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Phosphorylation of p53 at the casein kinase II site selectively regulates p53-dependent transcriptional repression but not transactivation

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ABSTRACT

The p53 tumour suppressor protein is a potent transcription factor which plays a central role in the defence of cells against DNA damage and the propagation of malignant clones. We have previously shown that phosphorylation of serine 386 in mouse p53 by the growth-associated protein kinase, casein kinase II (CKII), plays an important role in the ability of p53 to block the proliferation of drug-resistant colonies. In this paper we show that blocking phosphorylation of serine 386 through an alanine substitution, or placing a constitutive negative charge at this position in the form of aspartate, had no significant influence on p53-dependent transcriptional activation of a promoter containing 13 copies of a p53 consensus binding sequence, or of the p21^{WAF1} promoter which is a natural target for p53. In contrast, the alanine mutant showed a weak reduction in the ability of p53 to repress expression from the c-*fos* promoter, which is a target for p53-dependent repression *in vivo*. Strikingly, when the repression of the SV40 early promoter was examined, a reduction in the repression capacity of up to 5-fold was observed. Moreover, repression of the SV40 promoter could be partially restored by aspartic acid substitution at the phosphorylation site. These data indicate that phosphorylation at a specific C-terminal site can selectively regulate p53-dependent repression, but not transactivation.

INTRODUCTION

The p53 tumour suppressor protein (reviewed extensively by Donehower and Bradley; 1) is a potent transcription factor which is activated in response to a variety of DNA-damaging agents. Induction of p53 occurs at least in part by a post-translational mechanism leading to stabilisation of the normally rapidly-degraded protein (2). Activation of p53 leads to cell growth arrest at the G1/S boundary (3,4) or the induction of apoptosis (5,6), thereby blocking the survival of genetically damaged cells. Loss of p53 suppressor function through mutation is a common event in the development of a wide variety of human cancers and may contribute to an increase in the number of genetic abnormalities (7).

p53 transactivates a range of promoters through site-specific binding to a *cis*-acting DNA sequence element (8–11) and there is a clear correlation between the transactivation and growth suppression functions of p53 (12,13). Genes whose expression is stimulated by p53 include GADD45 (14), *mdm* 2 (15,16), and *bax* (17). The most well characterised target for p53-dependent transactivation is p21^{WAF1}, an inhibitor of cyclin dependent kinases (18), the induction of which prevents cell cycle progression by blocking phosphorylation-dependent inactivation of the retinoblastoma protein (19–21).

p53 can also repress a wide variety of cellular and viral promoters (22–25) including the c-*fos*, c-*jun*, b-*myb*, DNA polymerase α and SV40 early region promoters (23,24,26,27). Repression is not a general effect but shows promoter selectivity (23,27) and can be cell type specific (28,29). Like transactivation, repression is a function of wild type, but not mutant p53 proteins (26,30,31), suggesting that loss of p53-dependent repression may have significance in tumour development. Transactivation and repression are separable activities within the p53 polypeptide (32,33); the transactivation domain lies within the first 42 amino acids of p53 while the transrepression function is encoded within two regions, one of which overlaps with the transactivation domain, while the other lies within the C-terminal 66 amino acids. Other lines of evidence support the division of the transactivation and repression functions. For example, three oncoproteins (adenovirus E1B-19K, E1A and *bcl* 2) specifically control repression but not transactivation (34–36). Moreover, the transactivation function of p53 appears to be dispensible for p53-dependent apoptosis (37,38) but recent evidence has suggested that its transrepression function may be important (35).

p53 is phosphorylated at multiple sites *in vivo* and by several different protein kinases *in vitro* (reviewed recently by Meek; 39) including the protein kinase CKII (casein kinase II; 40). CKII is a ubiquitous cyclic nucleotide-independent serine/threonine protein kinase which targets many cellular proteins including several nuclear proteins involved in growth regulation (for reviews see 41,42). Phosphorylation by CKII potently activates the sequence-specific DNA binding function of p53 *in vitro* (43). In addition, we have shown previously that mutation of p53 at the CKII phosphorylation site to encode alanine (which cannot be phosphorylated) abolishes the growth suppressor

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activity of p53 when this mutant is expressed in several non-transformed or SV40-transformed mammalian cell lines, while partial suppressor activity can be restored by encoding aspartic acid at this position, suggesting that the additional negative charge may mimic, albeit weakly, the effect of phosphorylation (44). These data were confirmed using a temperature-sensitive mutant p53 which permitted the experiments to be internally standardised (44). Other groups have also examined the effects of introducing mutations at the CKII site on various activities encoded within the p53 polypeptide (12,45,46). These experiments indicated that phosphorylation by CKII has little effect on the regulation of p53-dependent transcriptional activation or the ability of p53 to block S phase entry. However, loss of the CKII site significantly reduced the ability of p53 to block cellular transformation by dominant oncogenes (12). The phosphorylation of p53 by CKII may therefore selectively regulate specific activities of p53.

In this paper we have explored further the role of phosphorylation in regulating p53 biological function, in particular p53-dependent transcriptional modulation. Our data show that loss of the CKII phosphorylation site has no detectable effects on the ability of p53 to transactivate promoters containing p53 repsonsive DNA elements including the p21^{WAF1} promoter which is a physiological target of p53. However, we demonstrate clear effects of the loss of phosphorylation at serine 386 on p53-dependent transcriptional repression. These results support a model in which transactivation and repression are not only separable activities within the p53 polypeptide, but are also regulated differentially by oncoproteins and by phosphorylation.

MATERIALS AND METHODS

Cell lines

The mammalian cell lines used in the study were as follows; SAOS-2 cells, which are a p53 null line derived from a human osteosarcoma (47) and a non-clonal population of p53-null murine fibroblasts which were obtained from David Lane (Dundee) and originally derived from p53 knockout mice (48). The human fibroblast cell line GM701 was a kind gift from M. Jacobson (University College, London); these cells express the SV40 large T antigen and grow well at low passage. All cell lines were maintained in Dulbecco/Vogt Modified Eagle's Medium with the addition of 10% foetal bovine serum and supplemented with 2 mM glutamine, and 100 IU/ml each of penicillin and streptomycin. The cells were grown as monolayers on plastic culture dishes at 37°C, 5% atmospheric CO_2 in a humidified incubator.

Plasmid DNAs and transfections

In all cases the reporter plasmids used to measure p53-dependent transcriptional effects employed the chloramphenicol acetyl transferase (CAT) gene. The plasmids used to measure p53-dependent transactivation were as follows. pPG13CAT, which consists of the polyomavirus early promoter and 13 upstream copies of a consensus p53 binding site (PG or polygrip) (9). A control plasmid containing 15 copies of a mutated version of the p53 binding site in place of the PG sequences was used in parallel experiments. This construct (pMG15CAT) is not activated by wild type p53. The other plasmid was pWAF1CAT, containing the WAF1 promoter which is a natural target for p53 transactivation (49). These plasmids were a kind gift from Dr B. Vogelstein (John Hopkins Oncology Center, USA).

The plasmids used to measure p53-dependent repression were as follows. pSV40CAT (Promega) contains the simian virus 40 (SV40) immediate early promoter/enhancer fused upstream of the CAT gene. This transcriptional unit has been shown to be repressed by wild type p53 *in vivo* (24,50). The plasmid pSVTKCAT was constructed by cloning the SV40 enhancer region from pSV40CAT (as a *Hin*cII fragment) upstream of the thymidine kinase promoter from herpes simplex virus in the p41X (pTKCAT) plasmid (a kind gift from Dr M. Jackson, Beatson Institute, Glasgow). The plasmid pc-*fos* CAT contains 0.7 kb of the human c-*fos* promoter region cloned upstream of the CAT gene and was a kind gift from Dr G. Glenn, Salk Institute, San Diego). The human c-*fos* promoter has also been shown to be repressed by wild type p53 (23,26). The plasmid pCMVβgal expresses β-galactosidase from the CMV early promoter and was obtained from Dr B. McStay (Biomedical Research Centre, Dundee).

The plasmids expressing wild type or mutant p53 proteins have been reported previously (44). These were pCMVNc9 (encoding wild type p53) and pCMVc5 (encoding a transforming mutant of p53); both plasmids were obtained from M. Oren, (Weizmann Institute, Israel). pCMVdel has p53 codons 1–330 deleted from pCMVNc9, while pCMVp53SA and pCMVp53SD have point mutations encoding alanine and aspartic acid residues, respectively, at the CKII site (serine 386) (44). The coding sequences of these plasmids have been checked to ensure that only the desired mutations are present and the expression of the p53 proteins from these plasmids has been checked in Rat1 and BHK cell lines (44). We have previously shown that the plasmids encoding the changes at the CKII phosphorylation site express similar levels of p53 to the wild type plasmid (pCMVNc9) in a number of cell lines (44).

The plasmids used in the transfections were all prepared by caesium chloride density centrifugation. Cells were seeded at 2×10^5 cells per 5 cm dish (or 5×10^5 cells for the slower growing lines such as SAOS-2) and the DNAs were transfected into the cells by calcium phosphate precipitation. A typical precipitation (to transfect a 5 cm diameter dish) contained a total of 10 µg DNA comprising 5 µg of reporter plasmid and 5 µg of a p53-encoding plasmid under control of the CMV early promoter (or vector-control plasmid). In some experiments 1 µg of pCMVβ-gal was also included, allowing measurement of β-galactosidase activity as an internal standard for transfection efficiency (see below). All precipitations were carried out in triplicate or quadruplicate. The cells were harvested 48 h after transfection and the monolayers were washed twice with chilled PBS to remove excess serum. The cells were lysed using Reporter Gene Lysis Buffer (0.2 ml per plate; Promega), and the lysates were heated to 65°C for 10 min to inactivate any endogenous deacetylase activities. The lysates were cleared by microcentrifugation and the soluble fractions were routinely stored at –80°C before assaying for CAT activity.

In order to examine the efficiency of transfection in this system, a β-galactosidase reporter plasmid was co-transfected, and a single plate from each quadruplicate transfection was stained with 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ in phosphate-buffered saline. The percentage of the cells in the dish whose nuclei stained blue gave an

indication of the efficiency of the transfection method. Routinely, 3–10% of cells took up enough of the β-gal plasmid to stain an intense blue (the fraction of cells stained was always consistent within a set of transfections but varied between experiments).

Assay of chloramphenicol acetyl transferase activity in cell lysates

Incubations for the measurement of CAT activity contained: 100 µl cell lysate, 3 µl [^{14}C]chloramphenicol (ICN; 100 µCi/ml, 105 mCi/mmol in 25 mM Tris pH 7.4), 14 µl 3.5 mg/ml acetyl coenzyme A and 10 µl distilled water. (The amounts of lysate in the reactions were corrected for protein concentration as described by others; 24,34.) Reactions were incubated at 37°C for periods varying from 10 to 19 h, depending on the levels of CAT activity obtained from the promoter construct under investigation and the cell type used. Reactions were extracted with 500 µl ethyl acetate to partition the chloramphenicol into the solvent layer, and 480 µl of the solvent phase was dried under vacuum. The dried reaction products were resuspended in 30 µl ethyl acetate and were applied to a TLC plate and allowed to dry completely. The products were separated by chromatography in a 95:5 chloroform:methanol mixture in a pre-equilibrated tank. After drying, the plates were subjected to autoradiography to visualise the reaction products, and to phosphorimage analysis using the BioRad GS-250 Molecular Imager and Molecular Analyst sofware to allow quantitation. In the graphs, all points are displayed as the mean of triplicate assays together with the standard deviation over the mean.

RESULTS

Transactivation of a p53-responsive reporter plasmid is not affected by the phosphorylation status of Ser386

To examine the requirement for phosphorylation by CKII in the transcriptional activation function of p53, plasmids expressing wild type or mutant p53 proteins encoding alanine or aspartic acid at the CKII phosphorylation site were co-transfected in triplicate into p53-null fibroblasts or SAOS-2 cells together with an equal amount of pPG13CAT or pMG15CAT. Following transfection, the cells were incubated for 48 h after which lysates were prepared and analysed for CAT activity. The data (Fig. 1A) show that expression of wild type p53 (lanes W) in the p53-null mouse fibroblasts gave rise to efficient transactivation of the pPG13CAT construct containing a multiple-site p53-responsive element, but not the pMG15CAT construct which contains a mutated version of the site. The negative control plasmid pCMVc5 (lanes M) failed to cause transactivation of the reporter, confirming that the effect observed is due to wild type p53 protein, and not a non-specific effect of p53 overexpression or of the CMV promoter. The p53 proteins altered at the CKII phosphorylation site to encode alanine or aspartic acid (lanes A and D, respectively) were also able to transactivate the reporter plasmid to an extent comparable to wild type p53 (see lanes for A and D co-transfected with pPG13CAT). These data indicated that, at the levels of DNA transfected, alteration of the CKII phosphorylation site did not significantly influence the ability of p53 to transactivate an artificial reporter construct containing multiple copies of the p53 consensus binding site. These results were confirmed using the human SAOS-2 cell line (data not shown) indicating that the effect was reproducible in other cell types.

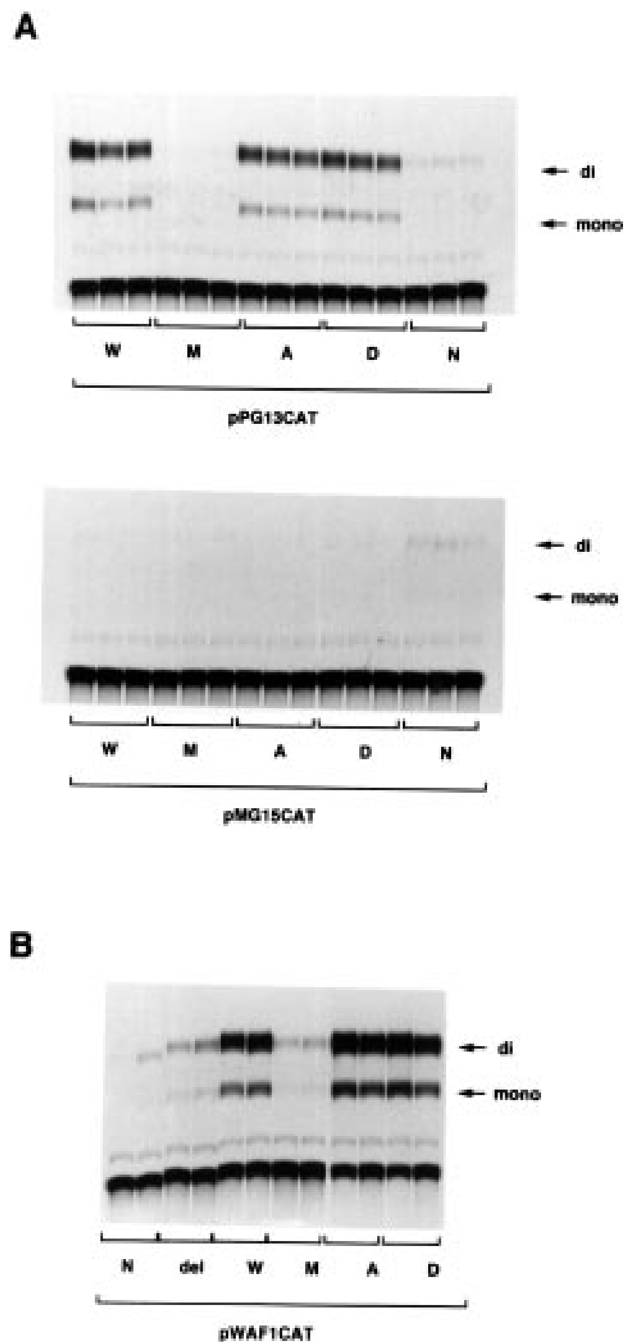

Figure 1. Transactivation of reporter constructs by p53 mutated at Ser386. p53-null murine fibroblasts were transfected with CAT-reporter plasmids and p53-expressing plasmids. The reporter plasmids were pPG13CAT and independently, pMG15CAT as control (**A**), and pWAF1CAT (**B**). In each case, these reporter plasmids were co-transfected with p53-expressing plasmids as follows: wild type (W), transforming mutant (M), alanine 386 (A), aspartate 386 (D) and an expression plasmid lacking the p53 coding region (del). Control transfections including only a reporter plasmid are denoted (N). Cells were harvested after 48 h and lysates assayed for CAT activity as described. The data are representative of several independent experiments.

The ability of the mutant proteins to influence transcription from a promoter which is transactivated physiologically by p53 was also examined. In these experiments the plasmids encoding the wild type or mutant p53 proteins were co-transfected with the reporter plasmid in which the CAT gene was under control of the p21^{WAF1} promoter (pWAF1CAT; 49). The effects of p53 on expression from the WAF1 promoter construct in p53-null mouse fibroblasts are shown in Figure 1B. The results were very similar to those obtained with the pPG13CAT construct: wild type p53 (lanes W) transactivated the WAF1 promoter effectively while the negative controls [pCMVdel (lanes del) and pCMVc5 (lanes M)] yielded background levels of CAT activity. The phosphorylation site-mutant proteins (lanes A and D) again displayed wild type activities. In general, the transactivation observed on the WAF1 promoter was stronger than that on the PG13CAT construct as reflected by higher CAT levels under identical conditions (~2–3-fold, perhaps indicating the importance of the context of the p53 binding site in the promoter and hinting at a possible role for interaction with other factors). The transfections with the WAF1 promoter were also carried out in SAOS-2 cells with similar results (data not shown), although the background levels of expression from the reporter plasmid were higher leading to less pronounced activation by wild type p53.

The lack of activity changes observed with the p53 phosphorylation site mutants is not attributable to an excess of transfected DNA

One possible explanation for the lack of activity changes observed with the phosphorylation site mutants was that the levels of DNA transfected gave saturating levels of p53 in the cells which could mask subtle changes in the level of transcriptional activity. For example, others have shown that the effects of deletion of the C-terminus of p53 are masked when high levels of plasmid are transfected and only become apparent when the amount of DNA transfected is reduced accordingly (51). In order to examine this possibility, the co-transfection experiments described above were repeated in the mouse p53-null fibroblasts using variable amounts of the pCMV-p53 constructs. The total amount of DNA transfected was equalised with the pCMV-del construct to avoid any potential confusion from the number of copies of the CMV promoter present in each cell. A range of amounts of the p53 constructs were tested, from 1 ng up to 5 µg. In a preliminary transfection using pCMVNc9 (encoding wild type p53), it was observed that maximal CAT activity was obtained using 0.5–1 µg of this plasmid and that higher levels of plasmid led to a slight reduction in the level of transactivation observed (data not shown), probably due to sequestration of the basal transcription factors machinery by excess p53 molecules (33,51). The results of the dose-response analysis for the wild type and mutant proteins are shown in Figure 2. Each data point shows the mean of triplicate transfections together with the standard deviation. The data for both pPG13CAT and pWAF1CAT indicate that the levels of transactivation observed are dependent on the amount of p53-expressing plasmid transfected, with approximately background levels being observed at the lowest dose (1 ng) and levels increasing up to the maximum of 100 ng shown here. When pPG13CAT was used as the reporter (Fig. 2A), the responses of wild type p53 and the two phosphorylation site mutants at each level of p53-expressing plasmid were essentially similar. A slight decrease in the level of transactivation from the alanine 386 mutant (in comparison with the wild type p53) was observed with the pPG13CAT plasmid (Fig. 2A) but when the errors in the measurements were taken into account, this difference was not significant. Moreover, when the pWAF1CAT reporter plasmid was examined (Fig. 2B), there were no significant differences in the behaviour of the wild type p53 in comparison with the alanine or aspartic acid 386 mutants. These data therefore confirm that the serine 386 mutations do not significantly influence the ability of p53 to transactivate transcription mediated by a p53 responsive element, even at suboptimal levels of p53 in the cell.

The transcriptional repression function of p53 is modulated by mutation of the CKII phosphorylation site

To determine whether phosphorylation by CKII played a role in transcriptional repression by p53, reporter constructs with the c-*fos* promoter or the SV40 early promoter fused to the CAT gene were used; both of these promoters are repressed by p53 *in vivo* (23,24,26,50). The effects of the CKII phosphorylation site mutations on p53-dependent repression of the c-*fos* promoter in p53-null murine fibroblasts are shown in Figure 3A. The data show that repression is mediated (although weakly) by wild type p53 (W) but not by the CMV vector alone (del). Lack of repression by the transforming p53 mutant (M), indicated that the effect was not due to non-specific squelching by an excess of p53 protein. The alanine and aspartic acid 386 mutants behaved much more like the transforming mutant in this assay, showing essentially no repressive activity. These data therefore suggested that loss of the phosphorylation site significantly reduced the ability of p53 to repress the c-*fos* promoter. The effects of these p53 proteins on repression of a hybrid promoter comprising the human herpes simplex virus thymidine kinase basal promoter with the SV40 enhancer element were were also examined in p53-null fibroblasts. Although this was a composite rather than a natural promoter, the level of transcription was higher (3–4-fold) than with the c-*fos* promoter (the level of transcription, and hence repression, was barely detectable with the thymidine kinase promoter alone; Fig. 3B). Once again, the phosphorylation site mutants were significantly less able to repress transcription than wild type p53. (Owing to errors in measuring the low levels of transcription observed with the Fos and SVTK promoter plasmids, it was difficult to discern differences between the alanine and aspartic acid mutants.) Similar results were obtained using SAOS-2 cells and when the complete SV40 early promoter was examined in both cell types (data not shown).

Repression of the SV40 early promoter was also examined in GM701 cells, a line of human fibroblasts transformed by SV40. The levels of expression of the SV40 promoter in this line were considerably higher than the c-*fos* promoter in the SAOS-2 or mouse fibroblast cells (by ~15-fold; data not shown). The level of wild type p53-dependent transcriptional repression of the SV40 promoter was of the order of 10-fold (Fig. 4; compare lanes N and del with lane W), while the transforming mutant p53 was unable to repress transcription. Strikingly, the level of repression observed with the alanine 386 mutant p53 was 5-fold lower than the wild type (compare lanes W and A), indicating that the presence of the serine at the CKII phosphorylation site was a significant factor in determining the level of transcriptional repression. When the aspartic acid 386 mutant was examined, once again there was a significant reduction on CAT activity (by

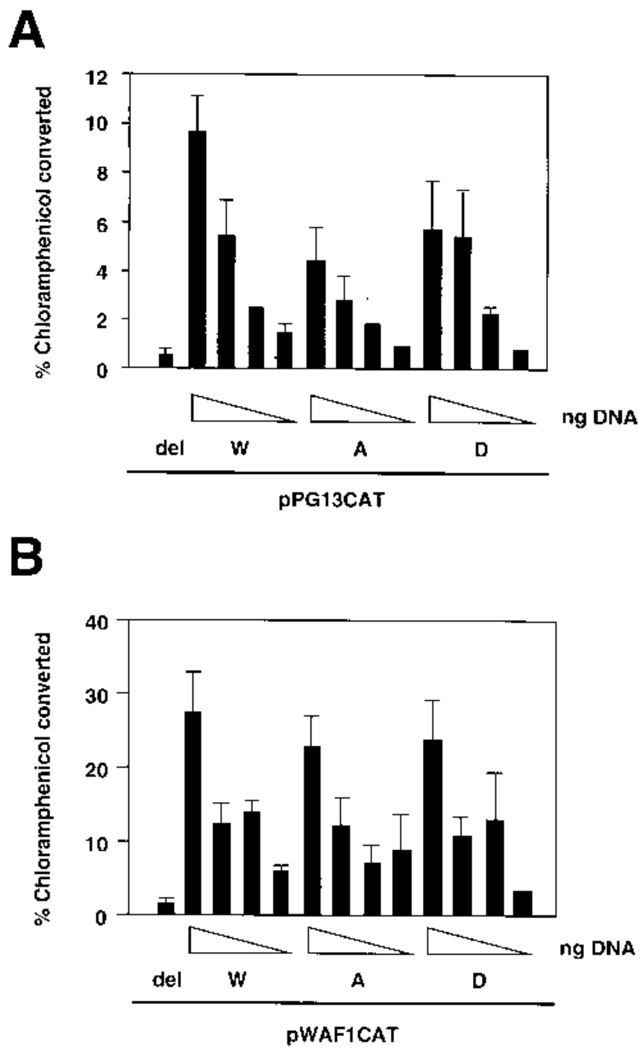

Figure 2. Dependence of p53 transactivation on levels of pCMV-p53 plasmid. p53-null murine fibroblasts were co-transfected with 5 µg of either pPG13CAT (**A**) or pWAF1CAT (**B**) and varying amounts of the plasmids encoding wild type p53 (W), or the alanine- (A) or aspartate-386 (D) mutant p53 proteins. The range of amounts of the p53-expressing plasmids were 100, 10, 5 and 1 ng, respectively. In each case the total amount of plasmid DNA was made up to 5 µg with pCMV-del DNA and the level of baseline activity was measured using 5 µg of the pCMV-del plasmid (del) in the absence of p53 expression. Cells were harvested after 48 h and lysates were assayed for CAT activity. Results are presented as a bar chart showing the mean of the percentage chloramphenicol converted to the acetylated form ± standard deviation from triplicate assays, corrected for the protein concentration of the lysates. The data are representative of several independent experiments.

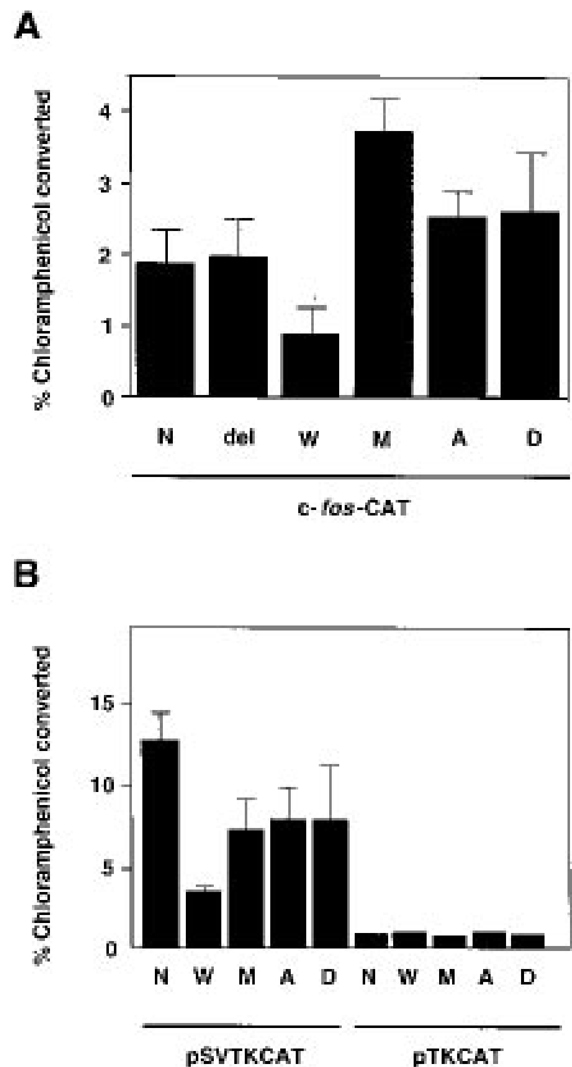

Figure 3. Transrepression of the human c-*fos* promoter by p53 mutated at Ser 386. p53-null fibroblasts were transfected with 5 µg of pc-*fos* CAT (**A**), pSVTKCAT or pTKCAT (**B**) and 5 µg of plasmid encoding wild type p53 (W), a transforming mutant p53 (M), the alanine- (A) or aspartate-386 (D) mutant p53 proteins, or with the p53 expression plasmid in which the p53 coding region had been deleted (del). Some cells were transfected with the reporter plasmid alone (N). Cells were harvested after 48 h and were assayed for CAT activity which was quantitated using a BioRad Phosphor-Analyst. Results are presented as a bar chart showing the mean of the percentage chloramphenicol converted to the acetylated form ± standard deviation from triplicate assays, corrected for the protein concentration of the lysates. The data are representative of several independent experiments.

~2-fold) compared with wild type p53 (compare lanes W and D). This result suggested that the presence of a negative charge at this site made a significant contribution towards restoring wild type levels of repression. In addition, the overall increase in sensitivity of measuring CAT activity in the GM701/pSV40CAT system allowed considerably more accurate quantitative measurements of p53-dependent repression and the results underpinned the observations in Figure 3 that the integrity of the CKII phosphorylation site in p53 is an important factor in this process.

DISCUSSION

In this paper, we describe the effects of introducing mutations at the CKII site in mouse p53, on the ability of the protein to behave as a transcriptional regulator. Two mutations were examined: the first of these replaced serine 386 with alanine which cannot be phosphorylated; the other change introduced an aspartic acid, and therefore a constitutive negative charge at this position. When expressed in murine fibroblasts or the human osteosarcoma line SAOS-2, both of which lack endogenous p53, the phosphoryla-

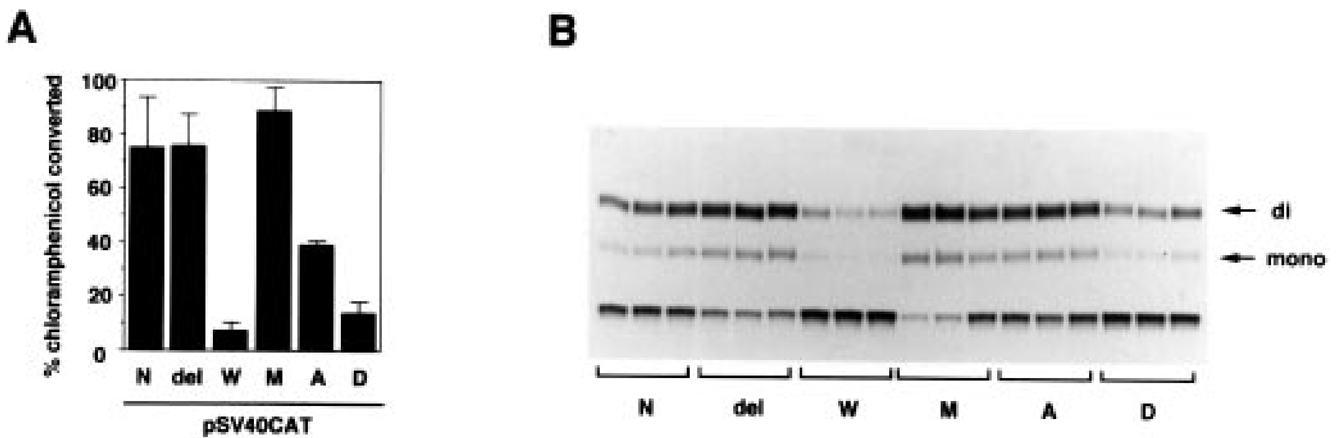

Figure 4. Transrepression of the SV40 promoter by Ser386 mutant p53s in GM701 cells. GM701 cells were transfected with the pSV40CAT reporter plasmid alone or with plasmids encoding wild type p53 (W), transforming mutant p53 (M), the alanine (A) or aspartate (D) 386 mutant p53 proteins, respectively, or with the p53 expression plasmid in which the p53 coding region had been deleted (del). Some cells were transfected with the reporter plasmid alone (N). The cells were harvested after 48 h and lysates were assayed for CAT activity as described. (**A**) Activities were quantitated using a BioRad PhosphorAnalyst and are represented as a bar chart showing the mean of the percentage chloramphenicol converted to the acetylated form± standard deviation from triplicate assays. The data are representative of several independent experiments. (**B**) A representative exposure showing triplicate measurements.

tion site mutant proteins were indistinguishable from wild type p53 in their abilities to transactivate a promoter containing tandem synthetic p53 DNA responsive elements, or the WAF1 promoter which is a natural target for p53 (Fig. 1). Moreover, even at progressively lower levels of p53 expression, where the amount of p53 available for transactivation becomes limiting, the mutant proteins were still able to mimic wild type p53 (Fig. 2), indicating that the change of residue at the phosphorylation site had no significant role in transcriptional activation. Several other groups have reported similar findings (12,45,46) and our data are therefore in full agreement with these publications. This result is perhaps surprising because activation of the sequence-specific DNA binding function of p53 through phosphorylation by CKII (43) might be expected to stimulate p53 as a transcriptional activator. However, it is possible that other mechanisms in the cell (e.g. phosphorylation of p53 at other sites by different enzymes) may be able to fully activate p53-dependent transactivation and overcome any possible contribution from phosphorylation of p53 at the CKII site. The CKII mutants have also been reported to efficiently mediate G1 growth arrest (12,45,46), an activity which is closely linked to the transactivation capacity of p53 (12,13).

In contrast to the lack of effects on transcriptional transactivation, the data in this paper consistently show that the presence of serine at position 386 is an important factor in achieving a high level of p53-dependent transcriptional repression (Figs 3 and 4). The magnitude of this effect (and indeed of the level of repression with wild type p53) appears to be promoter and cell type specific (other researchers have made similar observations; 28). Although the experimental errors were generally high in examining repression of the (weak) c-*fos* promoter, the general trend of the results was consistent, indicating that mutation of the CKII phosphorylation site resulted in lower repression activity of the p53 (Fig. 3). In contrast, the high levels of expression of the SV40 promoter in the GM701 cells and the potency of p53-dependent repression in this system allowed accurate quantitative measurement of the effects of the p53 mutants (Fig. 4). Moreover, the intermediate effect of the aspartic acid mutant was observed much more clearly and reproducibly in the GM701 cells (Fig. 4) and is consistent with an important role for phosphorylation of this residue. One potential contributing factor to the transcriptional measurement in the GM701 cells is the presence of SV40 which has a well-established role in the stabilisation and accumulation of p53 in the cell (including p53 uncomplexed with T antigen). This is a phenomenon which occurs as part of the activation of p53 in response to DNA damaging agents (2,3,52). SV40 may therefore stimulate molecular pathways which activate p53 and the effects of phosphorylation at the CKII site may well be intensified under such conditions. It is also possible that T antigen may itself have effects on repression of the SV40 promoter through its ability to block wild type p53 function. However, although T antigen is present in the GM701 cells, the differences in the levels of p53-mediated repression are still observed. Moreover, since the p53-mediated repression is stronger in these cells, we were able to accurately measure the effects of the loss of phosphorylation at serine 386. The mechanism by which phosphorylation of p53 at the CKII site contributes to efficient repression is not known, but may be mediated (in this case) at least partly through the interation of T antigen and p53 (our previously published data indicated that complex formation between p53 and T antigen was unaffected by phosphorylation of serine 386; 44). Similarly, the oligomerisation status of p53 is not affected by phosphorylation at serine 386 (data not shown). p53 is also able to bind directly to a number of basal and other transcription factors (including TFIID components, the CAAT-binding factor, Sp1 and AP-2; 31,53–57) and may repress transcription by preventing the interaction of certain factors with the promoter region (30,55). It is therefore possible that phosphorylation of the C-terminal tail of p53 signals a conformational shift which enables the protein to bind more efficiently to other transcription factors. A similar mechanism is thought to unmask the specific DNA binding domain of p53 in response to CKII phosphorylation (43) and it is possible that movement of this tail could additionally stimulate interaction with other transcription factors. The data presented in this paper, indicating that phosphorylation can regulate

repression independently of transactivation, are also consistent with the finding that domains of the p53 protein responsible for transactivation and repression overlap, but are not identical (32,33).

The finding that phosphorylation of a C-terminal site can selectively regulate repression but not transactivation is striking but not unprecedented. For example the repression function of the Fos protein is also controlled through the phosphorylation of C-terminal sites while the transactivation capacity remains unaltered (58). Moreover, other mechanisms exist which can control p53-dependent repression independently of transactivation. For example, three oncoproteins (adenovirus E1B-19K, E1A and *bcl* 2) specifically control repression but not transactivation (34–36). The finding that viral oncogene products, cellular oncogene products and phosphorylation each permit independent modulation of transactivation and repression implies a strong evolutionary pressure for the selective regulation of different activities within the p53 protein.

We and others have previously studied the effects of p53 proteins with alterations at the CKII phosphorylation site of p53. A number of different assays measuring separate activities within the p53 protein have been examined in these studies. Thus, the mutants were reported to be unaltered in specific DNA binding (59), transcriptional transactivation of various promoters (12,45,46), G1 growth arrest (45,46), inhibition of drug-resistant colony formation (46) and cellular transformation by mutant forms of p53 (60). In one study however, a minor reduction in inhibition of the growth of SAOS-2 cells was observed while there was a significant (5–10-fold) reduction in the ability to block transformation by activated *ras* and E7 oncogenes (12). Moreover, our own work has attributed an important role for phosphorylation at the CKII site to the ability of p53 to block the growth of G418-resistant colony formation, particularly in SV40-transformed cell lines (44) (this assay was used as a measure of the anti-proliferative activity of p53, but it did not discriminate between G1 arrest or apoptosis). Additionally, we have shown in the present study that repression is also dependent on the integrity of the CKII site. Taken together, these studies suggest that phosphorylation by CKII can selectively regulate particular functions within the p53 protein and that the magnitude of phosphorylation-dependent changes in activity may vary according to factors such as cell type and growth or transformation status. It will be important to determine the relationship between these regulated functions and to show whether phosphorylation by CKII *per se* is responsible for controlling p53 in the cell. Experiments are underway in our laboratory to address these issues.

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