p53 is phosphorylated *in vitro* and *in vivo* by the delta and epsilon isoforms of casein kinase 1 and enhances the level of casein kinase 1 delta in response to topoisomerase-directed drugs

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The p53 tumour suppressor protein plays a key role in the integration of stress signals. Multi-site phosphorylation of p53 may play an integral part in the transmission of these signals and is catalysed by many different protein kinases including an unidentified p53-N-terminus-targeted protein kinase (p53NK) which phosphorylates a group of sites at the N-terminus of the protein. In this paper, we present evidence that the delta and epsilon isoforms of casein kinase 1 (CK1δ and CK1ε) show identical features to p53NK and can phosphorylate p53 both *in vitro* and *in vivo*. Recombinant, purified glutathione S-transferase (GST)-CK1δ and GST-CK1ε fusion proteins each phosphorylate p53 *in vitro* at serines 4, 6 and 9, the sites recognised by p53NK. Furthermore, p53NK (i) co-purifies with CK1δ/ε, (ii) shares identical kinetic properties to CK1δ/ε, and (iii) is inhibited by a CK1δ/ε-specific inhibitor (IC261). In addition, CK1δ is also present in purified preparations of p53NK as judged by immunoanalysis using a CK1δ-specific monoclonal antibody. Treatment of murine SV3T3 cells with IC261 specifically blocked phosphorylation *in vivo* of the CK1δ/ε phosphorylation sites in p53, indicating that p53 interacts physiologically with CK1δ and/or CK1ε. Similarly, over-expression of a green fluorescent protein (GFP)-CK1δ fusion protein led to hyper-phosphorylation of p53 at its N-terminus. Treatment of MethAp53ts cells with the topoisomerase-directed drugs etoposide or camptothecin led to increases in both CK1δ-mRNA and -protein levels in a manner dependent on the integrity of p53. These data suggest that p53 is phosphorylated by CK1δ and CK1ε and additionally that there may be a regulatory feedback loop involving p53 and CK1δ.

Keywords: CK1δ; CK1ε; DNA damage; p53; phosphorylation; protein kinase

Introduction

The p53 tumour suppressor protein (for reviews see Gottlieb and Oren, 1996; Ko and Prives, 1996) is a potent transcription factor which is activated in response to a variety of cellular stresses including DNA damage, heat shock, hypoxia, cytokines, metabolic changes, viral infection and activated oncogenes, and is considered to be an integrator of these signals

(Hall *et al.*, 1996; Jacks and Weinberg, 1996). Activation of p53 can lead to cell growth arrest at the G1 (Kastan *et al.*, 1991, 1992; Kuerbitz *et al.*, 1992) or G2 (Aloni *et al.*, 1995; Cross *et al.*, 1995; Paules *et al.*, 1995) phases of the cell cycle, or the induction of apoptosis (Shaw *et al.*, 1992; Yonish *et al.*, 1991) and is thought to prevent the proliferation of genetically damaged cells (Lane, 1993). Consequently, loss of p53 function, through mutation, which is a common occurrence in the development of tumours (Hollstein *et al.*, 1991), is a central event in the progression of the disease (Kinzler and Vogelstein, 1996).

The activity of p53 is regulated at different levels and includes control by multi-site phosphorylation (reviewed by Meek, 1994). Multi-site phosphorylation of the N-terminus of p53 is thought to provide a synergistic mechanism which can stimulate p53-dependent transactivation, growth arrest and suppression of cellular transformation (Fiscella *et al.*, 1993; Lohrum and Scheidtmann, 1996; Mayr *et al.*, 1995). At least six sites within the N-terminal 83 amino acids of p53 are phosphorylated *in vivo* and several different protein kinases have been identified which phosphorylate these sites *in vitro*, including DNA-activated protein kinase (DNA-PK) (Lees-Miller *et al.*, 1990), MAP kinase (Milne *et al.*, 1994) and the u.v.- and stress-inducible kinase JNK1 (Milne *et al.*, 1995); p53 can also be phosphorylated by high levels of casein kinase 1α (the major isoform of the casein kinase 1 (CK1) family (Milne *et al.*, 1992)).

Five members of the CK1 family have now been cloned from mammalian cells (isoforms α, β, γ, δ, and ε; (Fish *et al.*, 1995; Graves *et al.*, 1993; Rowles *et al.*, 1991); reviewed by Christenson *et al.*, 1996). These show striking similarity within their kinase domains but can differ extensively in their C-terminal non-catalytic domains. The sites in p53 phosphorylated by CK1 (serines 4, 6 and 9 in mouse p53) and DNA-PK (serine 15) are the most highly phosphorylated sites in the protein (Meek, 1994), and are turned over rapidly, consistent with an acute role in regulation (our unpublished data). Although CK1α can phosphorylate these N-terminal sites *in vitro*, it is not thought to phosphorylate p53 *in vivo* (Milne *et al.*, 1992). Moreover, little is known about the signalling mechanism(s) which regulates the modification of p53 at these sites, nor are the stimuli to which such a signalling pathway responds known. Our finding that p53 is preferentially phosphorylated by a CK1-like enzyme, which is separable from CK1α by affinity chromatography, suggests that p53 is phosphorylated

in the cell by a CK1 isoform or a highly related kinase (Milne *et al.*, 1992). Moreover, the potential interaction of p53 with members of the CK1 family is of striking interest since CK1 family members play an integral role in resistance to radiation and DNA damage in yeasts and Drosophila (Dhillon and Hoekstra, 1994; Hoekstra *et al.*, 1991). Finally, our recent observation that p53 N-terminus-targeted protein kinase (p53NK) activity can be stimulated by anti-tumour drugs which elicit DNA damage (Knippschild *et al*, 1996) makes the identification of this kinase(s) and characterisation of its interaction with p53 a major goal in understanding the mechanisms which activate and control p53 function.

In this paper we present evidence that p53 can be phosphorylated *in vitro* by the delta and epsilon isoforms of CK1 (CK1δ and CK1ε) respectively which are highly related to each other (Fish *et al.*, 1995; Graves *et al.*, 1993) and show that the identity of the cellular p53NK is entirely consistent with CK1δ and CK1ε. We also provide compelling evidence that p53 is phosphorylated *in vivo* by these kinases. Furthermore, we show that level of CK1δ is influenced by the presence of wild type p53, suggesting the possibility that the levels of p53 and CK1δ may be interdependent.

Results

p53 is phosphorylated in vitro at N-terminal sites by the delta and epsilon isoforms of CK1

We have shown previously that serines 4, 6 and 9 of murine p53 are phosphorylated by a CK1-like enzyme(s) which we have termed p53-N-terminus-targeted kinase (p53NK; Knippschild *et al.*, 1996; Milne *et al.*, 1992). Analysis of partially purified p53NK (see Materials and methods) using a 'blotted protein kinase' assay with p53 as substrate (Ferrell and Martin, 1991) revealed a single species of kinase with a molecular weight of 49 000 (data not shown) consistent with the identity of the p53 kinase as either the delta (δ) or epsilon (ε) isoform of CK1, but not other CK1 isoforms which are considerably smaller (α, β and γ; Christenson *et al.*, 1996). CK1δ and CK1ε show both striking sequence similarity (98% within the kinase domains (Fish *et al.*, 1995; Graves *et al.*, 1993; DeMaggio AJ, Christenson E and Hoekstra MF, submitted) and functional similarity (DeMaggio AJ, Christenson E and Hoekstra MF, submitted). To determine whether p53 was a substrate for either of these enzymes, the ability of recombinant CK1δ or CK1ε to phosphorylate a number of GST-p53 fusion proteins was measured. The data indicate that both FP267 (N-terminal 64 amino acids of murine p53) and casein were substrates for the recombinant CK1δ (Figure 1c) and CK1ε (Figure 1a, b and c). Note that multiple bands are evident in the p53 proteins phosphorylated by the CK1 isoforms (see especially Figure 1c where the exposure is lighter); we have shown previously that the p53NK catalyses multisite phosphorylation of serine residues 4, 6 and 9 in murine p53 and generates multiple closely migrating bands in SDS gels (Knippschild *et al.*, 1996; Milne *et al.*, 1992). In addition, recombinant human CK1δ and CK1ε are
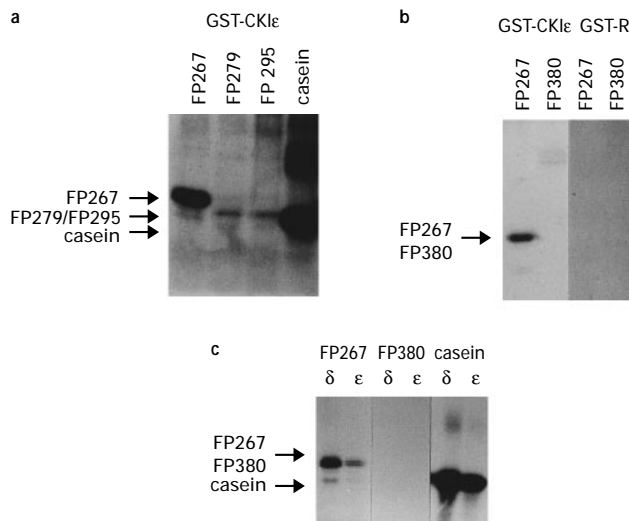
**Figure 1** Phosphorylation of p53 by the delta and epsilon isoforms of CK1. The GST-p53, GST-CK1δ and GST-CK1ε fusion proteins used in this experiment, which are described in Materials and Methods, were expressed in bacteria and purified by affinity chromatography on glutathione-sepharose 4B beads. Protein kinase assays were carried out as described in Materials and methods, and the phosphorylated products were separated by SDS–PAGE on 12.5% gels and detected by autoradiography. The positions at which casein and the GST-p53 fusion proteins migrated on the SDS gels are indicated. GST-R in panel B is a fusion protein in which the CK1ε cDNA was cloned into the vector in the anti-sense orientation. The product of this fusion was purified and used in this experiment as a control

capable of phosphorylating a peptide based on the N-terminal amino acids of human p53 (data not shown). Two fusion proteins lacking the first ten amino acids of p53 (FP279 and FP295) were poor substrates for CK1ε (Figure 1a). Similarly, a fusion protein, FP380, in which these residues were replaced with alanine residues, was no longer a substrate for either CK1δ (Figure 1c) or CK1ε (Figure 1b), consistent with these sites being the target residues. A control protein (GST-R) in which the CK1ε coding sequence was cloned in the anti-sense orientation with respect to GST, had no protein kinase activity (Figure 1b). Taken together, these data indicate that both CK1δ and CK1ε can phosphorylate p53 *in vitro* at the same sites as the cellular p53NK.

p53NK shows properties characteristic of CK1δ and CK1ε

To further explore the possibility that p53NK was an isoform(s) of CK1, three additional biochemical measurements were carried out. Firstly, the co-purification of cellular p53NK with ³⁵S-labelled CK1δ or CK1ε was investigated. Extracts from rat F111 cells were mixed with ³⁵S-labelled *in vitro*-translated rat CK1δ or human CK1ε, then partially purified using the procedure described in Materials and methods. The data shown in Figure 2 are taken from the first step, ion exchange chromatography on Mono Q H/R 5/5 columns. These elution profiles show that p53NK activity co-eluted with either radiolabelled CK1δ or CK1ε; (note that the difference in the positions at which the kinase eluted in Figure 2a *versus* b most
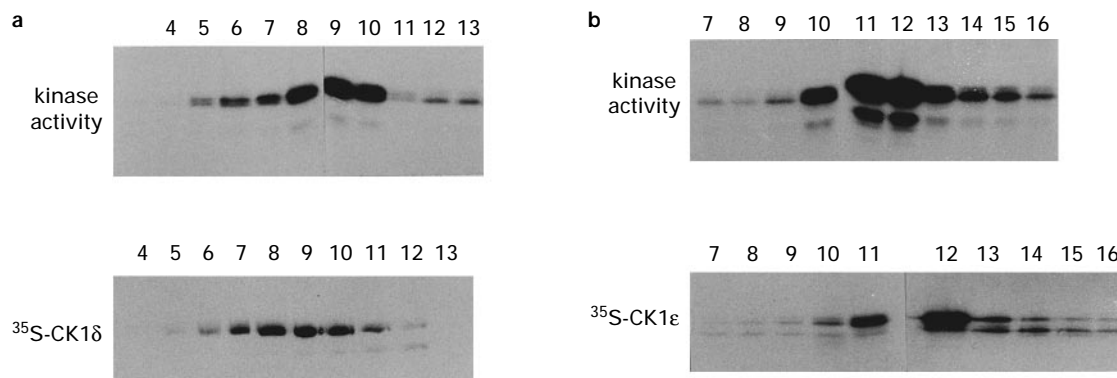
Figure 2 Co-purification of cellular p53NK with ^{35}S-labelled *in vitro*-translated CK1δ or CK1ε. Rat F111 cells were lysed, the extracts were mixed with ^{35}S-labelled *in vitro*-translated CK1δ or CK1ε, and the proteins were chromatographed on a Mono Q (H/R 5/5) column as described in Materials and methods. p53 kinase activity was measured using the GST-p53 fusion protein FP267 as substrate and the phosphorylated proteins were resolved by SDS–PAGE on 12.5% gels and detected by autoradiography (upper panels **a** and **b**). Aliquots of each fraction from the Mono Q column were also separated by SDS–PAGE and the gels were prepared for fluorography using Enhance (Amersham) then exposed to X-ray film at −80°C (lower panels in **a** and **b**). The different positions at which the peaks eluted in panel A as compared with panel B was the result of using two different Mono Q columns

likely occurred because two different Mono Q columns were used). Identical elution profiles were obtained if the cellular extracts and the radiolabelled proteins were not mixed but chromatographed separately (data not shown). In addition, the kinase activity and ^{35}S-radiolabel continued to co-elute during further purification on phosphvitin-sepharose 4B and Mono S columns (data not shown). (The similarity in the elution characteristics of CK1δ and CK1ε was not surprising since these isoforms show striking similarity to each other at the amino acid level, especially within their kinase domains (Fish *et al.*, 1995; Graves *et al.*, 1993; DeMaggio AJ, Christenson E and Hoekstra MF, submitted). In addition to co-purification with the radiolabelled proteins, partially purified fractions containing p53NK activity could be inhibited by micromolar concentrations of IC261, an inhibitor which is specific for CK1δ and ε (data not shown; DeMaggio AJ, Christenson E and Hoekstra MF, submitted).

The kinetic properties of the cellular p53NK (partially purified from SV52 cells by a three-step procedure (see Materials and methods)) and the bacterially-expressed CK1 isoforms were also measured (using Lineweaver-Burk plots) and the data are summarised in Table 1. When the p53 substrate FP267 was used, no significant differences were found in the K_m values for p53NK, CK1δ, CK1ε. Moreover, the K_m value was also similar for CK1εFS, which has an intact CK1ε kinase domain, but has a frame-shift in the C-terminal domain such that the C-terminal 63 amino acids are substituted with 59 different amino acids followed by a stop codon (Materials and methods). Similarly, when casein was used as substrate, the behaviour of each of the enzymes was identical.

p53NK is recognised by a CK1δ-specific monoclonal antibody

Immunological evidence also supports the idea that p53 and CK1δ interact physiologically. At each stage of the purification of p53NK from cells, the fractions were examined by Western blotting using 128A, a

Table 1 Kinetic properties of p53NK and CK1δ and CK1ε isoforms

| Kinase	Substrate	K_m (μM)
p53NK	casein	1.25
Ck1δ	casein	1.33
CK1ε	casein	1.30
CK1εFS	casein	1.54
p53NK	p53 (FP267)	4.00
CK1δ	p53 (FP267)	2.90
CK1ε	p53 (FP267)	3.33
CK1εFS	p53 (FP267)	2.90

monoclonal antibody specific for CK1δ which does not recognise the α, β, γ or ε isoforms (DeMaggio AJ, Christenson E and Hoekstra MF, submitted). An example is shown in Figure 3. Elution of p53NK from a Mono S column (the third stage in the purification procedure) showed that a single peak of kinase activity was detectable upon elution with a gradient of NaCl. The appearance of CK1δ by western analysis was observed only in the same fractions as those containing the kinase activity. Similar observations were made when fractions from earlier stages of the purification procedure were examined (i.e. following fractionation using the Mono Q or phosvitin-sepharose-4B columns; data not shown). The co-purification of CK1δ protein with p53NK protein kinase activity over several fractionation regimes strongly supports the identity of CK1δ as a p53-N-terminus-targeted protein kinase.

p53 is phosphorylated by CK1δ in vivo

To determine whether the interaction between p53 and CK1δ occurs physiologically, two approaches were taken. In the first, murine SV3T3 cells were treated with the CK1δ/ε-specific inhibitor, IC261 (DeMaggio AJ, Christenson E and Hoekstra MF, submitted); SV3T3 cells were chosen because they express high levels of p53 which is phosphorylated extensively at the N-terminus (Milne *et al.*, 1994, 1995). The cells were labelled with ^{32}P-orthophosphate for 2 h, then treated for 1 h with 3 μM IC261 in DMSO; (cells were treated

with DMSO alone as control). *In vitro* measurements of the inhibition of CK1δ and CK1ε by IC261 confirmed that, in each case, the kinase activities were
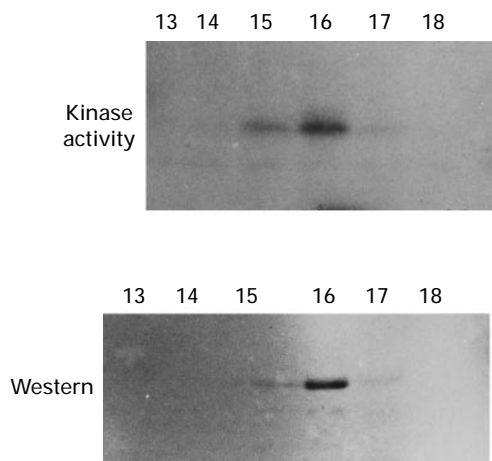

Figure 3 Partially purified p53NK can be detected by a monoclonal antibody specific for CK1δ. p53NK was partially purified through three different columns as described in Materials and methods. p53NK activity (measured using the GST-p53 fusion protein FP267 as substrate) elutes from the third column (Mono S) as a single peak; the kinase activity of fractions 13–18 are shown with p53NK eluting at fraction 16, corresponding to elution at approximately 320 mM NaCl. The Western blot showing the elution position of CK1δ is shown in the lower panel

inhibited by greater than 80% at a concentration of 3 μM (data not shown). The cells were then lysed and the phosphorylated p53 was immunoprecipitated and analysed by two-dimensional tryptic phosphopeptide mapping. Figure 4 shows the phosphopeptide pattern obtained in the mock treatment (Figure 4a). The first cleavage site for trypsin in murine p53 is after the lysine residue at position 24: phosphorylation of p53 at serines 4, 6 and 9 (by CK1) and 15 (by DNA-PK or related kinases) would therefore be expected to give rise to multiply phosphorylated species of this peptide. Each subsequent addition of a phosphate will increase the mobility of the peptide towards the anode and reduce its migration with the solvent front during chromatography. The peptides labelled 1–4 in Figure 4 therefore represent 1 to 4 moles of phosphate per mole of peptide respectively. (Mutation of serines 4, 6 and 9 abolished the appearance of peptides 2–4 (data not shown).) Note that these data show only the number of phosphates in the peptide but fail to show the positions at which any number of phosphates are located. Figure 4b shows the phosphopeptide pattern of the p53 from cells were treated for 1 h with 3 μM IC261. In this case peptides 3 and 4 were not detectable while the intensity peptide 2 was reduced by about 50%. No changes in any of the other phosphopeptides were apparent with the exception of the loss of an unidentified peptide migrating close to the origin. These data are entirely consistent with the phosphorylation of p53 by CK1δ and/or CK1ε *in vivo*.
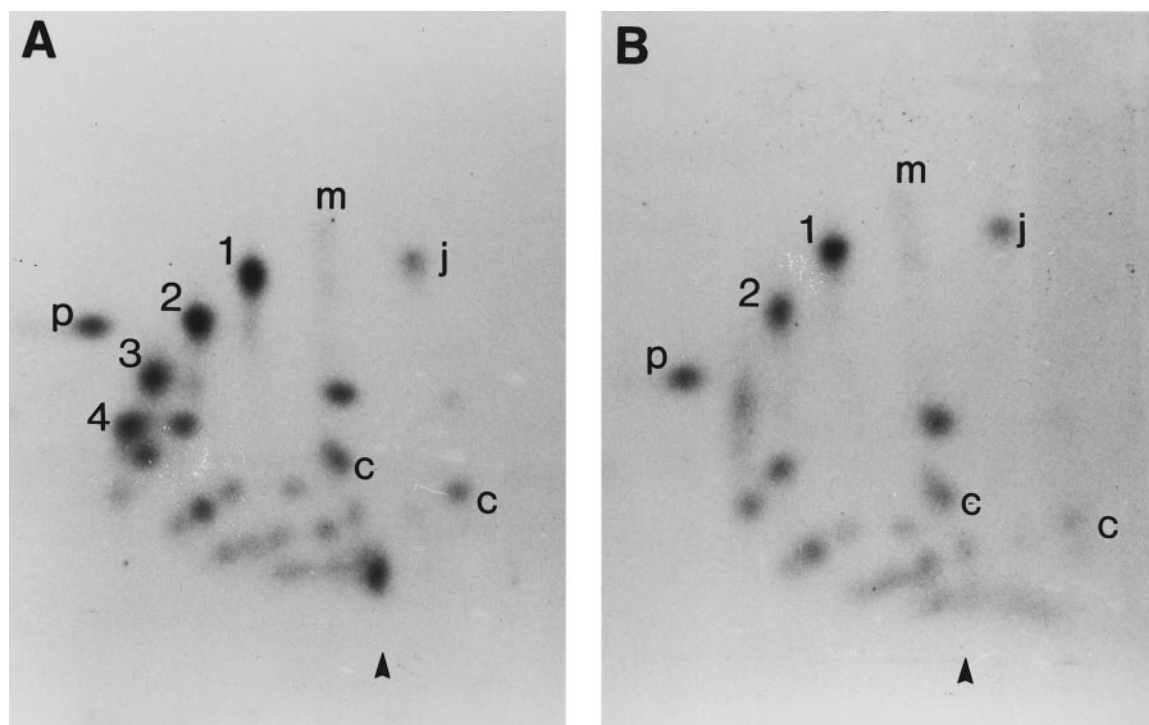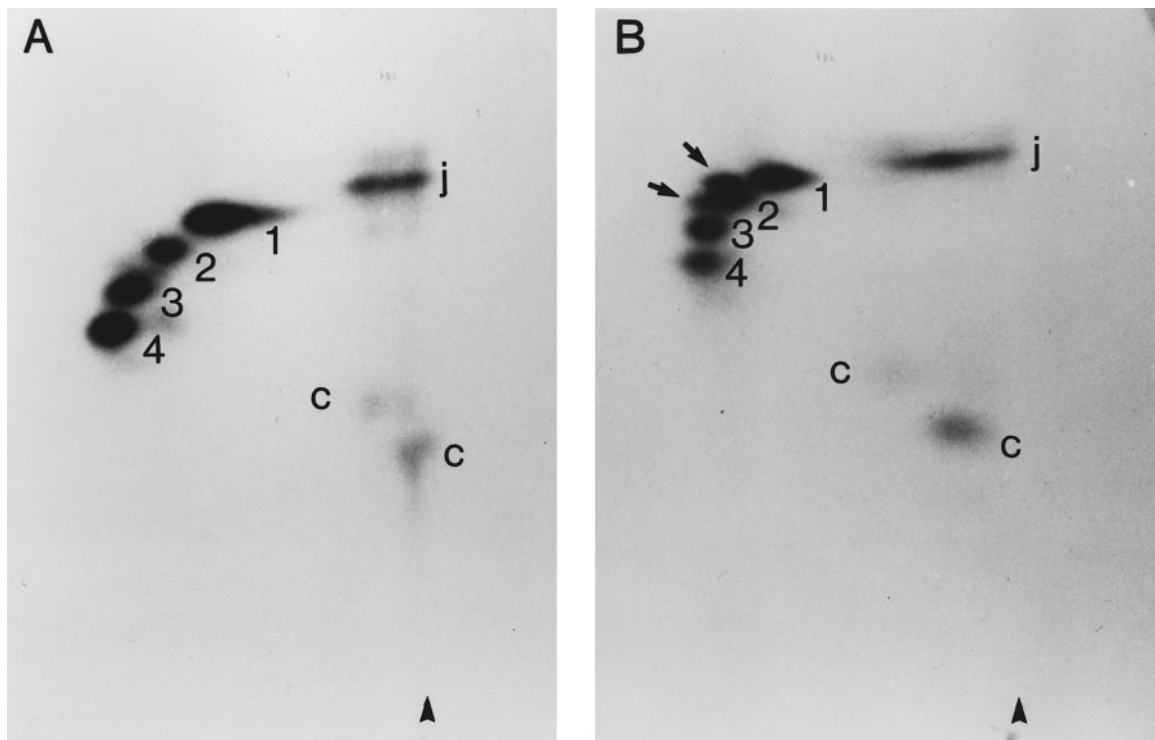

Figure 4 Phosphorylation of p53 at N-terminal sites *in vivo* is inhibited by the CK1δ-specific inhibitor IC261. SV3T3 cells were labelled using ^{32}P-orthophosphate as described in Materials and methods. One hour before harvesting, IC261 was added to the medium to a final concentration of 3 μM. An equal volume of DMSO, the solvent for IC261, was added to control plates. The cells were lysed and the radiolabelled p53 proteins isolated by immunoprecipitation and SDS–PAGE. Tryptic phosphopeptides were prepared and spotted onto thin layer plates at an origin (marked on each panel with an arrowhead). (**a**) shows p53 from untreated cells while (**b**) shows p53 from the IC261-treated cells. The lettering and numbering on the figure refer to individual phosphopeptides as follows: c, j and m are phosphopeptides arising from phosphorylation by CK2, JNK and MAP kinase respectively. p represents the position at which free phosphate migrates. Phosphopeptides 1 to 4 represent the N-terminus of p53 (amino acids 1–24) containing 1 to 4 phosphate groups respectively (see text for an explanation)

CK1δ CON

FP267 →
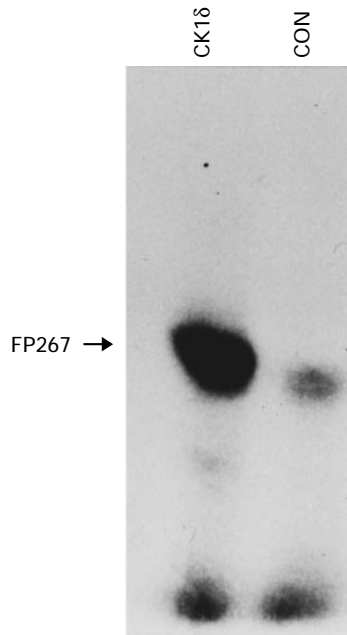
Figure 5 Protein kinase activity of a GFP-CK1δ fusion protein expressed in COS-1 cells and immunoprecipitated using an anti-GFP monoclonal antibody. COS-1 cells transfected with CK1δ, a plasmid expressing a GFP-CK1δ fusion protein (**b**), or pGFPcon which expresses only the GFP portion owing to a frame shift in the linker region (**a**). 60–72 h after transfection, the cells were harvested and the GFP proteins were immunoprecipitated and washed in kinase buffer as described in Materials and methods. The ability of the immunoprecipitated proteins to phosphorylate the p53 substrate FP267 was measured

Using a different approach, COS-1 cells were transiently transfected with plasmids expressing wild type murine p53 together with a plasmid expressing a GFP-CK1δ fusion protein (or GFP alone as control). To check that the CK1δ portion was active as a protein kinase, the cells were lysed and the GFP fusion proteins immunoprecipitated using a GFP-specific monoclonal antibody. The immobilised proteins were then used to phosphorylate the p53 substrate FP267. The data (Figure 5) show that the immunoprecipitated GFP-CK1δ protein did indeed possess p53 kinase activity whereas the GFP alone was not able to phosphorylate p53. When the transiently-transfected cells were labelled with ^{32}P-orthophosphate, two additional phosphopeptides were observed in the mouse p53 immunoprecipitated from the cells over-expressing GFP-CK1δ (Figure 6b, indicated with arrows), as compared with mouse p53 from the cells transfected with the GFP control plasmid (Figure 6a). These peptides migrate very closely to the position of N-terminal peptide 2; neither these peptides nor peptide 2, 3, and 4 were observed when the COS-1 cells had been transfected with GFP-CK1δ and p53 containing mutations at the phosphorylation sites, residues 4, 6 and 9 (data not shown). They are therefore likely to arise from phosphorylation at N-terminal residues including serines 4, 6 or 9. It is not clear why additional phosphopeptides are observed but a strong possibility is that high level expression of CK1δ disrupts sequential addition of phosphates to these residues, or may lead to the increased phosphor-


Figure 6 Hyper-phosphorylation of p53 at N-terminal sites in COS-1 cells following over-expression of a GFP-CK1δ fusion protein. COS-1 cells were transfected with pCMVNc9 which expresses wild type mouse p53 together with pGFP-CK1δ, a plasmid expressing a GFP-CK1δ fusion protein (panel B), or pGFPcon which expresses only the GFP portion owing to a frame shift in the linker region (panel A), were labelled using ^{32}P-orthophosphate as described in Materials and methods. Cell lysis, immunoprecipitation and tryptic phosphopeptide analysis was carried out. The lettering and numbering on the figure are as described in the legend to Figure 4. The two arrows in (B) highlight additional N-terminal phosphopeptides arising from over-expression of the kinase

ylation of sites which may normally be phosphorylated to only a very low level; (eg serines 6 then 9 appear to be the preferred sites *in vitro*, followed by serine 4 (Milne *et al.*, 1992)). This would generate species of the phosphopeptide which differed only in the positions of the phosphates and which could therefore have marginally different migration properties. Alternatively, constant on-phosphorylations could result in the formation of diester bonds between closely spaced phosphates or between phosphate residues and carboxyl side chains of neighbouring asp/glu residues.

CK1δ, but not CK1ε, is induced by etoposide and camptothecin

Previous analysis of p53NK in MethAp53(*ts*) cells indicated that the activity of the kinase could be enhanced both by p53 and, in a p53-dependent manner, by the drugs camptothecin and etoposide (Knippschild *et al.*, 1996); (this cell line expresses the valine 135 temperature-sensitive p53 which adopts a mutant conformation at 38°C but a wild type conformation at 28–32°C (Otto and Deppert, 1993)). To determine whether CK1δ or ε could be induced in a similar manner, the levels of mRNA for each of these kinases was measured by Northern analysis after p53 induction or treatment with the drugs in the MethAp53(ts) cells, at both the permissive and non-permissive temperatures. The data (Figure 7a) indicated that, as had been shown previously (Graves *et al.*, 1993), there were two species of CK1δ mRNA, at 1.9 kb and 2.2 kb respectively. There were no significant differences in the levels of CK1δ mRNA when comparing the untreated cells grown as monolayers at the two different temperatures. However, when the cells grown at 28°C were treated with either etoposide or camptothecin increases in the level of CK1δ mRNA of up to 2.5-fold were observed. In contrast, at the non-permissive temperature, the levels of CK1δ mRNA actually decreased slightly. These observations closely match the previously reported effects of wild type p53 conformation and DNA damage-inducing drugs on p53NK kinase activity (Knippschild *et al.*, 1996). When the levels of CK1ε mRNA were examined under identical conditions, no increases were observed (Figure 7B). The only notable difference was that CK1ε mRNA decreased following camptothecin treatment at 38°C.

When the levels of CK1δ protein were measured in this cell line at the different temperatures and in the presence or absence of the drugs, similar results were obtained. Following ion exchange chromatography on a Mono Q column, the kinase activity (from cells grown at 28°C) appeared predominantly in fraction 12, with some additional activity in fraction 13 (Figure 8a; Mono Q fractionation prior to immunodetection allowed us to confirm that the increased levels of CK1δ protein co-fractionated with the p53NK protein kinase activity (data not shown)). Treatment with etoposide or camptothecin showed increases in the levels of CK1δ in the peak fractions (fractions 11–13) in line with the increases observed in CK1δ mRNA. No such changes were observed when the cells were maintained at 38°C (Figure 8b; only fractions 12 from each treatment are shown). Taken together, the data from the Northern and Western analyses indicate that
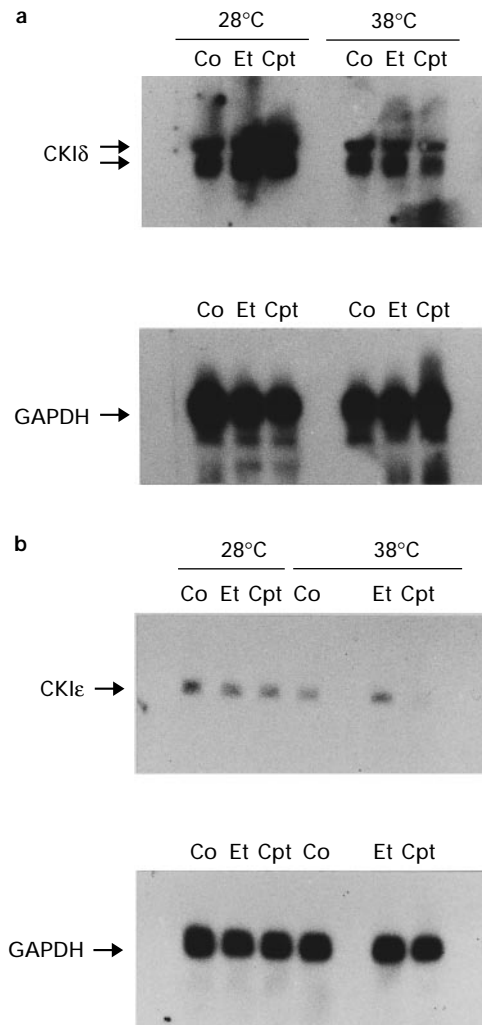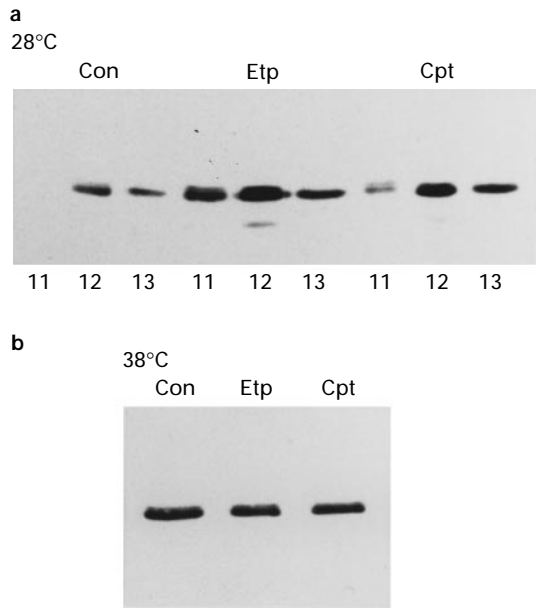

Figure 7 Northern analysis of CK1δ and CK1ε mRNAs from drug-treated MethAp53(*ts*) cells. MethAp53(*ts*) cells were grown as monolayers at 38°C, then shifted to 28°C for 24 h and treated with 150 μM etoposide (Et) or 10 μM camptothecin (Cpt) for 2 h. Control cells received no additions (Co). The cells were harvested and the RNA was extracted and analysed by Northern blotting using ^{32}P-labelled full length cDNA probes for CK1δ (rat, **a**) or CK1ε (human, **b**). After detection by autoradiography, the filters were stripped and re-probed with a full length cDNA for wild type GAPDH

treatment of MethAp53(ts) cells with topoisomerase-directed (DNA damage-inducing) drugs can enhance the level of CK1δ through a p53-dependent mechanism.

Discussion

Previously, we detected and characterised a p53- and DNA-damage-inducible p53 N-terminus-targeted protein kinase activity (p53NK) present in various cell lines including MethAp53(ts) cells (Knippschild *et al.*, 1996). In this paper, we show that two isoforms of the casein kinase 1 family, CK1δ and CK1ε (Fish *et al.*, 1995; Graves *et al.*, 1993), which are highly related to each other, display properties characteristic of p53NK and can indeed phosphorylate p53 in the cell. At the biochemical level, CK1δ and CK1ε co-purify with p53NK (Figure 2) and phosphorylate the same sites in

a
28°C

Con   Etp   Cpt

11 12 13   11 12 13   11 12 13

b
38°C

Con   Etp   Cpt

Figure 8 Western analysis of CK1δ from drug-treated MethAp53(*ts*) cells. MethAp53(*ts*) cells were grown as monolayers at 38°C, then shifted to 28°C for 24 h and treated with 150 μM etoposide (Etp) or 10 μM camptothecin (Cpt) for 2 h. Control cells received no additions (Con). The cells were lysed, and the proteins were fractionated by ion exchange chromatography using a Mono Q (H/R 5/5) column and eluting with a linear gradient of NaCl. The peak fractions were analysed by SDS-PAGE on 10% gels and subsequently transferred to membranes. Fractions containing the peak of p53NK activity (fractions 11, 12 and 13) from the cells grown at 28°C are shown in (**a**). The fraction containing the peak of p53NK activity (fraction 12) from the cells grown at 38°C is shown in (**b**). The presence of CK1δ was detected using the CK1δ-specific monoclonal antibody 128A

p53 *in vitro* (Figure 1); they are also indistinguishable from each other with respect to their kinetic behaviour using either p53 or casein as substrates (Table 1) and can be inhibited by micromolar concentrations of the CK1δ/ε-specific inhibitor, IC261 (DeMaggio AJ, Christenson E and Hoekstra MF, submitted; data not shown). In addition to this, the availability of the monoclonal antibody 128A, which is specific for CK1δ (DeMaggio AJ, Christenson E and Hoekstra MF, submitted) confirmed that the p53NK co-purifies with CK1δ through three different steps (Figure 3). However, we cannot determine whether this also holds for CK1ε as we do not have an isoform-specific antibody for CK1ε which does not cross-react with CK1δ. The data in this paper (Fig. 4) also support the idea that phosphorylation of p53 by CK1δ and/or CK1ε occurs *in vivo* since treatment of SV3T3 cells with the inhibitor IC261 leads to loss of phosphorylation at three sites within the N-terminus of p53. (Since the inhibitor IC261 can block the activity of both the CK1δ and CK1ε (but not α, β or γ) isoforms *in vitro* (data not shown), we cannot attribute the loss in phosphorylation of p53 to either one of these two isoforms individually.) We have also shown that transient over-expression of CK1δ leads to hyperphosphorylation of the N-terminus of p53 (Figure 6). (Experiments involving transient over-expression were carried out because we have been unable to observe any significant ectopic expression of CK1δ in isolated

clones, in spite of screening over 120 clones from four different cell types (data not shown). It is therefore possible that there is a strong selection against overexpression of this kinase in cells.) Strikingly, CK1δ and CK1ε (which map to different chromosomes (Fish *et al.*, 1995)) show a remarkable degree of similarity, especially within the protein kinase domain (98%) (Christenson *et al.*, 1996; Fish *et al.*, 1995; Graves *et al.*, 1993) and it may be that they are either redundant *in vivo*, or that they are responsive to different signals or interact with different regulatory proteins, thereby placing a common set of substrates under control of (at least) two different regimes. The selective enhancement of CK1δ by p53 in the MethAp53(*ts*) system (Figures 7 and 8) would tend to support the latter hypothesis. An additional factor to consider is that the levels of expression of the δ and ε isoforms of CK1 may be dependent upon cell or tissue type. Certainly in the MethAp53(*ts*) cells the level of CK1ε mRNA was barely detectable as compared with CK1δ mRNA and was not inducible (Figure 7). Moreover, we have observed different relative levels of CK1δ and CK1ε expression in a number of different cell lines (data not shown). Phosphorylation of p53 by both enzymes could therefore represent convergent regulation arising from different environmental conditions.

The data are also consistent with a model in which the levels of p53 and CK1δ may be interdependent, perhaps involving a regulatory loop. p53 is clearly phosphorylated by CK1δ *in vitro* and *in vivo* (Figures 1, 4 and 6) while the induction of wild type p53 in MethAp53(ts) cells leads to enhanced levels of CK1δ mRNA and protein (Figures 7 and 8), and p53NK protein kinase activity (Knippschild *et al.*, 1996). Similar increases in the levels of CK1δ protein were observed in primary murine fibroblasts upon drug treatment (data not shown) indicating that the effect is not restricted to the MethAp53(*ts*) cells. Moreover, the demonstration that CK1δ is a p53-responsive protein kinase adds weight to the evidence that identifies CK1δ as p53NK. The mechanism of the induction of the protein kinase is not clear and may occur either through p53-dependent transactivation of the CK1δ promoter or through a post-transcriptional effect. Alternatively, increased levels of CK1δ may reflect a p53-dependent enrichment of cells at specific stages of the cell cycle at which the kinase is present in an activated form.

The key issue which should now be addressed is the biological consequence of the interplay between p53 and CK1δ. CK1 isoforms are thought to be involved in the regulation of DNA repair and chromosomal segregation. For example, the *S. cerevisiae hrr* 25 gene encodes a protein kinase with striking similarity to CK1 family members: *hrr* 25 mutants show sensitivity to DNA damage and have defects in nuclear segregation and meiotic division (Hoekstra *et al.*, 1991). Two genes in *S. pombe*, *hhp* 1 and *hhp* 2, may play similar roles to *hrr* 25 and deletion of these genes generates large cells containing multiple nuclei (i.e. polypolidy; Dhillon and Hoekstra, 1994). DMCK1, (the homologue of CK1α in *D. melanogaster*) is activated following DNA damage and relocates to the nucleus (Santos *et al.*, 1996), while mammalian CK1α is both thought to be involved in controlling DNA replication (Cegielska and Virshup, 1993) and in events at the mitotic spindle (Brockman *et al.*, 1992).

CK1δ and CK1ε, (the mammalian CK1 isoforms which show the greatest degree of similarity to HRR25), but not CK1α, can complement *hrr* 25 mutations in yeast, suggesting that the mammalian and yeast proteins may have functional similarity (Graves *et al.*, 1993; DeMaggio AJ, Christenson E and Hoekstra MF, submitted). p53 is also a protein with established roles in the G1 (Kastan *et al.*, 1991, 1992; Kuerbitz *et al.*, 1992) and G2 (Aloni *et al.*, 1995; Cross *et al.*, 1995; Paules *et al.*, 1995) checkpoints which monitor chromosomal aberrations and segregation respectively. The activation of p53 at these checkpoints is strikingly co-incident with the proposed role of CK1 and the interaction of these two proteins may therefore play a pivotal role in mechanism(s) of sensitivity to DNA damage at these checkpoints.

Materials and Methods

Cell lines

The following cell lines were used in this study: COS-1, SV3T3, F111, FR(wt648) (a rat F111 fibroblast line transformed by SV40 (Pintel *et al.*, 1981)), SV-52 (a REF52 cell line transformed by SV40 (Graessmann and Graessmann, 1983)) and MethAp53(*ts*). The parental 'MethA' cell line was originally isolated as a methylcholanthrene transformant. MethAp53(*ts*) cells were established after transfection of BALB/c mouse MethA tumour cells with the 'valine 135' temperature-sensitive murine p53 in the plasmid vector pLTRcGval135 (Otto and Deppert, 1993). Cells were grown in Dulbecco-Vogt modified Eagle's medium (DMEM) supplemented with either 5% FBS plus 200 μg/ml G418 (MethAp53(*ts*)), or 10% FBS (COS-1 cells, FR(wt648), SV-52 and SV3T3 cells) and incubated at 37°C (COS-1 cells, FR(wt648), SV-52 and SV3T3 cells) or 39°C (MethAp53(*ts*)) in a humidified 5% carbon dioxide atmosphere. In some experiments, MethAp53(*ts*) cells were also shifted to 28°C for 24 h.

Oligonucleotides, PCR and plasmids

The two oligonucleotide primers used for polymerase chain reaction (PCR) amplification of human CK1ε were (5' to 3') ATGGAGCTACGTGTGGGGAAC (5') and TCACTTCCCGAGATGGTAAATG (3'); for rat CK1δ the primers were ATGGAGCTAGGGTCGGGAATAG (5') and TCAGTAGGTGGTACGTCGTGG (3'). A full length human CK1ε cDNA was isolated by PCR using a human liver cDNA library (kindly provided from P Hall, Dundee). RT–PCR was used to obtain a full length rat CK1δ cDNA using RNA extracted from FR(wt648) cells. The PCR conditions were annealing at 50°C (CK1ε) or 61.5°C (CK1δ) for 1 min, elongation at 72°C for 2 min and denaturation at 95°C for 30 s. The resulting fragments were cloned into pCRII (Invitrogen) and fully sequenced using the USB Sequencing Kit (Amersham) and appropriate sequencing oligonucleotides. DNA sequence analysis revealed that the predicted amino acid sequence of the CK1δ cDNA showed one change in comparison with the published sequence (Graves *et al.*, 1993), that of Lys 223 (AAG) to Arg (AGG). The predicted amino acid sequence for CK1ε showed a number of changes from the published sequence (Fish *et al.*, 1995); these were: Ser 101 to Ile, Gln 271 to His, Leu 272 to Val (all within the kinase domain); and Gly 332 to Ala; Pro 333 to Ala; Pro 348 to Gln; Val 370 to Ala and Pro 403 to Ser (all within the C-terminal non-catalytic domain). A further change from the published sequence, a point insertion of a C at codon

352, was also evident and when expressed yielded a truncated CK1ε product. (This clone, termed CK1εFS (frame shift), substitutes 59 different amino acids followed by a stop codon and therefore effectively deletes the C-terminal 63 amino acids. This clone was examined together with the other CK1 isoforms in kinetic experiments.) All of the CK1ε clones isolated by PCR showed these changes (data not shown). To obtain, a CK1ε product with the same C-terminus as the published sequence, the additional C at codon was deleted by oligonucleotide-directed mutagenesis using the oligonucleotide (5' to 3') CGAGAGGCAGGGGTAGAAGC. The template for mutagenesis was the pCRII-CK1ε product which had been rescued in single-stranded form following infection of the bacterial cells with the helper phage KO7.

Plasmid pCMVNc9 expresses a wild type murine p53 cDNA under control of the CMV early promoter (Eliyahu *et al.*, 1988, 1989). pCMV-triple is a derivative of this plasmid in which the codons for serine residues 4, 6 and 9 (this sites phosphorylated by CK1) have been mutated by oligonucleotide-directed mutagenesis to encode alanine residues. The mutagenesis was carried out as described previously (Milne *et al.*, 1992) using the oligonucleotide (5' to 3') GGAGGAGG-CACAGGCGGATATCGCCCTCGAG and a single-stranded p53 cDNA template in the M13 vector tg130 (Amersham). Complete sequence analysis of this cDNA revealed no other changes in the coding region (data not shown).

Plasmid pGFP-CK1δ, which expressed a green fluorescent protein (GFP)-CK1δ fusion protein using the CMV promoter, was constructed by cloning the full length PCR-generated CK1δ cDNA into a modified version of the vector phGFPS65T (Clontech Labs Inc). A control plasmid pGFPcon, is identical except that it has a point deletion in the linker between the GFP and CK1δ coding sequences which introduces an in frame stop codon immediately upstream of the CK1δ coding sequence.

Expression and purification of glutathione S-transferase fusion proteins

The recombinant glutathione S-transferase fusion proteins were expressed in *Escherichia coli* using the vector pGEX-2T (Amrad Corp.). The construction, expression and purification of recombinant glutathione S-transferase-p53 (GST-p53) fusion proteins has been described in detail elsewhere (Milne *et al.*, 1994, 1995). In short, FP267 contains a fragment encoding the first 1–64 amino acids of wild type mouse p53 with potential phosphorylation sites for CK1, DNA-PK and JNK1. FP222 contains codons 1–85 of wild type mouse p53 in the antisense orientation, FP279 expresses mouse p53 codons 11–63, and FP295 also expresses codons 11–63 but has a serine to alanine mutation at position 34. FP380 (which has not been reported previously) comprises amino acids 1–85 of murine p53 but with serine to alanine changes at residues 4, 6 and 9. The GST-protein kinase fusion protein expression plasmids were as follows: to construct pGST-CK1ε, an *Eco*RI fragment encoding the full length human CK1ε was cloned into pGEX-2T in the correct orientation; (a plasmid in which this fragment was cloned in the opposite orientation (pGST-R) was used as a control in some experiments; the CK1εFS cDNA was also cloned into pGEX-2T to give pGST-CK1εFS). Similarly, an *Eco*RI fragment encoding rat CK1δ (again isolated by PCR) was cloned in the sense orientation into pGEX-2T to give plasmid pGST-CK1δ.

Fusion proteins were isolated by incubation of the GST-p53 bacterial lysates with glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.) at 4°C for 1 h. The beads were washed three times with 50 mM Tris (pH 7.5). Fusion proteins were eluted from the beads by incubating with

50 mM Tris buffer (pH 7.5) and 5 mM reduced glutathione. Eluted fusion proteins were used immediately as significant loss of activity occurred upon various regimes of storage.

Purification of p53 N-terminus-targeted protein kinase

Mammalian cells (F111, SV-52 or MethAp53(*ts*)) were washed three times in ice-cold PBS then lysed in 20 mM Tris-acetate (pH 7.0) 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM sodium β-glycerophosphate, 50 mM NaF, 1 mM benzamidine, 4 μg/ml leupeptin and 0.1 % β-mercaptoethanol. Alternatively, nuclear extracts were prepared as follows. Cells were washed twice with KM buffer (10 mM MOPS (pH 6.8), 10 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol, 10% (v/v) glycerol) and then lysed in 2 ml per plate of KM buffer containing 1% (v/v) Nonidet P-40 (NP-40) for 30 min at 4°C The remaining structures were washed with KM buffer then lysed as described above. Whole cell extracts or nuclear extracts were passed through 0.22 μm filters and 4–6 mg applied to a MonoQ (H/R 5/5) column or 360–600 mg to a HiTrap Q column attached to the FPLC system (Pharmacia Biotech). The proteins were then eluted with a linear gradient of sodium chloride in buffer A: (comprising 50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 5% glycerol, 0.03% Brij35, 1 mM benzamidine, 4 μg/ml leupeptin, 0.1% β-mercaptoethanol). Fractions were screened for p53 kinase activity as described previously (Milne *et al.*, 1992). Peak fractions were diluted in buffer A and applied to a phosvitin-sepharose 4B column, then eluted stepwise using increasing concentrations of NaCl to 600 mM with 50 mM increments (Milne *et al.*, 1992). Fractions containing p53NK activity were diluted in buffer A and applied to a MonoS H/R 5/5 column (Pharmacia Biotech). As before the proteins were eluted with a linear gradient of increasing NaCl concentration. Attempts to further purify the kinase after this step consistently led to complete loss of kinase activity.

Blotted protein kinase assay

'Blotted protein kinase' assays were carried out using the procedure of Ferrell and Martin (1991). Peak fractions of kinase activity from MethAp53(*ts*) cell extracts chromatographed on a MonoQ (H/R 5/5) column (Pharmacia Biotech) were separated by SDS–PAGE on a 12.5% polyacrylamide gel containing 150 μg FP267, then transferred to a nylon membrane (Immobilon, Millipore) at 4°C in 25 mM Tris, 192 mM glycine (pH 8.3), without methanol as transfer buffer. The filter was washed several times in ice cold denaturation buffer (7 M guanidine hydrochloride, 50 mM Tris (pH 8.3), 50 mM dithiothreitol, 2 mM EDTA, 1% (w/v) BSA) and incubated in denaturation buffer for 16 h at 4°C. The filter was rinsed in 50 mM Tris (pH 7.4) and incubated in renaturation buffer (140 mM NaCl, 10 mM Tris (pH 7.4), 2 mM DTT, 1% (w/v) BSA, 0.1% (v/v) NP-40) at 4°C for 24 h. The filter was blocked by incubation in 30 mM Tris (pH 7.4), 1% (w/v) FP267 for 1 h at room temperature, then washed and finally incubated in reaction buffer (10 mM MgCl₂, 25 mM Tris (pH 7.4), 0.1 mM EDTA and 20 mM ATP containing 100 μCi/ml [γ-³²P]ATP) for 1 h at 30°C. The filter was washed twice with 30 mM Tris (pH 7.4), once with 30 mM Tris (pH 7.4), 0.05% NP-40, twice 30 mM Tris (pH 7.4), once with 1 M KOH, and twice with 30 mM Tris (pH 7.4) for 10 min each at room temperature. The filter was subsequently dried and exposed to Fuji RX film at −80°C with intensifying screens.

Standard protein kinase assays

p53 kinase assays were carried out using purified GST-p53 fusion proteins FP267, FP279, FP295 or FP380 as substrates. The substrate protein was resuspended in a total volume of 20 μl containing 25 mM Tris (pH 7.5) 10 mM MgCl₂, 0.1 mM EDTA, 1 mM sodium orthovanadate and 20 mM [γ-³²P]ATP (specific activity 5 Ci/mmol). The reactions were initiated by the addition of enzyme (usually 2 μl of 1 ml fractions eluted from Mono Q, HiTrap, or phosvitin-sepharose 4B columns, or 10 μl of recombinant GST-CK1δ, GST-CK1ε or GST-R) and incubated for 30 min at 30°C. Kinase assays were also performed in the presence of 3 μM IC261, a CK1δ specific inhibitor (DeMaggio AJ, Christenson E and Hoekstra MF, submitted). Alternatively transiently expressed GFP-CK1δ fusion protein was immunoprecipitated from COS-1 cell extracts using a GFP-specific monoclonal antibody (Clontech) and the immobilised fusion protein used as the source of kinase. The reactions were stopped by adding 5 μl of concentrated SDS sample buffer (75% (v/v) β-mercaptoethanol, 20% (w/v) SDS, 10% (v/v) glycerol and 0.02% (w/v) bromphenol blue). Reaction products were separated by SDS-PAGE on 12.5% gels and the phosphorylated proteins visualised by autoradiography. For quantitative analysis, the phosphorylated products were excised from the gel and Cerenkov radiation was measured using a scintillation counter.

Phosphopeptide analysis

SV3T3 cells were labelled with 5 mCi ³²P-orthophosphate per 10 cm plate for 3 h as described previously (Milne *et al.*, 1994). One hour prior to harvesting, the CK1δ-specific inhibitor IC261 (DeMaggio AJ, Christenson E and Hoekstra MF, submitted) was added to a final concentration of 3 μM; an equal volume of DMSO, the solvent for the inhibitor, was added to control plates. For the COS-1 cell labelling experiments, the cells were transiently co-transfected with plasmids expressing wt (pCMVNc9) or phosphorylation-site mutant (pCMV-triple) mouse p53 and either the full-length GFP-CK1δ fusion protein or the truncated fusion protein as control (pGFP-CK1δ and pGFP-con respectively). COS-1 cells were labelled with ³²P-orthophosphate for 30 min. In each case p53 was immunoprecipitated with PAb421 or PAb246 and separated by SDS–PAGE on 12.5% polyacrylamide gels. The gels were then blotted onto a membrane (Immobilon-P, Millipore) and the radioactive bands containing phosphorylated p53 were detected by autoradiography and excised for further analysis (Luo *et al.*, 1991). Excised membrane fragments were incubated in 100 mM acetic acid, 5% polyvinylpyrrolidone (PVP-360, Sigma) at 37°C, extensively washed with water and 50 mM ammonium bicarbonate buffer, digested with trypsin and oxidised with performic acid for 1 h. Phosphopeptides were separated in two dimensions on cellulose thin-layer plates by electrophoresis at pH 1.9, followed by ascending chromatography (Meek and Eckhart, 1988) and subsequently visualised by autoradiography.

Transient transfections

Cells were seeded at a density of 10⁶ per 10 cm plate. A total of 4 μg of plasmid DNA were transfected per 10 cm plates by mixing the DNA with Lipofectamine reagent (Gibco) in FBS-free MEM, according to the manufacturer's instructions. All subsequent procedures were carried out as described by the manufacturer. Analysis of transiently-transfected cells was performed 60–72 h after transfection.

In vitro *transcription and translation*

In vitro transcription (from the SP6 promoter) and translation (in the presence of [³⁵S]methionine) of pCRII-derivative plasmids encoding CK1δ and CKε cDNAs was

carried out using the TNT SP6 coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions.

Comparative kintetic analysis of CK1 isoforms

Kinetic analyses were carried out using recombinant GST-CK1δ, GST-CK1ε, GST-CK1εFS and p53NK which was partially purified from SV-52 cells using the regime described above. Kinase reactions were performed for each enzyme preparation at 30°C for 15 min in a total volume of 25 μl using different amounts of substrates: 8.2 μg, 4.1 μg, 2 μg, 1 μg, 0.5 μg and 0.25 μg of FP267; and 10 μg, 5 μg, 2.5 μg, 1.25 μg, 0.75 μg and 0.38 μg of casein (Sigma).

Northern blot analysis

Total cellular RNA was extracted as described by Sambrook *et al.* (1989). For Northern Blot analysis total RNA (5 μlg) was denatured with 6.3% formaldehyde and 50% formamide and separated on 1% agarose/formaldehyde gels. Transfer of RNA to Magnacharge nylon membranes (Micron Separations Inc) by capillary blotting and hybridisation were performed by standard methods (Sambrook *et al.*, 1989). The blots were hybridised with ^{32}P-labelled CK1δ or CK1ε cDNA probes or, alternatively with a ^{32}P-labelled GAPDH cDNA as control. Hybridisation was performed under stringent conditions (50% formamide, $5 \times$ SSPE, 0.5% SDS, $5 \times$ Denhardt's solution, 5% dextran sulphate and 100 μg/ml denatured salmon sperm DNA) at 48°C, and the blots were washed several times at 55°C in $2 \times$ SSC containing 0.1% SDS.

Western blotting

Steady state levels of CK1δ were determined by Western analysis. Gels were transferred to Immobilon-P membrane (Millipore) and probed with the CK1δ-specific monoclonal antibody 128A (DeMaggio AJ, Christenson E and Hoekstra MF, submitted). Detection was carried out using horse radish peroxidase-conjugated anti-mouse IgG as a secondary antibody, followed by chemiluminescence detection (ECL, Amersham).

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