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Mutation of the casein kinase II phosphorylation site abolishes the anti-proliferative activity of p53

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ABSTRACT

The p53 tumour suppressor protein is phosphorylated by several protein kinases, including casein kinase II. In order to understand the functional significance of phosphorylation by casein kinase II, we have introduced mutations at serine 386 in mouse p53, the residue phosphorylated by this kinase, and investigated their effects on the ability of p53 to arrest cell growth. Replacement of serine 386 by alanine led to loss of growth suppressor activity, while aspartic acid at this position partially retained suppressor function. These data suggest that the anti-proliferative activity of p53 is activated by phosphorylation at serine 386, and establish a direct link between the covalent modification of a growth suppressor protein and regulation of its activity in mammalian cells.

INTRODUCTION

The p53 tumour suppressor protein can block cellular transformation by dominant oncogenes [1,2], induce growth arrest [3–5] and activate apoptotic cell death [6,7] when expressed at high levels. Loss of p53 suppressor function, by mutation, is a universal step in the development of human cancer [8]. Similarly, the transforming proteins of some DNA tumour viruses target p53 and ablate its suppressor function [9–12]. Mice deficient for p53 develop normally but become susceptible to the spontaneous development of a variety of tumours by early adulthood [13], suggesting that p53 plays a major role in protecting against tumour development. At the molecular level, p53 can act as a potent transcriptional activator [14–16] and this effect is mediated through its interaction with a specific DNA sequence element [17–20]. However, with the exception of the muscle-specific creatine kinase promoter [21], p53 *down*-regulates most natural promoters tested to date including the interleukin-6, c-fos, β-actin, hsc70, c-jun and p53 promoters [22,23]. The current model for p53 action proposes that p53 monitors the integrity of the genome and arrests cell growth, or induces cell death, when extensive DNA damage occurs, thereby preventing the proliferation of malignant clones [24].

p53 is phosphorylated at multiple sites in vivo [25–27] and by several different protein kinases in vitro including $p34^{cdc2}$ [28,29], casein kinase I [30] double-stranded DNA-activated protein kinase [27,31] and casein kinase II [32–34]. However, the role of phosphorylation in regulating p53 function has not yet been established. In order to determine whether phosphorylation of p53 by casein kinase II at serine 386 [numbered according to ref. 35] plays a role in the ability of the protein to suppress cellular growth, two mutations were introduced at the codon for this residue by oligonucleotide-directed mutagenesis. Replacing serine by alanine provided a non-phosphorylatable residue with minimal structural change to the protein, while aspartic acid was introduced to mimic phosphorylation at this site. Wild type and mutant p53-expressing plasmids were introduced into mammalian cells to test whether the p53 proteins were able to suppress cell growth as measured by the formation of drug-resistant colonies [3–5]. The data suggest that phosphorylation of p53 at serine 386 is required for the growth suppressor activity of the protein.

MATERIALS AND METHODS

Cell lines and plasmid constructs

Baby hamster kidney cells transformed by simian virus 40 (SV-BHK cells), mouse 3T3 fibroblasts transformed by simian virus 40 (SV3T3 cells) and Rat-1 fibroblasts were routinely used and were maintained in Dulbecco-Vogt modified Eagle's medium (DMEM) plus 10% newborn calf serum. Plasmids pCMVNc9 and pCMVc5 (a gift from M.Oren) encode wild type and mutant mouse p53 respectively under control of the cytomegalovirus (CMV) immediate-early promoter [36,37]. These and derivative plasmids are listed in Table 1. Plasmid pSV2neo [38] was routinely used to confer G418 resistance during colony selection.

Oligonucleotide-directed mutagenesis

Mutagenesis involved transfer of the appropriate restriction fragments into M13tg130 (Amersham). Recombinant phage were purified and single-stranded DNA prepared for the mutagenesis reactions. Mutations were introduced using an oligonucleotide-

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Table 1. p53-expressing plasmids.

Plasmid name	mutations in p53*
pCMVNc9	none
pCMVp53SA	alanine at codon 386
pCMVp53SD	aspartic acid at codon 386
pCMVc5	glycine at 165 and isoleucine at 231 [Ref. 37]
pCMVdel	deletion of p53 codons 1–330
pCMV132W	valine at codon 132
pCMV132SA	valine at codon 132 and alanine at codon 386
pCMV132SD	valine at codon 132 and aspartic acid at codon 386

* codons are numbered according reference 35.

directed *in vitro* mutagenesis system according to the manufacturer's instructions (Amersham) and the desired mutants were identified by DNA sequencing. The mutant fragments were substituted into the pCMVNc9 plasmid and the entire p53 coding sequence was re-sequenced to ensure that only the desired mutations were present.

Transfection of cell lines and selection of drug-resistant colonies

Cells were seeded at 3×10^5 per 6 cm plate and transfected in quadruplicate with 0.5 μg pSV2neo, 4.5 μg pCMVNc9 (or derivative plasmids) and 5 μg of sonicated human placental DNA as carrier per plate using calcium phosphate precipitation [39]. Cells were maintained in DMEM plus 10% newborn calf serum and 300 μg/ml G418 in a humidified incubator at with 5% CO_2. The incubation temperatures are given in the figure legends. The medium was changed every three days and after 14 days, the plates were stained with crystal violet. In some cases, when 10 cm plates were used, these were seeded, each with 10^6 cells, and transfected with 1 μg pSV2neo, 9 μg pCMVNc9 (or derivative plasmids) and 10 μg of sonicated human placental DNA.

Radiolabelling, immunoprecipitation and SDS gel electrophoresis

Plates were seeded, transfected and maintained under drug selection at 37.5°C as described above. After 12 days the plates were shifted to 32°C for a further 24 hours. Prior to labelling with L-[^{35}S]methionine, the cells were rinsed twice with DMEM lacking methionine, then incubated at 32°C for two hours in 2 ml of methionine-free DMEM containing 5% dialysed calf serum and 200 μCi of L-[^{35}S]methionine (ICN; specific activity 1175 Ci/mmol). For [^{32}P]phosphate labelling, the cells were rinsed twice with DMEM lacking phosphate, then incubated at 32°C for four hours in 2 ml of phosphate-free DMEM containing 10% dialysed calf serum and 2.5 mCi of [^{32}P]orthophosphate (Amersham; carrier free). Plates were rinsed twice with ice-cold phosphate-buffered saline and the cells lysed in 0.5 ml of RIPA buffer (0.15 M NaCl, 1% [v/v] Nonidet P-40, 0.1% [w/v] SDS, 1% [w/v] sodium deoxycholate, 10 mM sodium phosphate [pH 7.0], 1 mM benzamidine and 1 mM phenymethylsulphonyl fluoride). Mouse p53 was immunoprecipitated using the mouse-specific monoclonal antibodies RA3-2C2 [40,41] or PAb242 [42] while the simian virus 40 (SV40) large T antigen was immunoprecipitated using monoclonal antibody PAb108 [43]. The immune complexes were washed four times in RIPA buffer, boiled in SDS sample buffer and separated by SDS polyacrylamide gel electrophoresis. For L-[^{35}S]methionine labelling, gels were treated with 'Amplify' (Amersham) according to the manufacturer's instructions and radiolabelled proteins were visualised by fluorography at −70°C using pre-sensitised Kodak XAR-5 film. [^{32}P]phosphate-labelled proteins were visualised by autoradiography at −70°C with intensifying screens using pre-sensitised Kodak XAR-5 film.

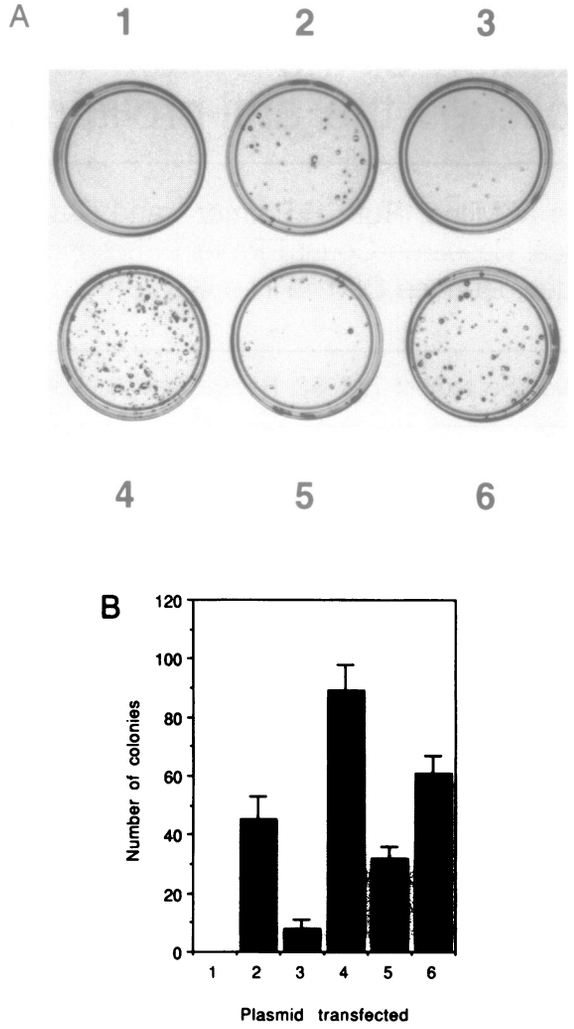

Figure 1. Effect of mutation at the serine 386 phosphorylation site on the ability of p53 to suppress SV3T3 cell growth. Assays were carried out in quadruplicate and a representative set of plates are shown in (A). The average (mean) number of colonies from the four separate transfections are shown as a bar chart in (B) with the standard deviations shown as error bars. The transfected plasmids were as follows: (1), carrier DNA only; (2), pCMVdel; (3), pCMVNc9; (4), pCMVp53SA; (5), pCMVp53SD; (6), pCMVc5.

RESULTS

In order to determine whether phosphorylation at serine 386 of p53 [numbered according to ref. 35] plays a role in the ability of the protein to suppress cellular growth, two mutations were introduced at the codon for this residue by oligonucleotide-directed mutagenesis. Replacing serine by alanine provided a non-phosphorylatable residue with minimal structural change to the protein, while the introduction of aspartic acid provided a

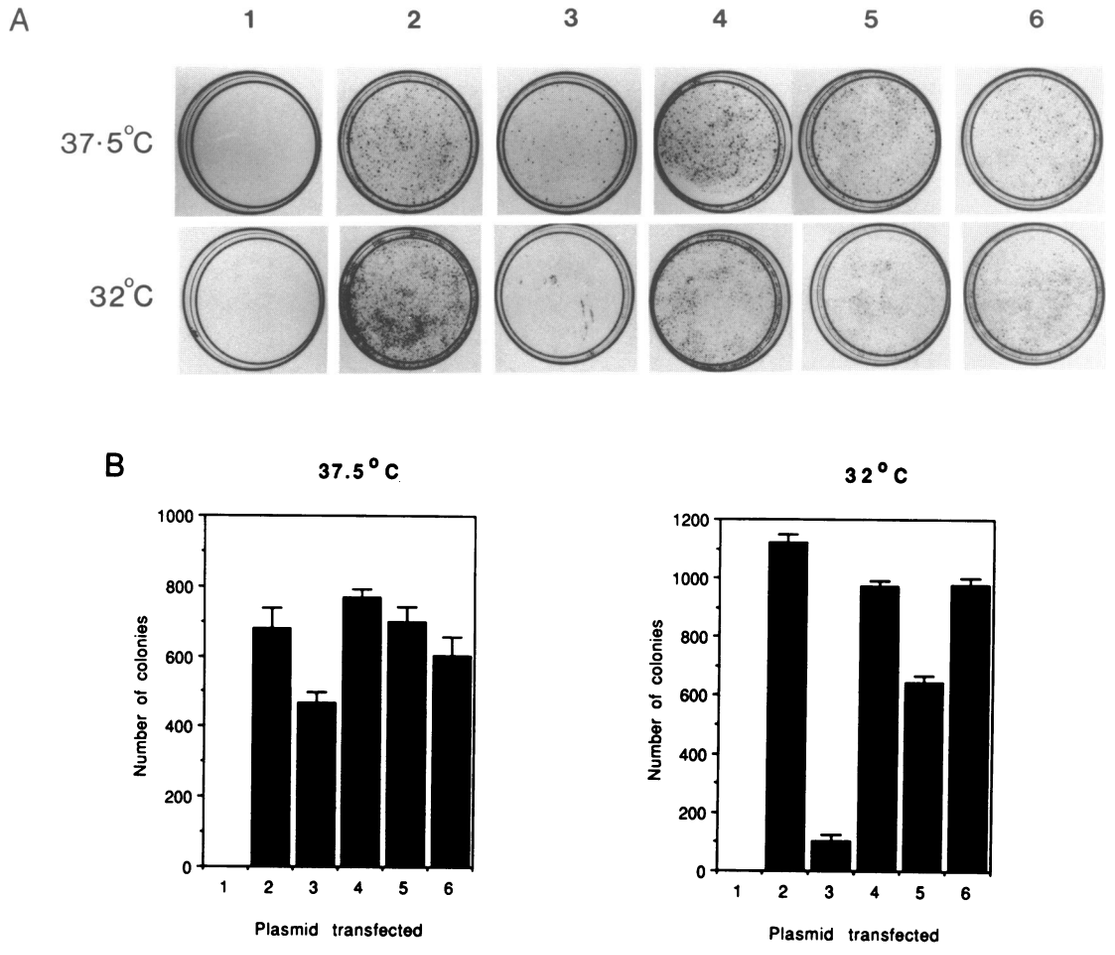

Figure 2. Effect of mutation of temperature sensitive p53 at the serine 386 phosphorylation site on the growth of SV40-transformed baby hamster kidney cells. After transfection, quadruplicate sets of plates for each transfected plasmid were placed at 37.5°C and 32°C. A representative set of plates are shown in (A). The average (mean) number of colonies from four separate transfections are shown as a bar chart in (B) with the standard deviations shown as error bars. The transfected plasmids were as follows: (1), carrier DNA only; (2), pCMVdel; (3), pCMV132W; (4), pCMV132SA; (5), pCMV132SD; (6), pCMVc5.

constitutive negative charge to mimic phosphorylation. The p53 mutations were substituted into the mouse p53 cDNA in plasmid pCMVNc9, as described in Materials and Methods. These and other constructs used in this study are listed in Table 1. The p53 coding region in each plasmid was sequenced completely to ensure that only the desired mutations were present (data not shown).

Plasmids encoding wild type, or mutant, p53 were co-transfected with pSV2neo (as a G418 selectable marker) into mouse SV3T3 cells in quadruplicate assays (i.e. 4 separate plates were transfected in each case). The principle of the assay is that discrete colonies should arise from cells which have taken up the selectable marker. Co-expression of wild type, but not mutant, p53 should lead to the arrest or down-regulation of cell growth and therefore prevent the formation of visible colonies (3–5). Fig. 1 shows a typical set of plates, with the colonies visualised by crystal violet staining (A), and a bar chart indicating the average number of colonies from quadruplicate assays (B). Colony formation was dependent upon the presence of the pSV2neo plasmid (compare plates/columns 1 and 2) and was unaffected when a plasmid expressing a p53 transforming double mutant [37] was present (plate/column 6). Very few colonies were recovered in the wild type p53 transfection (plate/column 3), confirming that wild type p53 can inhibit cell growth in this system. Strikingly, when the alanine 386 mutant was transfected in place of the wild type plasmid, there was no inhibition of growth (plate/column 4) suggesting that phosphorylation of serine 386 is important for the ability of p53 to suppress growth. A plasmid encoding aspartate at codon 386 (plate/column 5) retained some suppressor activity but was consistently less efficient than wild type p53. These data suggest that the presence of a negative charge at position 386 can partially restore the ability of p53 to suppress cellular growth. Similar results were obtained when SV40-transformed baby hamster kidney cells (SV-BHK) were used instead of SV3T3 cells or when the CMV promoter was replaced by the mouse mammary tumour virus (MMTV) promoter (data not shown).

The results in Fig. 1 could also be explained if the mutant constructs were unable to express p53 or if the efficiency of DNA uptake varied in each set of transfections. To control for these possible effects, an alanine to valine mutation at codon 132 was introduced into the plasmids expressing the wild type p53 or phosphorylation site mutants. This well-characterised mutation confers a temperature sensitive phenotype on p53 such that it behaves as a growth suppressor at 32°C but as an oncogene at 37.5°C [44]. Using the *ts* -p53, it is possible to test the effects

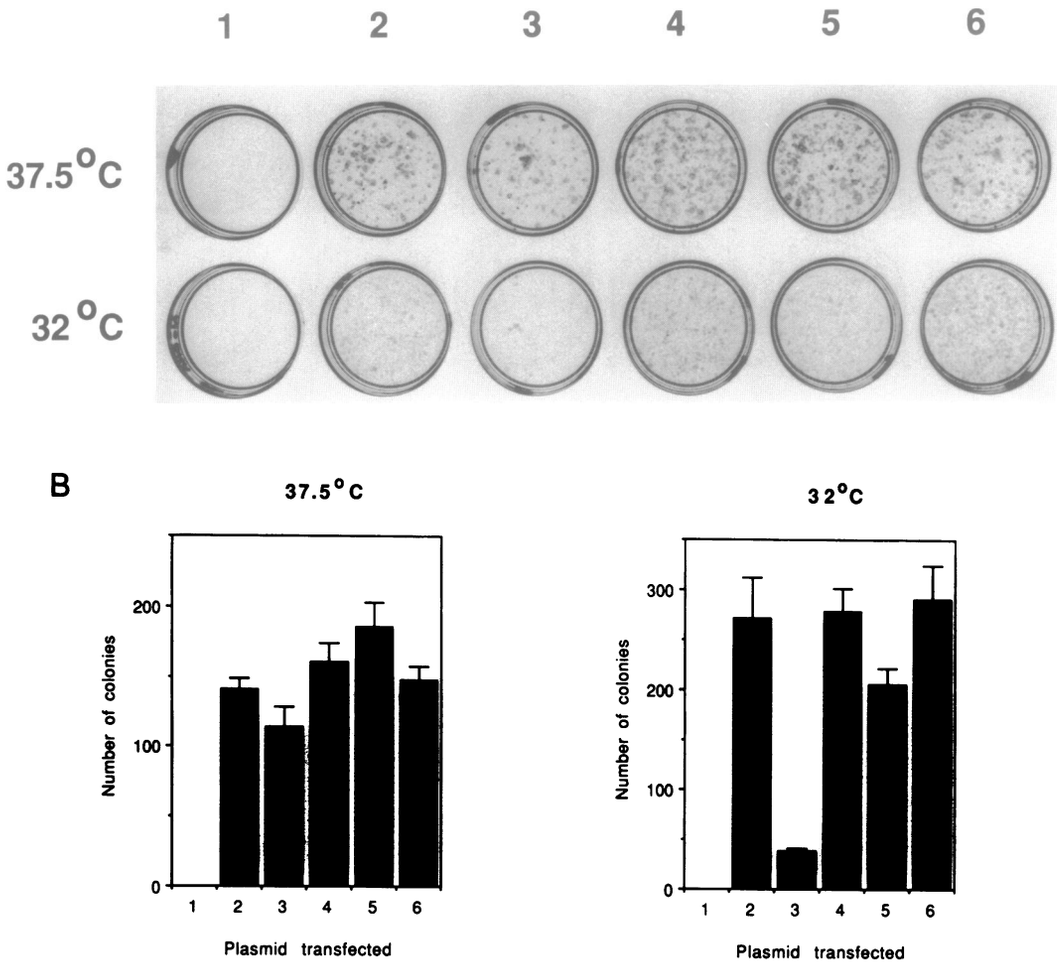

Figure 3. Effect of mutation of temperature sensitive p53 at the serine 386 phosphorylation site on the growth of Rat-1 cells. After transfection, quadruplicate sets of plates for each transfected plasmid were placed at 37.5°C and 32°C. A representative set of plates are shown in (A). The average (mean) number of colonies from four separate transfections are shown as a bar chart in (B) with the standard deviations shown as error bars. The transfected plasmids were as follows: (1), carrier DNA only; (2), pCMVdel; (3), pCMV132W; (4), pCMV132SA; (5), pCMV132SD; (6), pCMVc5.

of mutation at a phosphorylation site on the suppressor activity at 32°C, and measure the transfection efficiency at 37.5°C (at which temperature the *ts*-p53 does not suppress growth). In addition, by transfecting rat or hamster cells, analysis of expression can be carried out since mouse p53 can be distinguished from the endogenous p53 based on monoclonal antibody specificity. The effects of the phosphorylation site mutants in the valine 132 background were tested on the growth of SV-BHK cells (Fig. 2). At 37.5°C, similar numbers of colonies were observed on all plates except those transfected with 'wild type' p53 (plate/column 3) where slightly fewer colonies were consistently observed and those which appeared were generally smaller and less dense than in any of the other plates. (The inverted commas denote p53 that is wild type with respect to codon 386 but which has the valine 132 *ts* mutation.) This suggested that the efficiency of transfection was similar in each case but the *ts* p53 was slightly leaky at 37.5°C. When growth arrest was assayed in parallel at 32°C (Fig. 2), virtually no colonies appeared on the 'wild type' plate (plate/column 3) whereas very clear colony formation occurred with the deletion derivative (plate/column 2) or the p53 double transforming mutant (plate/column 6), indicating that the *ts*-p53 was able to arrest cell growth efficiently at this temperature. A striking result was observed with the alanine 386 mutant which was completely unable to suppress growth at 32°C (plate/column 4). When the aspartate 386 mutant was tested (plate/column 5), an intermediate level of suppression was again observed.

To determine whether the same effect would be observed with non-transformed cells, the experiment was repeated using Rat-1 cells (Fig. 3). Once again, suppression occurred when p53 encoded serine at codon 386, but not when this residue was changed to alanine. Moreover, as before, the aspartate 386 mutant displayed weak growth suppressor activity. These results are consistent with the data in Figs. 1 and 2, and support the idea that phosphorylation of p53 at serine 386 is required for growth arrest of both normal and transformed cells.

The levels of expression of p53 were examined in pooled cells from each transfection; (cells were selected at 37.5°C for 12 days, then shifted to 32°C for 24 hours prior to labelling). SV-BHK cells were labelled with L-[^{35}S]methionine and the p53 immunoprecipitated using monoclonal antibody PAb 242 which is specific for mouse p53 [42], or PAb108 which recognises T antigen [43]. The results (Fig. 4) show that mouse p53 was expressed only in those cells which had been transfected with plasmids encoding full length p53 (lanes 2−5). The levels of expression of the p53 proteins, whether wild type or the

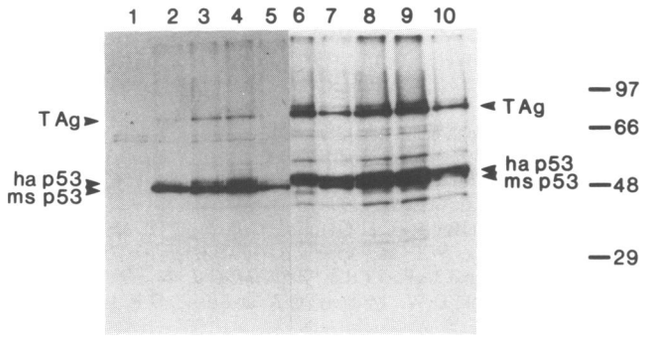

Figure 4. Expression of mouse p53 in SV40-transformed baby hamster kidney cells. L-[^{35}S]methionine-labelled mouse p53 was immunoprecipitated using the mouse-specific monoclonal antibody PAb242 (lanes 1–5) while the SV40 large T antigen was immunoprecipitated using monoclonal antibody PAb108 (lanes 6–10). The radiolabelled proteins were visualised by fluorography at −70°C using pre-sensitised Kodak XAR-5 film. The exposure time was 5 days. The transfected plasmids were as follows: pCMVdel (lanes 1 and 6), pCMV132W (lanes 2 and 7), pCMV132SA (lanes 3 and 8), pCMV132SD (lanes 4 and 9), pCMVc5 (lanes 5 and 10). The positions of mouse p53 (ms p53), hamster p53 (ha p53) and the SV40 large T antigen (T Ag) are shown together with molecular weight markers in the range 29–97 kD.

transforming mutant or the phosphorylation site mutants, were also very similar. (In the case of the Rat-1 cells, similar results were obtained [data not shown]). T antigen was observed to co-immunoprecipitate with the wild type p53 (lane 2) and with both the alanine and aspartate 386 phosphorylation site-mutant p53 proteins (lanes 3 and 4 respectively). Similarly, when T antigen was immunoprecipitated with PAb108, wild type mouse p53 (lane 7) and the two phosphorylation site mutants (lanes 8 and 9) were co-precipitated, but the transforming mutant p53 did not associate with T antigen (lane 10).

Transfected SV-BHK cells were also labelled with [^{32}P]orthophosphate and phosphopeptide analysis was carried out on the immunoprecipitated p53 proteins as described previously [45]. The results confirmed that mutation of the 386 phosphorylation site led to loss of phosphorylation at this site in vivo, while the other phosphorylation sites were unaffected (data not shown).

DISCUSSION

The data presented in this paper suggest that the anti-proliferative activity of p53 is stimulated by phosphorylation at serine 386, the residue which is targeted by casein kinase II in vitro and possibly in vivo [32]. Loss of phosphorylation at this site, by replacement of the serine with an alanine residue, results in a p53 molecule which is inactive as a suppressor of cell growth. Placing a constitutive negative charge at this site gives rise to a p53 molecule which retains some suppressor activity, but is less potent than wild type p53. Since phosphate is a large bulky group with two negative charges at physiological pH, the effect of placing a small group with a single negative charge at this site might be expected to be weaker. Therefore, the stimulatory effect of the asp 386 mutant supports the idea that phosphorylation of serine 386 favours suppressor activity and strongly suggests that a negative charge at this position is important. Serine 386 has also been proposed to be the attachment site of an unusual RNA moiety [46]. The issue of whether serine 386 is a site for a regulatory phosphorylation or another covalent modification has yet to be resolved. We therefore cannot rule out the possibility that lack of suppressor activity occurs when p53 is unable to interact with the putative RNA molecule, although the results obtained with the aspartate 386 mutant (Figs. 1, 2 and 3) favour the explanation of a regulatory phosphorylation at this site.

In assaying the biological activity of p53 we have employed a standard procedure used by several groups (suppression of neomycin resistant colony formation [3–5]) which effectively measures the anti-proliferative potential of p53. However, we cannot determine whether the anti-proliferative activity results from cell growth arrest or apoptotic cell death (since both of these effects would be expected to limit the recovery of colonies). Nevertheless, whether the basis of the anti-proliferative effect is growth arrest or apoptosis (or indeed both), this does not detract from the striking observation that altering the phosphorylation site has profound effects on p53's anti-proliferative activity.

The data are very exciting in light of two recent findings. Firstly, Ullrich and colleagues have reported that the ability of wild type p53 to suppress growth correlates with increased phosphorylation of p53 and loss of the epitope for monoclonal antibody PAb421 [47]. It is interesting that PAb421 (and PAb122 which recognises an overlapping epitope [48]) can block phosphorylation of p53 by casein kinase II in vitro [32, 49]. Conversely, it may be possible that phosphorylation of p53 at serine 386 prevents the association of p53 with PAb421. This would explain the concomitant loss of the PAb421 epitope, increased phosphorylation of p53 and activation of its suppressor activity. In addition, the results presented in this paper are entirely consistent with the finding that phosphorylation of human p53 by casein kinase II activates its DNA sequence-specific binding activity [49]. Since p53 is thought to act as a transcription factor [14–23], these data fit a model in which activation of p53 sequence-specific DNA binding, through phosphorylation by casein kinase II, promotes p53-dependent stimulation or repression of transcription of genes involved in growth control, leading to suppression of cell growth. It will therefore be important to determine whether regulation of the anti-proliferative activity of p53 is a direct consequence of regulation of its DNA binding activity by casein kinase II.

Recently, similar mutants to those reported in this paper were tested for their effects on suppression of the growth of *S. pombe*, but in this case no effect of changing the casein kinase II phosphorylation site was apparent [34]. However, it is possible that the effects of phosphorylation can be detected only under more physiological conditions, for example in a mammalian cell background. (No p53 gene has yet been reported in yeast.) Additionally, there is still some disagreement about the effects of other well-characterised mutants in yeast [34,50,51] and therefore this system, while providing much useful information, might not provide the ideal environment for examining the effects of phosphorylation site mutants. It will be interesting to see if any 'natural' mutations occur at the codon for the casein kinase II phosphorylation site, for example in tumor samples. No such mutations have been reported to date. However, these may have been missed since most surveys of mutations arising in the p53 gene in human cancer have focussed on the central highly conserved portion of the gene.

In conclusion, the results presented in this paper support the idea that the p53 growth suppressor protein is activated in the cell by phosphorylation.

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