oligonucleotide corresponding to the the −2,285/−2,255 p21 promoter region was labeled with [α-32P]dCTP and subjected to gel mobility shift assays using nuclear extracts from HepG2 cells in the absence or presence of the monoclonal anti-p53 antibody DO1 as described under “Materials and Methods.” The p53-DNA complexes formed in the presence or absence of the anti-p53 antibodies are shown with arrows.
Transactivation of the p21 Promoter by p53 and Sp1

**FIG. 2.** Transactivation of the p21 promoter by p53 requires the proximal Sp1-binding sites. **A,** human keratinocytes HaCaT cells were transiently transfected with the indicated p21 promoter-luciferase (luc) constructs (2 μg) along with an expression vector for the human p53 protein (+p53) or the empty vector (−p53) and the CMV β-galactosidase plasmid (1 μg) that was used for normalization of the transfections. Luciferase activity was determined in cell lysates 48 h later, and the values (± S.E.) from at least two independent experiments performed in duplicate are shown on the right. **B,** sequence of the human p21 promoter between nucleotides −130 and −33. Sequences shown in reverse color show consensus binding sites for the transcription factor Sp1 (sites 1–6). Nucleotide substitutions that were introduced into the p21 promoter are shown on top of the sequence and are designated mut 1–5,6. **C,** effect of mutations in Sp1-binding sites on the p53-mediated transactivation of the human p21 promoter. HaCaT cells were transiently transfected with the indicated wild type (wt) or mutated (Mut 1–5,6) −2,325/+8 p21 promoter-luciferase (luc) constructs (2 μg) along with an expression vector for the human p53 protein or the empty vector and the CMV β-galactosidase plasmid (1 μg) that was used for normalization of the transfections. Luciferase activity was determined in cell lysates 48 h later, and the values (± S.E.) from at least two independent experiments performed in triplicate are shown in the form of a bar graph. Gray bars, −p53; black bars, +p53. The fold transactivation of the p21 promoter by p53 is shown on top of the graph.

Luc (luc) that lacks the upstream p53-binding site of the p21 promoter (Fig. 3, B and C). In contrast, when the −2,325/+2,600 region that contains the p53-responsive element was placed in front of the p21 −215/+8 proximal promoter, strong synergistic transactivation was observed by Sp1 and p53 in SL2 cells which was 3-fold higher than the sum of transactivations achieved separately by Sp1 and p53 (Fig. 3, B and D).

The transactivation functions of p53 are also crucial for its functional cooperation with Sp1 since a mutant p53 protein containing a double point mutation in the transactivation domain (Leu22Gln, Trp23Ser) (Fig. 4A) was severely impaired in the transactivation of the p21 promoter in Saos-2 cells that do not express endogenous p53 protein (Fig. 4B). The mutant p53 protein also did not synergize with Sp1 in the transactivation of the p21 promoter following transfection in SL2 cells (Fig. 4C).

**p53 Interacts Physically with Sp1 in Vitro and in Vivo**—The functional cooperation between Sp1 and p53 proteins on the p21 promoter strongly suggested direct physical interactions between the two factors. Physical interactions between Sp1 and p53 were investigated in vitro and in vivo. In the in vitro approach, Sp1 (amino acids 83–778) fused with the GST at the N terminus (Fig. 5A) as well as the GST protein portion alone were expressed in bacteria, purified, and coupled to glutathione-agarose beads (Fig. 5B). The GST-Sp1 beads and the control GST beads were used as an affinity support for binding of human wt p53 protein or the p53 (residues 22 and 23) mutant containing the double amino acid substitution in the transactivation domain. The p53 proteins were transcribed and translated in vitro in the presence of [35S]methionine. As shown in Fig. 5C, both p53 and p53-(22,23) bound efficiently to the GST-Sp1 beads but did not bind to the GST beads indicating specific physical interactions between p53 and Sp1. To identify domains in Sp1 protein involved in physical interactions with p53, GST pull-down assays were performed using GST-Sp1 fusions that corresponded to different segments of Sp1 protein. Specifically the following Sp1 mutants were utilized (Fig. 5A): Sp1 A (amino acids 349–497) which lacks the transactivation domain A of Sp1; Sp1 Δint 349 (AB + C) which lacks the transactivation domain B as well as the regulatory domain C; and Sp1 N 619 (ΔD) which lacks the C-terminal transactivation domain D. All these Sp1 deletion mutants, fused at their N terminus with the GST protein, were expressed in bacteria, coupled to glutathione-agarose beads (Fig. 5D), and used in GST pull-down assays. As shown in Fig. 5E, all Sp1 mutants bound efficiently to p53 with affinity equal to the wild type protein. These data indicate that the physical interactions of p53 with Sp1 are possibly mediated by the 610–702 amino acid region that con-
contains the DNA binding domain of Sp1. This region is included in all mutants tested (Fig. 5A).

Physical interactions between p53 and Sp1 were also demonstrated by an in vivo coimmunoprecipitation experiment. Whole cell extracts from monkey kidney COS-7 fibroblasts that express abundantly both Sp1 and p53 proteins were subjected to immunoprecipitation using a polyclonal antibody that detects the endogenous Sp1 protein. In a parallel reaction, no anti-Sp1 antibodies were used as a control. The antibody-Sp1 complexes were subsequently bound to protein G-Sepharose, and the precipitated proteins were subjected to SDS-PAGE and Western blotting using a monoclonal antibody specific for p53. As shown in Fig. 6A, p53 protein was coimmunoprecipitated along with Sp1 protein, whereas no p53 protein could be detected in the control reaction performed in the absence of anti-Sp1 antibodies. The immunoprecipitation of Sp1 was monitored by Western blotting of the same membrane with an antibody specific for Sp1 (Fig. 6B). Furthermore, p53 could not be detected in the precipitate of a control coimmunoprecipitation reaction performed using an unrelated antibody (anti-GAL4) instead of the anti-Sp1 antibody. This finding strongly supports physical interactions between endogenous p53 and Sp1 proteins in vivo and are in agreement with the in vitro data of Fig. 5.

**DISCUSSION**

The −2,325/−2,260 Region Acts as a Transcriptional Enhancer in HepG2 Cells—One of the first effects of p53 expression in mammalian cells is to cause cell cycle arrest at least in part via induction of the p21WAF1/CIP1 (p21) CDK inhibitor. By activating the p21 gene, p53 causes a temporary block in G1 thus allowing cells to repair DNA breaks caused by ionizing radiation, UV light, or other chemicals that cause DNA damage (12, 23). p53 plays a central role in a protein network that controls cell cycle progression, differentiation, DNA repair, and apoptosis. Accumulation of wild type p53 in the cells causes an enhancement in the rate of transcription of several target genes (12, 23).

We have shown previously that the promoter of the human p21 gene is constitutively activated in the human hepatoma HepG2 cells, an observation that is consistent with the high steady state mRNA levels of this gene in these cells (37). We have also shown that an internal deletion of the p21 promoter between nucleotides −120 and −60 that contains four of the six
FIG. 4. Mutations in the N-terminal domain of p53 (amino acids 22 and 23) abolish functional cooperation with Sp1. A, schematic representation of the wt and p53 proteins used in the transactivation experiments of B and C. The double mutation in the transactivation domain of p53 in the p53 (residues 22 and 23) protein is shown. B, human osteosarcoma Saos-2 cells were transiently transfected with the −2,325/+8 p21 promoter-luciferase construct (2 µg) along with an expression vector for the wild type human p53 protein or a mutated form (p53 residues 22 and 23) and the CMV β-galactosidase plasmid (1 µg) that was used for normalization of the transfections. Luciferase activity was determined in cell lysates 48 h following the transfection, and the values (± S.E.) from at least two independent experiments performed in duplicate are shown in the form of a bar graph. C, Drosophila SL-2 cells were transiently transfected with the −2,325/+8 p21 promoter-luciferase construct (2 µg) along with an expression vector for Sp1, the wild type human p53 protein, a mutated form (p53 residues 22 and 23), or combinations of Sp1 and p53 proteins as indicated at the bottom of the graph and the CMV β-galactosidase plasmid (1 µg). Luciferase activity was determined in cell lysates 48 h following the transfection, and the values (± S.E.) from at least two independent experiments performed in duplicate are shown in the form of a bar graph. B and C, the fold transactivation of the p21 promoter by p53 and Sp1 and the fold enhancement in the transactivation achieved by a combination of the two proteins is shown on top of the graphs.

Sp1-binding sites resulted in a dramatic decrease in promoter activity in HepG2 cells (Ref. 37 and Fig. 1A). Furthermore, deletion in the upstream promoter region, between nucleotides −2,325 and −1,890, also resulted in significant loss of promoter activity suggesting that factors binding to the upstream region communicate with Sp1 or other factors bound to the proximal promoter.

In the current study, deletion analysis of the p21 promoter showed that the most upstream 60-bp region between nucleotides −2,325 and −2,260 is sufficient to enhance transcription from the homologous p21 promoter or the heterologous herpes simplex virus-thymidine kinase promoter. This region contains the sequence 5'-GAACATGTCCCAACATGTTG-3' which is homologous to the consensus Sp1-binding sites (sites 1–4). Our mutagenesis analysis showed that the most upstream 60-bp region between nucleotides −2,262 and 2,281, also resulted in significant loss of promoter activity suggesting that factors binding to the upstream region communicate with Sp1 or other factors bound to the proximal promoter.

Two Proteins and the Synergistic Transactivation of the p21 Promoter—HepG2 cells express constitutively functional p53 as shown in Fig. 1 and as reported previously (60). We hypothesized that functional cooperation between p53 bound to the distal p53 site and Sp1 or related factors bound to the proximal p21 promoter sites could be responsible for the high levels of constitutive p21 promoter activity in these cells as well as in other types that express both proteins. According to this hypothesis, the activity of the p21 promoter should be low in cells that express a mutant p53 protein or do not express p53 as well as in cells that do not express Sp1 proteins. Indeed, transient transfections in human HaCaT keratinocytes showed that p21 promoter activity is low in these cells. However, the p21 promoter activity can be greatly increased by overexpression of a functional p53 protein (Fig. 2). Furthermore, the p53-mediated transactivation of the p21 promoter was lost by deletion of the distal p21 promoter region or the proximal Sp1-binding sites. These findings strengthened the hypothesis that synergism between p53 and Sp1 occurs on the p21 promoter.

The −120/−60 region of the p21 promoter contains four Sp1-binding sites (sites 1–4). Our mutagenesis analysis
showed that site 3 which is localized between nucleotides 282/277 is essential for the p53-mediated synergistic transactivation of the p21 promoter (Fig. 2). In contrast, mutations in the other Sp1 sites (sites 1–6) either did not affect or caused a slight increase in p53-mediated transactivation of the p21 promoter (Fig. 2). These findings indicate strongly that the p53-mediated transactivation of the p21 promoter is achieved by the functional cooperation between p53 and Sp1 bound to site 3. Sp1 molecules bound to other sites in the proximal region may modulate the overall activity of the p21 promoter.

The Sp1 site 3 has been shown previously to mediate p21 induction by various agents such as TGF-β (33), butyrate (61), the histone deacetylase inhibitor trichostatin A (62), lovastatin (63), and Ca^{2+} (64) among others. In contrast, Sp1 sites 1 and 2 mediate transcriptional activation by phorbol esters and okadaic acid (30), the tumor suppressor protein BRCA1 (65), and the gut-enriched Kruppel-like factor (GKLF, KLF4) (66). No specific role has been attributed thus far to the most proximal and overlapping Sp1 sites 5 and 6. All the above observations suggest that specificity in Sp1 site utilization under different conditions of p21 regulation is important. The mechanism underlying this specificity is currently unknown. It is possible that the distance of the individual Sp1-binding sites from the TATA box of the p21 promoter that serves as a point of assembly of the factors of the basal transcription machinery determines the identity of the activators or coactivators that interact with each site, and this may determine its contribution to p21 promoter levels under constitutive or inducible conditions.

The experiments using the Sp1-deficient SL2 cells also confirmed that binding of p53 and Sp1 factors to their cognate elements on the p21 promoter is required for the synergistic transactivation of the p21 promoter by the two factors. How-
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Mutations in the N-terminal Transactivation Domain of p53 Do Not Interfere with p53/Sp1 Physical Interactions but Abolish Functional Cooperation between p53 and Sp1—The functional cooperation between p53 and Sp1 established in Figs. 1–3 strongly suggested physical interactions between the two proteins. Such interactions were established by both in vitro and in vivo approaches. In vitro, physical association between p53 and Sp1 was shown using GST pull-down assays. Specific interaction with Sp1 was also observed when a p53 protein was mutated in two amino acids (L22Q and W23S) in its N-terminal transactivation domain. It was shown previously that these mutations abolish the p53-mediated transactivation of p53 target promoters and interfere with its physical interaction and functional cooperation with the CBP coactivator (67) and the MDM2 protein (68). The CBP and p300 coactivators have been shown to participate in the regulation of G0-G1 cell cycle transitions by activating certain enhancers and by stimulating differentiation pathways (69, 70). Given that the transactivation of target genes by p53 correlates with its ability to suppress cell growth and induce differentiation and apoptosis, cell growth suppression by CBP could be mediated at least in part via synergistic interactions with p53.

In the case of the p21 promoter, overexpression of CBP or p300 in Drosophila SL2 cells caused a transcriptional activation of this promoter in a strict Sp1-dependent manner. However, no further transactivation of the p21 promoter was observed by p53 and Sp1 in the presence of p300 or CBP (data not shown). These findings along with the finding that a p53 mutant unable to bind CBP interacted with Sp1 efficiently (Fig. 5) suggest that CBP or p300 proteins are not necessary for the formation of the Sp1-p53 complex on the proximal p21 promoter. Detailed DNA affinity precipitation assays and chromatin immunoprecipitation assays are required to clarify this issue.

Critical Role of Sp1 and/or Related Activities in Processes Regulated by p21—The severe reduction in constitutive and p53-inducible p21 promoter activity by the mutations in the proximal Sp1-binding site 3 suggests that Sp1 or related factors must play crucial roles in biological processes regulated by p21 and p53 such as cell cycle progression, DNA repair, differentiation, and apoptosis. Based on these findings we could hypothesize that mutations in the Sp1 gene could also have dramatic consequences in cell growth, differentiation, and survival. The importance of Sp1 in development was established by the finding that targeted inactivation of the Sp1 gene by homologous recombination resulted in embryonic lethality at day 11.5 (41).

Our findings suggest that Sp1 bound to the proximal p21 promoter could play a pivotal role in the integration of multiple extracellular signals targeting p21, thus affecting cell cycle progression. Such a critical role of Sp1 in the regulation of the cell cycle is supported by a recent finding showing that the transcriptional activity of Sp1 is regulated by phosphorylation in a cell cycle-specific manner (42). It is tempting to speculate that cycling patterns of Sp1 phosphorylation during the cell cycle could determine the identity of the multiprotein complexes formed in the proximal p21 promoter and thus the strength of the p21 promoter. Whether phosphorylation of Sp1 affects its affinity for coactivators or other sequence-specific activators remains to be determined.

Sp1/p53 Synergism as a General Mechanism of Transcriptional Activation of p53 Target Genes—An increasing number of genes are transcriptional targets of p53 protein. Recently, using oligonucleotide arrays, Zhao et al. (71) examined a large number of genes for transcriptional response to p53. A total of 107 genes were found to be activated and 54 genes to be repressed. These genes encode for proteins that regulate diverse biological processes such as cell cycle arrest (p21WAP1, GADD45, 14-3-3 δ, Cdk2, Rhap46, and lamin B receptor), extracellular matrix production (collagen type II α1 and VI α1), angiogenesis (endothelin-2 and thrombospondin), apoptosis (Fas/APO-1), and cytoskeletal organization (actin, keratins 5, 17, and 19), among others. Yu et al. (72), using p53 conditional overexpression and serial analysis of gene expression, observed substantial heterogeneity in the transcriptional responses of 10,000 genes to p53. Many of these genes had not previously been known to be regulated by p53. Assuming that the majority of these genes contain p53 elements in their promoters, the question remains how many of these genes are transcriptionally dependent on Sp1 or Sp1 family members. Conditional formation of transcriptional Sp1-p53 regulatory complexes has been reported recently in a variety of promoters (73–76). In addition, many p53 target genes that contain Sp1-binding sites in their promoters (77–81) could also be regulated by synergistic interactions between Sp1 and p53. Thus, the functional synergism between p53 and Sp1 may be a widely utilized...
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mechanism of transcriptional regulation of genes that respond to various inducers via p53 activation.

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Sp1 Plays a Critical Role in the Transcriptional Activation of the Human Cyclin-dependent Kinase Inhibitor p21 \textit{WAF1/Cip1} Gene by the p53 Tumor Suppressor Protein

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