Serine Phosphorylation in the NH2 Terminus of p53 Facilitates Transactivation¹

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ABSTRACT

Mureine tumor suppressor p53 is phosphorylated in the NH2-terminal transactivating domain at serines 9, 18, and 37. Change of any one of these serines to either alanine or aspartic acid did not alter p53 suppression of transformation of rat embryo fibroblasts by activated ras and E1A. Change of any two of these serines to alanines, however, led to a significant decrease in suppressor function. Substitution of alanines for all three serines caused the most severe loss of suppression and also reduced transactivation functions. The triple substitution had no apparent effects in intracellular accumulation or localization of p53, oligomerization, DNA binding, or interaction with the TFIIID TATA-binding protein. In contrast, triple substitution of aspartic acid for serines 9, 18, and 37 had minimal effects on suppression and transactivation by p53. These results argue strongly that phosphorylation of serines 9, 18, and 37 facilitates the suppression and transactivation functions of p53.

INTRODUCTION

WT p53³ suppresses transformation of cells transfected with oncoproteins (1–3). Moreover, when WT p53 is introduced into cells derived from natural human tumors, it can suppress cellular growth (4). The ability of WT p53 to suppress transformation correlates with its ability to act as a transcriptional activator (5–8). Deletion of the NH2-terminal transactivation domain of p53 impairs its ability to suppress transformation (9). Replacement of the natural transactivation domain of p53 with the transactivation domain of the unrelated herpes virus protein VP-16 restores the suppression and transactivation functions of p53 (7, 8). Identification of the WAF1 gene as a target of p53 transactivation (10) underscores the role between the relations of p53 in transactivation and suppression of transformation; WAF1 inhibits a variety of cyclin-dependent kinases necessary for cell cycle progression (11).

Murine p53 consists of 390 amino acids (12) and has a distinct domain structure. The acidic NH2-terminal region is a strong transcriptional activator when targeted to promoters either as part of p53 or as part of a chimeric protein (5, 13–19). In the highly homologous human p53, amino acids 1–42 are required for transactivation and for interaction with the TFIIID basal transcription complex (20–26). The central domain of murine p53, consisting of amino acids 80–290, binds well to a consensus DNA recognition sequence (27–30). The COOH terminus of p53 includes a strong tetramerization domain (31, 32), nuclear localization signals (33–35), and regions that may regulate DNA binding and transactivation functions (36, 37). These COOH-terminal functions, however, are not required for the suppression and transactivation functions of p53 (32, 38, 39).

Both human and murine p53s are phosphorylated at multiple sites (40–43). Murine p53 is phosphorylated at serines 9, 18, and 37 in the NH2-terminal transactivation domain. Here, we use the original amino acid numbering system of Pennica et al. (12) to identify amino acids in murine p53. Serines 9, 18, and 37 would be equivalent to serines 6, 15, and 34 in murine p53, as numbered by Soussi et al. (44), and to phosphoserines 6, 15, and 33 in human p53. It is interesting that changing human p53 serine 15 to alanine alters the ability of the protein to inhibit cell cycle progression of transformed cells (45). Our study focused on the role of murine serines 9, 18, and 37 in suppression of transformation and in transactivation. We changed serines to alanines to mimic constitutively unphosphorylated p53 and to aspartic acids to mimic constitutive phosphorylation. We found that while phosphorylation of individual sites in the transactivation domain is not essential for wild-type function, phosphorylation of at least two of three serines in this region facilitates transactivation by p53.

MATERIALS AND METHODS

Expression Plasmids. We have described two plasmids for expression of WT and mutant p53s in animal cells. The first, p53MN (46), expresses murine p53 under the control of the relatively weak MSV promoter. The second, pCMH6K (30), expresses p53s under the control of the strong immediate-early promoter of CMV. pCMH6K encodes p53s with a 22-amino acid NH2-terminal tag containing 6-histidines for purification by metal affinity chromatography and a hemagglutinin epitope recognized by the mAb PAb12CA5. The authenticity of the cloned p53s was verified by DNA sequencing. For purification of p53, we moved the cloned p53s into baculovirus expression vectors using the shuttle plasmid pH6K as described previously (30). We used plasmids pSP72-RAS and pBS-E1A to express activated Ras and adenovirus E1A proteins in animal cells (7).

Site-directed Mutagenesis of p53. Mutations were made in WT p53 with the use of the Kunkel method of site-directed mutagenesis (47). All mutations were verified by sequencing. Alanine and aspartic acid mutations are designated by amino acid numbers, followed by an A and D, respectively.

p53 Purification from Sf9 Cells. High-titer stocks of WT and 9-18-37A mutant p53s were used to infect SF9 cells and overproduce p53 proteins. Proteins were purified using Ni-NTA-agarose from Qiagen (Chatsworth, CA) as described previously (30). The proteins were eluted from the Ni-NTA-agarose, and purity was verified by SDS-PAGE with Coomassie blue staining; the p53 preparations were greater than 90% pure.

Suppression of Transformation Assays. Fourth-passage Fisher REF cells cultured from whole rat embryo preparations were obtained from Whittaker. Cells were transfected using DOTAP (Boehringer Mannheim, Indianapolis, IN) in 5.5 ml of DMEM containing 10% FCS and antibiotics (complete DMEM). Dishes (10 cm) containing 3 × 10⁵ cells/dish were transfected with 2.5 μg each of pSP72-RAS and pBS-E1A, and 5 μg of a plasmid expressing WT or mutant p53 under the control of the MSV promoter. The cultures were refed with complete DMEM after 18 h and every 3–4 days. After 12–14 days of incubation at 37°C in a CO2 incubator, cells were washed and stained with 0.05% Coomassie blue in 50% methanol and 10% acetic acid for 1 h, and the foci were counted.

p53 Transactivation of a CAT Reporter Plasmid. The transactivation assays were performed under conditions consistent with a linear p53 dose-CAT response range. p53 null H358 cells (16) were seeded into 10-cm dishes at 60–70% confluence and transfected with 5 μg each of expressor and reporter plasmids with the use of DOTAP as described previously. The reporter plasmid pCAβGlo-TATA contains 26 consensus p53-binding sites and an E1B TATA box upstream of a CAT gene (46). The cells were harvested 40–44 h after transfection, washed with PBS, and pelleted by low-speed centrifugation. One-half of each cell pellet was resuspended in 150 μl of 250 mM Tris-HCl (pH 8.0), lysed by three consecutive freeze-thaw cycles, and centrifuged at

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³The abbreviations used are: WT p53, wild-type p53 protein; MSV, Moloney sarcoma virus; CMV, cytomegalovirus; Ni-NTA-agarose, Ni-nitrilo-triacetic acid-agarose; REF, rat embryo fibroblast; CAT, chloramphenicol acetyltransferase; β-ME, β-mercaptoethanol; ECL, enhanced chemiluminescence; TBP, TATA-binding protein; DOTTAP, N[1-ε-(2,3-diolioxy)propyl]4-NN-trimethylammonium methyl-sulfate.

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12,000 x g to remove cellular debris. CAT activities were measured by phase extraction with the use of a kit and reagents supplied by United States Biochemical (Cleveland, OH). Lysate protein concentrations were determined by a modified Bradford assay (Bio-Rad, Melville, NY), and equivalent amounts of total protein were used in each reaction. Assays were performed in duplicate with less than 10% variation, and each pair was averaged to give a percentage of WT transactivation.

Quantitation of p53 Expression Levels. The remaining one-half of the H358 cell pellets used for the CAT assays described previously was used to determine p53 expression levels. Each cell lysate was boiled in buffer containing 150 mM Tris (pH 9.0), 150 mM NaCl, 10% glycerol, 0.5% NP40, 1 mM phenylmethylsulfonyl, 1 mM benzamidine, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 10 μg/ml pepstatin A. The lysate was cleared, and 100 μg of total protein were added to 200 μl of Ni-NTA-agarose bead slurry (1:1, beads:buffer). The samples were tumbled overnight at 4°C. The samples were boiled for 5 min in an equal volume of loading buffer containing 4% (w/v) SDS and 5% (v/v) β-ME and electrophoresed in a 10% Laemmli SDS-PAGE gel. The proteins were transferred to nitrocellulose and processed for reaction was stopped by the addition of an equal volume of buffer containing 0.03, or 0.1% glutaraldehyde in a final reaction volume of 150 mM or 9-18-37A protein, purified from Sf9 cells, was cross-linked in 0.001, 0.01, or 0.1% glutaraldehyde in a final reaction volume of 60 μl of 150 mM Tris (pH 7.0)-150 mM NaCl-10% glycerol-5 mM β-ME for 30 min at 25°C. The antibodies against p53 was used to detect p53.

Oligomerization of WT and Mutant p53s. We have described previously an assay for oligomerization (32, 46). Briefly, 1 μg of WT p53 or 9-18-37A protein, purified from Sf9 cells, was cross-linked in 0.001, 0.01, 0.03, or 0.1% glutaraldehyde in a final reaction volume of 60 μl of 150 mM Tris (pH 7.0)-150 mM NaCl-10% glycerol-5 mM β-ME for 30 min at 25°C. The reaction was stopped by the addition of an equal volume of buffer containing 4% (w/v) SDS and 5% (v/v) β-ME (48) and boiling for 5 min. The samples were electrophoresed for 4 h at 200 V through a 4–15% polyacrylamide gradient gel. The proteins were transferred to nitrocellulose and processed for ECL as described above, except that a mixture of PAb240 and PAb246 antibodies against p53 was used to detect p53.

Specific DNA Binding. We have described previously a mobility shift assay for detection of p53-specific DNA binding (30). Each DNA-binding reaction contained 100 ng of protein, 2 ng of labeled p53-specific duplex DNA (5'-CCGGAGAGATCCGCTAGACATGCTCTCGGG-3') in 25 mM HEPES (pH 7.4), 50 mM KCl, 20% glycerol, 0.1% NP40, 1 mM DTT, and 1 mg/ml BSA (Sigma Chemical Co., St. Louis, MO; ELISA grade). The reactions were performed in the absence of competitor DNA, in the presence of 100 ng of nonspecific competitor DNA, or in the presence of 100 ng of specific competitor DNA. After 40 min on ice, the samples were run on a 4% polyacrylamide gel at 4°C for 2 h. The gels were dried and exposed to X-ray film at –70°C.

Cellular Localization. Multiple 20-mm glass coverslips were placed in a 10-cm dish, and 5 x 10⁵ REF cells were seeded into each dish. These cells were transfected with p53 expression plasmids with the use of CaPO₄ transfection in 10 ml of complete DMEM (49). Sixteen h after transfection, the cells were washed twice with 8 ml of complete DMEM and refed with 12 ml complete DMEM. Thirty-six h after transfection, the cells were washed, fixed, and permeabilized as described by Shaubsky et al. (50). The cells were probed with 400 ml of PBS containing 13.2 mg/ml of PAb212CA5, followed by anti-rabbit IgG FITC antibody. The coverslips were washed extensively and mounted to slides with the use of Gel Mount (Fisher Scientific). The cells were examined with a Nikon Labophot microscope and were photographed with Kodak Tmax 400 black and white film.

p53-TBP Interaction. TBP made in bacteria was purified partially by heparin-Sepharose chromatography (51). The TBP protein in this preparation represented approximately 5% of total protein. Purified p53s (100 and 800 ng) were combined with approximately 500 ng of TBP at 30°C for 30 min in 120 μl of 25 mM HEPES (pH 7.6)-0.1 mM EDTA-12.5 mM MgCl₂-10% glycerol-0.1% NP40-1 mM DTT-0.1 mM phenylmethylsulfonyl (HEMGN) buffer containing 0.1 mM KCl and 0.005% SDS (HEMGN/KCl/SDS). Forty ml of a 1:1 slurry of PAb421 cross-linked to Protein A-Sepharose CL4B beads in HEMGN/KCl/SDS buffer was added. The beads were washed extensively and mounted to slides with the use of Gel Mount (Fisher Scientific). The beads were examined with a Nikon Labophot microscope and were photographed with Kodak Tmax 400 black and white film.

RESULTS

Substitutions of Serine Residues in p53. Murine p53 serines 9, 18, and 37 are highly phosphorylated and are well conserved among other species (44). Because they are in the acidic transactivation region of p53, we mutated them to investigate their possible functions in transcriptional activation. The WT p53 gene was subjected to site-directed mutagenesis (47). Serines 9, 18, and 37 were changed individually to either alanine (A) to mimic unphosphorylated serines or to aspartic acid (D) to mimic constitutively phosphorylated serines. Additional mutants with two or three alanine or aspartic acid substitutions in the same p53 were also made. The WT and mutant p53s were placed under control of either the relatively weak MSV or the stronger CMV immediate early gene promoter (30). WT and 9-18-37A mutant p53s were also expressed in insect cells so that these proteins could be produced in quantities sufficient for purification and characterization in vitro.

Suppression of Transformation by p53. It is known that WT murine p53 suppresses the formation of foci by the transforming oncogenes ras and EIA in REF cells (1–3). We wished to determine whether the substitution of serine residues in the amino terminus of p53, either singly or in combination, would affect the ability of p53 to suppress transformation by ras and E1A. To this end, REF cells were triply transfected with plasmids expressing activated ras and E1A and either WT p53 or one of the mutant p53s. The p53s used in these assays were transfected under the control of the MSV promoter. Triple transfection of plasmids expressing activated ras and E1A and a control pBluescript KS+ that does not express p53 established the basal focus-forming potential of ras and E1A in the absence of any exogenous p53. The average basal transformation frequency was equated to 100%, and all other combinations within an assay were compared to this value. The results of multiple assays for each mutant p53 are summarized in Fig. 1.

The histogram shows that WT murine p53 suppressed the number of transformed foci formed by ras and E1A to about 37% of the number seen without p53. The substitution of alanine for any of the three phosphorylated serines in the NH₂ terminus did not significantly reduce suppression efficiency of p53. Double and triple substitutions of alanines for serines, however, did interfere with the suppression of transformation by ras and E1A. The triple 9-18-37A mutant had the greatest loss of suppressor activity. In contrast, neither single nor multiple substitutions of aspartic acid for serine residues significantly altered p53 suppression of ras and E1A transformation. Because aspartic acids probably mimic phosphorylated serines at least to a limited extent, these findings argue that NH₂-terminal phosphorylation facilitates the suppression function of p53.

When the alanine-substitution mutants of p53 were expressed under the control of the strong CMV promoter in similar experiments, they suppressed transformation nearly as well as WT p53 did (data not shown). We conclude that overexpression of serine mutant p53s, at levels well in excess of the expression of endogenous p53, can compensate for the partial loss of the suppression function.

Transactivation Potential and Expression Levels of WT and 9-18-37A Mutant p53s. Previous studies have shown that the NH₂-terminal region of p53 has a domain capable of transcriptional activation when bound to promoter regions by a natural or chimeric DNA binding domain (5, 13–18). Moreover, the suppressor function of p53 is dependent on the presence of this p53 domain or of another
To investigate the role of NH2-terminal phosphorylation in transactivation, we used the CMV promoter to express WT, 9-18-37A, and 9-18-37D p53s in a transactivation assay. We cotransfected the plasmids expressing p53 with a reporter plasmid containing twenty-six copies of a p53 consensus DNA binding site 25 bp upstream from a TATA sequence and the chloramphenicol acetyltransferase (CAT) gene. The assays were carried out in H358 human lung carcinoma cells which are null for functional p53 (16). After transfection, cell extracts were split so that p53 expression levels could be compared directly with p53 transactivation levels.

Fig. 2A compares protein expression levels of WT and 9-18-37 mutant p53s in p53-null H358 cells. Protein levels were determined by Ni-NTA-agarose affinity precipitation followed by western blotting and ECL. Under the control of the MSV promoter, p53 expression was below the level of detection. Under the control of the CMV promoter, WT and mutant p53 levels were similar in all of our assays; a representative example is shown. WT and mutant p53s do not comigrate perfectly; this difference is consistent with changes in protein phosphorylation as would be expected. Fig. 2B compares the transactivation potential of WT and 9-18-37 mutant p53s. The transactivation data represent averages derived from four independent analyses each consisting of duplicate samples. In each experiment, CAT activity induced by WT p53 was taken as 100% of the p53 transactivation potential. The H6K vector that does not express p53 was used to determine background in the assay, and values from these assays are shown in the column labeled "None." The graph indicates that the 9-18-37A mutant p53 induced a relatively low level of CAT activity. Interestingly, the 9-18-37D mutant p53 induced CAT activity to levels intermediate to those induced by the WT and 9-18-37A mutant p53s. These findings argue that NH2-terminal phosphorylation facilitates the transactivation function of p53 in parallel with the suppression function of p53.

Oligomerization of WT and 9-18-37A p53s. To determine whether the substitution of alanines for serines affected oligomerization of p53, baculovirus-produced WT and 9-18-37A proteins were cross-linked at glutaraldehyde concentrations from 0.001% to 0.1%. The cross-linked proteins were run on an SDS PAGE gradient gel, transferred to nitrocellulose, and analyzed by ECL as described in Materials and Methods. Fig. 3 shows that, at low glutaraldehyde concentrations, preformed oligomers were only partially cross-linked and, under SDS treatment, dissociated into monomers, dimers, trimers, and tetramers. At high glutaraldehyde concentrations, preformed oligomers were completely cross-linked, and tetramers and octamers were the predominant forms of both the WT and 9-18-37A mutant p53s. The
complete absence of nontetrameric forms at high glutaraldehyde concentrations argues strongly that glutaraldehyde does not lead to artifactual cross-linking. Furthermore, mutant p53s lacking the tetramerization domain consist predominantly of monomers under the same conditions (32). These results confirm that the quaternary structure of 19-18-37A is similar to that of WT p53.

**Comparison of Specific DNA Binding by WT and 9-18-37A p53s.** Because the transactivation potential of 9-18-37A was less than that of WT p53 and was not the result of differences in protein levels (Fig. 2), we compared specific DNA binding by WT and mutant p53s. We used baculovirus-produced proteins in a gel retardation assay (30) to determine whether the defect in 9-18-37A could reflect a loss of DNA binding ability. Fig. 4 demonstrates that the 9-18-37A mutant bound DNA with characteristics similar to those of WT p53. Both proteins bound labeled DNA containing a p53 consensus site in the absence of competitor DNA and in the presence of a 50-fold excess of nonspecific competitor DNA. Multiple bound species represent higher oligomeric structures bound to DNA (53). In contrast, a 50-fold excess of unlabeled specific DNA competitor competed effectively with the labeled probe. Thus, the 9-18-37A mutant is capable of binding specifically to the consensus p53 target site on DNA. Since the DNA specific binding domain is located in the central region of p53 (27-30), this finding is not unexpected. However, it argues that the reduced transactivation ability of 9-18-37A p53 is not associated with a major conformational change which alters DNA binding.

**Localization of Both WT and 9-18-37A to the Nucleus.** Shaulsky et al. have shown that the location of p53 in cells changes during the cell cycle and that p53's localization to the nucleus at the G1-S boundary may be related to its normal function (35). The nuclear localization domains are in the COOH terminus of p53 (33-35). We wanted to determine if the 9-18-37A mutant was capable of nuclear localization since a loss of nuclear localization could result in a loss of transactivation and suppression activity. Immunofluorescence assays were done after transient transfection of REF cells. The cells were grown on coverslips, fixed, permeabilized, and probed with antibody as described in Materials and Methods. We found that the 9-18-37A protein was capable of localizing preferentially to the nucleus of the cells. The results are summarized in Table 1, and representative examples are shown in Fig. 5. Fig. 5A shows that a plasmid expressing only the NH2-terminal histidine tag gives no significant background staining. The staining seen in Fig. 5B for WT p53 and in Fig. 5C, for 9-18-37A p53 shows specific staining of the antibody-tagged p53s. Because our studies were done in the presence of high serum concentrations without synchronization, we would expect cells to be in various stages of the cell cycle at the time of assay for p53 localization. It is, therefore, not surprising that both WT and mutant p53s were found in the cytoplasm, as well as in the nucleus as shown by Shaulsky et al. (54) in another system. The cells shown in the figure were selected to show a variety of p53 localization patterns rather than the most typical patterns summarized in Table 1. Although there may be a slight reduction in nuclear localization frequency for the mutant protein, this difference is not sufficient to explain the loss of transactivation potential by this mutant.

**Binding of WT and 9-18-37A p53s to TBP.** WT p53 may transactivate promoters containing specific p53 DNA-binding sites via an interaction with TBP (20, 22-26). To test the possibility that the defect in transactivating potential seen with the 9-18-37A mutant could be due to a loss in ability to interact with TBP, we performed a communoprecipitation assay using baculovirus-produced WT and 9-18-37A p53s and bacterially produced human TBP. The p53s and TBP were mixed with Protein A-Sepharose beads cross-linked to PAb421, an antibody against p53. p53 and TBP bound to p53 were eluted from PAb421 beads and run on an SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane, which was probed with the use of an antibody specific for TBP. Thus, only TBP that had coprecipitated with the p53 protein would be detected in the ECL image. The communoprecipitation assay in Fig. 6 shows that TBP coprecipitated with both WT and 9-18-37A p53s. The amount of TBP precipitated increased as the amount of p53 protein added to the reaction was increased from 100 to 800 ng. In the case of 9-18-37A p53, the amount of TBP precipitated was significantly less than that seen with WT p53. However, the difference was not large enough to explain the reduced transactivation ability of 9-18-37A p53.

![Image of gel electrophoresis](https://example.com/fig3.png)

**Fig. 3. Oligomerization of WT and 9-18-37A p53s.** Purified p53s in the presence or absence of glutaraldehyde (GA) cross-linking were electrophoresed in a 4-15% SDS-gradient gel, transferred to a membrane, and identified with primary antibody (PAb122CA5) with the use of ECL (Amer sham). Arrows, positions for monomer, dimer, trimer, tetramer, and octamer. These positions were determined with the use of molecular weight markers as shown previously (46).
p53 PHOSPHORYLATION AND TRANSACTIVATION

Non-specific

Specific

Bound ▲

Free ▲

WT p53 9-18-37A

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<tr>
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*Approximately 10% of cells had WT or mutant p53 in both the nucleus and cytoplasm.

We have examined the importance of phosphorylation at serines 9, 18, and 37 in the NH2 terminus of murine p53 for the normal function of p53. These serines are well conserved in mammalian species and are phosphorylated in vivo.

NH2-Terminal Phosphorylation of p53 Facilitates the Suppression of Transformation. Single alanine or aspartic acid substitutions for serines 9, 18, or 37 had no measurable effect on the transformation of cells by activated ras and E1A. These results are consistent with the absence of single amino acid changes at corresponding positions of p53 in human tumors; the vast majority of point mutations in human tumors affect the central DNA-binding domain of p53 (55, 56). It has been reported, however, that human tumors containing an isoleucine substitution at position 237 or an alanine substitution at position 143 had reduced phosphorylation of serine 15, a position that corresponds to serine 18 in murine p53 (57). This finding is consistent with the idea that normal levels of phosphorylation of serine 15 may be important for full wild-type function. Moreover, mutation of serine 15 of both the WT and 9-18-37A p53s, not all of the TBP was coprecipitated, even at 800 ng of p53 protein. This result may reflect the stoichiometry of TBP-p53 complexes, or it may indicate that only a subpopulation of TBP is able to bind p53. Bacterial proteins contaminating the TBP preparation were not coprecipitated (data not shown).

While it is possible that the serine to alanine substitutions might affect the way p53 or p53-TBP interact with other members of the transcriptional complex, the binding of the TBP protein to the 9-18-37A mutant remains intact under the conditions of our experiment.

DISCUSSION

We have examined the importance of phosphorylation at serines 9, 18, and 37 in the NH2 terminus of murine p53 for the normal function of p53. These serines are well conserved in mammalian species and are phosphorylated in vivo.

Fig. 4. DNA binding by WT and 9-18-37A p53s. Proteins were purified from Sf9 cells, and binding was measured by gel retardation of labeled specific DNA. One hundred ng of p53 protein was used to shift 2 ng of labeled probe in the absence of competitor, in the presence of 100 ng of nonspecific competitor DNA, or in the presence of 100 ng of unlabeled specific competitor DNA. The p53 is present in a 6:1 molar ratio of tetramer to DNA.

Fig. 5. Representative images of p53 immunofluorescence. WT p53 and 9-18-37A p53 were expressed under control of the CMV promoter. A, background staining using a control plasmid expressing no p53; B, cells expressing WT p53. Distinct nuclear or cytoplasmic staining can be seen in two different cells; C, cells expressing 9-18-37A p53. The left panel shows nuclear and cytoplasmic staining, and the right panel shows cytoplasmic staining alone. These images represent the types of cells counted in Table 1. Cells that stained in both the nucleus and the cytoplasm were counted as both in the table.
Lanes 2 and 3, levels of TBP coprecipitated by 100 and 800 ng of WT p53 protein, TBP were coprecipitated with the use of mAb PAb421 against p53. A 10% SDS-PAGE gel was used to separate the TBP from bound p53, and the gel was analyzed by western blotting with antibody against TBP. Lane 1, background amount of TBP nonspecifically coprecipitated by PAb421 linked to protein A-Sepharose with no p53 added to the reaction; Lanes 2 and 3, levels of TBP coprecipitated by 100 and 800 ng of WT p53 protein, respectively; Lanes 4 and 5, levels of TBP coprecipitated by 100 and 800 ng of 9-18-37A p53 protein, respectively; Lane 6, bacterially purified TBP (100 ng) from Promega, used as a marker. p53 protein without TBP gave no signal (lane not shown).

None WT p53 9-18-37A TBP

Fig. 6. Coimmunoprecipitation of TBP by WT and 9-18-37A proteins. p53 proteins were purified from Sf9 cells, and TBP was partially purified from bacteria. Approximately 500 ng of TBP was mixed with 100 or 800 ng of p53 for 30 min at 30°C. p53 and bound TBP were coprecipitated with the use of mAb PAb421 against p53. A 10% SDS-PAGE gel was used to separate the TBP from bound p53, and the gel was analyzed by western blotting with antibody against TBP. Lane 1, background amount of TBP nonspecifically precipitated by PAAb421 linked to protein A-Sepharose with no p53 added to the reaction; Lanes 2 and 3, levels of TBP coprecipitated by 100 and 800 ng of WT p53 protein, respectively; Lanes 4 and 5, levels of TBP coprecipitated by 100 and 800 ng of 9-18-37A p53 protein, respectively; Lane 6, bacterially purified TBP (100 ng) from Promega, used as a marker. p53 protein without TBP gave no signal (lane not shown).

To alanine in human p53 impaired p53 suppression of cell cycle progression (45).

Since single substitutions of serines 9, 18, or 37 were insufficient to alter significantly the tumor suppression abilities of the p53 protein, we made double and triple substitutions of alanines or aspartic acids for these serines to investigate possible complementary or additive effects among these phosphorylation sites. Lin et al. (58) showed that multiple substitutions of hydrophobic residues in the NH2-terminus of p53 have a cumulative effect on transactivation. A similar effect has been shown for the c-Jun protein, which has two serines in its transactivation domain that are phosphorylated in response to Ha-ras stimulation (59, 60). Change of either serine to alanine left partial transactivation activity, but substitution of both to alanines led to an inhibition of the c-Jun response to Ha-ras. In our present study of murine p53, we found that any combination of two alanine substitutions for serines 9, 18, or 37 resulted in reduced tumor suppressor function. Substitution of all three serines resulted in further loss of suppressor function. These findings suggest that the effect of phosphorylation of these three serines is cumulative. Changing serines 9, 18, and 37 to aspartic acids in double and triple combinations has no significant effect on suppressor function (Fig. 1). The fact that the triple aspartic acid mutant has wild-type suppression levels argues that negative charges at these sites are important for normal suppressor function.

We used the relatively weak MSV promoter to express p53 in our studies of the role of p53 phosphorylation in the suppression of transformation. The resultant low levels of mutant p53 expression mimic levels of endogenous p53 expression more closely than would p53 expressed under control of the strong CMV promoter. Endogenous p53 in REF cells or exogenous p53 expressed under the control of the MSV promoter are made in such small quantities that they are difficult to detect by Western blotting. WT and many mutant p53s expressed under control of the very strong CMV promoter are easily detectable by Western blotting and accumulate in large amounts. When expressed under the control of the MSV promoter, WT p53 suppressed transformation well. In contrast, the mutant 9-18-37A p53, which is not phosphorylated appropriately, suppressed transformation significantly less efficiently. When overexpressed under the control of the CMV promoter, mutant 9-18-37A p53 did suppress transformation efficiently (not shown). The difference in results with the use of the weak and strong promoters is not surprising. NH2-terminal phosphorylation facilitates but is not absolutely required for suppression. Restoration of mutant function by overexpression is not novel to our study and is, in fact, characteristic of partially functional mutant proteins.

NH2-Terminal Phosphorylation of p53 Facilitates Transactivation. Since the 9-18-37A triple mutant could not suppress transformation as well as WT p53, we attempted to identify the function or functions altered by loss of all three phosphorylation sites. We found that the 9-18-37A p53 (Fig. 2B) was not able to transactivate a p53 responsive reporter plasmid as well as did the WT protein. The 9-18-37D protein was less severely compromised in its ability to transactivate. Possibly, phosphorylation of all three sites involves more than simply imparting an acidic character to the protein. Interestingly, a VP-16-p53 construct, in which the first 80 amino acids of p53 were replaced by the transactivation domain of VP-16, suppressed transformation by ras and E1A more efficiently than did WT p53 (7). Perhaps some characteristic function shared by the phosphorylated p53 transactivation domain and the transactivation domain of VP-16 is retained only partially by the 9-18-37D mutant p53.

We used the CMV promoter to express p53 for our transactivation studies. Under these conditions, we demonstrated that WT and mutant p53s accumulated at similar intracellular levels. Even when overexpressed, the mutant 9-18-37A p53 was defective in transactivation. NH2-terminal phosphorylation, therefore, facilitates transactivation and suppression of transformation in parallel. Theses results are consistent with abundant evidence in the literature that p53-induced transactivation is required for suppression (7, 8). That phosphorylation appears to facilitate transactivation to a greater extent than it facilitates suppression is not surprising. The assays for transactivation and suppression use different cell types and measure events of quite different duration in time. It would, therefore, be unrealistic to expect a perfect quantitative correlation in the assays. Furthermore, suppression of transformation may require a threshold of transcription that can be achieved even at relatively low levels of p53 expression and function. This would be especially true of suppression assays using REF cells that already express endogenous WT p53. In contrast, the transactivation studies were done in H358 cells that express no endogenous p53.

Mutant 9-18-37A p53 Maintains Many Wild-Type Molecular Activities. The nuclear localization patterns of the triple alanine mutant showed that it was capable of reaching the nucleus and was not being excluded from participating in transactivation on the basis of location (Fig. 5). Although 9-18-37A p53 was not found in the nucleus quite as often as was WT p53 (Table 1), the difference does not appear to be sufficient to account for the phenotype associated with this mutation. Moreover, 9-18-37A forms both tetramers and multiples of tetramers (Fig. 3), a characteristic that is thought to be important for wild type function, possibly in aligning the protein along DNA (61). Previous studies have shown that many transforming mutants of p53 have altered DNA-binding abilities (17, 62–64). To determine whether the loss of transactivation function by 9-18-37A p53 was due to a loss of specific DNA binding, gel shift assays were done. Both WT and 9-18-37A p53 bound specifically to the p53 DNA consensus sequence (Fig. 4). This result argues that the loss of suppressor and transactivation functions by 9-18-37A p53 reflects neither a loss of DNA-binding function nor a major conformational change.

TBP is part of the transcriptional initiation complex thought to interact with acidic activators in transcription (65, 66). The binding
of TBP to the TATA box in type II promoters stimulates nucleation of transcriptional machinery on the DNA. Recent studies have indicated that p53 can bind to the TBP protein; the domain of p53 that binds to TBP has been mapped to amino acids 20–57 and the last 75 amino acids in human p53 (20–26). Moreover, a number of studies present evidence that p53 activates transcription by forming p53-TFIID complexes on target promoters (22, 23, 25, 67). Because the 9-18-37A p53 is mutated within the transcription and TBP-binding domains, loss of TBP binding could result in loss of protein transactivation function. The 9-18-37A mutant, however, has WT p53 TBP-binding ability in vitro (Fig. 6), an observation which suggests that phosphorylation of serines 9, 18, and 37 per se is not critical for binding of p53 to TBP. Nevertheless, our present studies strongly implicate phosphorylation of p53 in the activation of promoters with TATA sequences. Recent reports have identified additional interactions between p53 and the transcriptional machinery. These include the TAFII40 and TAFII60 subunits of the TFIID complex (67) and the TFIIH complex (68, 69). Phosphorylation could facilitate either protein-protein interactions or the functional consequences of those interactions. It will, therefore, be important to continue to examine the role of NH2-terminal phosphorylation in transactivation by p53 as transcription initiation complexes are analyzed further.

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Serine Phosphorylation in the NH$_2$ Terminus of p53 Facilitates Transactivation

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