Physical and functional interactions between members of the tumour suppressor p53 and the Sp families of transcription factors: importance for the regulation of genes involved in cell-cycle arrest and apoptosis

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In the present study, we have investigated mechanisms of transcriptional co-operation between proteins that belong to the tumour suppressor p53 and Sp (specificity protein) families of transcription factors. Such mechanisms may play an important role in the regulation of genes containing binding sites for both classes of transcription factors in their promoters. Two of these genes were analysed in the present study: the cyclin-dependent kinase inhibitor p21^{Cip1} gene and the PUMA (p53-up-regulated mediator of apoptosis) gene. We found that Sp1 and Sp3, but not Sp2, co-operate functionally with p53, p73 and p63 for the synergistic transactivation of the p21^{Cip1} promoter in *Drosophila* Schneider SL2 cells that lack endogenous Sp factors. We also found that Sp1 strongly transactivated the PUMA promoter synergistically with p53, whereas deletion of the Sp1-binding sites abolished the transactivation by p53. Using p53 mutant forms in GST (glutathione S-transferase) pull-down assays, we found

that the C-terminal 101 amino acids of p53, which include the oligomerization and regulatory domains of the protein, are required for the physical interactions with Sp1 and Sp3, and that deletion of this region abolished transactivation of the p21^{Cip1} promoter. Utilizing truncated forms of Sp1, we established that p53 interacted with the two transactivation domains A and B, as well as the DNA-binding domain. Our findings suggest that Sp factors are essential for the cellular responses to p53 activation by genotoxic stress. Understanding in detail how members of the p53 and Sp families of transcription factors interact and work together in the p53-mediated cellular responses may open new horizons in cancer chemotherapy.

Key words: apoptosis, cell cycle, p21, p53, p53-up-regulated mediator of apoptosis (PUMA), specificity protein 1 (Sp1).

INTRODUCTION

p53 is a sequence-specific transcription factor that enhances the expression of a large number of genes whose products are required for cell-cycle progression, differentiation, DNA repair and apoptosis [1–3]. p53 is encoded by one of the most frequently mutated genes in various forms of sporadic and inherited cancers [1,3]. Mice in which the p53 gene was inactivated by homologous recombination developed spontaneous tumours at a young age, thus establishing the tumour-suppressor functions of this protein [4].

p53 can be structurally and functionally divided into three regions: the N-terminal region which harbours an acidic TAD (transactivation domain) between amino acids 20 and 60, the central region that harbours a sequence-specific DBD (DNA-binding domain) between amino acids 102 and 292, and the C-terminal region that harbours a tetramerization domain between amino acids 324 and 355 [2]. Two additional regions of p53 that play functional roles are a proline-rich domain (residues 60–90) that contains five copies of the PXXP (Src homology 3) motif and plays a role in apoptosis [5,6], and a C-terminal basic region between amino acids 367 and 393 that may affect the DNA-binding function of the central region of p53 protein by steric or allosteric interactions, and contributes to recognition of DNA forms that resemble sites of DNA damage [7–9]. The tetrameric p53 protein (which is a dimer of two dimers) binds to four different repeats

of a consensus DNA sequence 5'-RRRC(A/T)-3' (where R is a purine) when this sequence is repeated in two pairs, each arranged as an inverted repeat [10]. The majority of human tumour-associated mutations in p53 that have been characterized to date are localized in the DBD and the C-terminal basic region of the protein [3].

Agents that cause DNA damage, such as UV radiation, hypoxia or certain carcinogens, activate p53 by inducing its phosphorylation at specific serine residues which results in the dissociation of MDM2 (murine double minute 2) protein and the subsequent stabilization of p53 [11]. p53 activity is also regulated by the peptidyl-prolyl isomerase Pin1, which binds to phospho-serine/proline motifs on p53 and isomerizes the peptide bond formed between the phosphorylated serine and the adjacent proline residue [12,13].

p53 belongs to a family of transcription factors that also includes the homologous proteins p63 and p73, with structures and functions similar to, but not identical with, p53 (Figure 1C) [14,15]. Most of the conserved residues in p53, p63 and p73 are clustered within the TAD, the DBD and the oligomerization domain [16–18]. However, p63 and p73 contain extended C-termini with particular functions, including a second TAD [19] as well as a SAM (sterile alpha motif) that is involved in protein–protein interactions [20,21]. Different p63 and p73 C-termini are generated as a result of alternative mRNA splicing [15].

Abbreviations used: ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; 5-FU, 5-fluorouracil; GST, glutathione S-transferase; HEK, human embryonic kidney; HRP, horseradish peroxidase; IPTG, isopropyl β -D-thiogalactoside; Ni-NTA, Ni²⁺-nitrilotriacetate; NP40, Nonidet P40; ONPG, o-nitrophenyl β -D-galactopyranoside; PUMA, p53-up-regulated mediator of apoptosis; Rb, retinoblastoma protein; SAM, sterile alpha motif; Sp, specificity protein; TAD, transactivation domain; TnT, transcription and translation.

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Figure 1 Transcriptional synergism between Sp and p53 family members regulates the activity of the human p21^{Cip1} promoter

(A) Schematic representation of the p21^{Cip1} reporter construct (–2,325/+8) p21-Luc that was used in the transactivation experiments of this and subsequent Figures. Characterized binding sites for members of the Sp and p53 family members are shown by white and grey ovals respectively. The arrow indicates the transcription start site. (B) Schematic representation of the three members of the Sp family of transcription factors (Sp1, Sp2 and Sp3) that were utilized in the transactivation experiments presented in this Figure. Different domains are shown with brackets and are designated A–D and DBD. The serine/threonine-rich regions of TADs A and B are shown as light grey boxes and the glycine-rich regions of the same domains as dark grey boxes. The zinc-fingers of the DBD are shown as three small black squares and –/+ indicates sequences in domain C that modulate the transcriptional activity of Sp factors. (C) Schematic representation of the structure of various p53 family members used in the present study. PXXP, Src homology 3 domain; OD, oligomerization domain; RD, regulatory domain. (D)–(F) *Drosophila* Schneider SL2 cells were transiently transfected with the (–2,325/+8) p21-Luc construct (2 µg) along with *Drosophila*-specific expression vectors for Sp1, Sp2 or Sp3 (25 ng) independently or in combination with expression vectors for p53 (D), p73 (E) and p63 (F) (1 µg), as indicated beneath each histogram. The hsp-lacZ plasmid expressing β-galactosidase (1 µg) was included in each sample for normalization of transfection variability. Luciferase activity was determined in cell lysates at 48 h after transfection, and the values (± S.E.M.) from at least two independent experiments performed in duplicate are shown as percentage relative luciferase activity. (G) Immunoblotting assays showing the expression of different p53 family members in *Drosophila* Schneider SL2 cells. WB, Western blot. The arrows show the position of the detected proteins.

A number of genes have been identified as encoding participants in the apoptotic responses of cells to p53 activation following DNA damage, including the Bax gene, the IGF-BP3 (insulin-like growth-factor-binding protein 3) gene, PIG (p53-induced gene) and, more recently, the gene encoding the cytochromic PUMA (p53-up-regulated mediator of apoptosis) protein [3,22,23]. On the other hand, current experimental evidence suggests that the cell-cycle-arrest effect of p53 is mediated mainly by the transcriptional activation of the cell-cycle inhibitor p21^{Cip1} gene. This gene codes for a ubiquitously expressed protein that is involved in the regulation of cell-cycle progression in mammalian cells [24,25]. p21^{Cip1} controls, in a concentration-dependent manner, the activity of complexes consisting of cyclins, cdks (cyclin-dependent kinases) and the subunit of DNA polymerase δ called PCNA (proliferating cell nuclear antigen), in a positive or a negative manner [26–28]. The suppression of the activity of these complexes causes the accumulation of hypophosphorylated Rb (retinoblastoma protein) and cell-cycle arrest in G₁ [25,29].

p53 regulates the expression of the p21^{Cip1} gene at the level of transcription. The promoter of the human p21^{Cip1} gene contains a set of six binding sites for members of the Sp (specificity protein) family of transcription factors in the proximal region (nucleotides –120 to –40) as well as two distal p53-binding sites (Figure 1A) [30]. The proximal Sp-binding sites were shown to be essential for the activation of the p21^{Cip1} promoter by p53 in human hepatoma HepG2 cells and in *Drosophila* Schneider SL2 cells [31].

Sp1 is the prototype member of a small family of transcription factors (Sp1, Sp2, Sp3 and Sp4) with homologous functional domains. These include two N-terminal glutamine- and serine/threonine-rich domains (domains A and B), which are essential for transcriptional activation, a highly charged regulatory domain (domain C), a DBD of the zinc-finger type that binds to GC-rich (Sp1, Sp3 and Sp4) or GT-rich (Sp2) DNA sequences and a C-terminal domain (domain D), which is involved in oligomerization as well as in interactions with other transcription factors (Figure 1B) [32–34]. Although Sp1 has been traditionally considered as a ubiquitous factor that is closely associated with core promoter activities, it has been shown recently that it participates in several cases of regulated gene transcription triggered by multiple signalling pathways and metabolic or differentiation conditions. To accomplish this task, Sp1 needs to interact transcriptionally with several sequence-specific activators, including NF- κ B (nuclear factor κ B), YY1 (Yin Yang-1) and Rb [35–37]. Mice in which the Sp1 gene was inactivated by homologous recombination die at approx. day 11.5 of gestation, indicating that Sp1 plays a pivotal role in development [38].

MATERIALS AND METHODS

Materials

Restriction enzymes and modifying enzymes (T4 DNA ligase, the Klenow fragment of DNA polymerase I and calf intestinal alkaline phosphatase) were purchased from Minotech, New England Biolabs or Life Technologies. L-[³⁵S]Methionine and [α -³²P]dCTP were purchased from Amersham Biosciences. All reagents for cell culture [DMEM (Dulbecco's modified Eagle's medium), foetal bovine serum, trypsin-EDTA and PBS] were purchased from Life Technologies. ONPG (*o*-nitrophenyl β -D-galactopyranoside), PMSF and 5-FU (5-fluorouracil) were purchased from Sigma. The anti-p73 (N-19) goat polyclonal antibody, the anti-p63 (4A4) mouse monoclonal antibody, the anti-p53 (DO-1) mouse monoclonal antibody, the anti-GST (glutathione S-transferase) (B-14) mouse monoclonal antibody, the anti-Sp1 (Pep-2) goat polyclonal antibody and the anti-Sp3 (D-20) goat polyclonal antibody were purchased from Santa Cruz Biotechnology. The

anti-His₆ mouse monoclonal antibody was purchased from R & D Systems. The anti- α -tubulin monoclonal antibody was a gift from Dr C. Stournaras (Department of Basic Sciences, University of Crete). The anti-PUMA antibody was a gift from Dr J. Yu (Department of Pathology, University of Pittsburgh, Pittsburgh, PA, U.S.A.). The luciferase assay kit and the coupled *in vitro* TnT (transcription/translation) kit were purchased from Promega.

Plasmid constructions

The p21^{Cip1} promoter plasmids (–2325/+8) p21-Luc, (–215/+8) p21-Luc and (–2325/–2260, –215/+8) p21-Luc have been described previously [31,39]. The *Drosophila* expression vectors pPac-Sp1, pPac-Sp2 and pPac-Sp3 were gifts from Dr John Noti (Laboratory of Molecular Biology, Guthrie Institute, Sayre, PA, U.S.A.). The *Drosophila* expression vectors pPac-Sp1 516C (Δ A), pPac-Sp1 Δ int 112 (Δ C) and pPac-Sp1 Δ int 349 (Δ B + C) have been described elsewhere [40]. The hsp-lacZ expression vector used for normalization of transfections in *Drosophila* Schneider SL2 cells was a gift from Dr C. Delidakis (Department of Biology, University of Crete and IMBB, Heraklion). The expression vectors for the human p73 α and p73 β were gifts from Dr William Kaelin, Jr (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, U.S.A.). The expression vectors for the mouse p63 α and p63 γ were gifts from Dr Giovanni Blandino (Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, Rome, Italy). The GST-Sp1 and GST-Sp3 vectors were constructed by subcloning the human Sp1 and Sp3 cDNAs from the corresponding pPac vectors into the EcoRI site of plasmid pGEX-4T1 (Amersham Biosciences). The bacterial vectors expressing different Sp1 domains (domains A, B, Bn, Bc, C and D) fused with GST were constructed by PCR amplification of the corresponding fragments of human Sp1 cDNA and subsequent cloning into the expression vector pGEX-4T1 at the BamHI and EcoRI sites. The sequence of the primers used in the amplification of individual domains is shown in Table 1. Bacterial expression vectors for the DBD of Sp1 fused with GST or the His₆-epitope tag were gifts from Dr M. Horikoshi (Laboratory of Developmental Biology, Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan). The mammalian vectors expressing the Sp1 domains A, B and D fused with GST were constructed by excising the above Sp1 cDNA fragments from the corresponding pGEX vectors by BamHI and NotI and subcloning them into the expression vector pEBG at the same restriction sites. Different p53 fragments cloned into the vector pGEM4 were kindly provided by Dr T. Halazonetis (Molecular and Cellular Oncogenesis Program, Wistar Institute, Philadelphia, PA, U.S.A.). These fragments were excised with HindIII and EcoRI and were subcloned at the corresponding sites of the mammalian expression vector pRK5. The plasmids pGEX-p53-(1–160) and pGEX-p53-(160–393) were gifts from Dr M. Murphy (Department of Pharmacology, Fox Chase Cancer Center, Philadelphia, PA, U.S.A.). The bacterial expression vector for wild-type p53 fused with the His₆ tag was constructed by subcloning the full-length human p53 cDNA into the EcoRI and HindIII sites of vector pRSETB (Invitrogen). The –336/+157 and –36/+157 PUMA-Luc reporter plasmids were gifts from Dr Bert Vogelstein (Johns Hopkins University, Baltimore, MD, U.S.A.). The (–336/+157 Δ –126/–25) PUMA-Luc plasmid was constructed by digesting the –336/+157 PUMA-Luc plasmid with SacII and SmaI, and re-ligation.

Cell cultures, transient transfections and luciferase assays

Human hepatoma HepG2 cells and COS-7 monkey kidney fibroblasts were cultured in DMEM supplemented with 10% foetal

Table 1 Oligonucleotides used as primers in PCRs

BamHI (GGATCC) and EcoRI (GAATTC) sites are in bold and underlined.

Name	Sequence	Purpose
Sp1-83 sense	5'-GCG <u>GGATCC</u> ACAGGTGAGCTTGACCTCACAGCCACA-3'	Used as 5' primer for the amplification of the 83–262 Sp1 region.
Sp1-262 antisense	5'-GCG <u>GAATTC</u> GACAGGTAGCAAGGTGATGTTCCATTCC-3'	Used as 3' primer for the amplification of the 83–262 Sp1 region.
Sp1-263 sense	5'-GCG <u>GGATCC</u> AACAGCGTTTCTGCAGCTACCTTGACT-3'	Used as 5' primer for the amplification of the 263–542 Sp1 region.
Sp1-542 antisense	5'-GCG <u>GAATTC</u> GCCTTGAATTGGGTGCACCTGGATTCC-3'	Used as 3' primer for the amplification of the 263–542 Sp1 region.
Sp1-543 sense	5'-GCG <u>GGATCC</u> CTGCCGTTGGCTATAGCAAATGCCCA-3'	Used as 5' primer for the amplification of the 543–610 Sp1 region.
Sp1-610 antisense	5'-GCG <u>GAATTC</u> CGAGCCCTTCTTCACTGTCTTTACA-3'	Used as 3' primer for the amplification of the 543–610 Sp1 region.
Sp1-702 sense	5'-GCG <u>GGATCC</u> CAGAATAAGAAGGGAGGCCAGGTGTA-3'	Used as 5' primer for the amplification of the 702–778 Sp1 region.
Sp1-778 antisense	5'-GCG <u>GAATTC</u> CAGAAGCCATTGCCACTGATATTAATGG-3'	Used as 3' primer for the amplification of the 702–778 Sp1 region.
PUMA 5' ChIP primer	5'-CTGTGGCCTTGTCTGTGAGTAC-3'	Used as 5' primer for the amplification of the –422/–211 region of the human PUMA gene.
PUMA 3' ChIP primer	5'-CCTAGCCCAAGGCAAGGAGGAC-3'	Used as 3' primer for the amplification of the –422/–211 region of the human PUMA gene.

bovine serum, L-glutamine and penicillin/streptomycin at 37°C, in a 5% CO₂ atmosphere. *Drosophila* Schneider SL2 cells were cultured in Schneider's insect medium supplemented with 10% insect-culture-tested foetal bovine serum and penicillin/streptomycin at 25°C. Transient transfections were performed by the Ca₃(PO₄)₂ co-precipitation method using 6 µg of DNA/well when the transfections were performed in six-well plates or 35 µg/plate when transfections were performed in P-100 plates. Luciferase assays were performed using the luciferase assay kit from Promega, according to the manufacturer's instructions.

Expression of proteins *in vitro*

Expression of proteins *in vitro* was performed using the coupled *in vitro* TnT system, according to the manufacturer's instructions. Labelling of *in vitro* expressed proteins was carried out by the inclusion of 20 µCi of L-[³⁵S]methionine in the TnT reaction mixture.

Bacterial expression of GST-fusion proteins from *Escherichia coli* and coupling to glutathione–Sepharose beads

The GST-fusion proteins were expressed in *E. coli* strains BL21 or DH5α. Bacteria were grown overnight, diluted 1:20 and, after reaching a *D*₆₀₀ of 0.7, were stimulated with 1 mM IPTG (isopropyl β-D-thiogalactoside) for 4 h at 37°C. Bacteria were then harvested, resuspended in 1/10 of the original culture volume of PBS, sonicated for 2 min in PBS on ice, lysed by the addition of Triton X-100 to final concentration of 1% and rotation at 4°C for 30 min. Lysates were subsequently cleared by centrifugation at 12 500 g for 10 min at 4°C. The pellets were redissolved in 500 µl of solubilization buffer (1 mM EDTA, 25 mM triethanolamine and 1.5% *N*-laurylsarcosine) 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 12 500 g for 10 min at 4°C. The solubilization of the expressed proteins was monitored by SDS/PAGE (10.5% polyacrylamide) and Coomassie Blue staining.

Glutathione–Sepharose 4B beads were equilibrated in PBS and mixed with 1 vol. of bacterially expressed GST-fusion proteins on a rotary shaker for 2 h at 4°C. The beads were washed once with an equal volume of 2× interaction buffer [40 mM Hepes, pH 7.9, 10 mM MgCl₂, 0.4% NP40 (Nonidet P40), 15% glycerol, 0.4% BSA, 2 mM PMSF and 10 µg/ml aprotinin]. Coupling efficiency was monitored by SDS/PAGE (10.5% polyacrylamide) and Coomassie Blue staining.

Purification of His₆-tagged proteins from *E. coli* and coupling to Ni-NTA (Ni²⁺-nitrilotriacetate) beads

The His₆-tagged fusion proteins were purified from *E. coli* strains BL21 or DH5α as follows. Bacteria were grown overnight, diluted 1:20 and, after reaching a *D*₆₀₀ of 0.7, were stimulated with 1 mM IPTG for 2–3 h at 37°C. Bacteria were then harvested, resuspended in 1/10 of the original culture volume of Buffer A (50 mM Hepes/KOH, pH 7.9, 0.15 M NaCl, 20% glycerol, 0.1% NP40, 5 mM imidazole, 0.5% BSA, 14.33 mM 2-mercaptoethanol and protease inhibitors), lysed by the addition of lysozyme (1 mg/ml) for 30 min on ice and were sonicated for 2 min. Lysates were subsequently cleared by centrifugation at 16 060 g for 30 min at 4°C. The pellets were redissolved in 500 µl of solubilization buffer 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 12 500 g for 10 min at 4°C. The solubilization of the expressed proteins was monitored by SDS/PAGE (10.5% polyacrylamide) and Coomassie Blue staining.

Ni-NTA beads were equilibrated in Buffer A and mixed with 10 vol. of bacterially expressed His₆-tagged proteins on a rotary shaker for 2 h at 4°C. The beads were washed seven times with 10 bead vol. of Buffer B (50 mM Hepes, pH 7.9, 0.5 M NaCl, 0.1% NP40, 10% glycerol, 50 mM imidazole and protease inhibitors). Bound proteins were eluted with an equal bead vol. of Buffer C (50 mM Tris/HCl, pH 8.0, 0.5 M NaCl, 0.1% NP40, 10% glycerol, 250 mM imidazole and protease inhibitors) for 10 min at 4°C. Beads were removed by centrifugation at 16 060 g for 1 min at 4°C. Purification was monitored at each step by SDS/PAGE (10.5% polyacrylamide) and Coomassie Blue staining or by Western blotting. The eluates were stored at –80°C until use.

GST pull-down assays

For the binding reaction, an appropriate amount of a 1:1 GST–Sepharose bead slurry in 2× interaction buffer was combined with 5–15 µl of a ³⁵S-labelled reticulocyte lysate or an appropriate amount of affinity-purified His₆-tagged proteins in a final volume of 400 µl on a rotary shaker for 2 h (His₆-tagged proteins) or overnight (*in vitro* translated proteins) at 4°C. The beads were then washed four times (15 min each) with 20 vol. of washing buffer (20 mM Hepes, pH 7.9, 100–350 mM KCl, 5 mM MgCl₂, 0.2% NP40 and protease inhibitors), and the bound proteins were eluted by boiling in Laemmli SDS/PAGE loading buffer and subjected to SDS/PAGE (10.5% polyacrylamide). Bound proteins were

visualized either by autoradiography or by Western blotting using a mouse monoclonal anti-His₆ antibody.

Co-immunoprecipitation assays

For co-immunoprecipitation experiments, COS-7 cells or HEK-293T (human embryonic kidney) cells were washed 2× with ice-cold PBS and collected in PBS. Cells were pelleted by centrifugation at 12 500 *g* for 10 min at 4 °C and resuspended in lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 % glycerol and 1 % Triton X-100) supplemented with protease inhibitors (1 mM PMSF, 13 µg/ml aprotinin and 6.7 µg/ml leupeptin). Lysates were collected by centrifugation at 12 500 *g* for 10 min at 4 °C, then pre-cleared by incubation with 30 µl of Protein A–Sepharose Fast Flow pre-equilibrated with lysis buffer on a rotating platform at 15 rev./min for 3 h at 4 °C, followed by 30 s of centrifugation and collection of the supernatant. Supernatants were incubated with a proper dilution of the first antibody on a rotating platform at 15 rev./min 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 an equal volume of 2× SDS loading buffer. Immunoprecipitated proteins were analysed by SDS/PAGE (8.5 % polyacrylamide) followed by electrotransfer on to 0.45 µm pore size Protran nitrocellulose transfer membrane (Schleicher and Schuell). Immunoblotting was performed with a proper dilution of the second antibody followed by incubation with HRP (horseradish peroxidase)-conjugated antibody and visualization by treatment with enhanced chemiluminescence substrate (Pierce).

Mammalian GST pull-down assays

Transfected HEK-293T cells were washed with ice-cold PBS and collected in PBS. Cells were pelleted by centrifugation at 6200 *g* for 5 min at 4 °C and resuspended in lysis buffer supplemented with protease inhibitors (1 mM PMSF and 1 mM benzamide). Lysates were collected by centrifugation at 16 060 *g* for 4 min at 4 °C, after incubation on a rotating platform for 30 min at 4 °C. Supernatants were incubated with glutathione–Sepharose 4B beads that had been pre-equilibrated in PBS on a rotating platform for 4 h at 4 °C. Beads were washed three times in lysis buffer, precipitated and resuspended in equal volume of 2× SDS loading buffer. Proteins bound to the beads were analysed by SDS/PAGE (10.5 % polyacrylamide), followed by electrotransfer on to 0.45 µm pore size Protran nitrocellulose transfer membrane. Immunoblotting was performed with a polyclonal anti-p53 antibody, followed by incubation with HRP-conjugated anti-mouse antibody and visualization by treatment with enhanced chemiluminescence substrate.

Gel EMSAs (electrophoretic mobility-shift assays)

Gel EMSAs were performed as described previously [41] using extracts from *Drosophila* Schneider SL2 cells transfected with different pPac-Sp1 expression vectors. Sense and antisense oligonucleotides were annealed to generate the double-stranded oligonucleotide probe and labelled with Klenow fragment of DNA polymerase I and [α -³²P]dCTP. The sequence of the sense oligonucleotide used as probe was p21 (−86/−70): 5′-GGGGGTC-CCGCTCCTTGA-3′.

ChIP (chromatin immunoprecipitation)

HepG2 cells grown in P-100 plates were treated with 5-FU (50 µg/ml) for 24 h or were left untreated. The ChIP assay was

performed as described previously [42] using a monoclonal antibody against p53. Immunoprecipitated chromatin was analysed by PCR using primers corresponding to the proximal region −422/−211 of the human PUMA gene promoter. The sequence of the primers is given in Table 1. The products of the PCR amplifications were analysed by agarose gel electrophoresis.

RESULTS

Functional co-operation between members of the Sp and p53 families of transcription factors enhances the activity of the human p21^{Cip1} promoter

To investigate the potential involvement of different Sp family members in the p53-mediated transcriptional induction of the p21^{Cip1} promoter, we performed transactivation assays in the *Drosophila* Schneider SL2 cell line [43]. These cells lack endogenous Sp1 or Sp1-related activities and thus they represent a very useful system to study Sp-dependent transcriptional responses [44]. *Drosophila* Schneider SL2 cells were transiently transfected with a reporter plasmid bearing the luciferase gene under the control of the human (−2325/+8) p21^{Cip1} promoter alone or together with expression vectors for wild-type human Sp1, Sp2, Sp3 and p53. As shown in Figure 1(D), Sp1 and Sp3, but not Sp2, transactivated the p21^{Cip1} promoter to a low degree under the specific experimental setting (3–5-fold). In contrast, p53 expressed alone was unable to transactivate the p21^{Cip1} promoter in *Drosophila* Schneider SL2 cells, despite the presence of two binding sites for this protein in the distal region (Figure 1A). The lack of p21^{Cip1} promoter transactivation by p53 was not due to inefficient expression of this protein in *Drosophila* Schneider SL2 cells (Figure 1G). Importantly, co-expression of Sp1 or Sp3 with p53 caused a strong synergistic transactivation of the p21^{Cip1} promoter (37-fold and 30.5-fold respectively). In contrast, Sp2 was unable to transactivate the p21^{Cip1} promoter in the presence of overexpressed p53 (Figure 1D).

We utilized the same system to investigate potential functional interactions between Sp family proteins with four additional members of the p53 family: p73 α , p73 β , p63 α and p63 γ (Figure 1C). For this purpose, expression vectors for the above p53 family members were utilized to allow efficient expression of these proteins in the *Drosophila* Schneider SL2 cells (Figure 1G). As shown in Figure 1(E), both p73 α and p73 β enhanced the transcription of the luciferase gene driven by the −2325/+8 p21^{Cip1} promoter only in the presence of Sp1 or Sp3, in a strongly synergistic manner. Synergistic interactions between Sp1 and p63 isoforms α and γ in the transactivation of the p21^{Cip1} promoter were also observed in *Drosophila* Schneider SL2 cells (Figure 1F).

In summary, the results shown of Figure 1 indicate that Sp factors are essential for the transactivation of the p21^{Cip1} promoter by members of the p53 family of proteins, and that high levels of p21^{Cip1} promoter activity can be achieved by co-operative interactions between members of the two families.

Transcriptional regulation of the PUMA gene by functional interactions between p53 and Sp1

To establish the importance of Sp1 and related factors for p53-mediated transcriptional responses, we investigated the mode of regulation of an additional gene that also contains binding sites for both classes of transcription factors in its promoter. The promoter of the gene encoding the cytochromic PUMA protein contains a set of 10 potential binding sites for Sp family proteins in the proximal region between nucleotides −120 and −8 flanked by two p53-recognition sequences at the 5′ end between nucleotides −229/−209 and −145/−126 (Figure 2A). PUMA protein was

Figure 2 Sp1 is required for the transactivation of the human PUMA promoter by p53

(A) Schematic representation of the PUMA 5' regulatory region and of the three PUMA-Luc reporter constructs that were used in the transactivation experiments of (D). Characterized or potential binding sites for members of the Sp and p53 family members on the PUMA promoter are shown by white and grey ovals respectively. The arrows show the position of the primers used in the ChIP experiment of (C). The deleted region -126/-25 is shown with a broken line. (B) Activation of PUMA gene expression by DNA-damaging agents. HepG2 cells were treated with 50 μg/ml 5-FU (+5-FU) or were left untreated (-5-FU) for 24 h. Whole-cell extracts were analysed for the expression of endogenous p53, PUMA and α tubulin by immunoblotting. WB, Western blot. (C) ChIP assay using HepG2 cells treated with 50 μg/ml 5-FU (+5-FU) or left untreated (-5-FU). A monoclonal antibody that recognizes p53 was used for the immunoprecipitation of the chromatin. PCR reactions were performed with primers corresponding to the -422 and -211 regions of the PUMA promoter (shown in A). (D) *Drosophila* Schneider SL2 cells were transiently transfected with the PUMA-Luc reporter plasmids shown in (A), along with a *Drosophila*-specific expression vector for Sp1 (25 ng) and an expression vector for wild-type human p53 protein (1 μg) independently or in combination as indicated beneath the histogram. The hsp-lacZ plasmid expressing β-galactosidase (1 μg) was included in each sample for normalization of transfection variability. Luciferase activity was determined in cell lysates 48 h after transfection, and the values (±S.E.M.) from at least two independent experiments performed in duplicate are presented as percentage relative luciferase activity.

shown to be mainly responsible for the apoptotic responses of colorectal cancer cells to p53 induction [22,23]. Activation of p53 by DNA-damaging agents such as 5-FU was followed by an induction of the PUMA gene expression (Figure 2B), and this induction was associated with enhanced recruitment of endogenous p53 protein to the PUMA gene promoter (Figure 2C). As in the case of p21^{Cip1}, overexpression of p53 in *Drosophila* Schneider SL2 cells activated the (-336/+157) PUMA promoter

only in the presence of Sp1 (Figure 2D) or Sp3 (results not shown), suggesting a functional co-operation between p53 and Sp factors in the regulation of PUMA gene expression. To support this conclusion, we constructed a mutant PUMA promoter lacking the majority of the proximal Sp-binding motifs that are clustered in the region between nucleotides -126 and -25 (-336/+157 Δ-126/-25 PUMA-Luc) (Figure 2A). As shown in Figure 2(D), very low levels of transactivation of this mutant PUMA promoter by Sp1 was observed which could not be enhanced further by p53, despite the presence of two p53-binding sites in the mutant PUMA promoter. Similar results were obtained when a PUMA mutant lacking both the Sp1- and the p53-binding sites (-36/+157 PUMA-Luc) (Figure 2A) was utilized in transactivation experiments (Figure 2D).

The results shown in Figure 2 suggest that, as in the case of p21^{Cip1}, p53 cannot activate the human PUMA promoter in the absence of Sp factors or when the Sp-binding sites are mutated. Thus the mechanism of transcriptional activation of the PUMA gene by DNA-damaging drugs or other stimuli could involve, at least in part, functional interactions between p53 and Sp factors that bind to adjacent regions of the PUMA promoter.

Domains in p53 that mediate physical interactions with Sp1 and Sp3

Physical interactions between p53 and Sp1 could account for the synergistic transactivation of the p21^{Cip1} and PUMA promoters by the two factors [31]. To identify domains in p53 that mediate physical interactions with Sp1, GST pull-down experiments were performed using the wild-type or mutant p53 forms shown in Figure 3(A). These proteins were produced by *in vitro* TnT in the presence of L-[³⁵S]methionine (Figure 3B) and allowed to interact with GST-Sp1 or GST alone expressed in bacteria and coupled to glutathione-Sephadex beads. As shown in Figure 3(C), specific physical interactions between Sp1 and wt p53 or the N-terminal p53 mutants Δ3-79, Δ13-19 and Δ34-70 was observed. On the other hand, mutations at the C-terminal part of p53 (Δ300-321, Δ293-393 and Δ363-393) affected, to a different extent, its interaction with Sp1. The most drastic inhibition of p53/Sp1 interactions was observed with the p53 mutant Δ293-393 that lacks the entire C-terminal 101-amino-acid region (Figure 3C). The utilization of two additional mutants of p53, p53-(1-160) and p53-(160-393) confirmed that the C-terminal half of the protein (amino acids 160-393) contains at least one interaction surface for Sp1.

Physical interactions between p53 and Sp1 were also investigated *in vivo* by co-immunoprecipitation assays. As shown in Figure 3(D), Sp1 expressed endogenously in HEK-293T cells interacted strongly with exogenous wild-type p53 and weakly with exogenous p53 Δ363-393 in accordance with the *in vitro* GST pull-down assays shown in Figure 3(C).

To investigate whether Sp3 can also interact physically and functionally with p53, protein-protein interaction assays were employed. First, physical interactions between Sp3 and p53 *in vivo* were established by a co-immunoprecipitation assay using endogenous proteins expressed in COS-7 fibroblasts. As shown in Figure 3(E), a monoclonal anti-p53 antibody co-immunoprecipitated endogenous Sp3 and p53 proteins, whereas no Sp3 immunoprecipitation was observed when an unrelated antibody (anti-myc) was utilized. To verify the physical interactions between Sp3 and p53, *in vitro* GST pull-down assays were also performed. As shown in Figure 3(F), wild-type p53 interacted specifically with GST-Sp3, but not with GST. To identify the domains of p53 that mediate the physical interactions with Sp3, representative truncated forms of p53 shown in Figure 3(A)

Figure 3 Domains in tumour suppressor p53 that mediate physical interactions with Sp1

(A) Schematic representation of the wild-type (wt) and mutant p53 proteins utilized in the GST pull-down experiments of (C) and (E). The name of each p53 mutant is shown on the left. Abbreviations are as in Figure 1(C). (B) Wild-type (wt) human p53 protein or the indicated mutant forms were transcribed and translated *in vitro* in the presence of L -[35 S]methionine. The products of each reaction were analysed by SDS/PAGE following by autoradiography. M, molecular-mass protein marker (sizes in kDa). (C) The wild-type and mutant p53 proteins produced by *in vitro* TnT were incubated with GST or GST-Sp1-coupled glutathione-Sepharose beads as described in the Materials and methods section. Bound proteins were analysed by SDS/PAGE and autoradiography. Input represents 20% of the initial protein sample used in the binding experiments. (D) Co-immunoprecipitation of Sp1 with wild-type (wt) p53 and the p53 Δ 363–393 proteins. HEK-293T cells were transfected with expression vectors for wild-type p53 (first and second lanes) or the p53 Δ 363–393 mutant (third lane) and subjected to immunoprecipitation (IP) using a polyclonal anti-Sp1 antibody (second and third lanes) or no antibody as a control (first lane). The immunoprecipitated proteins, as well as the starting material (WCE, whole-cell extract) were subjected to SDS/PAGE and were analysed by Western blotting (WB) using the monoclonal anti-p53 antibody. (E) Co-immunoprecipitation of endogenous Sp3 and p53 proteins. Total cell extracts from COS-7 cells were subjected to immunoprecipitation (IP) using a monoclonal anti-p53 antibody (anti-p53) or a non-related antibody as a control (anti-myc) as described in the Materials and methods section. The immunoprecipitates were subjected to SDS/PAGE and Western blotting (WB) using the polyclonal anti-Sp3 antibody or the same anti-p53 antibody used for immunoprecipitation. The positions of Sp3 and p53 detected by Western blotting are shown with arrows. (F) Domains in p53 involved in physical interactions with Sp3. Wild-type (wt) human p53 protein or the indicated mutant forms were transcribed and translated *in vitro* in the presence of L -[35 S]methionine and were incubated with GST or GST-Sp3-coupled glutathione-Sepharose beads as described in the Materials and methods section. Bound proteins were analysed by SDS/PAGE and autoradiography. Input represents 20% of the initial protein sample used in the binding experiments. A summary of the interactions of p53 mutants with Sp1 and Sp3 is shown in (A).

were utilized in GST pull-down assays. This analysis revealed that mutations at the N-terminal region of p53 (Δ 3–79 and Δ 34–70) did not affect its ability to associate physically with Sp3 (Figure 3F). In contrast, all mutations at the C-terminal part of p53

(Δ 300–321, Δ 293–393 and Δ 363–393) abolished the physical interactions between p53 and Sp3 (Figure 3F).

The results shown in Figure 3 indicated that p53 interacts physically and functionally with two members of the Sp family

Figure 4 Physical interactions between Sp and p73 proteins

Wild-type human p73 α and p73 β proteins were transcribed and translated *in vitro* in the presence of L-[³⁵S]methionine and were incubated with GST, GST-Sp1 or GST-Sp3-coupled glutathione–Sephadex beads as described in the Materials and methods section. Bound proteins were analysed by SDS/PAGE and autoradiography. Input represents 20% of the initial protein sample used in the binding experiments. The arrows show the electrophoretic position of the bound p73 proteins.

of ubiquitous transcription factors, Sp1 and Sp3, and that the C-terminal 101 amino acids of 53 were found to be essential for its physical interaction with both factors. A summary of the interaction data is shown in Figure 3(A).

Physical interactions between Sp family proteins and additional members of the p53 family, such as p73 α and p73 β , were also investigated by GST pull-down assays. For this purpose, the two p73 isoforms were produced by *in vitro* TnT in the presence of L-[³⁵S]methionine and allowed to interact with GST–Sp1, GST–Sp3 or GST alone expressed in bacteria and coupled to glutathione–Sephadex beads. As shown in Figure 4, specific physical interactions between Sp1 and Sp3 with both p73 α and p73 β were observed. p73 α interacted more strongly with Sp3 than with Sp1, whereas the reverse was true for p73 β . The physical interactions between Sp and p73 proteins could account for the transcriptional co-operativity between them in the transactivation of the p21^{Cip1} promoter (Figure 1E).

We next determined the transcriptional activity of each p53 mutant in transactivation assays using the human –2325/+8 p21 promoter. As shown in Figure 5(A), overexpression of wild-type human p53 in COS-7 fibroblasts caused a strong transactivation of the p21^{Cip1} promoter (23-fold). This transactivation was attenuated by internal deletions that removed the entire or part of the TAD of p53 (1.3-fold and 4.5-fold transactivation by the p53 mutants Δ 3–74 and Δ 34–78 respectively), whereas an internal deletion outside the TAD (p53 mutant Δ 13–19) had a less severe effect (16-fold transactivation). Deletions that removed different segments of the C-terminal region of p53 were also analysed for their ability to transactivate the p21^{Cip1} promoter in COS-7 cells. As shown in Figure 5(A), the internal deletions that removed a 22-amino-acid region immediately N-terminal to the tetramerization domain (p53 mutant Δ 300–321) or that removed the regulatory domain (p53 mutant Δ 363–393) had a minor effect on the transactivation of the p21^{Cip1} promoter (20-fold and 18-fold respectively). In contrast, deletion of the C-terminal 101 amino acids of p53 (p53 mutant Δ 293–393) drastically reduced, although not totally abolished, the transcriptional activity of this protein (3.4-fold transactivation). The differences in the transcriptional activity of the various p53 mutants could not be attributed to differences in the levels of expression of these proteins in COS-7 cells, as shown by the immunoblotting analysis of Figure 5(B).

To investigate further the importance of the C-terminal 101 amino acids of p53 (amino acids 293–393) for its functional co-operation with Sp1, transcriptional synergism assays were performed in *Drosophila* Schneider SL2 cells. In this set of experiments, a truncated p21^{Cip1} promoter–luciferase construct consisting of the proximal cluster of Sp1/Sp3-binding sites

Figure 5 Transactivation of the p21^{Cip1} promoter by wild-type and mutant p53 proteins

(A) COS-7 cells were transfected with the (–2325/+8) p21-Luc reporter plasmid (2 μ g) along with expression vectors for the indicated wild-type (wt) or mutant p53 proteins (1 μ g). Each sample included the CMV (cytomegalovirus)– β -gal plasmid expressing β -galactosidase (1 μ g) for normalization of transfection variability. The normalized relative luciferase activity of at least two independent experiments performed in duplicate is shown. (B) Monitoring the expression of wild-type (wt) and mutant p53 proteins in COS-7 cells by immunoblotting. COS-7 cells (100-mm-diameter dishes) were transfected with the expression vectors for the proteins indicated or were not transfected (–). Total extracts from COS-7 cells were subjected to SDS/PAGE and Western blotting (WB) using a monoclonal anti-p53 antibody. The position of the wt p53 is shown with an arrow and of the mutant p53 forms with a bracket. The asterisk shows a p53 mutant (Δ 3–74) that could not be detected by the anti-p53 antibody owing to the lack of the epitope that this antibody recognizes. The band in the untransfected cells and in cells transfected with the p53 (Δ 3–74) mutant corresponds to endogenous p53 protein which is also recognized by the anti-p53 monoclonal antibody.

(–215/+8), as well as the same promoter fragment linked to the far upstream (–2325/–2260) p53-binding site of the p21^{Cip1} promoter (Figure 6A) [31] were transfected into *Drosophila* Schneider SL2 cells alone or in the presence of expression vectors for Sp1, p53 and the p53 mutants Δ 363–393 and Δ 293–393. As shown in Figure 6(B), Sp1 expressed alone transactivated efficiently the proximal –215/+8 p21 promoter that lacks p53-binding sites, whereas p53 inhibited the Sp1-mediated transactivation of this promoter by 60%. Similar inhibition was observed using the p53 Δ 363–393 mutant lacking the C-terminal 31 amino acids. In sharp contrast, p53 mutant Δ 293–393 that lacks the 101 C-terminal amino acids could not inhibit the Sp1-mediated transactivation of the p21^{Cip1} promoter in *Drosophila* Schneider SL2 cells (Figure 6B). On the other hand, p53 and the Δ 363–393 mutant transactivated the p21^{Cip1} promoter bearing the distal p53-binding site close to the proximal Sp1/Sp3-binding sites independently of Sp1, whereas co-expression of Sp1 with

Figure 6 Importance of the C-terminal 101 amino acids of p53 for functional co-operativity with Sp1

(A) Schematic representation of the p21^{Cip1} promoter constructs used in the transactivation experiments of (B). Symbols are as in Figure 1(A). (B) *Drosophila* Schneider SL2 cells were transiently transfected with the (-215/+8) p21-Luc or the (-2325/-2260, -215/+8) p21-Luc constructs (2 µg) along with *Drosophila*-specific expression vectors for Sp1 (25 ng) independently or in combination with a vector for wild-type (wt) human p53 protein (1 µg), or the p53 mutants Δ363–393 and Δ293–393 protein (1 µg) as indicated. The hsp-lacZ plasmid (1 µg) was included in each sample for normalization of transfection variability. Luciferase activity was determined in cell lysates 48 h after the transfection and the values (± S.E.M.) from at least two independent experiments performed in duplicate are shown as percentage relative luciferase activity.

both p53 forms resulted in a strong synergistic transactivation of the p21^{Cip1} promoter (Figure 6B). In contrast, the p53 mutant Δ293–393 could not transactivate the p21^{Cip1} promoter independently or synergistically with Sp1 (Figure 6B).

In summary, the results shown in Figure 6 are in agreement with the findings shown in Figures 3 and 5, and confirm the importance of the C-terminal part of p53 (amino acids 293–393) for its physical and functional interactions with Sp1 and the transactivation of the p21^{Cip1} promoter.

Domains of Sp1 that mediate physical interaction with p53

To identify domains of Sp1 that are involved in physical interactions with p53, individual functional domains of Sp1 were cloned in-frame with GST and utilized in GST pull-down assays (Figure 7A). The domains of Sp1 that were fused with GST were the two glycine- and serine/threonine-rich TADs A (amino acids 83–262) and B (amino acids 263–542), the regulatory domain C (amino acids 543–610), the DBD (amino acids 611–701) and

the C-terminal domain D (amino acids 702–778). All the above GST-fusion proteins were expressed in *E. coli* and were coupled to glutathione–Sepharose beads (Figure 7B). As shown in Figure 7(C), *in vitro* transcribed and translated and ³⁵S-labelled wild-type p53 protein interacted with wild-type Sp1 (amino acids 83–78), as well as with individual domains A, B and the DBD. The strongest interaction was observed between p53 and domain A, whereas the weak interaction of p53 with the DBD of Sp1 could be attributed at least in part to the low levels of expression of this mutant Sp1 form in bacteria (Figure 7B).

Physical interactions between p53 and individual Sp1 domains were also investigated *in vivo*. For this purpose, mammalian expression vectors bearing Sp1 cDNA fragments corresponding to domains A, B and D fused with GST were transfected into HEK-293T cells along with wild-type p53. Extracts from the transfected cells were incubated with glutathione–Sepharose beads, and bound proteins were analysed by SDS/PAGE (10.5 % polyacrylamide) and immunoblotting using an anti-p53 antibody. As shown in Figure 7(D), wild-type p53 interacted strongly with Sp1 domain A and weakly with Sp1 domain B, but did not interact with Sp1 domain D, in accordance with the *in vitro* GST pull-down assays shown in Figure 7(C).

To confirm the interaction of p53 with multiple domains of Sp1, GST pull-down assays were performed using a p53 protein bearing a His₆-epitope tag at the N-terminus. The His₆-p53 protein was expressed in bacteria, purified to homogeneity by Ni-NTA affinity chromatography (Figure 7E) and was allowed to interact with GST, GST–Sp1-(83–778), GST–Sp1-(83–262) and GST–Sp1-(263–542) proteins. The interaction was monitored by immunoblotting using a monoclonal antibody raised against the His₆-epitope tag. As shown in Figure 7(F), affinity-purified wild-type p53 interacted specifically and efficiently with full-length Sp1 (amino acids 83–778), as well as with individual Sp1 domains A (amino acids 83–262) and B (amino acids 263–542), thus confirming the results shown in Figures 7(C) and 7(D). Further dissection of domain B of Sp1 showed that the serine/threonine-rich subregion of this domain, defined by residues 263–424, is sufficient by itself to mediate physical interaction with affinity-purified p53 (Figure 7G).

In a parallel experiment, the DBD of Sp1 fused with the His₆-epitope tag was expressed in bacteria, purified by Ni-NTA affinity chromatography (Figure 7H) and allowed to interact with GST or GST–p53 proteins. Again, the interaction was monitored by immunoblotting using a monoclonal antibody raised against the His₆-epitope tag. As shown in Figure 7(I), the affinity-purified DBD of Sp1 specifically and efficiently interacted with wild-type p53, thus confirming the results of Figure 7(C).

In summary, the results presented in Figure 7 indicate that p53 interacts with multiple domains of Sp1, including the two TADs A and B, as well as the DBD.

To investigate the contribution of individual domains of Sp1 to its functional synergism with p53 in the transactivation of the p21^{Cip1} promoter, deletion mutants of Sp1 lacking specific domains of the protein were transfected into *Drosophila* Schneider SL2 cells alone or together with p53, and their transactivation properties were monitored by luciferase assays using the (-2325/+8) p21-Luc vector as a reporter. The mutant Sp1 proteins that were utilized in these assays include Sp1 ΔC, ΔB + C and ΔA lacking domains C, B + C and A respectively (Figure 8A). The expression of wild-type Sp1, as well as of the three mutant forms in *Drosophila* Schneider SL2 cells was monitored by a gel EMSA using extracts from transfected *Drosophila* Schneider SL2 cells and a radiolabelled double-stranded oligonucleotide corresponding to Sp1/Sp3 site 3 (-86/-70) of the proximal p21^{Cip1} promoter [39] (Figure 8B). As shown in Figure 8(C), Sp1 ΔC mutant, which

Figure 7 Domains in Sp1 involved in physical interactions with p53 *in vitro*

(A) Schematic representation of the GST–Sp1 fusion proteins utilized in the GST pull-down experiments of (C), (F) and (G). Abbreviations and shadings are as in Figure 1(B). (B) Expression profile of the GST and the GST–Sp1 fusion proteins. GST and GST–Sp1 fusion proteins were expressed in *E. coli* DH5 α cells as described in the Materials and methods section. Following coupling to the glutathione–Sepharose beads and extensive washing, an aliquot of the beads was analysed for the presence of bound GST and GST–Sp1 proteins by SDS/PAGE and staining with Coomassie Brilliant Blue. The relative position of molecular-mass standards is shown on the left (in kDa). (C) Wild-type human p53 protein was transcribed and translated *in vitro* in the presence of L-[35 S]methionine and was incubated with glutathione–Sepharose beads coupled with GST or the different GST–Sp1 fusion proteins as indicated. Bound proteins were analysed by SDS/PAGE and autoradiography. Input represents 20% of the initial protein sample used in the binding experiments. The relative position of molecular-mass standards is shown on the left (in kDa). (D) Physical interactions between p53 and Sp1 domains A and B *in vivo*. HEK-293T cells were transfected with the vectors expressing the indicated Sp1 domains fused to GST or the GST alone along with an expression vector for wild-type p53. Extracts from the transfected cells were incubated with glutathione–Sepharose beads, and bound proteins were analysed by SDS/PAGE and immunoblotting using an anti-p53 antibody (top panel). The expression of p53 and of the GST–Sp1 fusion proteins were monitored by immunoblotting using antibodies against p53 and GST respectively (middle and bottom panels). WB, Western blot. (E) Purification of His $_6$ -tagged human wild-type p53 protein from *E. coli*. The purification of His $_6$ -p53 was monitored by SDS-PAGE and Coomassie Brilliant Blue staining. M, molecular-mass standards (sizes in kDa). (F, G) Purified His $_6$ -tagged wild-type human p53 protein was incubated with glutathione–Sepharose beads coupled with GST or the different GST–Sp1 fusion proteins as indicated. Bound proteins were analysed by SDS/PAGE and immunoblotting using a monoclonal antibody specific for the His $_6$ -epitope tag. Input represents 20% of the initial protein sample used in the binding experiments. The position of the His $_6$ -tagged p53 protein is shown with an arrow. WB, Western blot. (H) Purification of His $_6$ -tagged DBD of human Sp1 protein from *E. coli*. The purification of His $_6$ -Sp1 DBD was performed as described in the Materials and methods section, and was monitored by SDS/PAGE and Coomassie Brilliant Blue staining. M, molecular-mass standards (sizes in kDa). (I) Purified His $_6$ -Sp1 DBD was incubated with glutathione–Sepharose beads coupled with GST or GST–p53 as indicated. Bound proteins were analysed by SDS/PAGE and immunoblotting using a monoclonal antibody specific for the His $_6$ -epitope tag. Input represents 20% of the initial protein sample used in the binding experiments. The position of the His $_6$ -tagged Sp1 DBD protein is shown with an arrow. WB, Western blot.

lacks the regulatory domain C, transactivated the (–2325/+8) p21 promoter approx. 8 times more efficiently than wild-type Sp1 when transfected alone in *Drosophila* Schneider SL2 cells, and 3 times more efficiently than wild-type Sp1 when the two proteins were co-transfected with p53. Sp1 mutant $\Delta B + C$ transactivated the p21^{Cip1} promoter 3 times more efficiently than wild-type Sp1 in the absence of p53, whereas, in the presence of p53, transactivation was comparable with that of wild-type Sp1. Finally, the Sp1 mutant ΔA , which bears an internal deletion

of domain A, had very low transcriptional activity even in the presence of p53, in agreement with previous findings [45] (Figure 8C).

The findings shown in Figure 8 indicate that domain A of Sp1, which interacts physically with p53, is essential for its transcriptional activity and its functional synergism with p53 in the transactivation of the p21^{Cip1} promoter, whereas domain C, which does not interact physically with p53, seems to play an inhibitory role in both processes.

Figure 8 Contribution of individual domains of Sp1 to its functional synergism with p53 in the transactivation of the p21^{Cip1} promoter

(A) Schematic representation of wild-type Sp1 and the mutated forms used in the DNA-binding and the transactivation experiments of (B) and (C). Abbreviations are as in Figure 1(B). (B) Binding of wild-type and mutant Sp1 forms expressed in *Drosophila* Schneider SL2 cells to the proximal p21^{Cip1} promoter. A synthetic double-stranded oligonucleotide corresponding to the -86/-70 p21^{Cip1} promoter region was labelled with [α -³²P]dCTP and subjected to a gel EMSA using extracts from transfected *Drosophila* Schneider SL2 cells as described in the Materials and methods section. The Sp1-DNA complexes were analysed by native PAGE and detected by autoradiography. (C) *Drosophila* Schneider SL2 cells were transiently transfected with the (-2325/+8) p21-Luc construct (2 μ g) along with *Drosophila*-specific expression vectors for wild-type Sp1 or the Sp1 mutant forms (25 ng), as indicated beneath the histogram, or were not transfected (-), in the absence or the presence of an expression vector for wild-type human p53 (1 μ g). The hsp-lacZ plasmid (1 μ g) was included in each sample for normalization of transfection variability. Luciferase activity was determined in cell lysates 48 h after the transfection and the values ($\pm$ S.E.M.) from at least two independent experiments performed in duplicate are shown as percentage relative luciferase activity.

DISCUSSION

In the present study, we investigated the mechanism of transcriptional synergism between Sp family proteins and members of the p53 family of tumour-suppressor proteins. Such a mechanism could play an essential role in the p53-mediated transcriptional stimulation of genes containing binding sites for both classes of transcription factors in their promoters, as demonstrated in the case of the cell-cycle inhibitor p21^{Cip1} gene (Figure 1) and the pro-

apoptotic PUMA gene (Figure 2). The promoters of these two genes have a similar arrangement of p53- and Sp-binding sites, i.e. they both contain a cluster of GC-rich motifs flanked by two p53-recognition motifs at the 5' end (Figures 1A and 2A). In the case of the p21^{Cip1} promoter, the p53 sites are located at distal regions relative to the proximal Sp sites, suggesting that the intervening sequences may have to loop out in order to facilitate Sp-p53 interactions. In support of this hypothesis, we observed that p53 can activate an artificial p21^{Cip1} promoter containing the distal (-2325/-2260) p53 site placed next to the proximal (-215/+8) region in *Drosophila* Schneider SL2 cells in the absence of Sp factors, whereas it is unable to do so when the site is present in its natural position (Figure 6). Thus, in the case of the p21^{Cip1} promoter, p53-Sp interactions may be required in order to facilitate the recruitment of p53 to the proximal promoter region and its association with the basal transcription machinery.

Sp1 binds to DNA and activates transcription as an oligomer, and domain D of Sp1 promotes the functional co-operation between Sp1 molecules on promoters that contain multiple Sp1-binding sites, such as the p21^{Cip1} and the PUMA promoters [46]. Indeed, we have shown that Sp1 proteins activate the p21^{Cip1} promoter in a co-operative manner, whereas deletion of domain D abolished this co-operativity [39]. p53 proteins also bind to DNA as homotetramers (as dimers of dimers), and DNA binding is facilitated by the presence of two inverted pairs of the consensus DNA sequence 5'-RRRC(A/T)-3' in the promoters of target genes [10]. The possibility of the formation of multiprotein complexes consisting of oligomers of Sp1 and p53 is supported by our finding that p53 can interact with multiple domains of Sp1, including the two TADs A and B as well as the DBD (Figure 7). Whether domains A, B and DBD of Sp1 form an extended contact surface for p53 because of their proximity in space remains to be determined by crystallographic analyses. Alternatively, independent contacts of p53 with different domains of Sp1 could occur in the context of multiprotein complexes consisting of oligomers of each protein as proposed above.

In addition to p53, we found that Sp factors can interact physically and functionally with recently described additional members of the p53 family, such as p63 and p73 (Figures 1 and 4). This family, which includes several splicing isoforms of only three members (p53, p63 and p73), is characterized by a conservation of sequences within the TAD, the DBD and the oligomerization domain [16-18]. In p63 and p73 C-terminal regions, a second TAD [19], as well as a SAM that may be involved in protein-protein interactions [20,21], have been described. Interaction of Sp factors with additional members of the p53 family may have important regulatory roles in different cell types that differentially express the three genes [15].

In the present study, by utilizing a large panel of p53 and Sp1 mutants, we identified the domains in the two factors that mediate their physical and functional interactions *in vitro*, as well as *in vivo*. We found that the C-terminal region of p53, which includes the tetramerization domain of this protein, is essential for its optimal interaction with Sp1 and Sp3 (Figure 3). Our data can be interpreted as follows: (i) the oligomerization domain of p53 is involved directly in protein-protein interactions with Sp1 and Sp3; (ii) oligomerization of p53 is crucial to allow a different region of p53, possibly the C-terminal part of its DBD (amino acids 160-293), to interact with Sp1 or Sp3. The second hypothesis is in agreement with the data of Laguerre et al. [47] who showed that a region of p53 defined by amino acids 145-292 is capable of interacting physically with Sp1.

A number of intriguing observations were made by analysing a small set of Sp1 mutants for their ability to transactivate the p21^{Cip1} promoter independently or co-operatively with p53 (Figure 8).

This analysis showed that the deletion of domain C of Sp1 strongly enhanced its transactivation properties without affecting its synergism with p53. This finding suggested that domain C of Sp1 plays an inhibitory role, possibly by interfering with the transactivation functions of the adjacent domains A and/or B. Notably, an inhibitory domain that is a target for acetylation and sumoylation was identified in the similar region of Sp3 protein [48,49]. Given the extremely high levels of transactivation of the p21^{Cip1} promoter by a combination of p53 and the Sp1 Δ C mutant, we speculate that this mutant could be a useful tool for future anti-cancer therapies that are based on the transcriptional activation of p53 target genes such as p21^{Cip1}.

Another interesting observation was that p53 could not trans-activate the p21^{Cip1} promoter synergistically with the Sp1 mutant that lacks only domain A. This finding suggested that Sp1 does not serve as a mere attachment surface for p53 by facilitating its recruitment to the proximal region of the p21^{Cip1} promoter, but rather it plays an active role in the synergistic transactivation mechanism.

Based on the findings both of the present study and of previous studies [31,39], we propose that Sp1 and Sp3, and possibly other members of this family that may be found to interact with p53, are of equal importance to the decision of cells to cell-cycle arrest or apoptosis during p53 activation. We showed recently that deletion of the proximal Sp1/Sp3-binding sites of the proximal p21^{Cip1} promoter abolished the positive response of this promoter to the chemotherapeutic drug 5-FU [50]. We also found that mithramycin A, a DNA intercalator with preference for G/C-rich regions and an inhibitor of Sp1 DNA binding, drastically activated p53, without a concomitant increase in p21^{Cip1} gene expression in human hepatoma HepG2 cells [50]. Understanding in detail how members of the p53 and Sp1 families of transcription factors interact and work together in the p53-mediated cellular responses will be very important in the future design of molecules (small peptides or chemical inhibitors) that target these interactions. The potential utilization of such molecules in combination with established p53-activating drugs may open new horizons in cancer chemotherapy.

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