Multiple sites of in vivo phosphorylation in the MDM2 oncoprotein cluster within two important functional domains

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Abstract The MDM2 oncoprotein is a negative regulatory partner of the p53 tumour suppressor. MDM2 mediates ubiquitination of p53 and targets the protein to the cytoplasm for 26S proteosome-dependent degradation. In this paper, we show that MDM2 is modified in cultured cells by multisite phosphorylation. Deletion analysis of MDM2 indicated that the sites of modification fall into two clusters which map respectively within the N-terminal region encompassing the p53 binding domain and nuclear export sequence, and the central acidic domain that mediates $p14^{ARF}$ binding, p53 ubiquitination and cytoplasmic shuttling. The data are consistent with potential regulation of MDM2 function by multisite phosphorylation.

Key words: MDM2; Phosphorylation; p53; Deletion analysis

1. Introduction

p53 is a latent and highly labile transcription factor which is activated by, and is an integration point for, a wide range of cellular stresses including DNA damage, activated oncogenes and hypoxia (for reviews, see [1–6]). In response to an inducing signal, p53 accumulates in the nucleus and coordinates 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 directly activated by p53, many of which promote growth arrest or apoptosis [7].

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 [1–3,5]. These signals converge at the interaction of p53 with its negative regulatory partner, MDM2 (reviewed in [1,8]). The binding of MDM2 to the N-terminus of p53 (amino acids 19–26) represses p53 transcriptional activity [9,10], mediates ubiquitination of p53 by acting as an E3 ligase [11,12] and targets p53 to the cytoplasm for 26S proteosome-dependent degradation [13–16]. 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 [17]. The N-terminal 102 amino acids of MDM2 are responsible for binding to the N-terminus of p53 while regions within both the central acidic domain and C-terminus of MDM2 are required for targeting p53 to the proteosome [18].

Disruption of the p53–MDM2 complex is a pivotal event during the induction of p53 and is sufficient to invoke p53-mediated gene expression and cell cycle arrest [19]. The mechanism(s) by which this occurs physiologically is still unclear and recent efforts have been focussed intensely on understanding the role of multisite phosphorylation and acetylation of p53 in this process (reviewed in [1–3,5]). However, while p53 modifications may contribute to weakening the p53–MDM2 interaction, others have shown that phosphorylation of p53 is not sufficient for stabilisation induced by DNA damage or by dominant oncogenes [20–23]. These findings suggest that the MDM2 protein itself may be the principal target of signals which stabilise p53. In support of this idea, DNA damage-induced phosphorylation of MDM2 in vivo has recently been inferred on the basis of phosphatase-sensitive changes in the electrophoretic mobility of MDM2 in irradiated or NCS-treated cells [22]. This modification(s) is likely to be mediated by the ATM protein kinase, but the site(s) of modification has not yet been identified [22]. Moreover, although other reports indicate that cellular MDM2 is a phosphoprotein [24], and is an in vitro substrate for other protein kinases including DNA-activated protein kinase [25] and protein kinase CK2 [26,27], the full extent of MDM2 phosphorylation is not known nor is the role of MDM2 post-translational modification in controlling the p53–MDM2 interaction understood.

In this paper, we have used chymotryptic phosphopeptide mapping to analyse the phosphorylation of MDM2 in cell lines. The data reveal the multisite nature of MDM2 phosphorylation and localise two clusters of phosphorylation sites to two important functional regions of MDM2, the p53 binding domain and the ubiquitination/p53 degradation domain. These data provide a basis for the identification of regulatory modifications which may mediate MDM2 function in response to stress signals.

2. Materials and methods

2.1. Plasmids

Wild type murine MDM2 cDNA was cloned into the mammalian expression vector pcDNA3 (Invitrogen). A DNA fragment encoding four contiguous copies of the c-Myc 9E10 epitope (human c-Myc amino acids 408–439 [28]) was cloned immediately at either the 5′ or 3′ ends of the MDM2 coding sequence. Deletion derivatives were generated using oligonucleotide-directed mutagenesis which substituted specific codons with stop codons. These plasmids are all listed in Table 1.

2.2. Cell lines and transfections

MCF-7, HeLa, SAOS-2 and COS-7 cells were maintained in Dul-

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becco–Vogt modified Eagle's medium (DMEM: Gibco BRL) supplemented with 10% foetal bovine serum (Gibco BRL), 100 μg/ml Pen/strep (Gibco BRL) and 2 mM glutamine (Gibco BRL). The cells were incubated at 37°C in a humidified 5% carbon dioxide atmosphere.

For transfection, 10 cm plates were seeded with 5×10^5 cells in normal growth medium and incubated at 37°C for 24 h. Transfection of cells (8 μg of plasmid DNA per plate) was carried out using Lipofectamine reagent according to the manufacturer's instructions (Gibco BRL). The cells were then incubated at 37°C for 5 h before replacing the transfection reagent with complete growth medium. The following day, cells were washed three times with normal medium and maintained in this medium for a further 24 h, prior to preparation for [^{32}P]orthophosphate labelling.

2.3. Radiolabelling of cells with [^{32}P]orthophosphate and immunoprecipitation

Cells were pre-incubated with 8 ml of phosphate-free DMEM (Sigma) containing 10% dialysed foetal calf serum at 37°C for 1 h. The medium was replaced with the same volume containing 4 mCi of [^{32}P]orthophosphate per plate and incubated at 37°C for a further 3 h. After labelling, the cells were washed twice with 5 ml ice cold phosphate-buffered saline (PBS) and lysed in 1 ml of ice cold NP-40 buffer (10 mM NaH_2PO_4, 2 mM EDTA, 0.1 M NaCl, 1% NP-40 and 1 mM benzamidine). The samples were centrifuged at 13 000 rpm for 10 min and the supernatant fraction was collected. Immunoprecipitation of the Myc-tagged MDM2 from the lysates was performed overnight at 4°C on an Eppendorf wheel with 75 μl of protein A-Sepharose and 2–5 μg of 9E10 antibody [28]. The protein A-Sepharose 4B beads were subsequently washed three times, each with 1 ml ice cold RIPA buffer (10 mM NaH_2PO_4, 2 mM EDTA, 0.1 M NaCl, 1% NP-40, 1 mM benzamidine, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)) and once with 1 ml of ice cold 50 mM Tris pH 7.5. 25 μl of 2×SDS sample buffer was added and the samples were heat-denatured then separated on 10% SDS gels.

2.4. 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 [29] with two exceptions: (i) chymotrypsin was used to digest the protein in place of trypsin and (ii) the solvent used for the chromatographic separation comprised isobutyric acid (62.5%, v/v), *n*-butanol (1.9%, v/v), pyridine (4.8%, v/v) and glacial acetic acid (2.9%, v/v) in deionised water.

2.5. Western analysis

Proteins were transferred to Immobilon-P membranes (Millipore) using a Millipore SDE dry-blotting apparatus. The membranes were probed with the c-Myc 9E10 monoclonal antibody at 1 μg/ml in 5% (w/v) skimmed milk powder (Marvel) and 0.05% (v/v) Tween 20 in PBS. Detection was carried out using horseradish peroxidase-conjugated anti-mouse IgG (Scottish Antibody Production Unit), followed by chemiluminescence detection (ECL, Amersham).

3. Results

Labelling of established cells lines with [^{32}P]orthophosphate followed by immunoprecipitation of MDM2, SDS–PAGE and autoradiography produced only very weak signals with insufficient radiolabelled material to permit unequivocal phosphopeptide analysis (data not shown). Therefore, in order to analyse the phosphorylation of MDM2 in cultured cells, we expressed the murine MDM2 protein ectopically in a range of cell lines using a pcDNA3-based vector in which the MDM2 proteins are tagged either at the N- or C-termini with the Myc 9E10 epitope [28]. These plasmids directed expression of the MDM2 proteins in a range of cell lines. Representative data shown in Fig. 1A indicate that the transiently expressed proteins were clearly detectable in extracts of transfected COS-7 and MCF-7 cells using the 9E10 monoclonal antibody (expression was also detected in SAOS-2 and HeLa cells, although at a significantly lower level (data not shown)). COS-7 and MCF-7 cells transiently expressing N-terminally Myc-tagged MDM2 were also labelled with [^{32}P]orthophosphate and the recombinant MDM2 proteins were immunoprecipitated from the labelled extracts. Two-dimensional chymotryptic phosphopeptide analysis of these proteins revealed that in each case multiple phosphopeptides were present in MDM2 (Fig. 1B) consistent with the occurrence of multisite phosphorylation of MDM2 in vivo. Comparison of the maps from the COS-7 and MCF-7 cells indicated that the phosphopeptide patterns were strikingly similar, although there were some minor quantitative differences. These data

Table 1
MDM2 mammalian expression plasmids

Plasmid number	MDM2 status	Myc 9E10 epitope
pDWM487	full length wild type	not tagged
pDWM659	full length wild type	tag at N-terminus
pDWM667	stop at codon 194 (Δ1)	tag at N-terminus
pDWM668	stop at codon 294 (Δ2)	tag at N-terminus
pDWM669	stop at codon 370 (Δ3)	tag at N-terminus
pDWM794	full length wild type	tag at C-terminus

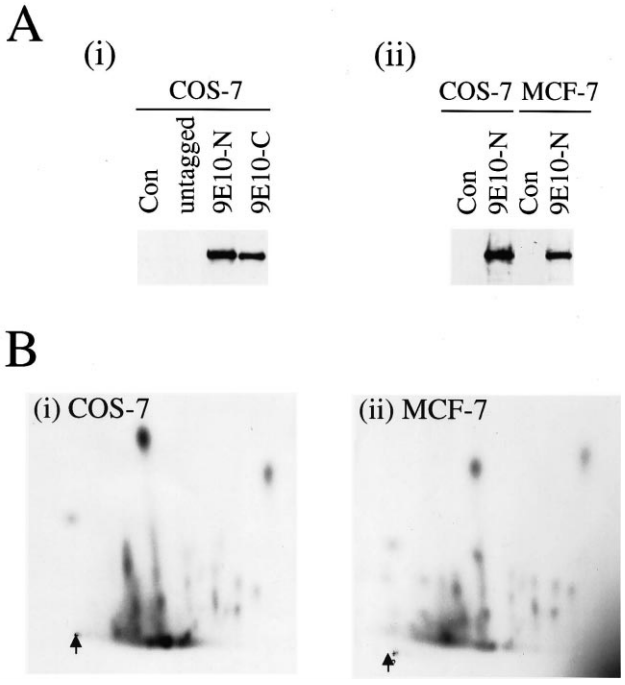

Fig. 1. Expression and chymotryptic phosphopeptide analysis of recombinant murine MDM2 in various cell lines. (A) The pcDNA3-derived plasmids pDWM487 (expressing untagged murine MDM2), pDWM659 and pDWM794 (expressing murine MDM2 tagged with the Myc 9E10 epitope at the N- or C-terminus, respectively) were transfected into different cell lines. 48 h post-transfection, extracts were made and expression of the recombinant MDM2 proteins was determined by Western analysis using the Myc 9E10 monoclonal antibody as probe. The panels are: (i) expression of untagged, N-terminally Myc-tagged MDM2, and C-terminally Myc-tagged MDM2 in COS-7 cells, (ii) expression of N-terminally Myc-tagged MDM2 in COS-7 and MCF-7 cells. In all cases Con refers to cells transfected with the empty pcDNA3 vector. (B) COS-7 or MCF-7 cells transiently expressing N-terminally Myc-tagged MDM2 were pulse-labelled with [^{32}P]orthophosphate. Extracts were prepared and the recombinant MDM2 proteins were immunoprecipitated using the 9E10 monoclonal antibody and analysed by chymotryptic phosphopeptide mapping. Representative maps from the COS-7 (i) and MCF-7 (ii) cells are shown. The origins at which the peptides were loaded are indicated by arrowheads.

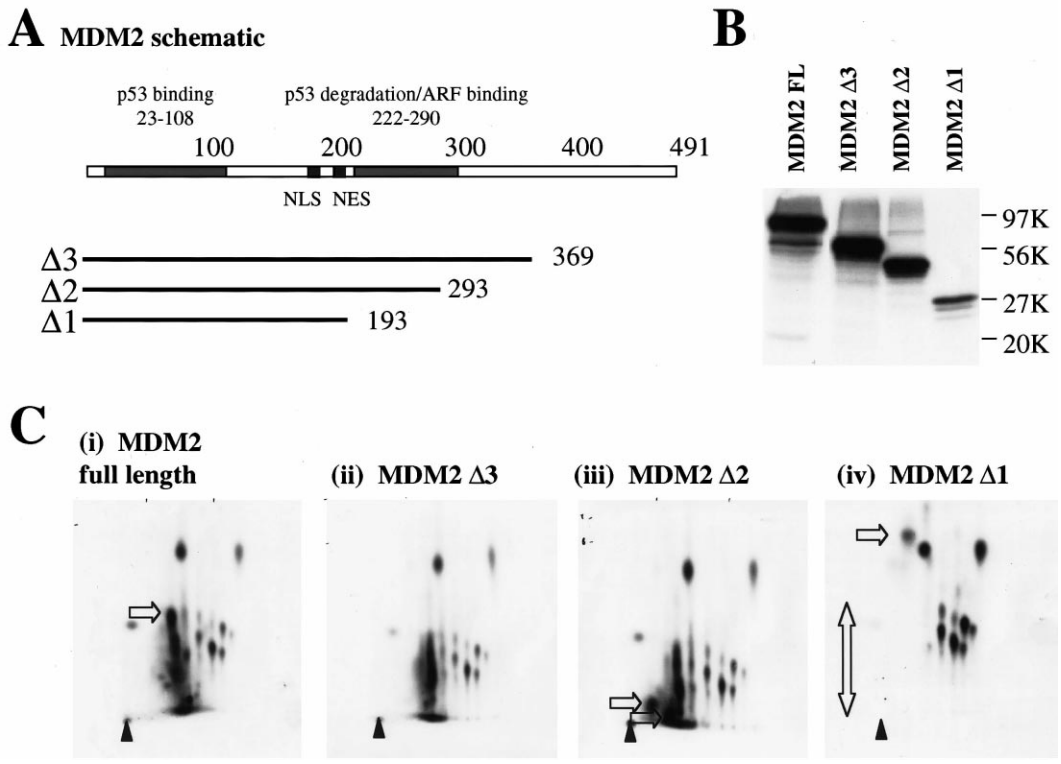

Fig. 2. ^{32}P-labelling and chymotryptic phosphopeptide analysis of full length recombinant murine MDM2 and various C-terminally truncated MDM2 mutants in COS-7 cells. (A) Schematic representation of MDM2 showing key functional domains and the C-terminally truncated mutants Δ1–3. The numbers refer to amino acid positions. (B) COS-7 cells transiently expressing N-terminally Myc-tagged MDM2 proteins (full length MDM2 and Δ1–3) were pulse-labelled with [^{32}P]orthophosphate. Extracts were prepared and the recombinant MDM2 proteins were immunoprecipitated using the 9E10 monoclonal antibody, resolved by SDS–PAGE and visualised by autoradiography. (C) The radiolabelled proteins in B were further analysed by chymotryptic phosphopeptide mapping. The origins at which the peptides were loaded are indicated by arrowheads. Points of interest in each of the panels, discussed in the text, are indicated by the broad open arrows.

indicated that the modifications on the MDM2 protein were not cell type-dependent.

In order to explore the localisation of these modifications, the phosphorylation status of full length MDM2 was analysed in comparison with three C-terminally truncated MDM2 mutants (Δ1–3; these mutants are described in Fig. 2A and Table 1). Immunoprecipitation of the ^{32}P-labelled MDM2 from transiently transfected COS-7 cells followed by SDS–PAGE revealed that, as with full length MDM2, each of the truncated proteins was phosphorylated in vivo (Fig. 2B). The Δ1 protein, however, showed a considerably reduced level of phosphorylation, suggesting that this protein lacked sites of phosphorylation (parallel Western analysis indicated that the levels of expression of the wild type and deletion mutant proteins were similar (data not shown)). Chymotryptic phosphopeptide analysis of these proteins highlighted differences in the modification status of the proteins (Fig. 2B (i–iv)). A major phosphopeptide in the full length MDM2 protein (indicated in (i) by an open arrow) was absent or very weak in the Δ3 mutant (ii), suggesting that there is a phosphorylation site(s) C-terminal to amino acid 369. An alternative explanation, however, would be that sequences in the 369–491 region are required to permit efficient phosphorylation of a site encompassed within the region encoded by the Δ3 mutant. At present, we cannot distinguish between these alternatives. All of the other major phosphopeptides resemble those observed in the full length protein, consistent with the idea that most of the phosphorylation sites lie between residues 1 and 369. Analysis of the Δ2 mutant (iii) narrowed down the location of these sites to within amino acids 1–293. However, two additional phosphopeptides appeared suggesting the possibility that this deletion had revealed cryptic (and possibly unphysiological) sites (indicated by the open arrows). Strikingly, the Δ1 mutant completely lacked the dense group of phosphopeptides on the left-hand-side of the map (indicated by the double-ended open arrow). These data suggest strongly that this group of peptides is located within amino acids 194–293. This is an important region of the MDM2 protein which is required for nucleo-cytoplasmic shuttling, ARF binding and ubiquitination of p53 [30,31]. Additionally, the Δ1 mapping data assign the remaining group of phosphopeptides to residues 1–193. Once again, these lie in a key functional region which encompasses the p53 binding domain (amino acids 23–108), the nuclear localisation sequence (176–182) and the nuclear export sequence (184–195). Interestingly, the Δ1 map shows the appearance of a novel, relatively hydrophobic phosphopeptide not present in the larger MDM2 proteins (indicated with an open arrow in (iv)). We believe that this phosphopeptide most likely arises from phosphorylation of serine 189 (which lies within the NES) for two reasons: (a) chymotryptic cleavage of the Δ1 protein on the C-terminal side of Phe-186 would produce a small negatively charged peptide with migrational properties (calculated according to [32]) similar to the novel peptide in (iv); and (b) this small phosphopeptide would never be observed in either the full length, Δ2 or Δ3 proteins because it would be part of a large 56 amino

acid peptide (187–243) which would have restricted mobility in the two-dimensional system and which is likely to contain additional sites of phosphorylation (unpublished data). However, although this is highly suggestive of phosphorylation within the NES, the alternative explanation, that the deletion may unmask a cryptic phosphorylation site, cannot be ruled out.

4. Discussion

There are three important conclusions which arise from the present study. Firstly, the data indicate that the MDM2 oncoprotein is modified by multisite phosphorylation in cultured cells. These data are consistent with multiple signalling pathways impinging on MDM2 and raise the possibility that p53 and MDM2 may be targeted in a coordinate manner by overlapping signal transduction mechanisms. The second point is that MDM2 phosphorylation patterns are similar in different cell lines (Fig. 1), indicating that the modifications are unlikely to be an artefactual consequence of using a particular cell line. The third conclusion is that the majority of the phosphopeptides arise from phosphorylation of MDM2 within two distinct important functional domains, the region responsible for p53 binding, and the central acidic region which mediates nucleo-cytoplasmic shuttling, ARF binding and ubiquitination of p53. The data suggest that the activities encompassed within these regions of MDM2 are likely to be under tight, and possibly independent, control through post-translational modification.

From a technical standpoint, the study offers a number of advantages. For example, the use of chymotrypsin to generate phosphopeptides, combined with the conditions of electrophoretic and chromatographic separation employed, permit very effective resolution of the phosphopeptides in two dimensions. One previous study has examined MDM2-derived phosphopeptides generated by digestion with trypsin followed by pronase [24]. Our own analyses using trypsin to digest phosphorylated MDM2 gave only very poor resolution of the peptides (data not shown) and this may be one reason why Deppert and co-workers employed a double digestion system [24]. We also believe that the use of chymotrypsin, which offers the advantage of specificity in determining sites of digestion which the use of pronase lacks, underscores the multisite nature of the phosphorylation of MDM2 (i.e. by eliminating the possibility of multiple peptides containing a single or few phosphorylation sites being generated through random partial cleavage). One caveat to the approach we have adopted is that we have analysed MDM2 molecules which are tagged and over-expressed. However, while we acknowledge that there is a slight possibility that tagging or over-expression may influence the phosphorylation status of the recombinant MDM2, the approach we have developed has not only overcome technical difficulties which are encountered from the analysis of endogenous MDM2 using currently available MDM2 antibodies (data not shown), but provides a powerful approach for further detailed analysis of MDM2 multisite phosphorylation, particularly when used in conjunction with other methods such as deletion or mutational analysis. Accordingly, this approach will be of considerable benefit in identifying residues phosphorylated in vivo (for example, by the use of site-directed mutagenesis of target residues) and in vitro by candidate protein kinases. Once the sites of modification have been rigorously identified, it will then be possible to generate phospho-specific antibodies which can discriminate between MDM2 molecules that are phosphorylated and those that are not phosphorylated at potential regulatory sites, on an individual basis. The availability of such reagents will be pivotal in discerning the induction and kinetics of these key modifications on endogenous MDM2 in response to a range of cellular stresses.

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