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Protein kinase CK1 is a p53-threonine 18 kinase which requires prior phosphorylation of serine 15

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Abstract p53 is a potent transcription factor which is regulated by sequential multisite phosphorylation and acetylation. In this paper, we identify threonine 18 of p53, a key site in regulating the interaction between p53 and its regulatory partner MDM2, as a novel site phosphorylated in vitro by purified recombinant casein kinase 1 (CK1) δ. Strikingly, phosphorylation of threonine 18 is dependent upon prior phosphorylation of serine 15. These data highlight an additional and physiologically important target residue for CK1 in p53 and suggest a potential mechanism by which sequential modification of a pivotal N-terminal residue in p53 may occur following stress-activated modification of serine 15.

Key words: p53; Phosphorylation; Casein kinase 1; Threonine 18; DNA-activated protein kinase; MDM2

1. Introduction

The p53 tumour suppressor protein is a latent transcription factor which is activated by a wide range of cellular stresses (for review, see [1,2]). Activation of p53 in response to DNA damage involves the release of p53 from complex formation with its negative regulatory partner, MDM2, and is accompanied by a sequential series of phosphorylation and acetylation events which include phosphorylation of serines 15, 20 and 33 at the N-terminus of the protein (for review, see [3]). The initial modification, phosphorylation of serine 15, is mediated by the ATM protein kinase in vivo [4,5]. (Serine 15 and serine 37 can also be modified in vitro by the DNA-activated protein kinase (DNA-PK) [6].) Serine 15 phosphorylation stimulates p53-dependent transactivation through increased interaction with p300/CBP [7,8] and may contribute to disruption of the p53-MDM2 association [9,10]. Serine 15 modification may also nucleate subsequent modifications such as the acetylation of the C-terminus through increased binding of p300/CBP [11]. Recent evidence has also established that serine 20, a key phosphorylation site which is modified following DNA damage [12], plays a critical role in the disassociation of p53 and MDM2. Similarly, threonine 18 is phosphorylated in vivo and can also contribute to the dissociation of the p53-MDM2 complex [13,14]. Moreover, extensive threonine 18 and serine 20 phosphorylation has been detected in a panel of human breast cancers with wild-type (WT) p53 status [14] underscoring their biomedical relevance. To date, however, the protein kinases which phosphorylate threonine 18 and serine 20 have not been established.

Protein kinase casein kinase 1 (CK1) comprises a family of highly related, constitutively active serine/threonine protein kinases (reviewed by [15]). CK1 is involved in controlling a wide variety of different cellular events including protein turnover [16,17], nuclear import [18] and the cellular response to DNA damage [19,20]. CK1 phosphorylates substrates which are generally acidic on the N-terminal side of the target residue and can utilise phosphorylated amino acids as recognition determinants [21,22]. Murine p53 is phosphorylated at serines 4, 6 and 9 by protein kinase CK1 in vitro and in cultured cells [23,24], but it has not yet been determined whether CK1 can phosphorylate p53 from other species. The involvement of CK1 in cellular events which influence p53 function suggests that phosphorylation of p53 by CK1 may be an important regulatory route by which p53 can sense changes in the environment.

In order to explore the phosphorylation of human p53 by CK1, we have utilised a series of well-characterised GST-p53 fusion proteins as substrates. The spacing between serine 15 and threonine 18 suggested that phosphorylation of serine 15 might generate a recognition determinant through which threonine 18 could become a CK1 substrate [21,22], the phosphorylation of which would be regulated through the serine 15 modification. In the present paper, we show that, in vitro, this is indeed the case. The data therefore provide a potential mechanism by which DNA damage-induced phosphorylation of serine 15 may nucleate additional and physiologically important modification of the p53 protein.

2. Materials and methods

2.1. Expression and purification of glutathione-S-transferase p53 proteins

The first 42 amino acids (WT or a series of serine or threonine to alanine mutants) of human p53 or the full length protein (WT or serine 15 and/or 37 to alanine) were cloned into a pGEX-6P1 vector (Amersham). The residues mutated in the GST-p53(1–42) proteins were serines 15, 20, 33, 37 and threonine 18. Two serine to alanine double changes at positions 6 and 9, and 15 and 37 were also included. Serines 6 and 9 are sites for phosphorylation by CK1 in murine p53 [23] whereas serines 15 and 37 are sites for phosphorylation in human p53 by DNA-PK [6]. The GST-murine p53 fusion proteins '267' (comprising amino acids 1–64) and '380' (in which serines 4, 6 and 9 are substituted by alanine residues) have been used extensively and are described in detail elsewhere [24]. The cloning, expression and purification of these proteins have been described in detail elsewhere [7].

2.2. Phosphorylation of p53 in vitro

Phosphorylation of p53 by recombinant DNA-PK was carried out according to the manufacturer's instructions (Promega) using 1–2 μg

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of the GST-p53 fusion protein as substrate. Control samples were treated following the same protocol but in the absence of the protein kinase (mock-treated). When labelling of the p53 was required, [γ-^{32}P]ATP was included in the reaction mix at a specific activity of 250 Ci/mmol. Phosphorylation of p53 by recombinant CK1 (delta isoform) was carried out according to the manufacturer's instructions (NEB) using 1–2 μg of GST-p53 fusion protein as substrate and 5 U of CK1. Double phosphorylation by DNA-PK and CK1 was carried out as follows. Following phosphorylation by DNA-PK using unlabelled ATP, the GST-p53 proteins were adsorbed onto glutathione-Sepharose 4B beads (Pharmacia Biotech) and the beads were washed twice in 25 mM Tris, pH 7.5, 50 mM NaCl, 5% (v/v) glycerol and once in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT. Phosphorylation of the adsorbed proteins by CK1 was carried out for 30 min according to the manufacturer's instructions, using [γ-^{32}P]ATP as phosphate donor.

Phosphorylated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography. Prior to drying, the gels were stained to confirm that the same amount of each GST-p53 protein is precipitated with glutathione-Sepharose 4B beads and that the protein was phosphorylated stoichiometrically (this was evident from the decrease in electrophoretic mobility of the phosphorylated forms of the protein (data not shown and see [7])). Quantitation was carried out using NIH Image software following scanning of appropriately exposed autoradiographs.

2.3. Phosphoamino acid analysis

Phosphoamino acid analysis was carried out as described by Kamps and Sefton [25].

2.4. Human MDM2 protein (HDM2) binding in vitro

The binding of the HDM2 to p53 in vitro was carried out as described recently [7] using GST pull-down assays in which the p53 was fused to GST (see above). The HDM2 was transcribed and translated in vitro using [^{35}S]methionine.

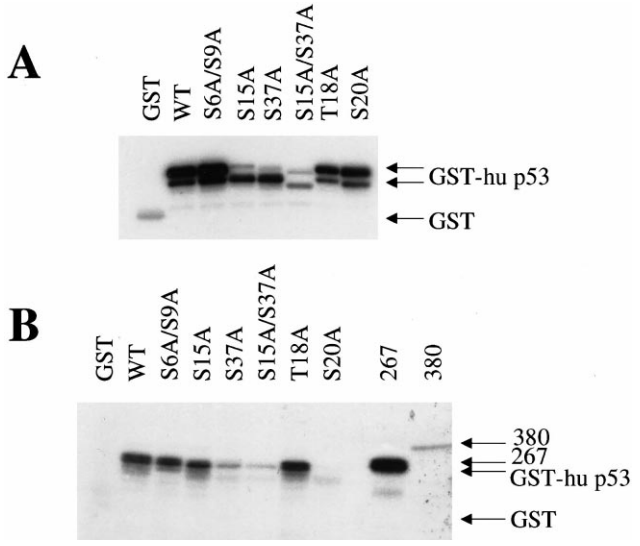

Fig. 1. Phosphorylation of GST-p53 fusion proteins by recombinant DNA-PK and CK1δ. GST-p53 proteins comprising the first 42 amino acids or human p53 (WT and a series of mutants in which known or potential phosphorylated residues were substituted by alanine) were phosphorylated in vitro using recombinant DNA-PK (A) or CK1δ (B) and [γ-^{32}P]ATP as phosphate donor. GST alone was included as a negative control. As additional controls for the CK1 phosphorylation assays, phosphorylation of two GST-murine p53 proteins was carried out. These proteins were '267' (comprising the first 64 amino acids or murine p53) and '380' (in which the CK1 phosphorylation sites serines 4, 6 and 9 had been substituted by alanine residues). The assay conditions were as described in Section 2. The positions at which the phosphorylated proteins migrate in SDS-PAGE are indicated.

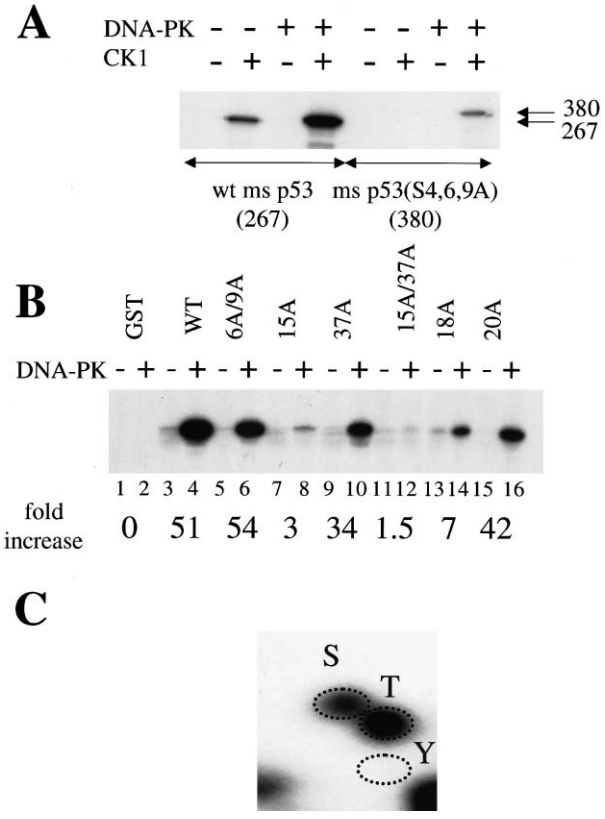

Fig. 2. Phosphorylation of GST-p53 fusion proteins by CK1δ, alone or following stoichiometric phosphorylation by DNA-PK. GST-p53 fusion proteins were mock-phosphorylated or phosphorylated using recombinant DNA-PK and unlabelled ATP as phosphate donor. Following removal of the kinase and excess ATP, the proteins were phosphorylated using recombinant CK1δ and [γ-^{32}P]ATP as phosphate donor. The panels are as follows. (A) The GST-murine p53 proteins 267 and 380. (B) GST alone, GST-WT human p53 and the series of phosphorylation site mutants. The fold increase in phosphorylation by CK1 after prior phosphorylation by DNA-PK over the phosphorylation by CK1 alone is indicated under the appropriate lanes. (C) Phosphoamino acid analysis of the GST-WT human p53 protein which was phosphorylated using recombinant DNA-PK and unlabelled ATP, followed by CK1 phosphorylation in the presence of [γ-^{32}P]ATP. The positions of unlabelled internal standards (phosphoserine [S], phosphothreonine [T] and phosphotyrosine [Y]) are indicated by the dotted circles.

3. Results

A series of GST-human p53 fusion proteins (comprising the first 42 amino acids of p53), in which known or potential phosphorylation sites were substituted with alanine residues, was used to investigate the phosphorylation of the N-terminus by DNA-PK and CK1. Fig. 1A shows that most of these proteins were substrates for DNA-PK. We have shown previously that the upper band in this experiment contains 2 mol phosphate per mol of protein while the lower band represents 1 mol phosphate per mol of protein [7]. Mutation of serine 15 or 37 led to loss of the upper band, confirming that these residues are DNA-PK target residues in vitro. When both serines 15 and 37 were mutated, the fusion protein was only a very poor DNA-PK substrate. GST was not phosphorylated at all by DNA-PK. These data confirm that DNA-PK phosphorylates serines 15 and 37 in human p53 [6,7]. The human p53 proteins were also phosphorylated in vitro by CK1 (Fig.

1B). Substitution of serine 37 reduced the level of phosphorylation while the S20A mutant could not be phosphorylated by CK1, suggesting that these residues are either in vitro CK1 targets or are required for interaction with the protein kinase. Strikingly, serines 6 and 9 of human p53, residues which are key targets of CK1 in murine p53, are not phosphorylated by CK1. GST-murine p53 proteins were also examined as controls. As expected, the 267 protein (comprising amino acids 1–64 of murine p53) was phosphorylated by CK1 whereas the 380 protein (in which serines 4, 6 and 9 are substituted by alanine) was not a CK1 substrate. In general, the human p53 proteins were much weaker substrates than the murine p53 protein.

To determine whether serine 15 phosphorylation could influence the ability of CK1 to phosphorylate p53, the GST-p53 fusion proteins (both murine and human) were phosphorylated stoichiometrically using recombinant DNA-PK and excess unlabelled ATP as phosphate donor. The proteins were bound to glutathione-Sepharose beads and washed prior to the addition of CK1 and [γ-^{32}P]ATP. Under the conditions used, the rate of murine p53 phosphorylation by CK1 was stimulated by up to 10-fold following prior phosphorylation of serine 15 (Fig. 2A). CK1-mediated phosphorylation of the 380 protein was also observed, only after prior phosphorylation of serine 15, indicating that the residue(s) phosphorylated by CK1 was neither serine 4, 6 or 9. WT human p53 and the

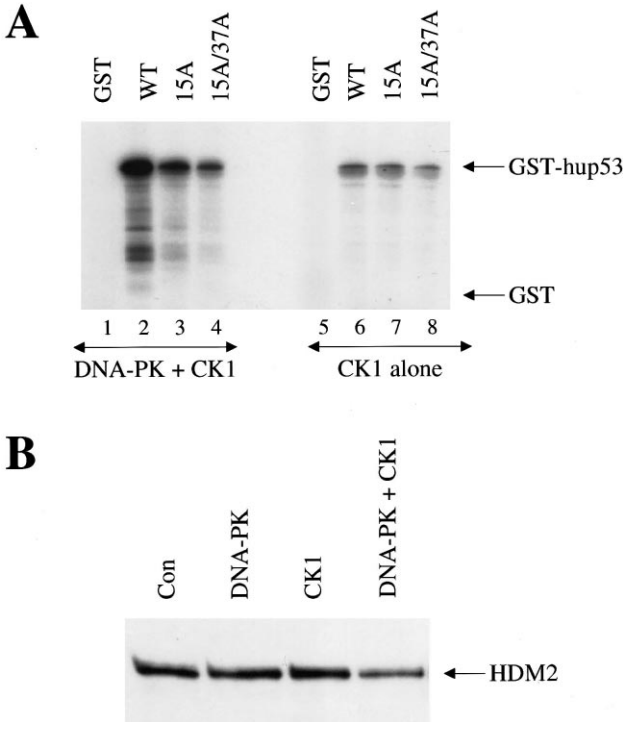

Fig. 3. DNA-PK/CK1δ-mediated phosphorylation of full length GST-p53 fusion proteins and interaction with HDM2 in vitro. (A) The GST-p53 fusion proteins comprising full length human p53 (WT, S15A or the S15,37A double mutant) were mock-phosphorylated (lanes 5–8) or phosphorylated using recombinant DNA-PK and unlabelled ATP as phosphate donor (lanes 1–4). Following removal of the kinase and excess ATP, the proteins were phosphorylated using recombinant CK1δ and [γ-^{32}P]ATP as phosphate donor. GST alone was included as a negative control. (B) Binding of in vitro-translated, radiolabelled HDM2 to phosphorylated and unphosphorylated GST-full length human p53.

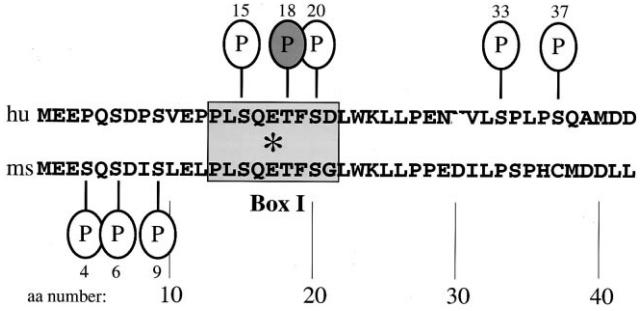

Fig. 4. Schematic representation of the N-terminal 42 amino acids of murine and human-p53. The amino acid sequences (in single letter code) of human p53 (hu, top) and murine p53 (ms, bottom) are shown in alignment. The highly conserved 'Box I' sequence [26] is indicated, as are the positions of known physiological phosphorylation sites (with the letter P inside an ellipse and the position of the phosphorylated residue indicated over the ellipse). The threonine 18 phosphorylation site is shown with a grey background. The CK1 phosphorylation sites in murine p53 at serines 4, 6 and 9 are also indicated. An asterisk denotes the negatively charged residue at position 17.

panel of phosphorylation site mutants were similarly examined (Fig. 2B). p53 which had previously been phosphorylated at serine 15 (hereafter designated p53-15P) was phosphorylated by CK1 at least 50-fold more effectively as compared with the unphosphorylated protein. The GST moiety was not phosphorylated by CK1 under these conditions. The role of serine 15 in this process was underscored by the observation that CK1-dependent phosphorylation of the 15A and 15A/37A mutants, but not the 37A alone mutant, was barely detectable and was only slightly higher as compared with the mock DNA-PK-phosphorylated protein. After phosphorylation by DNA-PK, there were some differences in the levels of phosphorylation of the WT and several of the mutant proteins. However, these differences could also be discerned at the basal level (i.e. when not phosphorylated by DNA-PK) and may reflect conformational differences or a partial requirement of specific serine residues to permit recognition or binding to the kinase. A convenient approach for determining the effect of serine 15 phosphorylation on subsequent phosphorylation by CK1 was to measure the ratio of CK1-mediated phosphorylation of p53-15P compared to the mock-phosphorylated proteins. These ratios are expressed as 'fold increase' under the autoradiograph in Fig. 2B. These data revealed that the 6A/9A, 37A and 20A mutants showed increases comparable to WT p53 in their ability to be phosphorylated by CK1 following prior phosphorylation of serine 15. Strikingly, however, the ability of DNA-PK phosphorylation to stimulate CK1-mediated phosphorylation of the 18A mutant was reduced by approximately 7-fold. This is consistent with phosphorylation of threonine 18 in p53-15P by CK1. To determine whether threonine 18 was indeed phosphorylated under these conditions, phosphoamino acid analysis of the GST-WT human p53 protein was carried out. The data (Fig. 2C) indicated that most of the radioactivity in the protein was present as phosphothreonine. There is only one threonine residue in the N-terminal 42 amino acids of p53 and this is located at position 18 (Fig. 4).

These data indicate that, in the context of the N-terminal 42 amino acids of p53, threonine 18 is a substrate for phosphorylation by CK1 as mediated by prior phosphorylation of serine 15. To determine whether this is a property of full length

p53, GST-p53 fusion proteins comprising the full length p53 protein, either WT, 15A or 15A/37A, were mock-phosphorylated or phosphorylated with DNA-PK using unlabelled ATP as phosphate donor. The data (Fig. 3) show that, in the absence of prior phosphorylation by DNA-PK, all three proteins were phosphorylated by CK1 to an equal extent (lanes 5–8). In contrast, the phosphorylation of WT p53 was stimulated by up to 50-fold when the protein had previously been phosphorylated by DNA-PK (lane 2). When the 15A or 15A/37A mutants were incubated with DNA-PK and unlabelled ATP prior to CK1 phosphorylation (lanes 3 and 4), the stimulation was considerably less effective than with the WT protein, although the levels of phosphorylation were slightly higher than those in the absence of any prior phosphorylation. As before, no phosphate was incorporated into the GST moiety. These data indicate that phosphorylation of full length p53 is stimulated by prior phosphorylation of the protein by DNA-PK. Fig. 3B shows that when the p53 had been phosphorylated by DNA-PK and subsequently by CK1, the binding to HDM2 in vitro was reduced by about 3-fold as compared with the unphosphorylated protein. Phosphorylation by DNA-PK alone or CK1 alone did not affect HDM2 binding. These data confirm the potential of threonine 18 modification for regulating the interaction of p53 with HDM2 [13,14].

4. Discussion

In this paper, we have explored the phosphorylation of human and murine p53 by the protein kinase CK1 in vitro. The most striking observation which is evident from our data is that prior phosphorylation of serine 15 in vitro can create a potent recognition determinant for subsequent phosphorylation by CK1 (this applies to both human and murine p53). Mutagenesis data and phosphoamino acid analysis indicate that the residue phosphorylated by CK1 is threonine 18 (Fig. 2). Previous studies have established the importance of phosphorylated residues in acting as recognition determinants for phosphorylation by CK1 [21,22] and our data therefore provide a potential mechanism by which serine 15 phosphorylation may nucleate threonine 18 phosphorylation in the cell. This would be consistent with an established role of serine 15 phosphorylation orchestrating sequential modification of other residues in p53 [11]. Such a role would be dependent on DNA damage-induced modification of serine 15 (which is mediated in vivo by ATM [4,5]) and would also be consistent with the indication from yeast studies that CK1 activity is pivotal to the DNA damage response [19,20]. Threonine 18 lies within a highly conserved element of the p53 protein (Fig. 4) which mediates p53 activation, regulation of the association with MDM2 and control of the interaction with transcriptional activators [4,5,7,8,10–12]. Moreover, recent evidence indicates that threonine 18 phosphorylation contributes significantly to abrogating the p53-MDM2 interaction and is therefore likely to be a significant factor in the p53 activation process [13,14]. Our data confirm that threonine 18 phosphorylation can partially block interaction of p53 with HDM2 (Fig. 3B). Threonine 18 modification may also be an important factor in the p53 response to tumour development [14]. The identification of CK1 as a potential threonine 18 kinase therefore opens up a new avenue of exploration to analyse the physiological modification of this important regulatory site.

While murine p53 is also a substrate for CK1 in the absence of serine 15 phosphorylation in vitro (at residues 4, 6 and 9 [23]), loss of the equivalent residues in human p53 does not affect the ability of CK1 to phosphorylate the protein (Fig. 1B). However, serine 20 (and possibly serine 37) may act as phosphate acceptor in the human protein in vitro. Comparison of the sequences of the first 42 amino acids of the murine and human proteins (Fig. 4) reveals differences which could account for these observations. For example, proline residues at positions 4 and 8 in the human protein may alter the secondary structure such that the kinase does not efficiently interact with p53. Similarly, a glutamic acid residue at position 17 (in both murine and human p53) may act as a recognition determinant for phosphorylation of serine 20 (we do not know whether serine 20 of murine p53 can also be phosphorylated by CK1). These differences may reflect genuine variance in the way in which p53 proteins from different species are regulated and add to a growing list of species-specific modifications of p53 (discussed by Meek [3]). Alternatively, when compared to the ability of CK1 to phosphorylate threonine 18 in p53-15P, which is many orders of magnitude greater than unphosphorylated p53, the physiological relevance of phosphorylation of these residues by CK1 may be questionable. This is not to say that the residues themselves are not of regulatory significance, only that the protein kinase(s) which modify these sites in vivo may well be different from CK1. A final issue concerns the isoform(s) of CK1 which can modify p53 in the cell. While previous data have indicated that the delta and epsilon isoforms are preferentially active towards murine p53 (at residues 4, 6 and 9 [24]), the use of a phosphorylated residue as a recognition determinant is not the property of any specific isoform(s) of CK1. It is therefore possible that other CK1 isoforms may modify threonine 18 in vivo. Future studies should resolve these issues.

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