The EMBO Journal Vol.18 No.24 pp.7002–7010, 1999

Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2

Nicolas Dumaz and David W.Meek[1]

Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, UK

[1]Corresponding author
e-mail: meek@icrf.icnet.uk

The p53 tumour suppressor protein is a labile transcription factor that is activated and stabilized in response to a wide range of cellular stresses, through a mechanism involving disruption of its interaction with MDM2, a negative regulatory partner. Induction of p53 by DNA damage additionally involves a series of phosphorylation and acetylation modifications, some of which are thought to regulate MDM2 binding. Here we report the effects of introducing mutations at several known or putative N-terminal phosphorylation sites on the transactivation function of p53. These studies highlight phosphorylation of Ser15, a key phosphorylation target during the p53 activation process, as being critical for p53-dependent transactivation. Biochemical data indicate that the mechanism by which phosphorylation of Ser15 stimulates p53-dependent transactivation occurs through increased binding to the p300 coactivator protein. The data also indicate that Ser15-dependent regulation of transactivation is independent of any involvement in modulating MDM2 binding, and that Ser15 phosphorylation alone is not sufficient to block the p53–MDM2 interaction.

Keywords: HDM2/p53/p300/phosphorylation/transactivation

Introduction

The p53 tumour suppressor protein is a latent and highly labile transcription factor that is activated by a wide range of cellular stresses including DNA damage (for reviews, see Levine, 1997; Prives and Hall, 1999). Following activation, p53 coordinates a change in the balance of gene expression leading to growth arrest or apoptosis, events that prevent the growth or survival of damaged cells. p53 is thought to stimulate transcription, at least in part, through its interaction with the multi-subunit transcription factor TFIID, and can directly contact components of this complex including the TATA-binding protein (TBP; Seto *et al.*, 1992; Chen *et al.*, 1993; Liu *et al.*, 1993; Mack *et al.*, 1993; Martin *et al.*, 1993; Truant *et al.*, 1993), $TAF_{II}31$ (Lu and Levine, 1995), $TAF_{II}40$ and $TAF_{II}60$ (Thut *et al.*, 1995; Farmer *et al.*, 1996). Interaction with each of these proteins occurs principally through the N-terminus of p53 (Liu *et al.*, 1993; Lu and Levine, 1995; Thut *et al.*, 1995). The p300/CBP proteins are also involved in p53 transactivation function, again through interaction with the N-terminus of p53 (Gu *et al.*, 1997; Lill *et al.*, 1997; Scolnick *et al.*, 1997). Moreover, p300 is thought to play a central role in mediating p53-dependent growth arrest and apoptosis (Avantaggiati *et al.*, 1997; Yuan *et al.*, 1999).

p53 is tightly controlled through a complex series of events including translational regulation, interaction with regulatory proteins such as MDM2 and 14-3-3, and a series of post-translational modifications including multi-site phosphorylation and acetylation (for reviews, see Giaccia and Kastan, 1998; Meek, 1999; Prives and Hall, 1999). p53 can be modified by a variety of different protein kinases and acetylases *in vitro* and, in some cases, in cultured cells. Several serines are phosphorylated in the C-terminal part of the protein, and phosphorylation (or in one case dephosphorylation) of these different sites can activate independently the site-specific DNA-binding function of the protein (Hupp *et al.*, 1992; Takenaka *et al.*, 1995; Wang and Prives, 1995; Lu *et al.*, 1997; Waterman *et al.*, 1998). Acetylation of Lys320 by PCAF, and Lys373 and/or Lys382 by p300/CBP, is also thought to contribute to the activation of site-specific DNA-binding function (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). The N-terminus of p53 contains its transcriptional activation domain (amino acids 1–42) and includes sites for phosphorylation *in vitro* by DNA-PK (Ser15 and Ser37; Lees-Miller *et al.*, 1992), ATM (Ser15; Banin *et al.*, 1998; Canman *et al.*, 1998), ATR (Ser15 and Ser37; Tibbetts *et al.*, 1999) and the CDK7–cyclin H–p36MAT1 complex (Ser33; Ko *et al.*, 1997). Based on sequence similarity with murine p53, it also contains putative phosphorylation sites for CK1 (Ser6 and Ser9; Milne *et al.*, 1992) and JNK (Ser33; Milne *et al.*, 1995). Recent studies using phospho-specific antibodies have established that serines 15, 20, 33 and 37 are sites of *de novo* phosphorylation in cells following DNA damage (Shieh *et al.*, 1997, 1999; Siliciano *et al.*, 1997; Banin *et al.*, 1998; Canman *et al.*, 1998; Sakaguchi *et al.*, 1998). Phosphorylation of N-terminal serines 15, 33 and 37 has also been proposed to permit subsequent modification of the distant C-terminal lysine residues through enhanced recruitment of p300/CBP/PCAF (Lambert *et al.*, 1998; Sakaguchi *et al.*, 1998).

Diverse signals that activate p53 converge at a single critical interaction, that of p53 and its negative regulator MDM2. MDM2, through complex formation with the N-terminus of p53 (amino acids 19–26), both represses p53 transcriptional activity (Oliner *et al.*, 1993) and mediates the degradation of p53 through the ubiquitin pathway (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Interfering with the interaction between p53 and MDM2 leads to the accumulation of p53 protein and the subsequent initiation of p53-dependent processes such as transactivation, cell cycle arrest or apoptosis (Bottger *et al.*, 1997). Recent studies have proposed that DNA damage-induced

phosphorylation of p53 at Ser15 and/or Ser20 attenuates the p53–MDM2 interaction (Shieh *et al.*, 1997; Unger *et al.*, 1999a). However, the role of these modifications is not clear cut, as other laboratories have published evidence suggesting that phosphorylation of p53 is not essential for DNA damage-induced stabilization (Ashcroft *et al.*, 1999; Blattner *et al.*, 1999). A further possibility is that phosphorylation of p53 could contribute to p53 activation without necessarily inducing an increase in the level of the protein. In accordance with this idea, phosphorylation of Ser15 has been shown to enhance the association of p53 with CBP/p300 *in vitro* (Lambert *et al.*, 1998). However, the relevance of this observation *in vivo* has not yet been established.

In order to understand the role of phosphorylation of the N-terminus of p53 in a cellular context, we have analysed a series of mutations at known or putative phosphorylation sites. Owing to the complex and interactive nature of the post-translational modification of p53, we initially incorporated these changes into a fusion protein comprising the transactivation domain of human p53 and a heterologous DNA-binding domain, that of the yeast GAL4 protein. This approach has allowed us to examine the regulation of a single function of the p53 protein, i.e. transactivation, in isolation from the other domains of the p53 protein and without inducing the downstream effects of p53 such as growth arrest or apoptosis. We show that both in this context, and in the context of full-length p53, the transactivation activity of p53 depends on the phosphorylation of Ser15, which *in vivo* increases the affinity of p53 for the transcriptional coactivator p300. The data also indicate that modification of Ser15 alone is not sufficient for attenuating the p53–MDM2 interaction.

Results

Transcriptional activity of phosphorylation site mutants within the transcriptional activation domain of p53

In order to analyse the role of the phosphorylation sites in the N-terminal part of the p53 protein in detail, a series of plasmids was constructed, each expressing the transactivation domain of human p53 (amino acids 1–42) fused to the DNA-binding domain of the yeast GAL4 protein (amino acids 1–147) (Unger *et al*., 1992). These plasmids encoded either the wild-type p53 N-terminus or a series of mutants with single amino acid changes at residues 6, 9, 15, 18, 20, 33 and 37, or double changes at residues 6 and 9 (sites for CK1; Milne *et al*., 1992) and 15 and 37 (sites for DNA-PK and ATR; Lees-Miller *et al*., 1992; Canman *et al*., 1998; Tibbetts *et al*., 1999). The transcriptional activity of the mutants was compared with that of the wild-type protein following transient transfection into p53-null fibroblasts ($p53^{-/-}$) together with the reporter plasmid pG5E1bCAT (which contains five copies of the GAL4 target sequence element). As negative controls, the reporter plasmid pG5E1bCAT alone or pG5E1bCAT plus the GAL4–p53(1–13) plasmid (containing only the first 13 amino acids of p53) were used. Under both of these conditions, no detectable CAT activity was observed (Figure 1A and B). Most of the single (serines 6, 9, 20, 33 and 37, and threonine 18) or double

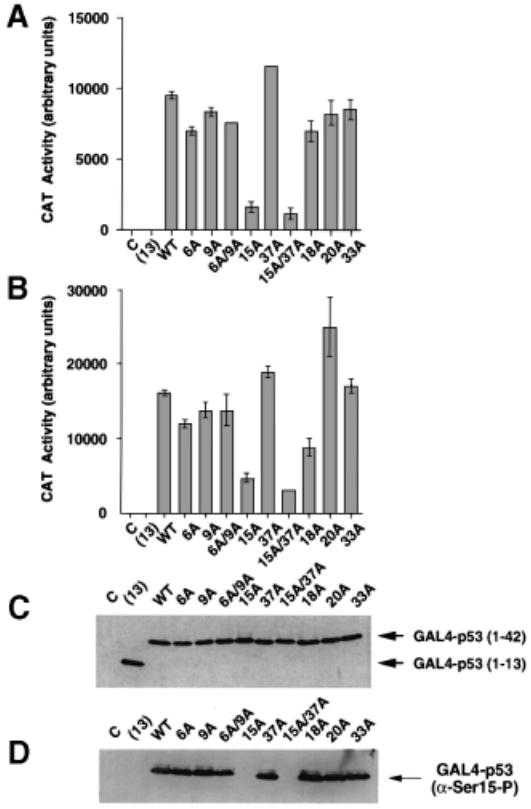

Fig. 1. Transcriptional activation by wild type or a series of phosphorylation site mutant GAL4–p53 proteins. p53-null murine fibroblasts ($p53^{-/-}$) (**A**) or COS-7 cells (**B**) were transfected with 1 μg of pG5E1bCAT and either no p53 (c), 50 ng of the GAL4–p53(1–13) plasmid or 50 ng of GAL4–p53(1–42) wild-type (WT) or mutant plasmids (as indicated under the data). A 50 ng aliquot of CMV-LUC was included to assess transfection efficiency. At 48 h after the beginning of transfection, the cells were harvested, lysed and assessed for luciferase and CAT activity. (**C**) Western blot analysis (using the GAL4 antibody) of COS-7 cells either untransfected (c) or transiently expressing GAL4–p53(1–13) or GAL4–p53(1–42) wild-type or mutant (as indicated above the panel), 24 h after transfection with 50 ng of the respective plasmid. (**D**) Western blot analysis (using the α-ser15P antibody) of COS-7 cells either untransfected (c) or transiently expressing GAL4–p53(1–13) or GAL4–p53(1–42) wild-type or mutant (as indicated above the panel), 24 h after transfection with 50 ng of the respective plasmid.

(serines 6–9) mutants showed transactivation activity that was indistinguishable from wild type (Figure 1A). However, mutation of Ser15 (15A) significantly reduced the transcriptional activity of the GAL4–p53(1–42) protein (Figure 1A). The double mutant, 15A/37A, showed the same activity as 15A alone. Similar observations were made when the mutants were expressed in other cell lines such as COS-7 (Figure 1B), the human p53-null line, SAOS-2 (data not shown) and the p53-competent line MCF-7 (data not shown), indicating that the effect could not be attributed to the influence of a particular cell line. Western analysis (Figure 1C) indicated that each of the mutant proteins was expressed efficiently to a level comparable to the wild-type protein.

Transient transfection of DNA represents an effective DNA damage signal, which stimulates a significant p53 induction (Renzing and Lane, 1995; Huang *et al*., 1996). The use of an anti-phosphoserine 15 antibody confirmed that Ser15 was phosphorylated in all but the p53 proteins where this residue was substituted (Figure 1D). Moreover,

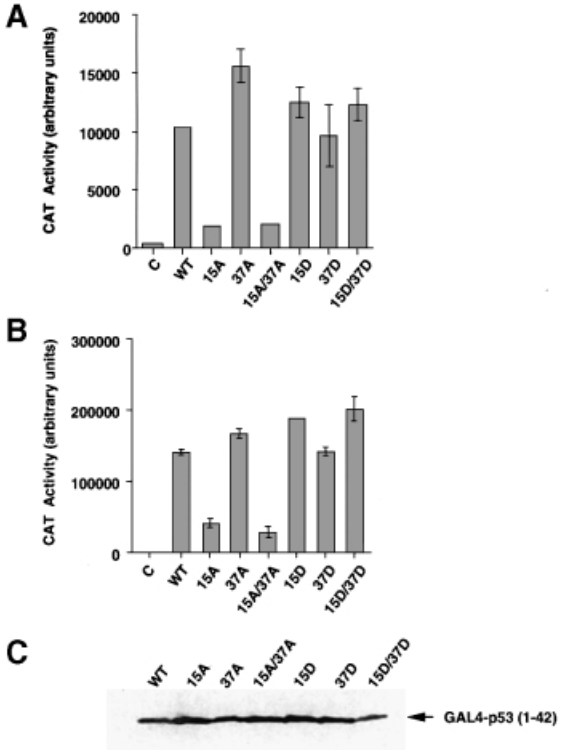

Fig. 2. Transcriptional activation by wild-type or Ser15/Ser37 phosphorylation site mutant GAL4–p53 proteins. $p53^{-/-}$ (**A**) or COS-7 (**B**) cells were transfected with 1 μg of pG5E1bCAT and either no p53 (c), 50 ng of GAL4–p53(1–13) or 50 ng of GAL4–p53(1–42) wild-type (WT) or mutant (as indicated under the data). A 50 ng aliquot of CMV-LUC was included to assess transfection efficiency. At 48 h after the beginning of transfection, the cells were harvested, lysed and assessed for luciferase and CAT activity. (**C**) Western blot analysis (using the GAL4 antibody) of COS-7 cells either untransfected (c) or transiently expressing GAL4–p53(1–42) wild-type or mutant (as indicated above the panel), 24 h after transfection with 50 ng of the respective plasmid.

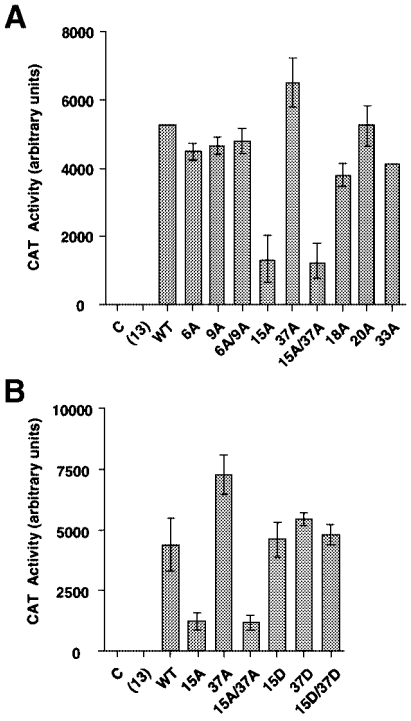

Fig. 3. Transcriptional activation by wild-type or phosphorylation site mutant GAL4–p53 proteins in p53-null, MDM2-null murine fibroblasts. $p53^{-/-}/mdm2^{-/-}$ cells were transfected with 1 μg of pG5E1bCAT and either no p53 (c), 50 ng of GAL4–p53(1–13) or 50 ng of GAL4–p53(1–42) wild-type (WT) or mutant. A 50 ng aliquot of CMV-LUC was included to assess transfection efficiency. At 48 h after the beginning of transfection, the cells were harvested, lysed and assessed for luciferase and CAT activity. The effects of Ser or Thr to Ala mutants are shown in (**A**), while the data with the Ala or Asp substitutions at positions 15 and 37 are shown in (**B**).

when the plasmids expressing the GAL4–p53 fusions were transfected into MCF-7 cells, Western analysis confirmed that the endogenous p53 was induced under these conditions (data not shown). These data indicate that the mechanism(s) responsible for inducing p53 were indeed active under our experimental conditions.

The loss of activity observed with the 15A mutant is consistent with the loss of an activating phosphorylation site. To explore this possibility, Ser15 was also changed to aspartate, thus substituting a constitutive negative charge at this site (since both Ser15 and Ser37 are substrates for DNA-PK and ATR, aspartate substitutions at residues 37 and 15/37 were also made). The transcriptional activities of the aspartate mutants 15D, 37D and 15D/37D were compared with that of the wild type and of the alanine mutants 15A, 37A and 15A/37A in transient transfection in $p53^{-/-}$ (Figure 2A) and COS-7 cells (Figure 2B). The 15D mutant restored the activity of the protein in both cell lines, underscoring the need for a negative charge at this residue for the full transcriptional activity of the protein. Although Ser15 and Ser37 are substrates for the same kinases, the mutations 37A and 37D did not change the activity of the protein significantly. Western analysis (Figure 2C) confirmed that each mutant protein was expressed efficiently to a level comparable to the wild-type protein. Taken together, these data support the idea that the phosphorylation of Ser15 activates the transactivation function of p53.

Interaction of the phosphorylation site mutants with MDM2 in cultured cells

Recent evidence has indicated that phosphorylation of Ser15 and Ser37 can attenuate the interaction between p53 and its inhibitor MDM2 *in vitro* (Shieh *et al.*, 1997). To test whether the lower transcriptional activity of the 15A mutant resulted from increased sensitivity to inhibition by the endogenous MDM2, the wild-type and mutant proteins were expressed in fibroblasts derived from $p53^{-/-}/mdm2^{-/-}$ double knock-out mice (Figure 3). In these cells, just as with the $p53^{-/-}$ cells, the 15A mutation significantly decreased the activity of the GAL4–p53(1–42) protein, whereas the 15D mutation restored the activity of the protein (Figure 3). These data indicate that the activity of the 15A mutant is independent of the endogenous MDM2.

To analyse further the role of Ser15 and Ser37 in the interaction between p53 and MDM2 in our system, the ability of HDM2 (the human homologue of MDM2) to block the transactivation function of the wild-type and the mutant proteins, 15D or 15D/37D, was measured in transient assays. Similar analyses were carried out with the 15A mutant, but the experimental errors were high owing to the already low level of activity with this mutant. In these experiments, the GAL4–p53(1–42) vectors were co-transfected into COS-7 cells with increasing amounts

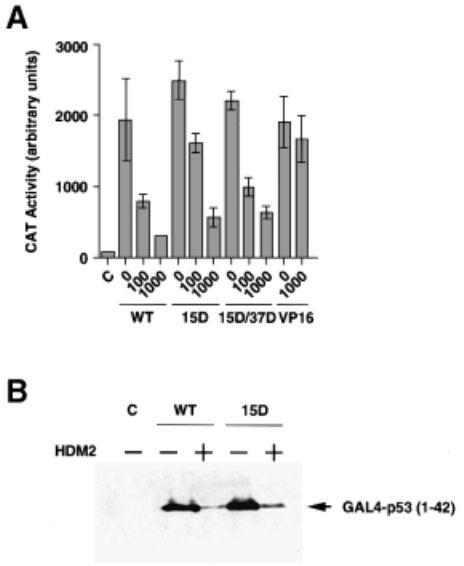

Fig. 4. Interaction of the phosphorylation mutant p53 proteins with MDM2 in cultured cells. (**A**) COS-7 cells were transfected with 1 μg of pG5E1bCAT and 50 ng of GAL4–p53(1–42) wild-type (WT) or mutant (as indicated under the graph) or 5 ng of the GAL4-VP16 plasmid. Where indicated, cells were co-transfected with either 100 ng (100) or 1 μg (1000) of a vector expressing HDM2. A 50 ng aliquot of CMV-LUC was included to assess transfection efficiency. At 48 h after the beginning of transfection, the cells were harvested, lysed and assessed for luciferase and CAT activity. (**B**) Western blot analysis (using the GAL4 antibody) of COS-7 cells either untransfected (c) or transfected with 100 ng of GAL4–p53(1–42) wild type or 15D together with (+) or without (–) 1 μg of a vector expressing HDM2. The cells were harvested 24 h after transfection.

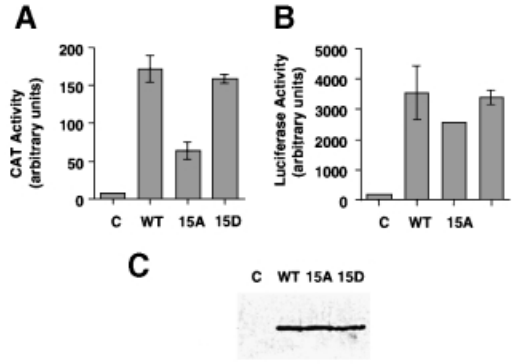

Fig. 5. Effect of mutation at Ser15 on the transcriptional activity of the full-length p53. (**A**) $p53^{-/-}$ cells were transfected with 1 μg of pWAF1CAT and 15 ng of CMV-p53 wild type, 15A or 15D. (**B**) $p53^{-/-}$ cells were transfected with 2 μg of pPG13LUC and 5 ng of CMV-p53 wild type, 15A or 15D. A 50 ng aliquot of CMV-LUC (A) or 150 ng of pCAT (B) were included to assess transfection efficiency. At 48 h after the beginning of transfection, cells were harvested, lysed and assessed for luciferase and CAT activity. (**C**) The levels of expression of full-length wild-type p53, the 15A and 15D mutants were measured by Western analysis using the DO1 antibody.

of a vector expressing HDM2. The data (Figure 4) showed that HDM2 inhibited wild-type p53 transcriptional activity in a dose-dependent manner. This inhibition was specific to p53 as the GAL4-VP16 protein was resistant to HDM2 inhibition. The substitution of aspartate residues at positions 15 and 37 did not significantly affect the susceptibility of the p53 protein to HDM2-mediated inhibition (Figure 4A).

Since MDM2 promotes the degradation of p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997), we tested whether the 15D mutation would render the GAL4–p53(1–42) protein resistant to HDM2-mediated degradation in cultured cells. Western analysis (Figure 4B) indicated that co-transfection of a plasmid expressing HDM2 induced a significant reduction of the GAL4–p53(1–42) steady-state levels and that the wild type and the15D mutant showed the same sensitivity to HDM2-mediated degradation. Taken together, these results indicate that although GAL4–p53(1–42) was sensitive to inhibition and degradation by HDM2, this sensitivity was not influenced by the substitution of aspartate for Ser15 (or Ser37).

Effect of mutation of Ser15 on the transcriptional activity of full-length p53

In order to determine whether modification of Ser15 could influence p53-dependent transactivation in a more physiological context, alanine and aspartate substitutions were made at residue 15 in full-length human p53. For this purpose, p53 was expressed under the control of the cytomegalovirus (CMV) immediate early promoter and, in order to achieve levels of expression comparable to the GAL4 vectors, 10-fold less CMV-p53 DNA was used in transfections. The CMV-p53 vectors expressing the wild-type or mutant p53 proteins (15A or 15D) were co-transfected with either the pWAF1CAT or the pPG13LUC reporter plasmids (Figure 5). The data show that substitution of alanine at position 15 resulted in a reduction in the capacity of full-length p53 to transactivate the WAF1 promoter, while p53 encoding aspartate at this position retained its transactivation function (Figure 5A). These data show that the effects of the Ser15 mutations are not artefacts arising from the presence of a heterologous DNA-binding domain. When the p53-dependent transactivation was measured in the context of the PG13 promoter, the impact of the Ser15 mutation was very low (Figure 5B), suggesting that there may be some promoter selectivity in the requirement for Ser15 modification. Western analysis confirmed that full-length wild-type p53 and the 15A and 15D mutants were expressed at similar levels in these experiments (Figure 5C).

Modulation of the ability of p53 to interact with components of the transcriptional machinery by phosphorylation on Ser15

The data in Figures 1 and 2 indicate that the low activity of the 15A mutant cannot be attributed to an enhanced instability of the protein. Therefore, to determine whether Ser15 phosphorylation could affect the ability to interact with transcriptional proteins, GST pull-down experiments were carried out using the first 42 amino acids of p53 fused to GST [GST–p53(1–42)]. DNA-PK, a protein kinase that phosphorylates human p53 on Ser15 and Ser37 *in vitro* (Lees-Miller *et al.*, 1992), was used to introduce phosphate groups at these residues. Figure 6 shows that recombinant GST–p53(1–42) was phosphorylated by DNA-PK in the presence of exogenous DNA *in vitro*. Mutations of Ser15 or Ser37 significantly decreased the phosphorylation of the protein by DNA-PK, while the double mutation 15A/37A completely abolished the phosphorylation by DNA-PK. This experiment confirmed that phosphorylation of the GST–p53(1–42) protein by

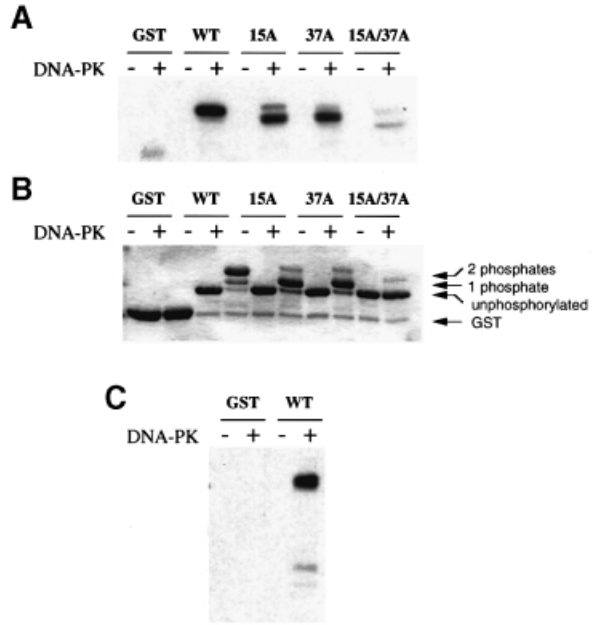

Fig. 6. Phosphorylation of GST–p53 by DNA-PK. (**A**) GST alone or GST–p53(1–42) wild type (WT) or mutant (as indicated in the figure) were phosphorylated (+) or not (–) with DNA-PK in the presence of [γ-^{32}P]ATP. (**B**) The Coomassie-stained gel from the autoradiograph in (A) showing a slower migrating form representative of 1 and 2 mol of phosphate incorporated. (**C**) GST alone or GST–p53(1–393) wild type were phosphorylated (+) or not (–) with DNA-PK in the presence of [γ-^{32}P]ATP. Stoichiometric phosphorylation was confirmed by measuring the amount of radioactive phosphate incorporated into the GST–p53(1–292) protein in this gel (data not shown).

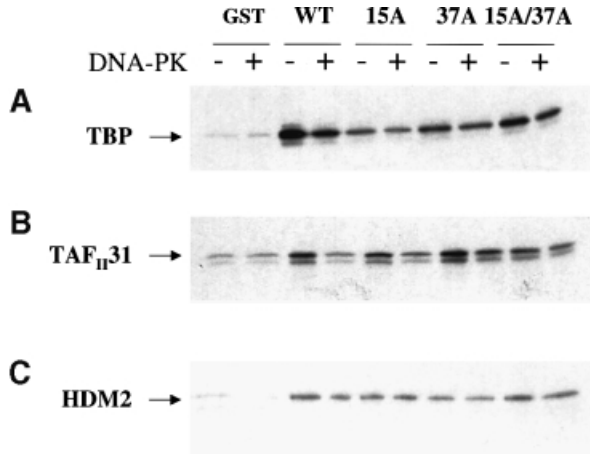

Fig. 7. Interaction of phosphorylated or unphosphorylated GST–p53(42) with TBP, TAF$_{II}$31 and HDM2. GST alone or GST–p53(1–42) wild type (WT) or mutant (as indicated in the figure) were stoichiometrically phosphorylated (+) or not phosphorylated (–) with DNA-PK. The ability of GST or phosphorylated or unphosphorylated GST–p53(1–42) to interact with ^{35}S-labelled TBP (**A**), TAF$_{II}$31 (**B**) or HDM2 (**C**) was assessed by GST pull-down.

DNA-PK was confined to Ser15 and Ser37. DNA-PK induced a shift in the migration of the GST–p53(1–42) protein in the stained SDS–gel, which allowed us to confirm that the proteins were phosphorylated stoichiometrically (i.e. 2 mol of phosphate per mol of protein, Figure 6B). GST–p53(1–393) was phosphorylated by DNA-PK under identical conditions to the GST–p53(1–42) protein. Phosphorylation of this full-length protein did not generate the striking mobility shift seen with the smaller protein. However, we were able to confirm stoichiometric phosphorylation of GST–p53(1–393) by measuring the incorporation of radioactive phosphate (using [γ-^{32}P]ATP of defined specific activity) into the band shown in Figure 6C (data not shown).

DNA-PK-phosphorylated GST–p53 proteins were tested for their ability to bind *in vitro* to ^{35}S-labelled TBP or TAF$_{II}$31, two key components of the transcriptional machinery with which p53 can interact (Seto *et al.*, 1992; Chen *et al.*, 1993; Liu *et al.*, 1993; Mack *et al.*, 1993; Martin *et al.*, 1993; Truant *et al.*, 1993; Lu and Levine, 1995). Complex formation between phosphorylated or unphosphorylated p53 and TBP or TAF$_{II}$31 proteins was determined by quantitating the amount of co-precipitated TBP or TAF$_{II}$31 protein. The data (Figure 7A) confirmed that the wild-type p53 transactivation domain interacts with TBP *in vitro*. Mutation of Ser15 slightly decreased the binding of p53 to TBP, whereas mutation of Ser37 had little effect on binding. Phosphorylation by DNA-PK did not stimulate this interaction and, if anything, weakened it slightly [this is consistent with a recent report by Pise-Masison *et al.* (1998)]. The interaction of the p53 transcriptional domain with TAF$_{II}$31 was studied using the same approach. While the wild-type p53 transactivation domain could bind to TAF$_{II}$31, mutations at Ser15 and/or Ser37 did not significantly modify this interaction (Figure 7B). However, phosphorylation of the Ser37 and, to a lesser extent, Ser15 caused a slight decrease in the binding of TAF$_{II}$31, which was more pronounced when both residues were phosphorylated. These data suggest that stimulation of transactivation by modification of Ser15 is not mediated by increased binding of p53 to either TBP or TAF$_{II}$31.

It has been reported that phosphorylation at Ser15 and Ser37 attenuates, *in vitro*, the interaction between p53 and its inhibitor MDM2 (Shieh *et al.*, 1997; Pise-Masison *et al.*, 1998). Our own data, in the cell culture system, show clearly that substitution of a constitutive negative charge at position 15, while able to restore effectively the transactivation function of p53, did not appear to change the ability of HDM2 to inhibit p53 transactivation or degradation (Figure 4). To study the effect of phosphorylation of Ser15 and Ser37 on the interaction between HDM2 and the N-terminal domain of p53, further GST pull-down analyses were carried out. The data show that, *in vitro*, unphosphorylated p53 and p53 that had been phosphorylated stoichiometrically on Ser15 and/or Ser37 bound HDM2 equally well (Figure 7C). These data suggest that Ser15 phosphorylation is not sufficient to interrupt the p53–HDM2 interaction.

These interactions were also analysed using full-length p53 [GST–p53(1–393)]. In this context, the data confirmed that phosphorylation of p53 on Ser15 and Ser37 did not significantly alter the binding of the protein to TBP (Figure 8A), TAF$_{II}$31 (Figure 8B) or HDM2 (Figure 8C).

Ser15 phosphorylation increased the affinity of p53 for p300 in cultured cells

An alternative mechanism by which Ser15 phosphorylation could regulate p53-dependent transactivation is by modulating the affinity of p53 for transcriptional coactivators such as p300/CBP. The transcriptional activity of p53

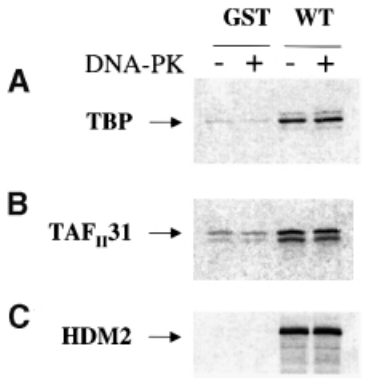

Fig. 8. Interaction of phosphorylated or unphosphorylated GST–p53(1–393) with TBP, $TAF_{II}31$ and HDM2. GST alone or GST–p53(1–393) wild-type were stoichiometrically phosphorylated (+) or not phosphorylated (–) with DNA-PK. The ability of GST, phosphorylated or unphosphorylated GST–p53(1–393) to interact with ^{35}S-labelled TBP (**A**), $TAF_{II}31$ (**B**) or HDM2 (**C**) was assessed by GST pull-down.

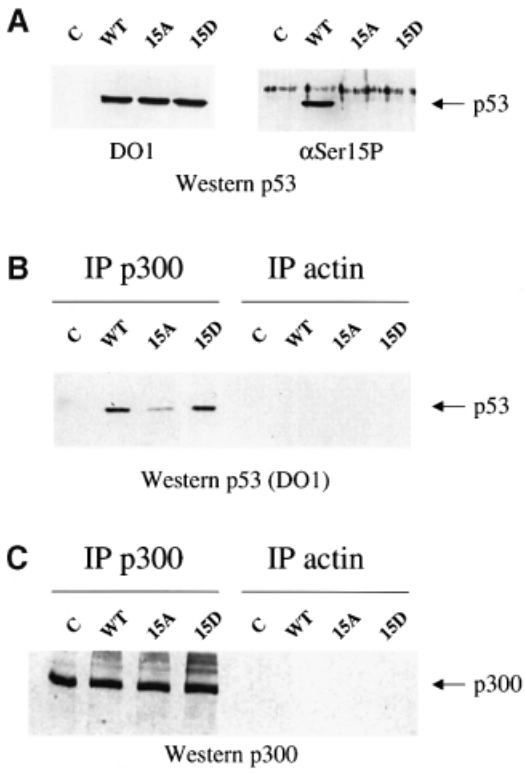

Fig. 9. Serine 15 phosphorylation increased the affinity of p53 for p300 in cultured cells. $p53^{-/-}$ cells were either untransfected (c), or transfected with 1 μg of CMV-p53 wild type, 15A or 15D. At 24 h post-transfection, the cells were harvested and lysed. (**A**) The expression level of the three proteins was analysed by Western blot (using the DO1 antibody) on equal aliquots of the lysates. The α-Ser15P antibody indicated that only the wild-type protein was phosphorylated at residue 15. (**B**) Half of each lysate was immunoprecipitated with an anti-p300 antibody (IP p300) and half with an anti-actin antibody (IP actin). The co-immunoprecipitated p53 was analysed by Western blot using the DO1 antibody. (**C**) Blots were reprobed with the anti-p300 antibody to assess the level of p300 immunoprecipitated.

has been shown to require the CBP/p300 coactivators (Avantaggiati *et al.*, 1997; Lill *et al.*, 1997; Scolnick *et al.*, 1997), and it has been shown recently that phosphorylation of p53 on Ser15 increases its interaction with CBP *in vitro* (Lambert *et al.*, 1998). To determine whether enhanced interaction of p53 with p300 could explain the requirement for a negative charge on Ser15 for the full activity of p53 *in vivo*, co-immunoprecipitation experiments were carried out. $p53^{-/-}$ cells were transfected with plasmids expressing either wild-type p53, 15A or 15D under the control of the CMV promoter. Western analyses indicated that the15A and 15D mutants were expressed at the same level as the wild-type protein, and that wild-type p53, but not the 15A or 15D mutants, was phosphorylated in the cell at Ser15 under these conditions (Figure 9A).

Immunoprecipitation of p300 indicated that the p300 levels were unaffected by transfection of either of the DNAs (Figure 9C). When the p300 was immunoprecipitated and the co-associated p53 measured by Western analysis, similar levels of the wild-type p53 and the 15D mutant were observed (Figure 9B). In contrast, very little of the 15A mutant protein was co-immunoprecipitated with p300 (Figure 9B). In control immunoprecipitations, no p300 or p53 was immunoprecipitated with an anti-actin antibody (Figure 9B and C). These data are consistent with the idea that modification of Ser15 can stimulate p53-dependent transactivation through increased association with p300.

Discussion

The p53 protein plays a critical role in the cellular response to a range of stresses including DNA damage. Activation of the p53 pathway can lead to cell cycle arrest or the induction of apoptosis, and the transcriptional transactivation function encoded within the N-terminus of p53 is important for both of these functions. In the present study, we have asked whether phosphorylation of p53 is essential for the transactivation function of the protein. To address this issue, we generated a series of mutations at known or putative sites of phosphorylation in human p53. In transient expression assays we showed that substitution of Ser15 with alanine lowered the transcriptional activity of p53, whereas substitution of asparate at this position restored the activity of the protein (Figures 1–5). This result can be demonstrated in multiple cell lines using either the transactivation domain of p53 fused to a heterologous DNA-binding domain, or the full-length protein. Together, these data demonstrate that a negative charge at position 15 is required for the full activity of the p53 protein, and are consistent with the idea that phosphorylation of Ser15 can stimulate p53-dependent transactivation *in vivo*. The data also indicated that there were no significant differences in the levels of expression or turnover of the mutant proteins compared with the wild-type protein. Moreover, the 15D mutant retained sensitivity to HDM2-dependent inhibition and degradation in a manner essentially similar to the wild-type protein. These experiments confirmed that the low transcriptional activity of the 15A mutant is independent of MDM2, and suggested that, since p53 must contact the transcriptional machinery in order to mediate its function, Ser15 modification could play a role in regulating association with key transcriptional components. Accordingly, we demonstrated that the 15D mutant shows enhanced ability to interact with the transcriptional coactivator p300 in cultured cells, whereas the 15A mutant interacts with this protein only very

weakly (Figure 9). p53 requires CBP/p300 to stimulate transcription of several responsive genes (Avantaggiati *et al.*, 1997; Lill *et al.*, 1997; Scolnick *et al.*, 1997) and, therefore, the increased affinity of the 15D mutant for p300 *in vivo* may explain the requirement for a negative charge on Ser15 for the full transcriptional activity of the protein. Moreover, recently published data indicate that phosphorylation of Ser15 stimulates the interaction of p53 with CBP *in vitro* (Lambert *et al.*, 1998). These data make a compelling case for the regulation of p53 transactivation function occurring through Ser15 phosphorylation-mediated interaction with p300/CBP.

Data from other laboratories have indicated that phosphorylation of Ser15 can reduce the ability of p53 to bind MDM2 *in vitro*, with the prediction that this might impair MDM2-mediated degradation *in vivo*, leading to stabilization of p53 (Shieh *et al.*, 1997; Pise-Masison *et al.*, 1998). Our own experiments have not detected any direct effect of Ser15 modification on the p53–MDM2 interaction either *in vitro* (Figures 3 and 4) or in the cell culture experiments (Figures 7 and 8) under conditions where we could show a clear effect of the modification on transactivation function. While we do not dispute that Ser15 phosphorylation may contribute to the regulation of MDM2 binding, these data underscore the idea that Ser15 modification alone may not be sufficient to block the interaction with MDM2. Other residues such as Ser20 have also been implicated in regulating MDM2 binding (Shieh *et al.*, 1999; Unger *et al.*, 1999a) and it is possible that Ser15 cooperates with these in blocking complex formation.

Several previous studies have examined the effects of mutating one or several phosphorylation sites within p53 on the biological activity of the protein, with varied results. Some analyses showed subtle alterations in the ability to modulate cell cycle progression or inhibition of cell growth. For example, simultaneous mutation of serines 9, 18 and 37 within the transactivation domain of murine p53 (which correspond to serines 6, 15 and 33 in human p53) significantly reduced the ability of p53 to suppress transformation of rat embryo fibroblasts transfected with E1A and Ras (Mayr *et al.*, 1995). In another study, overexpression of human p53S15A mutant protein resulted in partial failure compared with wild-type p53 to inhibit cell cycle progression (Fiscella *et al.*, 1993). More recently, Ser15 and Ser20 have been shown to play a role in p53-mediated apoptosis (Unger *et al.*, 1999b). These results, together with our own, support a physiological role for phosphorylation of Ser15 in the regulation of p53 activity. DNA damage-induced multi-site phosphorylation and acetylation of p53 is also a highly ordered and sequential process (Sakaguchi *et al.*, 1998; Shieh *et al.*, 1999), suggesting that these modifications have a key role in the activation mechanism. In contrast to these data, other reports have indicated that it is possible to strip p53 of many of its phosphorylation sites without significantly affecting either the induction or function of the protein (Ashcroft *et al.*, 1999; Blattner *et al.*, 1999). Consequently, there is still much uncertainty about the physiological roles of individual phosphorylation sites. We believe that both our approach and our data contribute significantly towards addressing this issue. For example, in using the transactivation domain of p53 fused to a heterologous DNA-binding domain, we were able to focus principally on regulation of a single function of p53 (i.e. transactivation), while excluding potential functional or regulatory contributions from other parts of the p53 molecule. Moreover, we were able to do this without inducing the downstream biological effects of p53 activation. This approach highlighted Ser15 as being critical for transactivation. Moreover, it showed that loss of Ser15 resulted in a 4- to 5-fold inhibition, but not a complete loss, of transactivation activity. Therefore, while such a change in the level of activity may well have significant effects on the balance of p53-dependent transactivation in the cell, it is entirely possible that other studies have missed this regulation, owing to either high levels of expression of the transfected p53 or contributions from other parts of the molecule that can assist or 'back-up' the transactivation domain.

Ser15 is emerging as a focal point for stress-targeted activation of p53. Based on our own data and those from other laboratories, it seems that the activation process at the N-terminus of p53 is highly structured and has several cooperating layers. The initiating event is phosphorylation of Ser15, which is mediated by the ATM protein kinase (Banin *et al.*, 1998; Canman *et al.*, 1998) and perhaps other related protein kinases, depending upon the activating stress (Tibbetts *et al.*, 1999). Subsequent N-terminal phosphorylation occurs at serines 20, 33 and 37 (Siliciano *et al.*, 1997; Sakaguchi *et al.*, 1998; Shieh *et al.*, 1999). Phosphorylation-dependent attenuation of the p53–MDM2 complex (Shieh *et al.*, 1997; Unger *et al.*, 1999a) is accompanied by increased recruitment of transcriptional coactivators such as p300 and CBP (Lambert *et al.*, 1998; the present study). These in turn not only cooperate directly in the transactivation process, but, once recruited, can acetylate key C-terminal residues involved in stimulating the site-specific DNA-binding function of p53 (Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). The challenge for the future will be to understand the temporal and functional nature of each of these modifications, how they cooperate to fine-tune the p53 activation process and how they may vary, depending on the type and strength of the activating signal, to tailor the outcome of the p53 response.

Materials and methods

Plasmids

Plasmids that express fusion proteins with the GAL4 DNA-binding domain (amino acids 1–147) and the first 42 or 13 amino acids of human p53 [GAL4–p53(1–42) and (1–13), respectively] were a gift from Dr T.Unger (Weizmann Institute, Israel) (Unger *et al.*, 1992). The GAL4-VP16 plasmid was a gift from Dr N.Perkins (University of Dundee, UK); this plasmid contains the activation domain of the herpes simplex virus in fusion with the GAL4 DNA-binding domain. Plasmids expressing p53 under control of the CMV promoter were obtained by cloning the full-length human p53 (wild type or mutant) into the vector pcDNA3 (Invitrogen). Three different reporter plasmids were used: pG5E1bCAT (a gift from Dr T.Unger, Weizmann Institute, Israel) comprises five GAL4 DNA-binding sites upstream of the adenovirus E1b promoter and linked to the CAT gene (Unger *et al.*, 1992); pWAF1CAT comprises the CAT reporter gene under the control of the WAF1 promoter (el Deiry *et al.*, 1993); and pPG13LUC consists of the polyomavirus early promoter and 13 copies of a synthetic consensus p53-binding site (el Deiry *et al.*, 1992) followed by the luciferase gene. All of the mutations were generated by oligonucleotide-directed mutagenesis using the method described by Deng and Nickoloff (1992).

Cell culture and transfections

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in an atmosphere

of 5% CO_2 in air. For transient transfection, 10^5 cells were plated per well in 6-well plates and transfected on the following day by calcium phosphate precipitation with an expression vector and a reporter construct. The exact amount of plasmid DNA used in each experiment is indicated in the figure legends. When required, carrier DNA was added to maintain a constant amount of DNA in each transfection mix. To assess the effects of MDM2, cells were co-transfected with different amounts of the pSG5 expression vector (Stratagene) containing the human homologue of MDM2 (HDM2) cDNA. To monitor transfection efficiency, transfections also included either CMV-Luc (kindly provided by Dr R.Kay, University of Dundee, UK) or pCAT (Promega) vectors. Cells were washed on the day following transfection and harvested after a further 24 h for Western blot or CAT and luciferase analyses. CAT activity was assayed as described previously (Hall *et al.*, 1996) and quantitated using the Bio-Rad GS-250 Molecular Imager and Molecular analysis software. Luciferase assays were performed according to the manufacturer (Promega). In the graphs, all points are displayed as the mean and standard deviation of triplicate assays.

Western blotting

To assess the level of expression of the p53 proteins, 10^5 p53-null fibroblasts or COS-7 cells were transiently transfected exactly as described above. At 24 h post-transfection, nuclear extracts were prepared by resuspending the cells in lysis buffer [20 mM HEPES–KOH pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Igepal], followed by incubation on ice for 10 min and centrifugation for 5 min at 800 *g*. The pellets were then resuspended in SDS sample buffer and the protein extracts were fractionated by SDS–10% PAGE and electrophoretically transferred to PVDF. Antibodies against p53 (DO1; Santa Cruz Biotechnology), p53 phosphorylated at Ser15 (α-ser15P; NEB) or GAL4 DBD (RK5C1; Santa Cruz Biotechnology) were used for immunochemical detection using chemiluminescence.

Expression and purification of GST–p53 proteins

The first 42 amino acids (wild-type or Ser15, and/or Ser37 mutated to alanine) of human p53 or the wild-type full-length protein were cloned into a pGEX-6P1 vector (Amersham). The GST–p53 fusion proteins, named GST–p53(1–42) (first 42 amino acids) or GST–p53(1–393) (full-length) were prepared from *Escherichia coli* DH5α cells induced for 2 h at 37°C with 0.5 mM isopropyl-β-D-thiogalactopyranoside. The bacteria were pelleted, resuspended in a solution of 50 mM Tris pH 7.5, 10% sucrose, 500 mM NaCl, 0.1 mg/ml lysozyme, 0.05% Igepal plus protease inhibitors (Protease Inhibitor Cocktail Set I; Calbiochem) and then lysed by sonication. After removal of the debris by centrifugation, the fusion protein was adsorbed onto glutathione–Sepharose 4B beads (Pharmacia Biotech). The beads were washed three times with 50 mM Tris pH 7.5 for GST–p53(1–42) or twice in 50 mM Tris pH 7.5, 1 M NaCl, 0.5% Igepal and once in 50 mM Tris pH 7.5 for GST–p53(1–393). The GST–p53 fusion protein was eluted from the beads by incubation in 50 mM Tris pH 7.5 and 5 mM reduced glutathione, and then dialysed overnight against a solution of 25 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol and 1 mM benzamidine.

Phosphorylation of p53 in vitro and in vitro association assays

Phosphorylation of p53 by DNA-PK was carried out according to the manufacturer's instructions (Promega) using 1–2 µg of the GST–p53 fusion protein as substrate. Control samples were treated following the same protocol but in the absence of the protein kinase. When labelling of the p53 was required [γ-^{32}P]ATP was included in the reaction mix at a specific activity of 250 Ci/mmol. To determine the ability of each mutant protein to bind TBP, $TAF_{II}31$ and HDM2 *in vitro*, GST pull-down assays were performed using *in vitro* translated proteins. For these experiments the human proteins were translated in the presence of [^{35}S]methionine using a reticulocyte lysate *in vitro* transcription/translation kit as directed by the manufacturer (Promega). The binding/washing buffers were as follows. TBP/HDM2 binding/washing buffer: 50 mM Tris pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 2 mM DTT plus protease inhibitors (Calbiochem). $TAF_{II}31$ binding buffer: 20 mM Tris pH 8.0, 0.5 mM EDTA, 20% glycerol, 60 mM KCl, 0.1% Igepal, 0.5 mM DTT plus protease inhibitors (Calbiochem); the $TAF_{II}31$ washing buffer was as above but with 100 mM KCl. Following phosphorylation, the GST–p53 proteins were adsorbed onto glutathione–Sepharose 4B beads (Pharmacia Biotech) and the beads were washed twice with the appropriate washing buffer. The GST–p53 proteins were incubated at 4°C with ^{35}S-labelled proteins in 200 µl of the appropriate binding buffer for 2 h. Complexes were washed twice in the washing buffer, resuspended in SDS sample buffer and fractionated by SDS–10% PAGE. To assess ^{35}S-labelled protein, gels were treated with Amplify (Amersham) prior to drying then exposed to X-ray films at –80°C.

Immunoprecipitation

For immunoprecipitations, 6×10^5 cells were transiently transfected with CMV-p53 (wild type or mutant) as described above. At 24 h post-transfection, the cells were lysed in 20 mM HEPES–KOH pH 7.4, 0.5% Triton X-100, 150 mM NaCl, 2 mM DTT and protease inhibitors (Calbiochem). Co-immunoprecipitations were performed at a final NaCl concentration of 100 mM. Typically, half of the total extract was immunoprecipitated with an anti-p300 antibody (N15; Santa Cruz Biotechnology) and half with an anti-actin antibody (20-33; Sigma). Immunoprecipitations were carried out at 4°C for 2 h, following which the precipitates were washed twice in lysis buffer and eluted in SDS sample buffer. Protein extracts were fractionated by SDS–7.5% PAGE and electrophoretically transferred to PVDF. Antibodies against p53 (DO1; Santa Cruz Biotechnology), p53 phosphorylated at Ser15 (α-ser15P; NEB) or p300 (N15, Santa Cruz Biotechnology) were used for immunochemical detection using chemiluminescence.

Acknowledgements

We are very grateful to Tamar Unger for reagents and to Neil Perkins for reagents and advice. This work was supported by the Biotechnology and Biological Sciences Research Council. D.W.M. is a Medical Research Council Senior Fellow.

References

Ashcroft,M., Kubbutat,M.H.G. and Vousden,K.H. (1999) Regulation of p53 function and stability by phosphorylation. *Mol. Cell. Biol.*, **19**, 1751–1758.

Avantaggiati,M.L., Ogryzko,V., Gardner,K., Giordano,A., Levine,A.S. and Kelly,K. (1997) Recruitment of p300/CBP in p53-dependent signal pathways. *Cell*, **89**, 1175–1184.

Banin,S. *et al*. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*, **281**, 1674–1677.

Blattner,C., Tobiasch,E., Litfen,M., Rahmsdorf,H.J. and Herrlich,P. (1999) DNA damage-induced p53 stabilisation: no indication for an involvement of p53 phosphorylation. *Oncogene*, **18**, 1723–1732.

Bottger,A., Bottger,V., Sparks,A., Liu,W.-L., Howard,S.F. and Lane,D.P. (1997) Design of a synthetic Mdm2-binding mini protein that activates the p53 response *in vivo*. *Curr. Biol.*, **7**, 860–869.

Canman,C.E., Lim,D.-S., Cimprich,K.A., Taya,Y., Tamai,K., Sakaguchi,K., Appella,E., Kastan,M.B. and Siliciano,J.D. (1998) Activation of the ATM kinase by ionising radiation and phosphorylation of p53. *Science*, **281**, 1677–1679.

Chen,X., Farmer,G., Zhu,H., Prywes,R. and Prives,C. (1993) Cooperative DNA binding of p53 with TFIID (TBP): a possible mechanism for transcriptional activation [published erratum appears in *Genes Dev.*, 1993, **7**, 2652]. *Genes Dev.*, **7**, 1837–1849.

Deng,W.P. and Nickoloff,J.A. (1992) Site-directed mutagnesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.*, **200**, 81–88.

el Deiry,W., Kern,S.E., Pietenpol,J.A., Kinzler,K.W. and Vogelstein,B. (1992) Definition of a consensus binding site for p53. *Nature Genet.*, **1**, 45–49.

el Deiry,W. *et al*. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

Farmer,G., Colgan,J., Nakatani,Y., Manley,J.L. and Prives,C. (1996) Functional interaction between p53, the TATA-binding protein (TBP) and TBP-associated factors *in vivo*. *Mol. Cell. Biol.*, **16**, 4295–4303.

Fiscella,M., Ullrich,S.J., Zambrano,N., Shields,M.T., Lin,D., Lees-Miller,S., Anderson,C.W., Mercer,W.E. and Appella,E. (1993) Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. *Oncogene*, **8**, 1519–1528.

Giaccia,A.J. and Kastan,M.B. (1998) The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.*, **12**, 2973–2983.

Gu,W. and Roeder,R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the C-terminal domain. *Cell*, **90**, 595–606.

Gu,W., Shi,X.-L. and Roeder,R.G. (1997) Synergistic activation of transcription by CBP and p53. *Nature*, **387**, 819–823.

Hall,S.R., Campbell,L.E. and Meek,D.W. (1996) Phosphorylation of p53 at the casein kinase II site selectively regulates p53-dependent transcriptional repression but not transactivation. *Nucleic Acids Res.*, **24**, 1119–1126.

Haupt,Y., Maya,R., Kazaz,A. and Oren,M. (1997) Mdm2 protein promotes rapid degradation of p53. *Nature*, **387**, 296–299.

Huang,L.C., Clarkin,K.C. and Wahl,G.M. (1996) Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G_1 arrest. *Proc. Natl Acad. Sci. USA*, **93**, 4827–4832.

Hupp,T.R., Meek,D.W., Midgley,C.A. and Lane,D.P. (1992) Regulation of the specific DNA binding function of p53. *Cell*, **71**, 875–886.

Ko,L.J., Shieh,S.-Y., Chen,X., Jayaraman,L., Tamai,K., Taya,Y., Prives,C. and Pan,Z.-Q. (1997) p53 is phosphorylated by CDK7–cyclin h in a p36MAT1-dependent manner. *Mol. Cell. Biol.*, **17**, 7220–7229.

Kubbutat,M.H.G., Jones,S.N. and Vousden,K.H. (1997) Regulation of p53 stability by Mdm2. *Nature*, **387**, 299–303.

Lambert,P.F., Kashanchi,F., Radonovich,M.F., Shiekhattar,R. and Brady,J.N. (1998) Phosphorylation of p53 serine 15 increases interaction with CBP. *J. Biol. Chem.*, **273**, 33048–33053.

Lees-Miller,S., Sakaguchi,K., Ullrich,S.J., Appella,E. and Anderson,C.W. (1992) Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol. Cell. Biol.*, **12**, 5041–5049.

Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.

Lill,N.L., Grossman,S.R., Ginsberg,D., DeCaprio,J. and Livingston,D.M. (1997) Binding and modulation of p53 by p300/CBP coactivators. *Nature*, **387**, 823–826.

Liu,L., Scolnick,D.M., Trievel,R.C., Zhang,H.B., Marmorstein,R., Halazonetis,T.D. and Berger,S.L. (1999) p53 sites acetylated *in vitro* by PCAF and p300 are acetylated *in vivo* in response to DNA damage. *Mol. Cell. Biol.*, **19**, 1202–1209.

Liu,X., Miller,C.W., Koeffler,P.H. and Berk,A.J. (1993) The p53 activation domain binds the TATA box-binding polypeptide in holo-TFIID and a neighboring p53 domain inhibits transcription. *Mol. Cell. Biol.*, **13**, 3291–3300.

Lu,H. and Levine,A.J. (1995) Human $TAF_{II}31$ protein is a transcriptional coactivator of the p53 protein. *Proc. Natl Acad. Sci. USA*, **92**, 5154–5158.

Lu,H., Fisher,R.P., Bailey,P. and Levine,A.J. (1997) The CDK7–cyclin H–p36 complex of transcription factor TFIIH phosphorylates p53, enhancing its sequence-specific DNA binding activity *in vitro*. *Mol. Cell. Biol.*, **17**, 5923–5934.

Mack,D.H., Vartikar,J., Pipas,J.M. and Laimins,L.A. (1993) Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. *Nature*, **363**, 281–283.

Martin,D.W., Munoz,R.M., Subler,M.A. and Deb,S. (1993) p53 binds to the TATA-binding protein–TATA complex. *J. Biol. Chem.*, **268**, 13062–13067.

Mayr,G.A., Reed,M., Wang,P., Wang,Y., Schwedes,J.F. and Tegtmeyer,P. (1995) Serine phosphorylation in the NH_2 terminus of p53 facilitates transactivation. *Cancer Res.*, **55**, 2410–2417.

Meek,D.W. (1999) Mechanisms of switching on p53: a role for covalent modification? *Oncogene*, in press.

Milne,D.M., Palmer,R.H., Campbell,D.G. and Meek,D.W. (1992) Phosphorylation of the p53 tumour-suppressor protein at three N-terminal sites by a novel casein kinase I-like enzyme. *Oncogene*, **7**, 1361–1369.

Milne,D.M., Campbell,L., Campbell,D.G. and Meek,D.W. (1995) p53 is phosphorylated *in vitro* and *in vivo* by an ultra-violet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK-1. *J. Biol. Chem.*, **270**, 5511–5518.

Oliner,J.D., Pietenpol,J.A., Thiagalingam,S., Gyuris,J., Kinzler,K.W. and Vogelstein,B. (1993) Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature*, **362**, 857–860.

Pise-Masison,C.A., Radonovich,M., Sakaguchi,K., Appella,E. and Brady,J.N. (1998) Phosphorylation of p53: a novel pathway for p53 inactivation in human T-cell lymphotropic virus type 1-transformed cells. *J. Virol.*, **72**, 6348–6355.

Prives,C. and Hall,P.A. (1999) The p53 pathway. *J. Pathol.*, **187**, 112–126.

Renzing,J. and Lane,D.P. (1995) p53-dependent growth arrest following calcium phosphate-mediated transfection of murine fibroblasts. *Oncogene*, **10**, 1865–1868.

Sakaguchi,K., Herrera,J.E., Saito,S., Miki,T., Bustin,M., Vassilev,A., Anderson,C.W. and Appella,E. (1998) DNA damage activates p53 through a phosphorylation–acetylation cascade. *Genes Dev.*, **12**, 2831–2841.

Scolnick,D.M., Chehab,N.H., Stavridi,E.S., Lien,M.C., Caruso,L., Moran,E., Berger,S.L. and Halazonetis,T.D. (1997) CREB-binding protein and p300/CBP-associated factor are transcriptional coactivators of the p53 tumor suppressor protein. *Cancer Res.*, **57**, 3693–3696.

Seto,E., Usheva,A., Zambetti,G.P., Momand,J., Horikoshi,N., Weinmann,R., Levine,A.J. and Shenk,T. (1992) Wild-type p53 binds to the TATA-binding protein and represses transcription. *Proc. Natl Acad. Sci. USA*, **89**, 12028–12032.

Shieh,S.-Y., Ikeda,M., Taya,Y. and Prives,C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, **91**, 325–334.

Shieh,S.-Y., Taya,Y. and Prives,C. (1999) DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerisation. *EMBO J.*, **18**, 1815–1823.

Siliciano,J.D., Canman,C.E., Taya,Y., Sakaguchi,K., Appella,E. and Kastan,M.B. (1997) DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.*, **11**, 3471–3481.

Takenaka,I., Morin,F., Seizinger,B.R. and Kley,N. (1995) Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases. *J. Biol. Chem.*, **270**, 5405–5411.

Thut,C.J., Chen,J.L., Klemm,R. and Tjian,R. (1995) p53 transcriptional activation mediated by coactivators $TAF_{II}40$ and $TAF_{II}60$. *Science*, **267**, 100–104.

Tibbetts,R.S., Brumbaugh,K.M., Williams,J.M., Sarkaria,J.N., Cliby,W.A., Shieh,S.-Y., Taya,Y., Prives,C. and Abraham,R.T. (1999) A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev.*, **13**, 152–157.

Truant,R., Xiao,H., Ingles,C.J. and Greenblatt,J. (1993) Direct interaction between the transcriptional activation domain of human p53 and the TATA box-binding protein. *J. Biol. Chem.*, **268**, 2284–2287.

Unger,T., Nau,M.M., Segal,S. and Minna,J.D. (1992) p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. *EMBO J.*, **11**, 1383–1390.

Unger,T., Juven-Gershon,T., Moallem,E., Berger,M., Vogt Sionov,R., Lozano,G., Oren,M. and Haupt,Y. (1999a) Critical role for Ser20 of human p53 in the negative regulation of p53 by MDM2. *EMBO J.*, **18**, 1805–1814.

Unger,T., Vogt Sionov,R., Moallem,E., Yee,C.Y., Howley,P.M., Oren,M. and Haupt,Y. (1999b) Mutations in serines 15 and 20 of human p53 impair its apoptotic function. *Oncogene*, **18**, 3205–3212.

Wang,Y. and Prives,C. (1995) Increased and altered DNA binding of human p53 by S and G_2/M but not G_1 cyclin-dependent kinases. *Nature*, **376**, 88–91.

Waterman,M.J.F., Stavridi,E.S., Waterman,J.L. and Halazonetis,T.D. (1998) ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nature Genet.*, **19**, 175–178.

Yuan,Z.-M. *et al*. (1999) Role for p300 in stabilisation of p53 in response to DNA damage. *J. Biol. Chem.*, **274**, 1883–1886.

Received August 20, 1999; revised and accepted October 25, 1999