Phosphorylation of murine double minute clone 2 (MDM2) protein at serine-267 by protein kinase CK2 *in vitro* and in cultured cells

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Murine double minute clone 2 oncoprotein (MDM2) is a key component in the regulation of the tumour suppressor p53. MDM2 mediates the ubiquitination of p53 in the capacity of an E3 ligase and targets p53 for rapid degradation by the proteosome. Stress signals which impinge on p53, leading to its activation, promote disruption of the p53–MDM2 complex, as in the case of ionizing radiation, or block MDM2 synthesis and thereby reduce cellular MDM2 levels, as in the case of UV radiation. It is therefore likely that MDM2, which is known to be modified by ubiquitination, SUMOylation and multi-site phosphorylation, may itself be a target for stress signalling (SUMO is small ubiquitin-related modifier-1). In the present study we show that, like p53, the MDM2 protein is a substrate for phosphorylation by the protein kinase CK2 (CK2) *in vitro*. CK2 phosphorylates a single major site, Ser²⁶⁷, which lies within

the central acidic domain of MDM2. Fractionation of cellular extracts revealed the presence of a single Ser²⁶⁷ protein kinase which co-purified with CK2 on ion-exchange chromatography and, like CK2, was subject to inhibition by micromolar concentrations of the CK2-specific inhibitor 5,6-dichlororibofuranosylbenzimidazole. Radiolabelling of cells expressing tagged recombinant wild-type MDM2 or a S267A (Ser²⁶⁷ → Ala) mutant, followed by phosphopeptide analysis, confirmed that Ser²⁶⁷ is a cellular target for phosphorylation. Ser²⁶⁷ mutants are still able to direct the degradation of p53, but in a slightly reduced capacity. These data highlight a potential route by which one of several physiological modifications occurring within the central acidic domain of the MDM2 protein can occur.

Key words: p53 pathway, signalling, oncogene.

INTRODUCTION

p53 is a latent and highly labile transcription factor which contributes significantly to the maintenance of genomic stability and the prevention of tumour development (reviewed in [1]). Loss of p53 function, mainly through mutation of the p53 gene, is a common genetic occurrence during the development of a wide range of tumours and is thought to contribute significantly to the progression of the disease. The p53 protein is activated by, and is an integration point for, a wide range of cellular stresses, including DNA damage, activated oncogenes, hypoxia and spindle damage (reviewed in [2]). In response to an inducing signal, p53 accumulates in the nucleus and co-ordinates a change in the balance of gene expression leading to growth arrest or apoptosis, events which prevent the growth or survival of damaged cells. At present, there are more than 100 genes known to be activated by p53, many of which are involved in the growth arrest or apoptotic pathways [3].

Signals arising from cellular stress initiate a complex series of regulatory events in the p53 pathway, leading to increased stability of p53 and activation of its biochemical functions [2]. These signals converge at the interaction of p53 with its negative regulatory partner, murine double minute clone 2 oncoprotein (MDM2) (reviewed in [4,5]). The binding of MDM2 to the N-terminus of p53 (amino acids 19–26) represses p53 transcriptional activity, mediates ubiquitination of p53 in the capacity of an E3 ligase [6], and targets p53 to the cytoplasm for 26 S-proteasome-dependent degradation [7–10]. The *mdm2* gene is itself activated by p53, and the two proteins therefore form an autoregulatory loop in which p53 positively regulates MDM2 expression while

MDM2 negatively regulates p53 levels and activity [5]. The N-terminal 102 amino acids of MDM2 are responsible for binding to the N-terminus of p53, while regions within both the acidic central domain and C-terminus of MDM2 are required for targeting p53 to the proteasome [11].

Disruption of the p53–MDM2 complex is a pivotal event during the induction of p53, leading to the accumulation of active p53 in the nucleus. Artificial disruption of this complex in cultured cells is sufficient to invoke p53-mediated gene expression and cell-cycle arrest [12] underscoring the central role of MDM2 in controlling p53. However, the mechanism(s) by which this occurs physiologically is still unclear. Previous attempts to understand the mechanism of p53 induction have focused primarily on p53 itself. In particular, key attention has been given to the role a complex series of phosphorylation and acetylation modifications of p53 which accompany p53 induction by stresses such as DNA damage (reviewed in [2]). However, while such modifications may contribute to weakening the p53–MDM2 interaction, other groups have shown that phosphorylation of p53 is not essential for DNA-damage-induced stabilization [13–15]. Moreover, the induction of p53 by dominant oncogenes, which occurs through the sequestration of MDM2 by the p14^{ARF} protein, is independent of p53 phosphorylation [16], underscoring the fact that p53 can be stabilized physiologically without the need for post-translational modification of p53, depending of course upon the initiating event(s). Taken together, these findings also suggest the possibility that the MDM2 protein itself may be the principal target of signals which lead to p53 stabilization. In support of this idea, MDM2 has been shown to be subject to multi-site phosphorylation, with most of the sites of modification

Abbreviations used: ARF, alternative reading frame; ATM, ataxia telangiectasia mutated; DNA-PK, DNA-activated protein kinase; DRB, 5,6-dichlororibofuranosylbenzimidazole; ECL* (Amersham), enhanced chemiluminescence; (E)GFP, (luminescence-enhanced) green fluorescent protein; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; MDM2, murine double minute 2; SUMO, small ubiquitin-related modifier-1; TNFα, tumour necrosis factor-α; CK2, protein kinase CK2; S267A etc., Ser²⁶⁷ → Ala mutant etc.; SAPK, p38-stress-activated protein kinase.

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clustered within the p53-binding domain and the important central acidic domain required for the degradation of p53 [17]. Moreover, MDM2 has recently been shown to be a DNA-damage- and protein-kinase-ATM-dependent target for phosphorylation, an event which is thought to contribute to the p53 activation mechanism [15] (ATM is ataxia telangiectasia mutated). In addition to phosphorylation, modification by the small ubiquitin-related modifier-1 (SUMO), can stimulate the ubiquitin ligase activity of MDM2 towards p53, while abrogating its self-ubiquitination function [18]. Reduced SUMOylation of MDM2 in response to DNA damage is also thought to be part of the route by which DNA damage contributes to p53 stability [18]. At a biochemical level, other reports have indicated that MDM2 can be phosphorylated by protein kinase CK2 (CK2) *in vitro* [19] and by DNA-activated protein kinase (DNA-PK) *in vitro* [20], but knowledge of the physiological relevance or significance of these (and other) phosphorylation events is entirely lacking. At the present time, therefore, surprisingly little is known about the regulation of MDM2 by multi-site phosphorylation, its potential role in governing MDM2 function in stress signalling, its relationship with other types of post-translational modification, and whether p53 and MDM2 may be co-ordinately regulated through post-translational modifications mediated by the same signalling pathways and enzymes.

CK2 (formerly termed 'casein kinase II') is a ubiquitous protein kinase comprising two α or α' catalytic subunits complexed in a tetrameric holoenzyme with two regulatory β subunits (reviewed in [21]). CK2 is a predominantly nuclear protein kinase which is required for cell viability and cell-cycle progression. Over 160 putative CK2 substrates have been characterized, and they include many proteins involved in transcription, growth control (such as Myc, Myb and Jun) and manipulation of DNA (such as topoisomerase II and DNA ligase). Of particular interest to the present study, the p53 tumour suppressor protein is phosphorylated at a single C-terminal site by CK2 (Ser³⁹² in human p53), leading to activation of the site-specific DNA binding function of p53 [22]. When assayed in cell extracts, CK2 is constitutively active, but there have been a number of reports showing that several growth factors can have a modest effect on CK2 activity (reviewed in [23]). More recently, CK2 has been reported to be responsive to cellular stress, in the form of heat-shock [24] or agents which activate the p38-stress-activated protein kinase (SAPK) pathway [25]. The interaction of p38 α with CK2, and the concomitant activation of CK2 reported in this latter study [25], is of particular interest with respect to the regulation of the p53 pathway because this and previous reports have shown that p53 is phosphorylated at Ser³⁹² (the site targeted by CK2) in response to agents such as tumour-necrosis factor- α (TNF α) and UV [25–28] in a manner that is highly sensitive to p38-specific inhibitors such as SB203580 [25,28]. Recent evidence has also linked CK2 with MDM2 [19,29] and raises the possibility that the two partner proteins may be co-ordinately regulated. However, to date, the link between CK2 and MDM2 has only been explored at a superficial level.

In the present study, we report that MDM2 is a substrate for the protein kinase CK2 and confirm the results of a recent study identifying Ser²⁶⁷ as the major site of *in vitro* phosphorylation by CK2 [29]. We extend this knowledge by showing that the sole Ser²⁶⁷-directed protein kinase activity detected in cell extracts is CK2-like, consistent with a physiological interaction between the two proteins, and demonstrate that Ser²⁶⁷ is indeed a physiological site of phosphorylation in the MDM2 protein. Our analyses of phosphorylation-site mutants indicate that, unlike other recently identified sites of phosphorylation in this region of MDM2, Ser²⁶⁷ phosphorylation on its own may play only a weak

role in regulating p53 turnover. These data highlight a potential mechanism by which one of several physiological modifications within the central acidic domain of MDM2 can occur.

MATERIALS AND METHODS

Cells

MCF-7, H1299 and COS-7 cells were maintained in Dulbecco–Vogt modified Eagle's medium (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 10 % (v/v) fetal-bovine serum (Life Technologies), 100 μ g/ml penicillin/streptomycin (Life Technologies) and 2 mM glutamine (Life Technologies). The cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO₂.

Plasmids

All of the plasmids used in the present study are listed in Table 1. Plasmids encoding murine MDM2 cDNA in Bluescript II KS+ and in pH6EX3 were generously given by Professor Dr. Wolfgang Deppert, Heinrich-Pette-Institut für Experimentelle Immunologie und Virologie, University of Hamburg, Hamburg, Germany. The presence of *mdm2* in the pH6EX3 plasmid permitted isopropyl β -D-thiogalactopyranoside (IPTG)-inducible expression in *Escherichia coli* and subsequent purification of MDM2 by nickel-agarose chromatography. Wild-type MDM2 cDNA was also cloned into the mammalian expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands) [17]. A DNA fragment encoding the c-Myc 9E10 epitope (human c-Myc amino acids 408–439 [30]) was cloned immediately at either the 5' or 3' ends of the MDM2 coding sequence such that the integrity of the MDM2 start and stop codons respectively were maintained. An untagged MDM2 cDNA expressed in pcDNA3 was used in the p53 degradation assays (see below) and was generously provided by Dr. Christine Blattner (Department of Surgery and Molecular Oncology, University of Dundee, Dundee, Scotland, U.K.). A plasmid expressing human p53 under control of the cytomegalovirus promoter has been described previously [31]. A plasmid expressing a glutathione S-transferase (GST)–wild-type human p53 fusion protein was also prepared using the IPTG-inducible vector pGEX-2T. The luminescence-enhanced green fluorescent protein (EGFP; as an internal standard in trans-

Table 1 Plasmids used in the present study

Plasmid	Description
pDWM411	MDM2 cDNA in Bluescript KS+
pDWM415	MDM2 cDNA in pH6EX3
pDWM927	S267A mutant of MDM2 cDNA in pH6EX3
pDWM659	MDM2 cDNA tagged at N-terminus with Myc 9E10 epitope in plasmid pcDNA3
pDWM930	S267A mutant of MDM2 cDNA tagged at N-terminus with Myc 9E10 epitope in plasmid pcDNA3
pDWM931	S267D mutant of MDM2 cDNA tagged at N-terminus with Myc 9E10 epitope in plasmid pcDNA3
pDWM932	S258,260,267A triple mutant of MDM2 cDNA tagged at N-terminus with Myc 9E10 epitope in plasmid pcDNA3
pDWM979 (1473)	Untagged MDM2 cDNA in pcDNA3
pDWM974	Untagged S267A mutant of MDM2 cDNA in pcDNA3
pDWM977	Untagged S267D mutant of MDM2 cDNA in pcDNA3
pDWM1051	Untagged S258,260A mutant of MDM2 cDNA in pcDNA3
pDWM715	Wild-type human p53 full-length in pcDNA3
pEGFP-C1 (ClonTech)	Expresses EGFP
pDWM721	Full-length human wild-type p53 in pGEX-2T

Table 2 Oligonucleotides used for mutagenesis of MDM2

Oligonucleotide	Sequence
Oligo MH2 (S267A)	CACGAGCTCGCAGATGAGG
Oligo MH1 (S267D)	GAAGGGCAGCAGCTCGACGATGAGGATGAG
Oligo THJ51 (S258A, S260A and S267)	CTCGGAAGATTACGCCCTGGCTGACGAAGGGCAC

fection experiments) was expressed from the plasmid pEGFP-C1 (ClonTech Laboratories UK Limited, Basingstoke, Hants., U.K.).

Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis was carried out using a modification of the *dut⁻ ung⁻* system described previously [32]. To prepare single-stranded DNA template, the Bluescript-based plasmid pDWM411 (containing the MDM2 cDNA) was used to transform CJ236 (*dut⁻ ung⁻*) bacterial cells to ampicillin resistance. Cultures were infected with the helper phage M13KO7 to generate replication-defective phage containing single-stranded copies of the plasmid. Bacterial cells were removed from the culture by centrifugation and the phage was subsequently purified from the medium by precipitation in 20% poly(ethylene glycol) 6000 (6 kDa molecular-mass cut-off) in 3.5 M ammonium acetate following removal of the bacterial cells by centrifugation. Single-stranded DNA was prepared by phenol/chloroform extraction of the resuspended phage pellet.

Oligonucleotides used for mutagenesis are listed in Table 2. To allow extension of the primer, 200 pmol of the mutagenic oligonucleotide was phosphorylated by incubating a solution containing 10 mM Tris, pH 8, 1 mM MgCl₂, 0.5 mM dithiothreitol, 0.43 mM ATP and 10 units of T₄ Polynucleotide kinase (MBI Fermentas, Flamborough, ON, Canada) in a total of 30 µl at 37 °C for 45 min. The oligonucleotides were heat-denatured at 65 °C for 10 min and subsequently stored at -20 °C. A 0.2 µg portion of the single-stranded DNA were incubated at 95 °C for 5 min and afterwards immediately put on ice. A 1 µl portion of the phosphorylated oligonucleotide was added to the single-stranded DNA in annealing buffer [20 mM Tris (pH 7.4)/2 mM MgCl₂/50 mM NaCl] in a final volume of 8 µl and incubated at 70 °C for 2 min, and then cooled slowly to 30 °C and placed on ice. Extension was carried out in a final volume of 50 µl containing 0.5 mM of each dNTP, 1 mM ATP, 10 mM Tris, pH 7.4, 5 mM MgCl₂, 2 mM dithiothreitol, 5 units of T₄ DNA ligase (Life Technologies) and 5 units of T₄ DNA polymerase (New England Biolabs, Hitchin, Herts., U.K.). The sample was incubated for 5 min on ice, 5 min at 25 °C and 90 min at 37 °C. The DNA was transformed into competent DH5α cells, plated out on to ampicillin plates and grown at 37 °C overnight. Recombinant plasmids were isolated and mutants identified by DNA sequencing. The entire coding region of each cDNA was checked by DNA sequencing to confirm the presence of the mutations and to ensure that no undesired changes had been incorporated.

Preparation of cell extracts for protein kinase assays and ion-exchange chromatography

Cells were grown on 10 cm-diameter plates to 70% confluency, then washed twice with 5 ml of ice-cold PBS and harvested in 500 µl of Lysis Buffer [20 mM Tris/acetate (pH 7)/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/0.1% β-mercaptoethanol/1 mM sodium orthovanadate]. For chromatography, five 15 cm-diameter plates were used as the starting material. The extracts were transferred to Eppendorf tubes, vortex-mixed and cleared by centrifugation for 15 min at 4 °C at 12 000 g. Extracts were divided into portions, snap-frozen and kept at -80 °C at this stage. For ion-exchange chromatography, the extracts were filtered using a 0.22 µm-pore-size filter. Ion-exchange chromatography was carried out using a MonoQ H/R 5/5 column (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) equilibrated with Buffer A [50 mM Tris (pH 7.5)/1 mM EDTA/1 mM EGTA/5% (v/v) glycerol/0.03% Brij 35/1 mM benzamidine/4 µg/ml leupeptin/0.1% β-mercaptoethanol] prior to loading the samples. The proteins were eluted into 24 fractions using a linear gradient of 0–600 mM NaCl in Buffer A. Both the fractions and the unbound 'flow-through' material were screened for protein kinase activity using casein and recombinant MDM2 as substrates. CK2 subunits were detected by Western blotting.

Preparation of cell extracts used for Western blotting

Cells were washed as described above, then harvested in 100 µl of 2 × SDS sample buffer [0.123 M Tris (pH 6.8)/4% (w/v) SDS/50% (v/v) β-mercaptoethanol/0.04% Bromophenol Blue/20% (w/v) sucrose/glycerol], placed at 100 °C for 3 min and subsequently sonicated [Soniprep 150 instrument (Sanyo Gallenkamp PLC, Loughborough, Leics., U.K.); amplitude 10 µ; two bursts of 30 s each]. Samples were then loaded on SDS-containing gels.

Transfection of cells in culture

Plates (10 cm diameter) were seeded with 5 × 10⁵ COS-7 cells in normal growth medium and incubated at 37 °C for 24 h. For each transfection, 8 µg of plasmid DNA and 40 µl of LIPO-FECTAMINE[™] reagent were diluted into a final volume of 8 ml OPTI-MEM I Reduced Serum Medium (Life Technologies). The cells were incubated at 37 °C for 5 h before replacing the medium with complete growth medium.

Recombinant CK2

CK2 α and β subunits were expressed in a bacterial expression system [pT7-7/BL21(DE3)] and purified to homogeneity as described in [33]. After mixing the subunits, CK2 holoenzyme (α₂β₂) was spontaneously reconstituted.

Recombinant MDM2

Wild-type and mutant MDM2 proteins were expressed in *E. coli* BL21 cells induced for 2 h at 37 °C with 1 mM IPTG. The bacterial pellet was resuspended in 10% (w/v) sucrose/50 mM Tris (pH 7.5)/1 mM benzamidine and lysed by sonication. His-tagged MDM2 was purified by chromatography on a nickel-agarose column equilibrated with 1 mM imidazole/50 mM Tris (pH 7.5)/100 mM NaCl/10 mM β-mercaptoethanol/5% (v/v) glycerol/0.02% Nonidet p40/10 µM ZnCl₂. The proteins were eluted into ten fractions using stepwise increasing concentrations of imidazole in the above buffer. All the fractions were checked by Western blotting and Coomassie Blue staining of SDS/10%-(w/v)-polyacrylamide gels.

Measurement of protein concentrations

Protein concentration was determined by the Bradford method using BSA as the standard and reagent supplied by Bio-Rad, Hemel Hempstead, Herts., U.K.

Western analysis

Following electrophoretic separation by SDS/PAGE, proteins were transferred to Immobilon P PVDF membranes (Millipore, Watford, Herts., U.K.) by semi-dry blotting at 150 mA for 45 min at room temperature. To assess the expression level of Myc-tagged MDM2, the monoclonal antibody 9E10 [30] (2.5 µg/ml) was used for immunochemical detection using an enhanced-chemiluminescence (ECL[®]; Amersham) kit. CK2 subunits α and β were detected by the monoclonal antibodies 1AD9 (0.5 µg/ml) [34] and 6D5 (0.5 µg/ml) [35].

Protein kinase assays

MDM2 kinase assays were carried out using purified recombinant wild-type or mutant MDM2 as substrate. Recombinant-CK2 assays were performed with 100 ng of CK2 in 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂ and 20 µM [γ -³²P]ATP (Amersham Pharmacia Biotech; sp. radioactivity 12.5 Ci/mmol) in a total volume of 20 µl. Assays using cell extracts were performed with 2 µl of cell extracts in 25 mM Tris, pH 7.5, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM sodium orthovanadate and 20 µM [γ -³²P]ATP (sp. radioactivity 12.5 Ci/mmol) in a total volume of 20 µl. The reactions were initiated by addition of enzyme and incubated at 30 °C for 30 min. A 5 µl portion of concentrated sample buffer (900 µl of β -mercaptoethanol, 200 µl 10% [w/v] SDS and 100 µl of saturated Bromophenol Blue solution) were added to the samples which were subsequently separated on SDS/10%-polyacrylamide gels. The gels were then stained with Coomassie Blue and dried on to Whatmann 3 MM filter paper. The proteins were revealed by autoradiography and quantified by determination of Cerenkov radiation using a liquid-scintillation counter.

Labelling cells with H₃³²PO₄

At 48 h after transfection, COS-7 cells were labelled with H₃³²PO₄ (carrier-free; ICN Pharmaceuticals Ltd., Basingstoke, Hants., U.K.). Cells were pre-incubated with 8 ml of phosphate-free medium (Sigma) containing dialysed 10% (v/v) fetal-bovine serum at 37 °C for 1 h. The medium was replaced with the same volume containing 4 mCi of H₃³²PO₄/plate and incubated at 37 °C for a further 3 h. After labelling, the cells were washed twice with 5 ml of ice-cold PBS and lysed in 1 ml of cold Nonidet P40 buffer (10 mM NaH₂PO₄/2 mM EDTA/0.1 M NaCl/1% Nonidet P40/1 mM benzamidine). The extracts were cleared by centrifugation. Immunoprecipitation of the Myc-tagged MDM2 from the extracts was performed overnight at 4 °C on an Eppendorf wheel with 2–5 µg of 9E10 antibody and excess Protein A–Sephacrose 4B. The Sepharose beads were subsequently washed three times with 1 ml of ice-cold RIPA Buffer (10 mM NaH₂PO₄/2 mM EDTA/0.1 M NaCl/1% Nonidet P40/1 mM benzamidine/1% sodium deoxycholate/0.1% SDS) and once with 1 ml of ice-cold 50 mM Tris, pH 7.5. A 25 µl portion of 2 × sample buffer was added and the samples were separated on SDS/10%-polyacrylamide gels.

Chymotryptic-phosphopeptide mapping

Two-dimensional thin-layer phosphopeptide analysis of MDM2 was carried out using essentially the same procedure as has been used previously for p53 [36] with two exceptions: (i) chymotrypsin was used to digest the protein in place of trypsin and (ii) the solvent used for the chromatographic separation was 4-methylpropanoic acid/butan-1-ol/pyridine/acetic acid/deionized water (62.5:1.9:4.8:2.9:27.9, by vol.).

Phosphoamino-acid analysis

The SDS/PAGE-fractionated proteins were transferred to a PVDF membrane by semi-dry blotting. The membrane was exposed, the band of interest was excised, and the MDM2 protein on the membrane fragment was then hydrolysed in 6 M HCl at 110 °C for 1 h. The sample was subsequently freeze-dried and the Cerenkov radiation determined. The amino acids were resuspended in 5 µl of pH 1.9 buffer (117 ml of acetic acid, 37.5 ml of formic acid in a total volume of 1.5 litres) containing 2 µg each of phosphoserine, phosphothreonine and phosphotyrosine as internal standards. The samples were then applied to cellulose thin-layer plates. Electrophoresis in the first dimension was carried out in pH 1.9 buffer at 550 V for 1 h. After drying the plate, electrophoretic separation in the second dimension was carried out in pH 3.5 buffer (75 ml of glacial acetic acid, 7.5 ml of pyridine and 1.5 ml of 0.5 M EDTA) at 500 V for 50 min. The unlabelled phosphoamino acids were revealed by spraying the plate with 0.2% ninhydrin in acetone, and the radiolabelled phosphoamino acids detected by autoradiography.

In vitro association assays

To determine the ability of the wild-type and phosphorylation site-mutant proteins to bind p53 *in vitro*, GST pull-down assays were performed using *in-vitro*-translated proteins. The MDM2 cDNAs were transcribed and translated (using the pcDNA3-based plasmids described in Table 1) in the presence of [³⁵S]methionine using a reticulocyte-lysate *in vitro* transcription/translation kit as directed by the manufacturer (Promega, Chilworth, Southampton, U.K.). The binding/washing buffer was 50 mM Tris (pH 7.4)/250 mM NaCl/0.1% Triton/5 mM EDTA/2 mM dithiothreitol/protease inhibitors. The GST–p53 protein was adsorbed on to GSH–Sepharose 4B beads (Amersham Pharmacia Biotech) and the beads were washed twice with the above buffer. The GST–p53 protein was incubated at 4 °C with ³⁵S-labelled proteins in 200 µl of buffer for 2 h. Complexes were washed twice in washing/binding buffer, resuspended in sample buffer and separated by SDS/10%-PAGE. Before being dried, the gels were stained to confirm that the same amount of GST–p53 protein was precipitated with GSH–Sepharose 4B beads. ³⁵S-labelled protein was detected by fluorography.

MDM2-directed degradation of p53

H1299 cells (p53-null lung carcinoma cell line) were seeded at 2 × 10⁵ cells/5 cm-diameter plate and co-transfected with plasmids expressing wild-type human p53 (100 ng), green fluorescent protein (GFP) [pEGFP-C1 (ClonTech); 200 ng] and increasing amounts of plasmids expressing wild-type or phosphorylation-site-mutant MDM2 proteins. At 24 h after transfection, the cells were rinsed three times in ice-cold PBS and harvested in 50 mM Tris (pH 7.5)/150 mM NaCl/1% (v/v) Igepal/1 mM dithiothreitol/Protease Inhibitor Cocktail (Calbiochem, San Diego, CA, U.S.A., according to the manufacturer's instructions). Proteins were resolved by SDS/PAGE and analysed by Western blotting. MDM2, p53 and GFP were detected using the antibodies SMP14, DO1 and anti-GFP (8362-1 from ClonTech) respectively.

RESULTS

To determine whether MDM2 could be phosphorylated by CK2 *in vitro*, purified recombinant His-tagged MDM2 was incubated with recombinant CK2 holoenzyme in the presence of [γ -³²P]ATP, and the phosphorylated proteins were resolved by

Figure 1 Phosphorylation of MDM2 *in vitro* catalysed by recombinant protein kinase CK2 holoenzyme

(A) Schematic representation of MDM2 showing the full-length MDM2 protein and the deletion derivatives $\Delta 1$ –3 used as substrates. The numbers refer to amino acid positions. (B) Full-length recombinant His-tagged MDM2 and C-terminally truncated mutants $\Delta 1$ –3 were purified and incubated in the presence of [γ - 32 P]ATP and 100 ng of recombinant CK2 holoenzyme as described in the Materials and methods section. The phosphorylated proteins were resolved by SDS/PAGE on 10% polyacrylamide gels and the recombinant MDM2 proteins identified by staining with Coomassie Blue (results not shown). The gel was dried and exposed to X-ray film at -80°C in the presence of intensifying screens. The positions of molecular-mass markers, the MDM2 proteins and the β subunit of CK2 (which becomes autophosphorylated *in vitro*) are shown. (C) Two-dimensional phosphoamino-acid analysis of the phosphorylated MDM2 was carried out as described in the Materials and methods section, in the presence of unlabelled phosphoserine [S], phosphothreonine [T] and phosphotyrosine [Y]. The ellipses indicate the positions to which these unlabelled standards migrate. The radiolabelled phosphorylated amino acid in MDM2 was detected by autoradiography. (D) The phosphorylated proteins from (B) were excised from the gel, oxidized, digested with chymotrypsin and the resultant phosphopeptides separated in two dimensions by thin-layer electrophoresis at pH 1.9 followed by chromatography. The plates were exposed to X-ray film at -80°C in the presence of intensifying screens. The origins (the points at which the phosphopeptides were loaded on to the maps) are indicated with vertical arrows. The angled arrow shows the migration position of the major chymotryptic phosphopeptide.

SDS/PAGE (Figure 1). CK2 catalysed the incorporation of phosphate into full-length MDM2 (Figure 1B). Phosphoamino-acid analysis indicated that the phosphorylated residue(s) was serine (Figure 1C). CK2 also catalysed the incorporation of phosphate into two C-terminally truncated deletion derivatives of MDM2, $\Delta 2$ and $\Delta 3$, but not into a shorter species, $\Delta 1$ (Figures 1A and 1B). This localized the site(s) of phosphorylation to the region encompassing amino acids 194–294. Interestingly, this is a highly acidic domain which contributes to the ability of MDM2 to promote the degradation of p53. The reduced level of incorporation of phosphate into the deletion derivatives could not be explained by differences in the level of substrates (results

Figure 2 Ser²⁶⁷ is the major CK2 target in MDM2

(A) The region of MDM2 encompassing amino acids 195–294 contains four potential target sites for CK2, at Ser²¹⁸, Ser²⁵⁸, Ser²⁶⁷ and Ser²⁸⁴. These residues, plus their respective C-terminal flanking regions, are shown in **bold**. Long arrowheads indicate the sites of chymotryptic digestion. (B) Wild-type MDM2, an S267A mutant and an S258,260,267A triple mutant were phosphorylated *in vitro* by recombinant CK2 using [γ - 32 P]ATP as phosphate donor. The phosphorylated proteins were separated by SDS/PAGE and exposed to X-ray film at -80°C in the presence of intensifying screens. The positions of MDM2, the autophosphorylated CK2 β subunit and the molecular-mass markers are indicated. (C) Two-dimensional chymotryptic-phosphopeptide analysis of the wild-type, S267A and S258,260,267A mutant MDM2 proteins was carried out. The vertical arrows indicate the origins at which the peptides were loaded, and the angled arrows show the migration position of the major chymotryptic phosphopeptide.

not shown), but may reflect a requirement for the protein kinase to interact with elements of tertiary structure within the MDM2 C-terminus. To explore the nature of the CK2-mediated phosphorylation further, the *in-vitro*-phosphorylated proteins were analysed by two-dimensional chymotryptic-phosphopeptide mapping ([36]; Figure 1D). This procedure showed that there was a single major chymotryptic phosphopeptide generated by CK2-mediated phosphorylation and several minor species. When the $\Delta 3$ and $\Delta 2$ phosphorylated proteins were examined, the maps were essentially similar, confirming that the sites lay within amino acids 194–294.

The consensus recognition determinant for phosphorylation by protein kinase CK2 is a serine or threonine target residue which is flanked on its C-terminal side by clusters of negatively charged residues [37]. In particular, a glutamate or aspartate residue at the +3 position, relative to the phosphorylated residue, is highly favourable [37]. The region encompassing amino acids 195–294 of MDM2 contains four serines residues which lie within amino acid sequences containing potential recognition determinants for CK2 phosphorylation (Figure 2A; Ser²⁰⁹, Ser²¹⁸, Ser²³⁸, Ser²⁵⁸, Ser²⁶⁷ and Ser²⁸⁴). Of these six, Ser²⁶⁷ best matches,

Figure 3 Phosphorylation of MDM2 *in vitro* using recombinant protein kinase CK2 holoenzyme or extracts of MCF-7 cells

Recombinant His-tagged MDM2 was purified and incubated in the presence of [γ - 32 P]ATP and either 10 ng of recombinant CK2 or 2 μ l of MCF-7 extract, as described in Materials and methods section. The phosphorylated proteins were isolated by SDS/PAGE, oxidized, digested with chymotrypsin and the resultant phosphopeptides separated in two dimensions by thin-layer electrophoresis at pH 1.9, followed by chromatography. The plates were exposed to X-ray film at -80°C in the presence of intensifying screens. The panels are as follows. (A) MDM2 phosphorylated *in vitro* using recombinant CK2 holoenzyme; (B) MDM2 phosphorylated *in vitro* using MCF-7 whole-cell extracts; (C) schematic representation of the phosphopeptides observed following labelling of the MDM2 *in vitro* using the MCF-7 whole-cell extract. A total of 22 discernible phosphopeptides (numbered 1–22) were observed. P represents the position at which free phosphate migrates. (D) mixture of the peptides obtained from the procedures represented in (A) and (B); (E) MDM2 phosphorylated *in vitro* using MCF-7 whole-cell extracts in the presence of 10 μ M DRB.

by far, these requirements. To determine whether this residue is the target residue for phosphorylation by CK2, the phospho-acceptor serine residue was replaced by alanine (which cannot be phosphorylated) using oligonucleotide-directed mutagenesis. The S²⁶⁷A mutant was subsequently tested as a CK2 substrate. The data indicate that substitution of Ser²⁶⁷ reduced the ability of CK2 to phosphorylate MDM2 by greater than 5-fold (Figure 2B). Chymotryptic-phosphopeptide mapping (Figure 2C and 2D) confirmed that this partial loss of phosphorylation occurred within the major chymotryptic phosphopeptide, consistent with the idea that Ser²⁶⁷ is the major CK2 target. The finding that phosphorylation of this peptide was not abolished suggested that there may be a second CK2 site within the peptide. Ser²⁵⁸ lies within the same chymotryptic peptide as Ser²⁶⁷ (Figure 2A). Consistent with this idea, a mutant in which Ser²⁵⁸, Ser²⁶⁰ and Ser²⁶⁷ were replaced with alanine further reduced the ability of CK2 to phosphorylate MDM2 (Figure 2B) and abolished the major phosphopeptide on the two-dimensional maps (Figure 2C).

If MDM2 is phosphorylated by CK2 in a physiological manner, whole-cell extracts should contain an MDM2-targeted protein kinase activity which phosphorylates Ser²⁶⁷ and has the properties of the recombinant CK2. To test this idea, extracts were prepared from MCF-7 cells and used as a source of protein kinase activity for the phosphorylation of MDM2 *in vitro*. The data from this experiment revealed that MDM2 is subject to multi-site phosphorylation by presumably many different protein kinase activities in whole-cell extracts (Figure 3B). [Qualitatively

similar data were obtained using extracts from HeLa, COS-7 and NIH3T3 cells (results not shown). Detectable phosphopeptides are numbered and shown schematically in Figure 3C.] Chymotryptic phosphopeptides produced from MDM2 that had been phosphorylated *in vitro* by recombinant CK2 (Figure 3A) were mixed, prior to loading on the thin-layer plate, with phosphopeptides generated from MDM2 phosphorylated by protein kinase activities in the cell extracts. When these peptides were separated in two dimensions (Figure 3D), the major CK2-generated phosphopeptide co-migrated with a phosphopeptide (designated peptide 7 in Figure 3C) that was generated when the cell extract was used as the source of protein kinase activity. Thus the major phosphopeptide generated from the phosphorylation of MDM2 by CK2 was represented following the phosphorylation of MDM2 by unfractionated activities in whole-cell extracts. To determine whether the activity which was responsible for modifying this site was CK2-like, the phosphorylation of MDM2 by the extract was carried out in the presence of micromolar amounts of the CK2 inhibitor, 5,6-dichlororibofuranosylbenzimidazole (DRB; [38]). The data (Figure 3E) indicated that peptide 7 was absent following phosphorylation in the presence of 10 μ M DRB, consistent with the idea that this phosphorylation event is catalysed by CK2. There was also some influence of this inhibitor on three other phosphopeptides (3, 4 and 5, marked with asterisks in Figure 3E), indicating that either DRB can affect other MDM2-directed protein kinase activities, or that these peptides may be derivatives containing the Ser²⁶⁷ phosphorylation site together with additional sites of phosphorylation (see below).

To explore further the phosphorylation of MDM2 *in vitro*, MCF-7 cell extracts were fractionated by ion-exchange chromatography using a MonoQ (H/R 5/5) column and applying a linear gradient of 0–600 mM NaCl. To detect casein kinase activity, fractions were assayed for their ability to phosphorylate casein *in vitro*. Activities corresponding to CK1 and CK2 were eluted in fractions 7–9 and fractions 18–19 respectively (Figure 4A), consistent with previous findings (for example, see [39]). Western analysis using antibodies which recognize the α and β subunits of CK2 confirmed the presence of CK2 only in fractions 18 and 19 (Figure 4B). MDM2 protein kinase activity was eluted over many fractions, consistent with the idea that there are multiple MDM2-targeted protein kinases, with the bulk of this activity appearing in fractions 4–10. When MDM2 proteins phosphorylated by protein kinase activities, either in the material which did not bind to the Mono Q resin, or in different fractions across the gradient, were examined by chymotryptic-phosphopeptide mapping, the data confirmed that several of these activities were resolved very effectively by anion-exchange chromatography (results not shown). Strikingly, only fractions 18 (and weakly 19) contained an activity capable of generating a phosphopeptide showing migration properties similar to those of the recombinant CK2-generated phosphopeptide (Figure 4D), consistent with the idea that cells contain a single detectable Ser²⁶⁷-directed protein kinase activity. When this fraction was used to phosphorylate the S267A and S258,260,267A mutant MDM2 proteins, as with the recombinant CK2, phosphorylation of the S267A mutant was significantly less than wild-type MDM2, while phosphorylation of the S258,260,267A protein was reduced even further (Figure 4C). Phosphopeptide mapping confirmed, once again, that the predominant phosphopeptide was essentially abolished when the Ser²⁶⁷ phosphorylation site was replaced by alanine (Figure 4D). Taken together, these data are highly consistent with the idea that cells contain an MDM2-Ser²⁶⁷ protein kinase activity which is characteristic of protein kinase CK2.

Figure 4 Fractionation of casein kinase activities and MDM2-directed protein kinase activities by ion-exchange chromatography

MCF-7 cell extracts were prepared and fractionated on a MonoQ (H/R 5/5) column as described in the Materials and methods section. (A) Fractions were assayed for the presence of casein kinase (using casein as substrate; ○) and MDM2 kinase (using recombinant purified His-tagged MDM2 as substrate; ●). (B) The presence of CK2 was detected in the individual fractions by Western blotting using the α - and β -subunit antibodies 1AD9 and 6D5 respectively. (C) Recombinant wild-type MDM2, the S267A mutant or the S258,260,267A triple mutant proteins were phosphorylated *in vitro* using fraction 18 as the source of MDM2-Ser²⁶⁷ protein kinase activity and [γ -³²P]ATP as phosphate donor. The phosphorylated proteins were separated by SDS/PAGE and exposed to X-ray film at -80°C in the presence of intensifying screens. (D) The phosphorylated wild-type MDM2, the S267A mutant or the S258,260,267A triple mutant proteins were analysed by chymotryptic-phosphopeptide mapping.

To determine whether Ser²⁶⁷ is a site of physiological phosphorylation, a plasmid expressing Myc-tagged MDM2 was transfected into COS-7 cells. At 48 h after transfection, the cells were transiently labelled with $\text{H}_3^{32}\text{PO}_4$ and the radiolabelled recombinant MDM2 protein isolated by immunoprecipitation and analysed by two-dimensional chymotryptic-phosphopeptide mapping. The data (Figure 5) confirmed previous analyses showing that MDM2 is subject to multi-site phosphorylation in cultured cells [17,40] and showed phosphopeptide patterns which were remarkably similar to those obtained when MDM2 was phosphorylated by protein kinase activities present in cell extracts (for example, compare with Figure 2B). A phosphopeptide was observed (marked 'a' in Figure 5A) with migrational properties identical with those of the phosphopeptide generated following phosphorylation of MDM2 by CK2 or cell extracts *in vitro*. When the S267A mutant was analysed in this system, this phosphopeptide was no longer present, consistent with Ser²⁶⁷ being a physiologically phosphorylated residue. This peptide appeared only to be weakly phosphorylated in the COS-7 cells, initially suggesting that it might be phosphorylated only at a low

Figure 5 Phosphorylation of wild-type and phosphorylation-site-mutant MDM2 proteins in COS-7 cells

COS-7 cells were transfected with plasmids expressing Myc-tagged wild-type, or phosphorylation-site-mutant, MDM2 proteins, then labelled with $\text{H}_3^{32}\text{PO}_4$. The phosphorylated MDM2 proteins were immunoprecipitated from cell extracts and analysed by two-dimensional chymotryptic-phosphopeptide mapping. The maps are as follows. (A) Wild-type MDM2; (B) S267A mutant; (C) S258,260A double mutant; (D) S258,260,267A triple mutant. (E) Schematic representation of the MDM2 amino acid sequence between residues 248 and 274, showing the CK2 phosphorylation site (marked P), sites of potential serine or tyrosine phosphorylation (marked ●) and the sites of chymotryptic digestion. In (A), phosphopeptides proposed to represent amino acids 258–274 are marked 'a' (having a single site phosphorylated) and 'b' (having two sites phosphorylated). In (C) the localization of a novel chymotryptic fragment observed when Ser²⁵⁸ and Ser²⁶⁰ were replaced by alanine is marked 'c'.

level. However, in addition to the loss of peptide 'a', the intensity of a second peptide (marked 'b' and lying within a densely populated and overlapping group of phosphopeptides) was also diminished, suggesting that either the mutation of Ser²⁶⁷ could affect modification of a distant site, or, more plausibly, that the chymotryptic peptide containing Ser²⁶⁷ has potentially greater than one physiological phosphorylation site (Figure 5E shows schematically the locations of possible phosphorylation sites and chymotrypsin digestion sites within this region of MDM2). Under the two-dimensional separation conditions the presence of an additional (negatively charged) phosphate would cause a phosphopeptide to migrate further during electrophoresis towards the anode (left-hand side of the map) and reduce its mobility in the second dimension (chromatography). The migrational properties of peptide 'b' fit these criteria well. In order to test the idea that additional independent phosphorylation events could give rise to multiple species containing

Figure 6 Degradation of p53 mediated by wild-type, S267A or S267D mutant MDM2 proteins

(A) The binding of immobilized GST–human p53 to wild-type, S267A or S267D mutant MDM2 proteins expressed *in vitro* using reticulocyte lysates was measured in GST pull-down assays as described in the Materials and methods section. (B–D) p53-null H1299 cells were transfected with plasmids expressing wild-type human p53, EGFP (as internal standard) and increasing amounts of plasmids expressing wild-type, S267A or S267D MDM2 proteins (B). As a control, the cells were transfected with a plasmid expressing the S258/260A mutant, which degrades p53 only very weakly (C). In each case the p53 blots were stripped and reprobed to determine the levels of EGFP protein (results not shown). The dose-dependence of p53 degradation by MDM2 was measured after quantification of the autoradiographs by densitometry (D). The data in (B)–(D) are representative of three independent experiments.

phosphorylated Ser²⁶⁷, the S258,260,267A mutant was used, since all of the serine residues within the region flanked by the chymotrypsin digestion sites are replaced by alanine in this protein. When the phosphorylation status of this MDM2 mutant was examined, there was now a strong signal from a novel phosphopeptide (marked 'c'), which migrated close to, but with slightly higher chromatographic mobility than, the wild-type Ser²⁶⁷-containing peptide (Figure 5C; the presence of the more hydrophobic alanine residues replacing serine residues at positions 258 and 260 would account for the increased mobility of the phosphopeptide during the chromatographic separation). When Ser²⁶⁷ was replaced by alanine within this S258,260A background, phosphopeptide 'c' was no longer present, consistent with this peptide representing phosphorylation of Ser²⁶⁷. Taken together, these data provide strong support for the idea that Ser²⁶⁷ is phosphorylated in a cellular context.

In order to determine whether Ser²⁶⁷ is required for MDM2-mediated p53 degradation, replacement of Ser²⁶⁷ by alanine or aspartate (to place a constitutive negative charge at the phosphorylation site) was carried out using an untagged MDM2

cDNA, and subsequently cloned into the vector pcDNA3. *In vitro* expression of the wild-type and mutant proteins showed that the nucleotide substitutions did not affect the ability of the cDNA to be translated, nor influence the ability of the MDM2 proteins to complex with wild-type p53 in pull-down assays (Figure 6A). The MDM2 proteins were also expressed at approximately equal levels when expressed in the p53-null human cell line H1299 (Figure 6B). When a plasmid expressing wild-type p53 (together with a plasmid expressing EGFP as an internal standard) were co-transfected with plasmids expressing the wild-type or phosphorylation-site-mutant MDM2 proteins, each of the MDM2 proteins was able to direct the degradation of p53 in a dose-dependent manner [Figure 5B; reprobing of the membrane initially probed for p53 expression indicated that there was little significant variation in the EGFP levels (results not shown)]. While the data from the autoradiographs suggested that both the S267A and S267D mutant had a reduced capacity to drive p53 degradation, quantitative analysis (shown in Figure 6D) indicated that this was only a weak effect (the data shown in this Figure are typical of three independent experiments). In comparison, a S258,260A phosphorylation-site double mutant, which cannot direct p53 degradation (C. Blattner, T. Hay, D. Meek and D. P. Lane, unpublished work), showed a striking loss of its capacity to mediate p53 turnover (Figures 6C and 6D).

DISCUSSION

The MDM2 protein is a key regulator of the p53 tumour suppressor protein. MDM2 represses p53 transcriptional activity [41,42], mediates ubiquitination of p53 in the capacity of an E3 ligase [6,43] and targets p53 to the cytoplasm for 26 S-proteasome-dependent degradation [7–10]. Like p53, MDM2 is subject to control by ubiquitination [6,43], SUMOylation [18] and multi-site phosphorylation [17,40], raising the possibility that signals impinging on the p53 'pathway' may be focused on MDM2 itself or may regulate MDM2 and p53 (and possibly other components of the pathway) in a co-ordinated manner. However, the extent to which MDM2 is targeted by different signalling pathways and the role of specific post-translational modifications in regulating its function are relatively unexplored.

In the present study we show that recombinant MDM2 is a substrate for phosphorylation by protein kinase CK2 *in vitro* (Figure 1), in agreement with previous observations [19]. While the present work was being undertaken, Gotz and colleagues [29] identified Ser²⁶⁷ as a site phosphorylated by CK2 *in vitro* and in insect cells, and again we have independently confirmed the identity of this target residue (Figure 2). Moreover, the present study has advanced our knowledge of the interaction between MDM2 and CK2 in several ways. (1) We show that Ser²⁶⁷ is by far the major target in MDM2 for phosphorylation by CK2 *in vitro*. Mutation of this residue results in a greater than 80% decrease in the ability to phosphorylate MDM2 by CK2 *in vitro* (Figure 2B); additional mutation of Ser²⁵⁸ almost abolishes the ability of MDM2 to be phosphorylated by CK2, suggesting the possibility that Ser²⁵⁸ may be an additional and possibly minor CK2 target. (2) Our data indicate that there is a single detectable Ser²⁶⁷-directed protein kinase activity present in cell extracts. The fractionation properties of this activity in ion-exchange chromatography, and its inhibition by DRB, are consistent with its identity being CK2 and strongly support the notion that CK2 is a physiological MDM2-directed protein kinase (Figures 3 and 4). We cannot rule out the possibility, however, that there may be other Ser²⁶⁷-directed protein kinase activities which are not detectable under our experimental conditions. For example, it is possible that such a protein kinase

could be present in an inactive state and might be activated only under certain conditions or in response to a given set of signals. (3) Our analyses of the phosphorylation of MDM2 using cell extracts as the source of protein kinase activity, combined with the phosphopeptide analyses, indicate that there are many MDM2-directed protein kinase activities which are detectable *in vitro*. Strikingly, the phosphopeptides generated from these *in vitro* experiments match very closely the number and migrational properties of phosphopeptides obtained following radiolabelling of MDM2 in cultured mammalian cells. The implication of this finding is that these protein kinase activities are likely to be physiologically relevant, and their detection in the cell extracts opens up a clear route for their analysis and identification. This is particularly encouraging, since we have been able to fractionate several of the activities by conventional methods (e.g., see Figure 3). (4) We show that Ser²⁶⁷ is a physiological site of phosphorylation in mammalian cells (Figure 5). We also confirm the presence of another physiological phosphorylation site(s) at Ser²⁵⁸ and/or Ser²⁶⁰ (C. Blattner, T. Hay, D. Meek and D. P. Lane, unpublished work), but we cannot yet show conclusively whether only one or both of these residues are physiologically modified. Interestingly, while Ser²⁵⁸ is only a weak CK2 target *in vitro*, it is possible that additional negative charge provided through potential phosphorylation of Ser²⁶⁰ could influence its modification by CK2. (5) We show that the Ser²⁶⁷ phosphorylation site does not appear to play a major role in the ability of MDM2 to direct the degradation of p53. As a control in this analysis, a S258/260A double mutant shows a striking loss of ability to target p53 for degradation, confirming the importance of these sites (C. Blattner, T. Hay, D. Meek and D. P. Lane, unpublished work) and underscoring the sensitivity of the assay in detecting changes in MDM2 function in a cellular context. One potential caveat with this approach is that, even although CK2 is a constitutively active protein kinase, we cannot yet show conclusively whether MDM2 is phosphorylated at Ser²⁶⁷ in the H1299 cells under our experimental conditions. While we show clearly using the COS-7 cells that Ser²⁶⁷ is phosphorylated in a cellular context, these cells are transformed by simian virus 40, which promotes the phosphorylation of p53 and may likewise affect the phosphorylation of MDM2. Moreover, owing to the nature of the expression system, COS-7 cells express high levels of the transfected MDM2, making effective phosphopeptide analysis possible. On the other hand, while the p53-null H1299 cells are ideal for carrying out the p53 degradation analyses, they express relatively much less MDM2, making it difficult to ascertain the MDM2 phosphorylation status by radiolabelling. Additionally, radiolabelling itself can also affect the status of the p53 protein, and it is therefore possible that the modification status of MDM2 could be affected in a similar way. For example, ³²P or ³⁵S labelling of normal human fibroblasts can induce a phosphorylation-dependent epitope concomitant with a p53-dependent G₁ arrest [44], clearly underscoring the possibility that the labelling conditions may deviate from a normal physiological situation. On the other hand, the fact that we detect Ser²⁶⁷ through the labelling procedure indicates within itself that this is a residue which is targeted within a cellular environment, albeit one which may possibly reflect a stress response. Clearly, the ability to resolve the issue of whether, and to what extent, Ser²⁶⁷ in MDM2 is phosphorylated under a given set of conditions and in a given cell type must await the development of a phospho-specific antibody specific for this site.

One final point concerns the relevance of the phosphorylation of MDM2 by CK2. p53 itself is phosphorylated at a single C-terminal site by CK2 (Ser³⁹² in human p53) [22,45], leading to

activation of the site-specific DNA-binding function of p53 [22], inhibition of the renaturation of cDNA strands mediated by p53 [46] and stabilization of tetramer formation [47]. CK2-dependent modification of p53 is therefore an important contributory event in the integrated control of this transcription factor. Recently, CK2 has been reported to be responsive to heat-shock [24] and other agents which activate the SAPK pathway [25]. Stress-induced complex formation between p38 α and CK2, and concomitant activation of CK2, may underlie the UV- and TNF α -responsiveness and p38 inhibitor-sensitivity of p53-Ser³⁹² phosphorylation [25–28]. It is therefore possible that MDM2 is also a target of this stress-induced pathway, and that p53 and MDM2 may be regulated in some sort of co-ordinated manner. Evidence suggests that MDM2 levels are decreased in response to UV radiation, most likely through reduced levels of mRNA expression [48], but it is also possible that an additional layer of regulation is present in the form of post-translation modification of existing protein. Once again, the development of a phospho-specific antibody to MDM2-Ser²⁶⁷ should permit these issues to be addressed and may reveal the circumstances under which modification of MDM2 through the CK2 pathway may be modulated and consequently have regulatory significance.

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