Protein Kinase CK1δ Phosphorylates Key Sites in the Acidic Domain of Murine Double-Minute Clone 2 Protein (MDM2) That Regulate p53 Turnover†

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ABSTRACT: Murine double-minute clone 2 protein (MDM2) is an E3 ubiquitin ligase that regulates the turnover of several cellular factors including the p53 tumor suppressor protein. As part of the mechanism of p53 induction in response to DNA damage, a cluster of serine residues within the central acidic domain of MDM2 become hypophosphorylated, leading to attenuation of MDM2-mediated p53 destruction. In the present study, we identify the protein kinase CK1δ as a major cellular activity that phosphorylates MDM2. Amino acid substitution, coupled with phosphopeptide analyses, indicates that several serine residues in the acidic domain, including Ser-240, Ser-242, and Ser-246, as well as Ser-383 in the C-terminal region, are phosphorylated by CK1δ in vitro. We also show, through expression of a dominant negative mutant of CK1δ or treatment of cells with IC261, a CK1δ-selective inhibitor, that MDM2 is phosphorylated by CK1δ in cultured cells. These data establish the identity of a key signaling molecule that promotes the phosphorylation of a major regulatory region in MDM2 under normal growth conditions.

The murine double-minute clone 2 (MDM2)[1] protein is a ubiquitin ligase that has been characterized mainly through its ability to promote the ubiquitylation and proteasome-dependent degradation of the p53 tumor suppressor protein (*1*), a highly labile transcription factor that is stabilized and accumulates in the nucleus following a variety of stresses including genotoxic stress and hyperproliferation (*2*). Induction of p53 involves mainly interruption of its ubiquitylation or degradation by MDM2 or both, leading to p53 accumulation. Activated p53 coordinates a change in the balance of gene expression leading to growth arrest or apoptosis, events that prevent the proliferation of damaged cells.

The MDM2 protein contains a number of functional elements including a p53 binding domain (N-terminal 102 amino acids), a C-terminal RING finger domain that mediates ubiquitin transfer, and a central acidic region critical for MDM2 function (*3*−*6*). This acidic region mediates interaction with a range of important regulatory proteins including p300, which may be required for p53 degradation potentially in the role of an E4 polyubiquitin ligase (*7*, *8*), and the MDM2 inhibitor protein, $p14^{ARF}$, which blocks p53 ubiquitylation and subsequent degradation (*6*).

We recently identified a cluster of phosphoserine residues within the acidic domain of MDM2 comprising serines 240, 242, 246, 253, 260, and 262. These phosphorylation sites, which are located close to the p300 and 14^{ARF} binding sites (*9*, *10*), are phosphorylated under normal (i.e., unstressed) conditions but become rapidly dephosphorylated in response to ionizing radiation (*9*). Alanine substitution of these residues (either individually or in pairs) gives rise to mutant MDM2 proteins that are still able to promote ubiquitylation of p53 but fail, to varying degrees, in mediating p53 degradation (*9*). These data are consistent with a model in which, under nonstressed conditions, MDM2-mediated p53 degradation requires phosphorylation of this group of serines, possibly through the action of a constitutively active protein kinase(s). Accordingly, stress-induced hypophosphorylation of one or more of these sites (either through inactivation of a protein kinase(s) or activation of a protein phosphatase) may be required for, or contribute toward, down-regulation of MDM2-mediated p53 degradation. However, the mechanism by which these phosphorylation events regulate p53 degradation is not known, nor have the signaling pathways that govern the phosphorylation and dephosphorylation of these residues been identified.

The protein kinase CK1 (formerly termed casein kinase 1) comprises a family of serine/threonine protein kinases that are ubiquitous in eukaryotic systems (for review, see ref *11*). The CK1δ isoform and the highly related CK1ε isoform are involved in regulating a variety of cellular events including transduction of the Wnt signaling pathway (*12*, *13*), regulation of circadian rhythms (*14*−*16*), the DNA damage response, and late cell cycle progression (*17*−*20*). CK1 isoforms are thought to be constitutively active, at least when

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[1] Abbreviations: MDM2, murine double-minute clone 2 protein; CK1, (formerly) casein kinase I.

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measured in vitro, and little is known about their physiological regulation.

Further understanding of the mechanisms that regulate the phosphorylation status of the acidic domain of MDM2 may be achieved through biochemical detection and purification of protein kinases that specifically phosphorylate established sites of physiological phosphorylation within this domain. Using this approach, we have identified the protein kinase CK1δ as a major cellular activity that phosphorylates several serine residues in the acidic domain in vitro and in cultured cells. These data are consistent with the idea that CK1δ is a key regulator of MDM2 under normal growth conditions.

EXPERIMENTAL PROCEDURES

Plasmids. The plasmids pDWM659 (encoding full-length murine MDM2 tagged at the N-terminus with the Myc 9E10 epitope), pDWM470 (encoding human CK1δ tagged at the N-terminus with the FLAG epitope), pDWM864 (plasmid pDWM470 encoding a K38M mutant CK1δ), and pDWM984 (plasmid pDWM470 encoding a T176I mutant CK1δ) have been described previously (*9*, *21*). Glutathione S-transferase (GST)-MDM2 fusion proteins were constructed by amplifying MDM2 by PCR using wild-type or mutant human MDM2 as a template. The sequence of primers is available on request. The PCR fragments were cloned in the pCR-BluntII-TOPO vector, digested with *Eco*RI and *Not*I, and cloned in frame in the pGEX-4T-2 vector. The GST−MDM2 fusion protein comprising amino acids 196−282 of MDM2 has been described elsewhere (*22*).

Cell Lines and Their Treatments. COS-7, MCF-7, HeLa, and OSA (SJSA-1) cells (*23*) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and 100 units/mL penicillin/streptomycin at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were transiently transfected using lipofectamine (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation. Cells were washed twice with ice-cold PBS and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris (pH 8), 5 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT) plus 25 mM NaF and 25 mM β-glycerophosphate. For immunoprecipitation, 1−2 μg of the appropriate antibody precoupled to protein A−Sepharose (Amersham) was mixed with up to 300 μg of the lysate and rotated at 4 °C for 2 h. The immune complexes were washed three times with NP-40 lysis buffer and subjected to kinase assays and two-dimensional peptide mapping.

Western Blotting and Antibodies. Cell extracts were subjected to SDS−PAGE and Western blotting using standard conditions. Detection of MDM2 and proliferating cell nuclear antigen (PCNA) was performed as described (*24*). Detection of the α, γ, δ, and ϵ isoforms of CK1 was carried out using the antibodies sc-6477, sc-6476, sc-6474, and sc-6472 (Santa Cruz Biotechnology), respectively. Alternatively, the δ isoform of CK1 was detected using the monoclonal antibody, 128A (*19*). Horseradish peroxidase-coupled goat anti-mouse and rabbit anti-goat IgG (DAKO) were used as secondary antibodies.

In Vitro Kinase Assays, Radiolabeling of Cells, and Two-Dimensional Peptide Mapping. GST−MDM2 was expressed in *Escherichia coli* and purified according to the instructions of the manufacturer of the GST-vector (Amersham). CKIδ in vitro kinase assays were performed according to the manufacturer's instructions for recombinant CKIδ (New England Biolabs). After incubation, the reactions were subjected to SDS−PAGE, and phosphorylated proteins were detected by autoradiography. Quantitation was carried out by scintillation counting following excision of the appropriate bands from the dried gels.

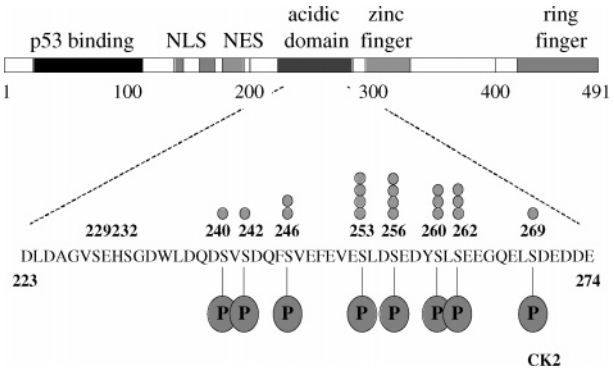

FIGURE 1: Schematic representation of MDM2. The upper section shows the salient features of MDM2, and the lower section shows the amino acid sequence of the acidic domain. The serine residues within this region are indicated. Those phosphorylated in vivo under normal unstressed conditions are highlighted by the letter P encompassed within an ellipse. Phosphoserine residues that can promote the degradation of p53 are indicated by gray circles: the strength of their influence on p53 degradation is indicated by the number of circles (1 = weak, 4 = strong). Serine 269 (serine 267 in murine MDM2) is an established target for phosphorylation by the protein kinase CK2.

Pulse labeling of COS-7 and OSA cells with ^{32}P-orthophosphate (5 mCi per 10 cm dish), gel elution, and phosphopeptide mapping have been described elsewhere (*9*, *25*).

Fractionation of Cell Extracts. Five 15 cm plates of normally growing MCF-7 or HeLa cells at 70% confluence were washed three times in ice-cold phosphate-buffered saline and lysed in 1 mL per plate of 20 mM Tris (pH 7.5), 0.27 M sucrose, 1 mM EDTA, 1% Triton X-100, 10 mM sodium β-glycerophosphate, 50 mM NaF, 1 mM benzamidine, 4 μg/mL leupeptin, and 0.1% β-mercaptoethanol. The extract was passed through a 0.22 μm filter and loaded onto a 5 mL HiTrap Q column (Amersham Pharmacia Biotech). The proteins were eluted with a linear gradient of sodium chloride in 50 mM Tris (pH 7.5), 1 mM EDTA, 5% glycerol, 0.03% Brij, 1 mM benzamidine, 4 μg/mL leupeptin, and 0.1% β-mercaptoethanol. Two microliter aliquots of the fractions were screened for their ability to phosphorylate casein and the GST−MDM2 fusion protein comprising amino acids 196−282 of human MDM2.

RESULTS

MDM2 Is Phosphorylated by Protein Kinase CK1δ in Cell Extracts and by Purified Recombinant CK1δ. The acidic domain of MDM2 contains a cluster of phosphorylation sites each of which contributes, to varying degrees, to the ability of MDM2 to mediate the degradation of the p53 protein under normal unstressed conditions (*9*), Figure 1. To identify protein kinase activities that phosphorylate this region of MDM2, extracts were prepared from unstressed MCF-7 cells and fractionated by ion exchange chromatography on a HiTrap Q column. Protein kinase activity was determined

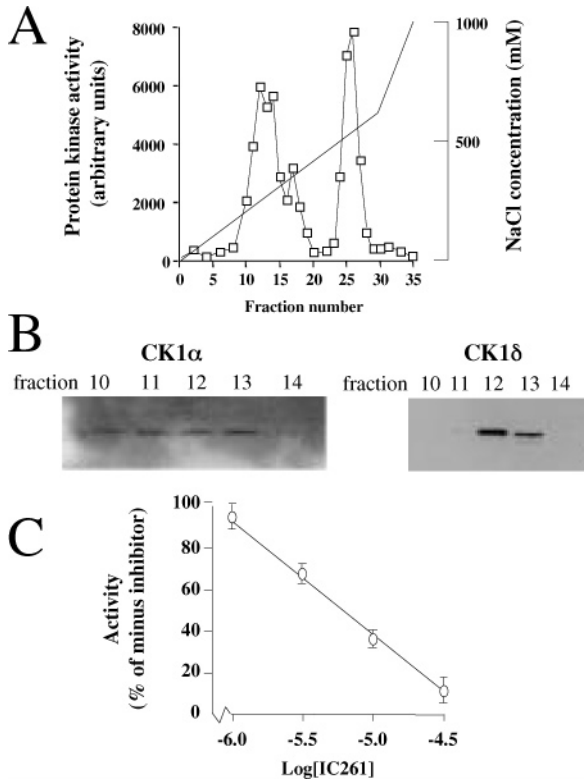

FIGURE 2: Fractionation of MDM2 (acidic domain) protein kinase activities from MCF-7 cell extracts by HiTrap Q-based ion exchange chromatography. In panel A, normally growing MCF-7 cells were harvested at 70% confluence and fractionated by ion exchange chromatography on a 5 mL HiTrap Q column (Amersham) as described in Experimental Procedures. Fractions were assayed for their ability to phosphorylate a GST−MDM2 fusion protein comprising amino acids 196−282 of human MDM2. In panel B, the presence of the α and δ isoforms of protein kinase CK1 in the fractions was determined by Western blotting using the antibodies sc-6477 (Santa Cruz Biotechnology) and 128A, respectively. In panel C, MDM2 (aa 196−282) protein kinase activity in fraction 12 was assayed in the presence of increasing concentrations of the CK1-selective inhibitor, IC261.

using a GST−MDM2 fusion protein containing only the central acidic domain of MDM2 (amino acids 196−282 of human MDM2). Two major peaks of activity were observed: a broad peak comprising possibly three overlapping activities eluting in the range of 200−300 mM NaCl and a sharp peak eluting at 500 mM NaCl (Figure 2A). The activity eluting at 500 mM NaCl was confirmed as protein kinase CK2 and was shown to specifically phosphorylate Ser-269 of human MDM2 (data not shown), confirming previously published data (*26*, *27*). Protein kinase CK1 was detected in fractions 10−14 both by measuring casein kinase activity (data not shown) and by Western analysis of the fractions in which the α and δ isoforms, but not the γ or ϵ isoforms, could be detected (Figure 2B). The δ isoform eluted as a particularly sharp peak in fractions 12 and 13. To determine whether the MDM2 kinase activity in these fractions was indeed CK1, MDM2 protein kinase activity in fraction 12 was assayed in the presence of increasing amounts of IC261, a CK1-selective inhibitor that has a 10- to 20-fold greater potency toward the δ and ϵ isoforms as compared with the other CK1 isoforms (*28*). Inhibition of MDM2 phosphorylation at low micromolar concentrations of IC261 confirmed that the MDM2 kinase activity in this fraction was indeed CK1δ. The half-maximal activity achieved at 5 μM IC261

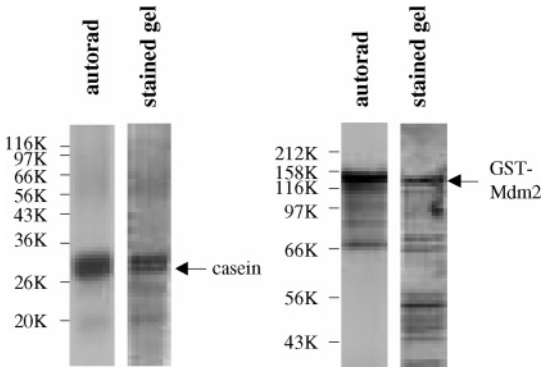

FIGURE 3: Recombinant CK1δ phosphorylates MDM2 in vitro. Purified casein (1 μg) or GST−full-length MDM2 (1 μg) was phosphorylated in the presence of 100 ng of recombinant CK1δ (New England Biolabs) and [γ-^{32}P]ATP as described in Experimental Procedures.

is consistent with CK1δ being the major activity since IC261 inhibits CK1δ potently in the low micromolar range (*28*). However, this does not rule out a contribution by the α isoform, particularly since (a) a significant degree of inhibition of CK1α would be expected at the higher concentrations of IC261 used in the titration curve (Figure 2C) and (b) the different CK1 isoforms recognize similar consensus motifs for phosphorylation and share many substrates (*11*). A similar set of data were obtained when HeLa cell extracts were examined (data not shown).

Incubation of full-length MDM2, as a GST-fusion protein, in the presence of recombinant CK1δ (NEB) and [γ-^{32}P]-ATP, confirmed that MDM2 is a substrate for CK1δ in vitro (Figure 3). GST alone was not phosphorylated by CK1δ (data not shown); similarly, no incorporation of ^{32}P was observed in the absence of the protein kinase (data not shown). The phosphorylation of GST−full-length MDM2 compared well under identical conditions with the phosphorylation of casein (Figure 3). Comparative kinetic analysis of these two substrates (in which the initial rates of phosphorylation were determined over a range of concentrations of GST−full-length MDM2 or casein) indicated that the V_{max} observed with GST−full-length MDM2 as substrate was 2-fold less than that observed with casein as substrate, whereas the K_m for MDM2 was approximately 1.5 μM as compared with 20 μM for casein.

MDM2 Is Phosphorylated by Recombinant CK1δ on Several Residues within the Acidic Domain, Including Ser-240, Ser-242, and Ser-246. To localize the site(s) of CK1δ-dependent phosphorylation of MDM2, a series of GST−MDM2 fusion proteins comprising amino acids 1−206, 216−305, and 296−491 were generated. When these proteins were used as substrates for phosphorylation by CK1δ in vitro, phosphorylation occurred overwhelmingly within the central region of MDM2 (within amino acids 216−305), although some phosphorylation of the C-terminal region (amino acids 296−491) could also be detected (Figure 4A,B). There was no detectable phosphorylation of amino acids 1−206.

Further analysis of the phosphorylation of the acidic domain, using GST fusion proteins comprising amino acids 220−252, 248−268, and 263−305, revealed that the targets of CK1δ were located within amino acids 220−263, principally within aa 220−252 but with some residual phosphorylation between aa 248 and 263 (Figure 4C,D).

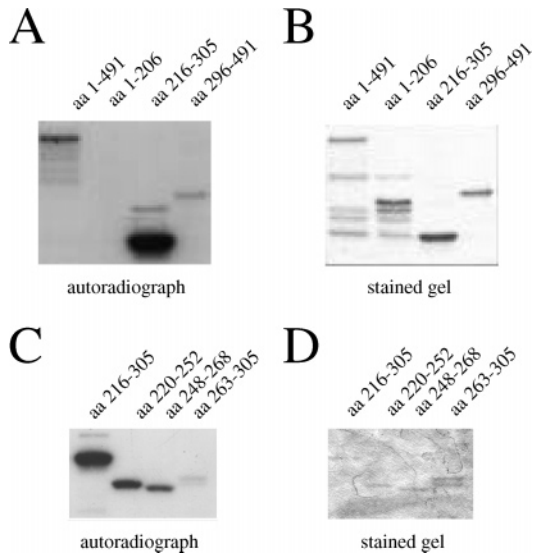

FIGURE 4: CK1δ phosphorylates the central acidic and the carboxyl terminal domains of MDM2. In panels A and B, GST-fusion proteins comprising full-length MDM2, the amino terminal domain of MDM2 (aa 1−206), the central domain of MDM2 (aa 216−305), or the carboxyl terminal domain of MDM2 (aa 296−491) were phosphorylated by recombinant CKIδ in vitro. In panels C and D, GST-fusion proteins comprising the acidic domain of MDM2 (aa 216−305) and fragment 1 (aa 220−252), fragment 2 (aa248−268), and fragment 3 (aa 263−305) of the central domain of MDM2 were phosphorylated by CK1δ in vitro under the same conditions as the experiment in panels A and B.

Given that members of the CK1 family normally phosphorylate serine or threonine residues flanked by acidic residues, these data are consistent with the occurrence of phosphorylation within the highly acidic region encompassing amino acids 237−274.When a GST−MDM2 fusion protein comprising amino acids 196−282 of MDM2 was used as substrate in a time course reaction, the data indicated that approaching 3 mol of phosphate were incorporated per mole of protein indicating that at least three sites within this region of MDM2 are phosphorylated in vitro by CK1δ (Figure 5A). Partial inhibition of the phosphorylation reaction by 5 μM IC261 confirmed that the recombinant enzyme catalyzing these modifications was indeed CK1. Similarly, when the GST−full-length MDM2 protein was used as substrate, 2−3 mol of phosphate could be incorporated by recombinant CK1δ.

To check the stoichiometry of phosphorylation by an alternative approach, a glycine to lysine substitution was made (in the GST−MDM2 (aa 196−282) protein) at residue 233, upstream of the acidic domain. The presence of this lysine (in a region that normally lacks any basic residues) permitted trypsin cleavage giving rise to a single phosphopeptide, amino acids 234−282, encompassing the core acidic domain. When separated in two dimensions by electrophoresis and chromatography, the consecutive attachment of additional phosphate groups to this peptide will increase the negative charge on the peptide (thereby shifting its electrophoretic migration further toward the anode [horizontal direction] and decreasing its migration with the organic solvent front during chromatography [vertical direction]). These modifications would therefore be expected to give rise to a series of phosphopeptide species of incrementally increasing charge that lie, equally spaced, on a diagonal following two-dimensional separation on thin layer plates.

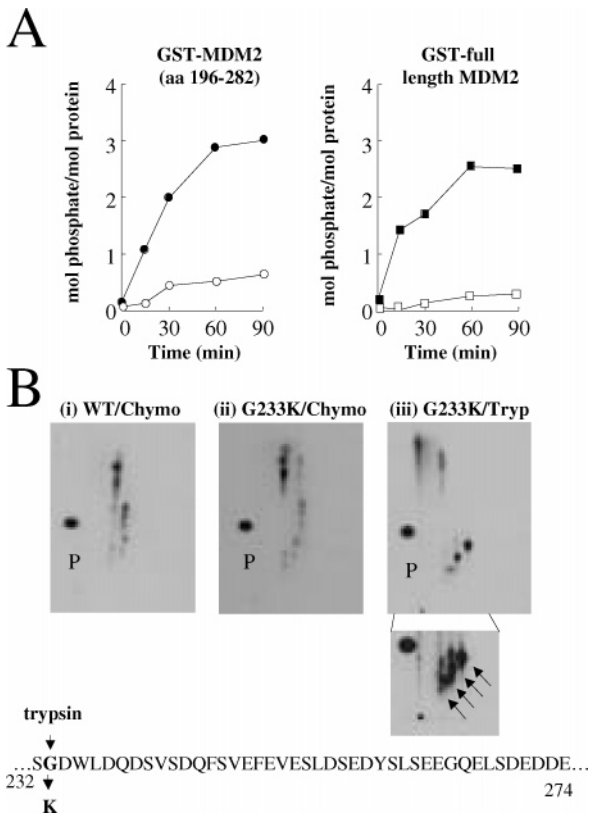

FIGURE 5: CK1δ phosphorylates up to three residues in the acidic domain of MDM2. Panel A presents a time course of the phosphorylation of a GST−MDM2 fusion protein comprising amino acids 196−282 (left panel) or GST−full-length MDM2 (right panel). Aliquots of the fusion proteins were phosphorylated in vitro by recombinant CK1δ for periods of up to 90 min. The phosphorylation reactions were carried out in the presence (○,□) and absence (●,■) of the CK1 inhibitor, IC261. In panel B, a GST−MDM2 fusion protein comprising amino acids 196−282 or a mutant of this protein in which Gly-233 of MDM2 was substituted with lysine, thereby generating a trypsin digestion site, was phosphorylated in vitro by recombinant CK1δ. The radiolabeled proteins were digested with chymotrypsin (panels i and ii) or trypsin (panel iii) and analyzed by two-dimensional phosphopeptide mapping. The letter P shows the position to which free phosphate migrates. The enlarged section under panel iii shows a longer exposure of the region to which the major phosphopeptides migrate (i.e., the bottom left-hand side of panel iii). The arrows indicate multiple phosphopeptides in diagonal alignment. The location of the G233K mutation is indicated at the bottom of the panel.

When this approach was adopted, phosphopeptide maps generated by chymotrypsin cleavage of phosphorylated wild-type or G233K mutant fusion proteins were identical (Figure 5B, compare panels i and ii), indicating that phosphorylation of the acidic domain by CK1δ was unaffected by the lysine substitution. (Chymotryptic digestion, incidentally, gave rise to multiple phosphopeptides consistent with multisite phosphorylation of this region; Figure 5B, panel i.) Strikingly, however, trypsin digestion of the G233K mutant gave rise to a series of up to four (three major and one very minor) phosphopeptides that adopted the predicted diagonal migration (Figure 5B, panel iii). These phosphopeptides were not observed when the wild-type fusion protein was subjected to trypsin digestion (data not shown). The phosphopeptide data are therefore consistent with the conclusion that CK1δ can phosphorylate at least three different sites within the central domain of MDM2.

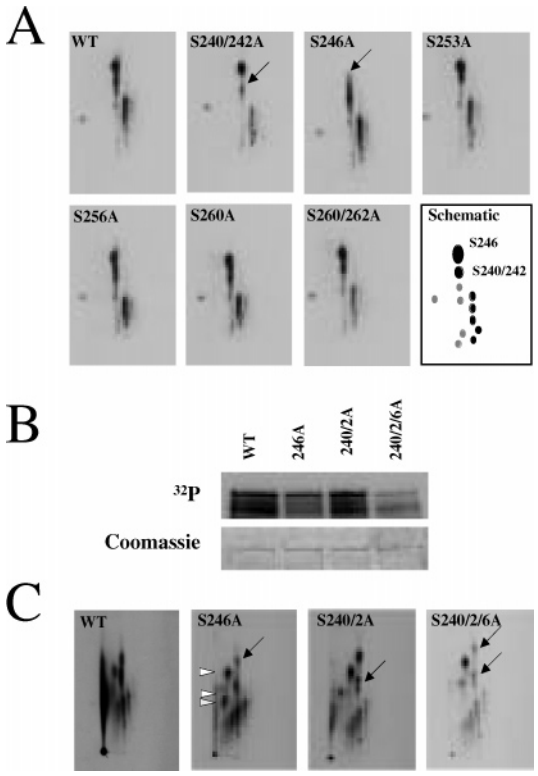

FIGURE 6: CK1δ phosphorylates serines 240, 242, and 246 in the acidic domain of MDM2. In panel A, GST-fusion proteins comprising amino acids 196−282 of wild-type MDM2 or of MDM2 with the indicated mutations were phosphorylated in vitro by recombinant CKIδ. The radiolabeled proteins were digested with chymotrypsin and analyzed by two-dimensional phosphopeptide mapping. The identities assigned to the major phosphopeptides are indicated schematically. In panel B, GST−full-length MDM2, wild-type or with the indicated mutations, was phosphorylated in vitro by CK1δ. In panel C, the radiolabeled proteins in panel B were subjected to two-dimensional chymotryptic phosphopeptide mapping.

To identify the sites of phosphorylation in this region, a series of single and double serine to alanine substitutions were made at residues 229/232, 240/242, 246, 253, 256, 260, and 260/262 within the context of the GST−MDM2 fusion protein comprising MDM2 amino acids 196−282. These were phosphorylated in vitro by CK1δ and analyzed by chymotryptic phosphopeptide mapping. As previously observed in Figure 5B (panels i and ii), chymotryptic digestion gave rise to a major phosphopeptide accompanied by two groups of phosphopeptides displaying less intense signals (Figure 6A, WT). The loss of the major phosphopeptide in the S246A mutant was consistent with Ser-246 being a major site of CK1δ phosphorylation. The loss of a second phosphopeptide in the S240/242A mutant suggested that either or both of these residues could be phosphorylated by CK1δ. None of the other mutants showed any major differences in their phosphopeptide patterns, even though a low level of phosphorylation had been observed within residues 248−268 (Figure 4C). However, CK1δ may be able to catalyze the incorporation of low levels of phosphate into several of the other serine residues in this region, making it very difficult to rigorously identify individual modifications using the phosphopeptide mapping approach. Additionally, serines 229 and 232, and tyrosine 259 were not observed to be targets for phosphorylation by CK1δ (data not shown).

To determine whether the GST−full-length MDM2 protein was phosphorylated at the same sites as the small fusion proteins, GST−full-length MDM2 and mutant derivatives were compared as substrates for phosphorylation by CK1δ in vitro and subsequently analyzed by two-dimensional chymotryptic phosphopeptide mapping. The data from the phosphorylation reactions (Figure 6B) confirmed that full-length MDM2 was phosphorylated by CK1δ, but there was no detectable difference in the phosphorylation of the S240/242A mutant. There was a small but significant reduction in the phosphorylation of a S246A mutant confirming that Ser-246 is an in vitro target of CK1δ. When the S240/242/246A mutant was examined, the level of phosphorylation was significantly lower than that with the S246A mutation alone suggesting that some phosphorylation of Ser-240, Ser-242, or both was indeed occurring. Significantly, however, loss of these three residues only accounted for about 50% of the radioactivity incorporated into MDM2, indicating that, in the context of the full-length protein, there were additional sites of CK1δ-dependent modification. Phosphopeptide analysis of the phosphorylated full-length MDM2 (Figure 6C) revealed phosphopeptides similar to those observed in the phosphorylated GST−MDM2(196−282) protein (indicated by black arrows, compare with Figure 6A) and highlighted the presence of additional phosphopeptides (indiated by white arrowheads). Analysis of the mutant MDM2 proteins showed a reduction (but not complete loss) of major phosphopeptides containing serines 240, 242, and 246 (Figure 4C) consistent with these residues being phosphorylated by CK1δ in the full-length protein. Clearly, however, the remaining presence of a significant number of phosphopeptides suggests that CK1δ is likely also to phosphorylate other sites in the full-length MDM2 protein.

The phosphorylation of the C-terminus of MDM2 was also analyzed in further detail. A series of GST−MDM2 fusion protein substrates comprising overlapping regions of MDM2 from amino acids 296−491 were generated for this purpose (Figure 7A). Phosphorylation of these proteins (Figure 7B) indicated that the phosphorylation site(s) lies within amino acids 374−402, a regulatory region that encompasses sites of phosphorylation for ATM and c-Abl (*29*, *30*). Analysis of mutant proteins in which the serine residues in this region were substituted, individually or in groups, with alanine suggested that Ser-383 is the predominant site of CK1δ-mediated phosphorylation in this region (Figure 7B).

MDM2 Is Phosphorylated by CK1δ in Cultured Cells. Analysis to determine whether MDM2 is phosphorylated by CK1δ in a cellular context was carried out on the basis that treatment of cells with the CK1δ inhibitor, IC261, or overexpression of a catalytically inactive, dominant-negative CK1δ mutant should prevent phosphorylation of the CK1δ-targeted sites in MDM2 in vivo. Two experimental approaches were used to examine the effects of these inhibitors. The first approach was carried out on the principle that, if MDM2 is un- or underphosphorylated in the cell, it should be possible to immunoprecipitate this unphosphorylated (or poorly phosphorylated) MDM2 from cell extracts and show that it can be phosphorylated by recombinant CK1δ in vitro (in the presence of [γ-^{32}P]ATP). Conversely, if the phosphorylation sites are modified in the cells prior to immunoprecipitation of the MDM2 (e.g., in the absence of any inhibitor), it should not be possible to incorporate any further

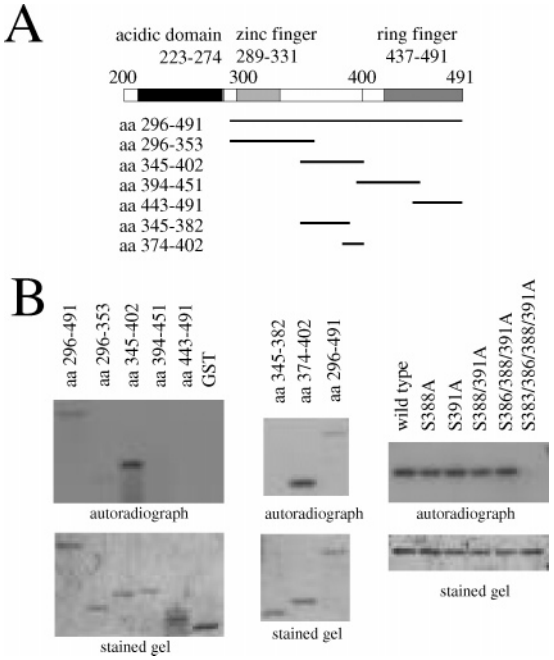

FIGURE 7: The carboxyl terminal CK1δ phosphorylation is located between aa 374 and aa 402 of the MDM2 protein. In panel A, GST-fusion proteins comprising the carboxyl terminal domain of MDM2 or fragments from within this domain (fragment 4, aa 296−353; fragment 5, aa 345−402; fragment 6, aa 394−451; fragment 7, aa 443−491; fragment 8, aa 345−382; fragment 9, aa 374−402) are shown schematically. In panel B, The GST−MDM2 substrates shown in panel A, together with a series of serine to alanine mutants in the GST−MDM2(374−402) protein, were phosphorylated by CK1δ in vitro.

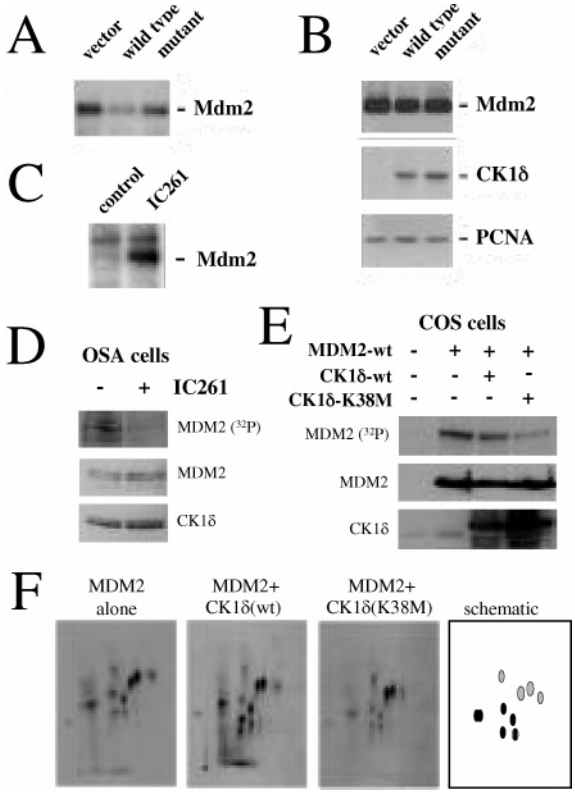

FIGURE 8: CKIδ phosphorylates MDM2 in vivo. In panels A and B, COS-7 cells were transiently transfected with a cDNA expressing myc-tagged MDM2 together with wild-type CKIδ, an inactive mutant (K38M), or an empty vector. Forty hours after transfection, cells were lysed and aliquots of the lysate were examined for the expression of myc-tagged MDM2 (antibody 9E10) and CKIδ (antibody sc-6474, Santa Cruz) by Western blotting (panel B). Myc-tagged MDM2 protein was precipitated from the remaining lysates using the 9E10 (anti-myc) antibody. The MDM2 antibody complexes were subsequently phosphorylated in vitro by recombinant CKIδ and detected by autoradiography (panel A); the principle of this assay is that phosphorylation in vivo inhibits the subsequent in vitro phosphorylation by CK1δ. In panel C, COS-7 cells were transiently transfected with a cDNA expressing myc-tagged MDM2. Forty hours after transfection, cells were treated with 10 μM IC261 for 1 h or left untreated for control. Immunoprecipitation of MDM2 and phosphorylation by CK1δ was carried out as described for panel A. Phosphorylated proteins were detected by autoradiography. In panel D, OSA cells were radiolabeled with ^{32}P-orthophosphate and mock treated or treated with 30 μM IC261 for 30 min. Following lysis, MDM2 was immunoprecipitated using monoclonal antibodies D12 and SMP14 and detected by SDS−PAGE and autoradiography (upper panel). The levels of MDM2 (middle panel) and CK1δ (lower panel) were determined by Western blotting using antibodies D12 and SMP14 for MDM2 and 128A for CK1δ. In panel E, COS-7 cells were transfected with plasmids expressing wild-type MDM2, wild-type CK1δ, or the K38M CK1δ mutant. Radiolabeling and detection was carried out as described in the legend to panel D. In panel F, the phosphorylated proteins from panel E were subjected to two-dimensional chymotryptic phosphopeptide analysis. The phosphopeptides were detected by autoradiography. In the schematic representation (4th panel), the black spots represent the cluster of phosphopeptides influenced by the expression of wild-type or mutant CK1δ.

phosphate (or very little phosphate) into these sites in vitro. When MDM2 was overexpressed and immunoprecipiated from COS-7 cell extracts, it was possible to phosphorylate the immunoprecipitated protein in vitro using recombinant CK1δ suggesting that in a proportion of the MDM2 molecules, the phosphorylation sites for CK1δ were not already modified (Figure 8A). However, when catalytically active CK1δ was coexpressed with MDM2, significantly less phosphorylation of the immunoprecipitated MDM2 was observed, consistent with a higher degree of phosphorylation of MDM2 in the cells overexpressing the kinase. In contrast, coexpression of MDM2 with a catalytically inactive mutant of CK1δ (K38M) did not significantly reduce the ability of CK1δ to phosphorylate the MDM2 in vitro. The active and inactive forms of the protein kinase were expressed to equal levels and neither had any direct effect on the levels of MDM2 expressed (Figure 8B). In support of these findings, a significant degree of phosphorylation of immunoprecipitated MDM2 could be achieved in vitro when the cells had been treated with 10 μM IC261 prior to harvesting (Figure 8C), consistent with the idea that inhibition of CK1δ in the cells resulted in inhibition of MDM2 phosphorylation.

As an alternative approach to investigating CK1δ-dependent phosphorylation of MDM2 in cultured cells, OSA cells (which express high levels of MDM2) were metabolically labeled with ^{32}P-orthophosphate. Immunoprecipitation of the radiolabeled MDM2 from cell extracts confirmed that MDM2 is phosphorylated in a cellular context. Treatment of the cells with the CK1δ-selective inhibitor, IC261, led to a significant decrease in the level of ^{32}P incorporation (Figure 8D). Treatment with this inhibitor did not affect the levels of endogenous MDM2 or CK1δ (Figure 8D). These data support the idea that endogenous MDM2 is phosphorylated by endogenous CK1δ. However, it was not possible to recover sufficient radioactive material from the radiolabeled MDM2 to carry out phosphopeptide analysis. To examine the cellular phosphorylation of MDM2 in further detail, COS-7 cells were transfected with plasmids expressing wild-

type MDM2 in the presence or absence of wild-type CK1δ or the inactive K38M mutant of CK1δ. ^{32}P-Orthophosphate labeling and immunoprecipitation indicated that coexpression of the K38M mutant led to a signficant decrease in the phosphorylation of MDM2 (Figure 8E). No effect of overexpression of active protein kinase was discernible at this stage in the procedure (Figure 8E). In contrast, however, subsequent phosphopeptide analysis revealed that a group of MDM2-derived phosphopeptides was enhanced when the MDM2 was coexpressed with active CK1δ (Figure 8F compare the first and second panels). The black dots in the schematic panel show the positions to which this group of phosphopeptides migrate; these show remarkable similarity to the group of phosphopeptides that we previously identified as occurring within the acidic domain (*10*). Strikingly, the intensity of this group of phosphopeptides was diminished in MDM2 that had been coexpressed with the K38M mutant of CK1δ (Figure 8F third panel). Taken together, these data provide compelling evidence that MDM2 is phosphorylated by CK1δ in a physiological context.

DISCUSSION

The MDM2 protein plays a pivotal role as the master regulator of the p53 tumor suppressor protein and is a focal point for many cellular signals including those initiated by DNA damage, survival factors, hyperproliferation, hypoxia, and the interruption of ribosomal biogenesis (*1, 2*). Many of these signals act through, or are influenced by, protein phosphorylation events that impinge directly on the MDM2 protein (*31*). As part of the multisite nature of MDM2 modification, the acidic domain of MDM2 contains a cluster of serine residues that are phosphorylated under normal, unstressed conditions. Individually, these modifications contribute, to varying degrees, to the ability of MDM2 to mediate the turnover of p53 (*9*). However, the mechanism of this regulation is not understood, nor is it yet known whether phosphorylation can influence interactions with other partner proteins. Moreover, very little is known about the signaling enzymes that normally phosphorylate the acidic domain, nor the chain of events that lead to their dephosphorylation in response to DNA damage.

In the present study, we have taken a biochemical approach toward exploring the signaling pathways that focus on the acidic domain. Fractionation of cell extracts indicates that a number of activities that phosphorylate sites in the region of amino acids 196−282 can be detected and resolved. One of these is the protein kinase CK1, which comprises a family of up to 14 isoforms (including splice variants) in mammalian cells (*11*). Our data strongly favor the idea that MDM2 is a target of the δ isoform of CK1 since (a) CK1δ coelutes with a peak of MDM2 kinase activity (Figure 1A), (b) the high degree of sensitivity of inhibition by IC261 is in keeping with phosphorylation by CK1δ (Figure 1C (*28*)), and (c) MDM2 can be phosphorylated very efficiently by recombinant CK1δ. Our data also support the idea that MDM2 and CK1δ interact physiologically on the basis that (a) the CK1δ-selective inhibitor, IC261, inhibits phosphorylation of endogenous MDM2 in OSA cells, (b) overexpression of wild-type CK1δ in COS cells stimulates the phosphorylation of MDM2, and (c) overexpression of a catalytically inactive mutant of CK1δ or treatment of cells with IC261 reduces or prevents the phosphorylation of CK1δ-targeted sites on MDM2 in cells (Figure 8). Attempts to explore further the phosphorylation of the acidic domain and define precisely the residues that are modified by CK1δ in vivo have been limited by the difficulty of generating appropriate phospho-specific antibodies, particularly those directed against the Ser-246 site. We continue to pursue the development of these reagents.

Time course analyses show that at least three residues within the acidic domain can be phosphorylated by CK1δ in vitro (Figure 5). Mutational analysis of the short GST−MDM2 fusion proteins indicates that serines 240, 242, and 246 are major sites for phosphorylation by CK1δ in vitro, as is Ser-383 in the C-terminus. Analysis of full-length MDM2 supports the idea that serines 240, 242, and 246 are CK1δ targets and suggests that additional residues, most likely from within the acidic domain (as judged by the migrational properties of the additional phosphopeptides), are also phosphorylated by this enzyme. The minimal consensus sequence for phosphorylation by members of the CK1 family comprises an acidic residue, or preferentially a phosphorylated residue, located three residues on the N-terminal side of the serine or threonine phospho-acceptor residue (i.e., S(P)/T(P)/D/E-X-X-S/T) (*32*−*34*). Serines 240, 242, 246, and 383 each have aspartate residues at the $n - 3$ position and therefore conform to this minimal consensus. Physiologically phosphorylated CK1 substrates often have additional or multiple acidic residues on the N-terminal flank of the phosphoacceptor site(s). This also holds true for serines 240, 242, 246, and 383. In addition to these minimal consensus motifs, two other important recognition elements have been described that favor interaction with and phosphorylation by CK1 (*35, 36*). Pinna's and Allende's groups have provided evidence that a configuration comprising SLS (where the first serine is the target for phosphorylation), followed two to five residues on the C-terminal side by a cluster of acidic residues, is an important CK1 recognition sequence in several physiological substrates, especially those that lack a canonical negatively charged residue at the $n - 3$ position (*35*). Strikingly, such a motif is present in the acidic domain of MDM2 and encompasses residues 260−274 (see Figure 1). Others have identified the motif, F-X-X-X-F, as a potential CK1 docking site (*36*). Once again this is represented within the acidic domain of MDM2, located within residues 245−249. The presence of these motifs therefore suggests that there may be atypical phosphoacceptor sites in MDM2 for CK1 or that these docking elements may enhance interaction of CK1 with MDM2 and is consistent with the idea that MDM2 is phosphorylated by CK1δ physiologically. One challenge will now be to determine precisely which of this group of regulatory phosphorylation sites within the acidic domain are indeed bona fide targets for CK1δ in vivo.

Another challenge will be to determine the mechanism by which hypophosphorylation of these sites occurs in response to DNA damage (*9*). One possibility is that the interaction of CK1δ with MDM2 may be curtailed following a stress signal, for example, through changes in the composition of MDM2 or CK1δ-containing complexes. Alternatively, dephosphorylation of the acidic domain may occur through activation or recruitment of a DNA damage-inducible phosphatase. The response to ionizing radiation is very rapid, and one would expect the induction or activiation of such a

phosphatase to precede p53 induction. There is certainly a precedent for this in the p53 pathway as Ser-376 of p53 itself becomes dephosphorylated in response to DNA damage through the action of an as yet uncharacterized ATM-dependent phosphatase (*37*). It is entirely plausible that MDM2 may be dephosphorylated by the same phosphatase, but this remains to be tested. The identification of protein kinases that phosphorylate the acidic domain of MDM2 and the consequent ability to generate high levels of phosphorylated MDM2 in vitro will provide a valuable tool with which to pursue the identity of the acidic domain phosphatase(s).

Previously, we showed that mutants of MDM2 in which Ser-240, Ser-242, or Ser-246 were substituted by alanine had partially lost their ability to mediate the degradation of p53 in vivo (*9*). This effect was, however, appreciably less than with mutants of neighboring phosphorylation sites (namely, S253/256A) suggesting that Ser-240, 242, or 246 phosphorylation may play more peripheral or auxiliary roles in this function as compared with a more direct influence by Ser-253 and Ser-256. Nevertheless, it is surprising that we have not been able to observe any significant influence of blocking CK1δ activity on p53 induction, using either IC261 or RNAi (data not shown), especially since the breadth of CK1-dependent phosphorylation may extend to other residues in this region. There are several possible reasons for this: First, overexpression of the CK1δ dominant negative mutant or treatment of cells with the inhibitor IC261 may not inhibit MDM2 phosphorylation completely, and consequently, any proportion of MDM2 molecules retaining phosphorylated Ser-240, 242, or 246 may provide sufficient activity to maintain efficient p53 turnover; this would not be the case for the S246A mutant, which cannot be phosphorylated at this site (*9*). However, without the availability of a set of suitable phosphospecific antibodies, it is difficult to determine precisely the effectiveness of blocking phosphorylation of these sites by these treatments. Second, while our data strongly favor the idea that MDM2 is phosphorylated by CK1δ physiologically, we cannot rule out the possibility that key serine residues may also be phosphorylated by other protein kinases, operating through converging signaling pathways, that could maintain a low but sufficient level of phosphorylation to promote p53 degradation. Finally, CK1δ is likely to have a multitude of substrates in the cell (including the p53 protein itself (*18*−*20*)). Blocking or significantly reducing cellular CK1δ activity is therefore likely to have direct and indirect effects on a range of different pathways or cellular events, possibly in a manner that inhibits or diminishes p53 induction. Such pleiotropic events are entirely plausible: for example, our attempts to determine whether inhibition of CK1δ can influence E2F-1 turnover have highlighted a direct and MDM2-independent involvement of CK1δ in regulating E2F-1 levels (unpublished data). The challenge will therefore be to develop a means of blocking the MDM2/CK1δ interaction in a manner that is highly specific and does not influence significantly the ability of CK1δ to phosphorylate its other cellular targets. Attempts to develop such a system are currently underway.

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