Critical roles for the serine 20, but not the serine 15, phosphorylation site and for the polyproline domain in regulating p53 turnover

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The p53 tumour suppressor protein is a short-lived transcription factor that becomes stabilized in response to a wide range of cellular stresses. Ubiquitination and the targeting of p53 for degradation by the proteasome are mediated by Mdm2 (mouse double minute clone 2), a negative regulatory partner of p53. Previous studies have suggested that DNA-damage-induced phosphorylation of p53 at key N-terminal sites has a pivotal role in regulating the interaction with Mdm2 but the precise role of phosphorylation of serines 15 and 20 is still unclear. Here we show that replacement of serine 15 and a range of other key N-terminal phosphorylation sites with alanine, which cannot be phosphorylated, has little effect on the ubiquitination and degradation of full-length human p53. In contrast, replacement of serine 20 makes p53 highly sensitive to Mdm2-mediated turnover. These results define distinct roles for serines 15 and 20, two sites previously demonstrated to be dependent on phosphorylation through mechanisms mediated by DNA damage and ATM (ataxia telangiectasia mutated). We also show that the polyproline region of p53, a domain that has a key role in p53-induced apoptosis, exerts a critical influence over the Mdm2-mediated turnover of p53.

Key words: DNA damage, tumour suppressor, ubiquitination.

INTRODUCTION

The p53 tumour suppressor protein is a short-lived transcription factor that is activated in response to a wide range of cellular stresses (reviewed in [1–6]). p53 is tightly controlled through its association with a negative regulatory partner, Mdm2 (mouse double minute 2). The Mdm2 protein represses p53 transcriptional activity [7,8], mediates the ubiqitination of p53 in the capacity of an E3 ligase [9,10] and targets p53 to the cytoplasm for 26 S proteasome-dependent degradation [11–14]. The key initiating event in the activation of p53 is thought to be the disruption of p53–Mdm2 association, which occurs in response to various stresses, albeit through different molecular mechanisms [15]. The physical separation of p53 and Mdm2 is proposed to permit the accumulation of p53 in the nucleus, where it co-ordinates a change in the balance of gene expression, leading to growth arrest or apoptosis, events that prevent the propagation or survival of damaged cells. It is currently estimated that in excess of 100 genes are regulated by p53, many of which can promote growth arrest or apoptosis [16].

The p53 protein becomes highly modified by sequential multi-site phosphorylation and acetylation in response to stresses such as ionizing radiation and UV radiation (reviewed in [1–3,5,17]). Pivotal to this series of events is the phosphorylation of serine 15 by the ATM (ataxia telangiectasia mutated) or ATR (ATM and Rad3 related) protein kinases [18,19]. We have shown previously that the phosphorylation of serine 15 in p53 is important in p53-dependent transcriptional transactivation [20] but, in contrast to other reports [18,21,22], our results did not reveal a direct role for the phosphorylation of serine 15 in regulating the ability of p53 to complex with Mdm2 or to mediate the degradation of p53 [20]. In addition to serine 15, several laboratories have now shown that phosphorylation of serine 20, which also occurs in response to ionizing radiation and UV radiation [23,24], has a central role in blocking the interaction of p53 with Mdm2, leading to attenuation of p53 degradation and stimulation of p53-dependent apoptosis [23,25–27]. Serine 20 has also been shown to be a substrate for the Chk1 and Chk2 protein kinases, placing this key residue, like serine 15, downstream of the ATM or ATR pathways [28–30].

We showed recently that a fusion protein encompassing the yeast GAL4 DNA-binding domain (residues 1–147) linked to the N-terminus of the transactivation domain of p53 (residues 1–42) was efficiently degraded by ectopically expressed Mdm2 [20]. When the role of serine 15 phosphorylation was assessed by mutation of this site in the context of the fusion protein, we concluded that the ability of Mdm2 to degrade p53 was independent of this modification [20]. However, other laboratories have presented evidence that regions of the p53 protein distinct from the transactivation domain contribute significantly towards Mdm2-mediated p53 degradation [31,32], raising the possibility that serine 15 phosphorylation could have a role in the degradation of the full-length protein that is not apparent in studies employing the N-terminus of p53 linked to a heterologous DNA-binding domain. Consequently, on the basis of these findings, we considered it important to determine the relationship between serine 15 phosphorylation and degradation in the context of the full-length p53 protein.

In the present study we tested a series of full-length p53 proteins, containing single-amino-acid changes at established N-terminal phosphorylation sites, as substrates for Mdm2-mediated ubiquitination and degradation. We show conclusively that serine 20, but not serine 15, has an important role in regulating p53 turnover. We also show that the ability to discern effects of altered phosphorylation sites is heavily dependent on the levels of expression of the p53 and Mdm2 proteins, underscoring the need for future studies to adopt more physiological approaches for the analysis of these regulatory events. Further-

Abbreviations used: ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3 related; CMV, cytomegalovirus; GST, glutathione S-transferase; mdm2, mouse double minute 2 protein.

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more, we find that loss of the polyproline domain of p53 (residues 62–91), which is thought to have a role in p53-mediated growth suppression [33] and apoptosis [34–36], makes p53 acutely sensitive to Mdm2-mediated degradation.

EXPERIMENTAL

Plasmids

The plasmid expressing a fusion protein with the GAL4 DNA-binding domain (amino acid residues 1–147) and the first 42 residues of human p53 [GAL4–p53(1–42)] was a gift from Dr T. Unger (Weizmann Institute, Rehovot, Israel) [37]. Plasmids expressing full-length p53 under control of the cytomegalovirus (CMV) promoter were obtained by cloning the full-length human p53 (wild-type or mutant) into the vector pcDNA3 (Invitrogen). A CMV-based plasmid expressing His_6-tagged ubiquitin [38] was obtained from Dr Christine Blattner (University of Dundee, Dundee, U.K.). Two plasmids were used for Mdm2 expression, a pcDNA3-based plasmid expressing murine Mdm2 (obtained from Dr Blattner) and a pSG5 (Stratagene)-based plasmid expressing the human homologue of Mdm2 (pDWM598 [20]). All of the human p53 point mutations used in this study were generated by oligonucleotide-directed mutagenesis with the method described in [39] and have been described elsewhere [20]. The same approach was used to delete the polyproline domain (residues 62–91) from human p53; this deletion was identical to that used in Debussche's and Levine's laboratories [33,35]. The p53-coding sequences in all of these plasmids were checked by automated DNA sequencing to ensure that only the desired changes were present.

Cell culture and transfections

This study used COS-7 cells and H1299 cells (H1299 cells are derived from a human lung carcinoma and lack the expression of endogenous p53 [40]). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum in an air/CO_2 (19:1) atmosphere. For transient transfection, 10^5 cells were plated per well in six-well plates (or 5×10^5 per 10 cm plate) and transfected on the following day by precipitation with calcium phosphate. The exact amount of plasmid DNA used in each experiment is indicated in the Figure legends. When required, carrier DNA was added to maintain a constant amount of DNA in each transfection mix. To assess Mdm2 effects, cells were co-transfected with different amounts of plasmid expressing human Mdm2 (see above). In some experiments, in which both human and murine Mdm2 were tested, the results were identical.

Ubiquitination assay

To show directly that p53 is ubiquitinated in an Mdm2-dependent manner, the method described by Rodriguez et al. [41] was used. Essentially, H1299 cells were seeded at 8×10^5 per 10 cm dish and transfected with 5 μg of a CMV-based plasmid expressing His_6-tagged ubiquitin [38], 3 μg of the wild-type human p53-expressing plasmid, pDWM715 [20], and various amounts of the Mdm2-expressing plasmid, pDWM598 (see above). At 42 h after transfection the cells were washed twice in PBS and lysed in 8 ml of guanidinium buffer [6 M guanidinium/HCl/0.1 M sodium phosphate (pH 8.0)/0.01 M Tris/HCl (pH 8.0)/5 mM imidazole/10 mM 2-mercaptoethanol] per plate. A suspension of nickel-agarose beads (100 μl; Qiagen) was added to the lysate and incubated at room temperature for 4 h on a rotating wheel. The agarose–protein complexes were collected by centrifugation and then washed successively as follows: once in guanidinium lysis buffer, twice in urea buffer [8 M urea/0.1 M sodium phosphate (pH 8.0)/0.01 M Tris/HCl (pH 8.0)/10 mM 2-mercaptoethanol] containing 0.2 % (v/v) Triton X-100 and twice in the same buffer but containing 0.1 % Triton X-100. The beads were then resuspended in 40 μl of SDS sample buffer, denatured by heat at 100 °C and loaded on a 7.5 % (w/v) polyacrylamide gel. The ubiquitinated proteins were subsequently analysed by Western blotting as described below.

Analysis of p53 ubiquitination and degradation by Western blotting

To assess the level of expression of the p53 and Mdm2 proteins, H1299 cells or COS-7 cells were transiently transfected as described above. At 24 h after transfection, nuclear extracts were prepared by resuspending the cells in lysis buffer [20 mM Hepes/KOH (pH 7.6)/20 % (v/v) glycerol/10 mM NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/1 mM dithiothreitol/0.1 % Igepal], followed by incubation on ice for 10 min and centrifugation for 5 min at 800 ***g***. The pellets were then resuspended in SDS sample buffer and the protein extracts were fractionated by SDS/PAGE [10 % (w/v) gel] and transferred electrophoretically on to a PVDF membrane (Westran; Schleicher and Schuell). Antibodies against the GAL4 DNA-binding domain (RK5C1; Santa Cruz Biotechnology), Mdm2 (SMP14; Santa Cruz Biotechnology) and p53 (DO1 and PAb1801; Santa Cruz Biotechnology) were used for immunochemical detection by chemiluminescence. For the serine 20 to alanine mutant p53 protein, only PAb1801 could be used because mutation of this serine weakens the interaction of DO1 with its epitope [25]. p53 turnover in the presence of Mdm2 expression was assessed by the decrease in the chemiluminescence signal. p53 ubiquitination was detected by overexposure of the autoradiographs to the chemiluminescence signal.

Glutathione S-transferase (GST) pulldown assays

To determine the ability of the wild-type or ΔPro p53 proteins to bind Mdm2 *in vitro*, GST pull-down assays were performed with proteins translated *in vitro*. For these experiments the human proteins were translated in the presence of [^{35}S]methionine with a reticulocyte-lysate *in vitro* transcription/translation kit as directed by the manufacturer (Promega). Essentially, the GST–p53 proteins were adsorbed on glutathione–Sepharose 4B beads (Pharmacia Biotech) and the beads were washed twice with buffer A [50 mM Tris/HCl (pH 7.4)/250 mM NaCl/0.1 % (v/v) Triton X-100/5 mM EDTA/2 mM dithiothreitol plus protease inhibitors (Calbiochem)]. The GST–p53 proteins were incubated for 2 h at 4 °C with ^{35}S-labelled proteins in 200 μl of buffer A. Complexes were washed twice in buffer A, resuspended in SDS sample buffer and fractionated by SDS/PAGE [10 % (w/v) gel]. Before being dried, the gels were stained to confirm that the same amount of GST–p53 protein was precipitated with glutathione–Sepharose 4B (results not shown). To assess ^{35}S-labelled protein, gels were treated with Amplify (Amersham) before drying, then exposed to X-ray films at −80 °C.

RESULTS

To confirm that fusion proteins comprising the DNA-binding domain of the GAL4 protein (residues 1–147) linked to the N-terminus of human p53 (residues 1–42) were subject to degradation as mediated by Mdm2, plasmids expressing GAL4–p53 were transiently expressed in p53-null H1299 cells in the absence and in the presence of a plasmid expressing Mdm2. The results show clearly that the levels of expression of each of these

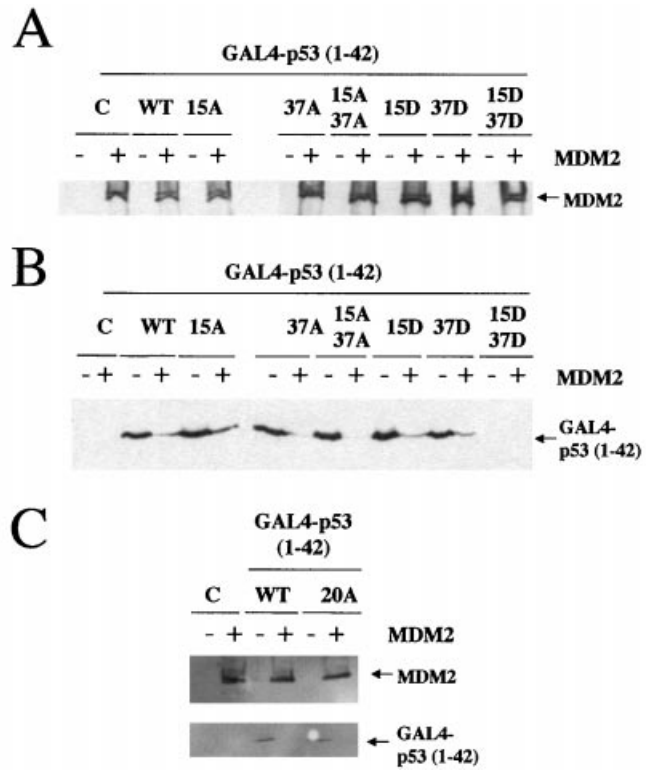

Figure 1 Mdm2 induces turnover of GAL4–p53 fusion proteins in p53-null H1299 cells

H1299 cells were transfected, in the presence or absence of 1 μg of a plasmid expressing the Mdm2 protein, with plasmids expressing fusion proteins comprising the DNA-binding domain of the *Saccharomyces cerevisiae* GAL4 protein (residues 1–147) linked to the N-terminal 42 residues of human p53. The p53 component of this fusion protein comprised either wild-type p53 (WT) or a series of mutant proteins in which three key physiological sites of phosphorylation (serine residues 15, 20 and 37) were replaced either independently or jointly with alanine or aspartate. The mutants were as follows: (**A**, **B**) serine 15 to alanine (15A), serine 37 to alanine (37A), serines 15 and 37 to alanine (15A/37A), serine 15 to aspartate (15D), serine 37 to aspartate (37D) and serines 15 and 37 to aspartate (15D/37D); (**C**) serine 20 to alanine (20A). In each case, 100 ng of the GAL4–p53-expressing plasmid was transfected. In control experiments (lanes C), the p53-expressing plasmids were replaced with carrier DNA. Cells were harvested 24 h after transfection and the proteins were examined by Western blot analysis. The Mdm2 protein was detected with the Mdm2-specific monoclonal antibody SMP14 (**A**, **C**). The GAL4–p53 fusion proteins were detected with the p53-specific monoclonal antibody DO1 (**B**) or the anti-GAL4 antibody RK5C1 (**C**) (RK5C1 was used because the mutation of serine 20 weakens the interaction of DO1 with its epitope [25]).

GAL4–p53 proteins (with the exception of the 15D/37D mutant) were very similar in the absence of co-expressed Mdm2 (Figures 1B and 1C). (The 15D/37D mutant showed only negligible expression; we do not yet know the basis of this low level of expression.) In the presence of co-expressed Mdm2, the levels of each of the GAL4–p53 fusion proteins were diminished significantly (Figure 1), confirming our previous observations [20]. Replacement of serine 15 (which is phosphorylated physiologically by ATM and ATR) and/or serine 37 (which is phosphorylated physiologically by ATR) with alanine or aspartic residues had little effect on the ability of Mdm2 to promote the disappearance of the GAL4–p53 fusion protein, again confirming our previous observations [20]. We have shown previously that the transfection of DNA is sufficient to induce p53 accumulation and serine 15 phosphorylation, indicating that the signalling mechanisms that activate p53 are operative under our experimental conditions [20]. Replacement of serine 20 also had apparently little effect on the degradation of GAL4–p53 by Mdm2 (Figure 1C). However, serine 20 lies within the epitope for the antibody DO1 and its substitution diminishes DO1 reactivity significantly [25]. An antibody against the GAL4 DNA-binding domain was therefore used to detect both wild-type Mdm2 and the 20A mutant (Figure 1C). Although this approach permitted the detection of the GAL4–p53 proteins and confirmed their degradation in the presence of Mdm2, the signal obtained with this antibody was consistently weak, making it difficult to discern possible differences between the behaviour of the wild-type and 20A mutant proteins with confidence. The most plausible explanation for the observation that Mdm2 promotes the degradation of the GAL4–p53 fusion proteins (Figure 1) is that Mdm2 did indeed bind to its docking site on the p53 moiety (at residues 19–26) and mediated the degradation of the fusion protein (this might occur through the ubiquitination of residues in the GAL4 moiety; alternatively, auto-ubiquitination of Mdm2 might be sufficient to promote the export and degradation of both itself and the GAL4–p53 protein). Accordingly, the lack of effect with the serine 15 and −37 mutations could be interpreted such that these residues were not directly involved in regulating the Mdm2 interaction or p53 degradation. However, because the GAL4–p53 protein is an artificial protein we cannot yet rule out more trivial explanations. Moreover, because other regions of the p53 protein are thought to contribute to its ubiquitination by Mdm2 in a physiological context [31,32], it was important to examine the role of these, and other, phosphorylation sites in the degradation of full-length p53.

To confirm that Mdm2 could promote the turnover of full-length p53, increasing amounts of Mdm2-expressing plasmid were transiently co-transfected into H1299 cells with a fixed amount of plasmid expressing the full-length p53 protein. Analysis of expression of these proteins confirmed that increasing levels of Mdm2 expression led to a corresponding decrease in the cellular p53 levels (Figures 2A and 2Bi). Strikingly, a longer exposure of the autoradiography in Figure 2(Bi) revealed a ladder of bands of increasing molecular mass as detected with the anti-p53 antibody (shown in Figure 2Bii). The occurrence of these bands was dependent on the presence of Mdm2 and was reminiscent of the appearance of ubiquitinated forms of p53. To check whether this was so, p53, Mdm2 and His_6-tagged ubiquitin were co-expressed in H1299 cells and the ubiquitinated proteins were isolated by nickel–agarose chromatography. Detection of p53 by Western blotting confirmed that the upper bands were indeed ubiquitinated forms of p53 and that their presence was dependent on the level of Mdm2 (Figure 2C). Under our assay conditions, ubiquitinated p53 could be detected 24 and 40 h after transfection (Figure 2D). Moreover, by carefully adjusting the amounts of the p53-expressing and Mdm2-expressing plasmids transfected into the cells (to give a relatively high level of Mdm2), it was possible to maximize the appearance of the ubiquitinated forms of p53 (see below).

To determine whether any of the established N-terminal phosphorylation sites in the p53 protein had any influence on the interaction of p53 with Mdm2, plasmids expressing wild-type p53 or a series of p53 phosphorylation-site mutants were co-transfected into H1299 cells in the absence and in the presence of Mdm2-expressing plasmid. The endpoint of the assay was the appearance of ubiquitinated p53. The results from these experiments revealed that the levels of expression of the wild-type protein and each of the mutant p53 proteins were very similar in the absence of co-expressed Mdm2 (Figure 3). Similarly, each of these p53 proteins was able to direct similar levels of expression of a luciferase reporter gene under the control of a polyomavirus promoter containing 13 copies of a consensus p53-binding site

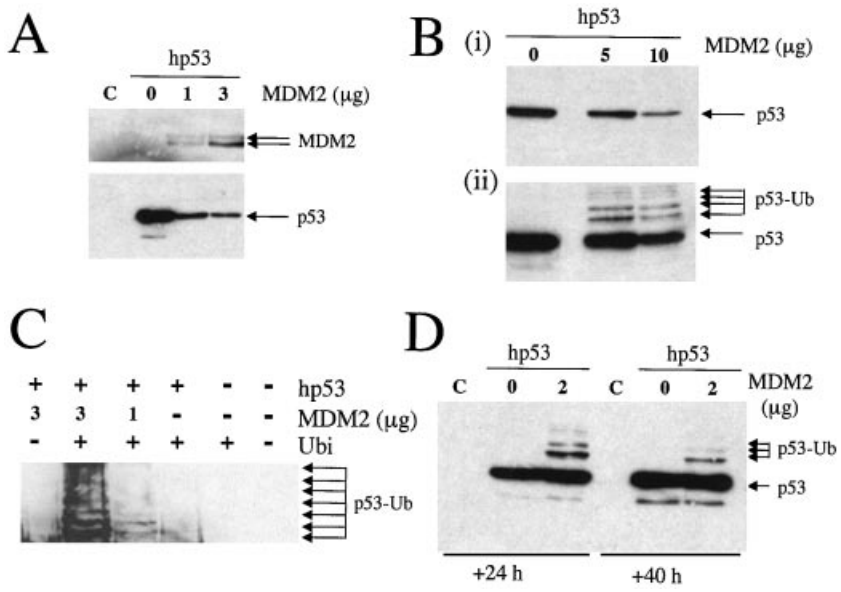

Figure 2 Mdm2 induces the ubiquitination and degradation of full-length human p53

Human H1299 cells were transfected with plasmids expressing full-length human wild-type p53 (100 ng) and increasing amounts of a plasmid expressing wild-type Mdm2, as indicated. Cells were harvested 24 h (**A**–**D**) or 40 h (**D** only) after transfection; the p53 proteins were examined by Western blot analysis with the monoclonal antibodies DO1 to detect the p53, and with 4B2 to detect the Mdm2. The positions of the unmodified and ubiquitinated p53 proteins are indicated. (**A**, **Bi**, **Bii**) Dose response of Mdm2 levels on p53 ubiquitination and turnover; (**Bii**) is a longer exposure of the results shown in (**Bi**). (**C**) Cells were additionally transfected with 5 μg of a plasmid expressing His_6-tagged ubiquitin. Cells were lysed in guanidinium lysis buffer as described in the Experimental section and the ubiquitin-conjugated proteins were isolated by nickel–agarose affinity chromatography. The ubiquitinated p53 proteins were analysed by Western blotting and detected with the monoclonal antibody DO1. (**D**) Comparison of Mdm2-induced ubiquitination of p53 at 24 or 40 h after transfection. In control experiments (lanes C), the p53-expressing plasmids were replaced with carrier DNA.

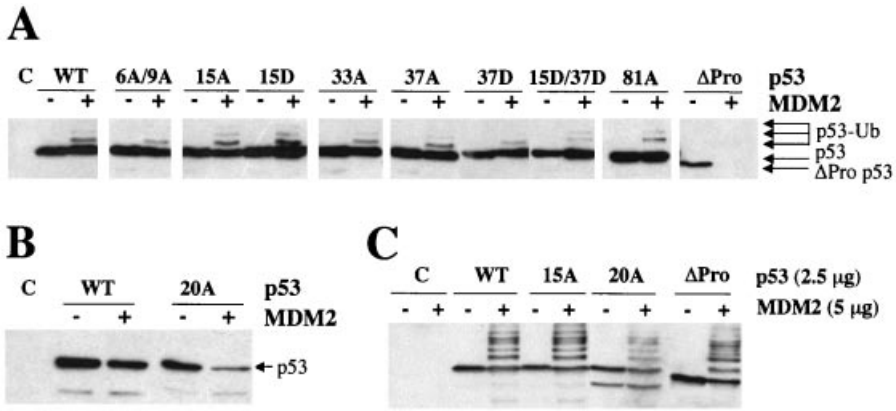

Figure 3 Mdm2-mediated ubiquitination and degradation of human wild-type and N-terminal mutant p53 proteins

(**A**, **B**) Human H1299 cells were transfected with plasmids expressing wild-type (WT) or mutant p53 proteins (100 ng), in the presence or absence of 2 μg of a plasmid expressing the Mdm2 protein. The cells were harvested 24 h after transfection and the p53 proteins were examined by Western blot analysis with the monoclonal antibodies DO1 (**A**) and PAb1801 (**B**) to detect the p53. The mutant p53 proteins were serines 6 and 9 replaced with alanine (6A/9A), serine 15 replaced with alanine (15A) or aspartate (15D), serine 33 replaced with alanine (33A), serine 37 replaced with alanine (37A) or aspartate (37D), serines 15 and 37 replaced with aspartate (15D/37D), threonine 81 replaced with alanine (81A), serine 20 replaced with alanine (20A), and deletion of residues 62–91 comprising the polyproline domain (ΔPro). (**C**) Human H1299 cells were transfected with plasmids expressing wild type or mutant p53 proteins (2.5 μg) together with a plasmid expressing mdm2 (5 μg). The cells were harvested 24 h after transfection and the p53 proteins were examined by Western blot analysis with a mixture of the monoclonal antibodies DO1 and PAb1801. In control experiments (lanes C), the p53-expressing plasmids were replaced with carrier DNA.

(results not shown; we have previously shown that the phosphorylation of serine 15, which regulates contact with p300, has little influence on the ability of p53 to direct expression of this particular promoter [20]). In comparison with wild-type p53, most of the mutant proteins showed no significant differences in their levels of ubiquitination or degradation as mediated by Mdm2: these mutants were serines 6 and 9 to alanine (6A/9A), 15A, serine 15 to aspartate (15D), 33A, 37A, 37D, 15D/37D and 81A (Figure 3A). Strikingly, however, under identical conditions only the 20A mutant promoted a rapid loss of the p53 protein (Figure 3B). These results confirm previously published observations that serine 20 has a pivotal role in regulating p53 turnover and are consistent with the model that phosphorylation of serine 20 inhibits the Mdm2-mediated degradation of p53

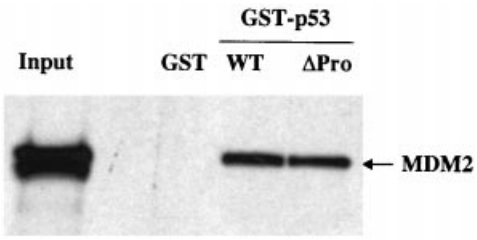

Figure 4 GST–p53 fusion proteins comprising wild-type p53 or the ΔPro mutant show no difference in their ability to bind to Mdm2 translated *in vitro*

The ability of GST–p53 fusion proteins, comprising either the full-length wild-type p53 or the ΔPro p53 proteins, to interact with ^{35}S-labelled Mdm2 translated *in vitro* was performed as described in the Experimental section. The samples were GST alone (GST), GST fused to full-length p53 (WT) and GST–ΔPro p53 (ΔPro). The lane labelled Input shows a sample of the ^{35}S-labelled Mdm2 translated *in vitro* before being mixed with the GST proteins.

[23,25–27]. The results also underscore the fact that, as in the context of the GAL4–p53 fusion protein, serine 15 does not seem to have any direct role in regulating the interaction of Mdm2 with full-length p53 or in the subsequent degradation of p53 [20], a point that is emphasized by the striking effect of the 20A mutant. Interestingly, one additional mutant, from which the entire polyproline domain of p53 (residues 62–91) had been deleted (ΔPro), was also analysed. This domain has been proposed to have a role in p53-mediated growth suppression [33] and apoptosis [34–36]. Strikingly, as with the 20A mutant, the ΔPro mutant p53 protein was acutely sensitive to Mdm2-mediated degradation (Figure 3A).

An additional interesting point is that the distinction between the 20A and ΔPro mutants in comparison with wild-type p53 was highly dependent on achieving the correct ratio of expression of the Mdm2 and p53 proteins. When the amount of the p53-expressing plasmid was increased from 0.1 to 2.5 μg (with the Mdm2 plasmid still in excess), the 20A and ΔPro mutants behaved in a manner indistinguishable from wild-type p53 (Figure 3C). There were no significant differences in the degradation rates of the 15A or 15D mutants in comparison with wild-type p53, regardless of the levels of expression of these proteins.

The finding that the ΔPro mutant was rapidly turned over suggested that its interaction with Mdm2 might be influenced by the absence of the polyproline domain. To test this idea, the binding of ^{35}S-labelled mdm2, translated *in vitro*, to GST–p53 fusion proteins comprising full-length p53 or the ΔPro mutant was examined as described in the Experimental section. As shown in Figure 4, there were no discernible differences in the ability of these different p53 proteins to interact with Mdm2 *in vitro*.

DISCUSSION

We and others have shown that a fusion protein encompassing the yeast GAL4 DNA-binding domain (residues 1–147) linked to the N-terminus of the transactivation domain of p53 (residues 1–42) is efficiently degraded in the presence of Mdm2 expressed ectopically [12,20]. Replacement, with alanine or aspartate residues, of the p53 phosphorylation site at serine 15 in this fusion protein has profound effects on its capacity to activate transcription but has no detectable effect on Mdm2-mediated degradation, suggesting that the phosphorylation site at serine 15 is dispensible for the regulation of the interaction with Mdm2 [20] (see also Figure 1). However, two other studies have reported that elements of the p53 protein distant from the Mdm2-binding region (residues 19–26) have key roles in determining the sensitivity of p53 to Mdm2-mediated degradation [31,32]. Accordingly, any influence of serine 15 phosphorylation on these additional elements would not be detected in experiments employing the GAL4–p53(1–42) chimaeric proteins alone. To address this issue, we examined the role of serine 15, and a series of other N-terminal phosphorylation site mutants, in the degradation of full-length p53. Thus the replacement of serines 6, 9, 15, 33 or 37 or threonine 81 had no detectable influence on the ability of Mdm2 to orchestrate the degradation of p53 (Figure 3). The lack of effect of these mutants is underscored by the finding that, under identical conditions, replacement of serine 20, a residue that has an established role in regulating p53 turnover [23,25–27]), made the p53 protein acutely sensitive to degradation through Mdm2 (Figure 3). Our results therefore strongly support the idea that serines 15 and 20 might have distinct, or only partly overlapping, roles in the p53 activation process. Taken together with our previous studies and those of other laboratories, these results are consistent with a model in which the DNA-damage-induced phosphorylation of serine 15 (through the ATM protein kinase pathway) stimulates p53-dependent transactivation by promoting the recruitment of p300/CBP but does not necessarily influence the p53–Mdm2 interaction directly [20,42]. In contrast, phosphorylation of serine 20, mediated by Chk2 (which is in turn activated by ATM), blocks the Mdm2-dependent degradation of p53 [23,24,26,28–30] and, as suggested by recent evidence, might actually contribute to the co-activator recruitment attributed to serine 15 phosphorylation [43,44]. Thus ATM can simultaneously co-ordinate the recruitment of a transcriptional co-activator and the attenuation of a negative regulator by promoting the modification of two functionally distinct residues.

An important issue arising from this study is that achieving the correct relative levels of expression of p53 and Mdm2 (and presumably levels of expression that match those observed physiologically) is necessary to observe the effects of perturbing regulatory modifications (see Figure 3). This might indicate that the contribution of residues such as serine 20 is subtle, reflecting a level of fine tuning in the regulation of p53 turnover as opposed to an on–off mechanism. The results also suggest that a step in the export/degradation process, rather than ubiquitination itself, is rate-limiting under these conditions because, at higher levels of p53 expression, ubiquitinated products accumulate (equally with both wild-type and mutant p53 proteins). The results are therefore consistent with the regulation by serine 20 of a step downstream of p53 ubiquitination. This dependence on expression levels might also highlight possible reasons why there has been disagreement in the literature about the contribution of p53 phosphorylation to p53 turnover.

In addition to our analysis of the phosphorylation-site mutants, we find that deletion of the polyproline domain makes p53 acutely sensitive to degradation, thus implying that this region has an inhibitory or attenuating role in p53 degradation. The mechanism by which this occurs in not clear and remains to be established. However, we find that ΔPro p53 shows no apparent difference from wild-type p53 in its ability to activate transcription (results not shown) or to bind to Mdm2 *in vitro* (Figure 4). The finding that its absence stimulates p53 turnover would argue that its ability to be ubiquitinated by Mdm2 is unaffected; this is supported by the results in Figure 3(C). The polyproline domain is thought to have a role in p53-mediated growth suppression [33] and apoptosis [35,36]. The increased sensitivity of ΔPro p53 to degradation would be consistent with the published evidence that p53 function is impaired by deletion of this domain [33,35,36]. While the present paper was in preparation, Berger et al. [34] also reported that ΔPro p53 is highly

susceptible to degradation, which is consistent with our own findings. However, in contrast with our analysis, the results in this study indicate that ubiquitination of ΔPro p53 is enhanced and suggest that the export of ΔPro p53 is correlated with an enhanced interaction with Mdm2 in a cellular context. The interaction of p53 and Mdm2 in cells is likely to be complex, involving the interplay of several factors including the p300 coactivator [45]; this could explain why we do not observe a significant difference between the wild-type and ΔPro p53 proteins *in vitro*. Clearly, therefore, the polyproline domain of p53 has a pivotal role in governing the turnover of the protein but the mechanism of its involvement must await further detailed analysis.

This work was supported by the Biotechnology and Biological Sciences Research Council (U.K.), the Association for International Cancer Research and the Medical Research Council (U.K.). D.W.M. is a Medical Research Council Senior Fellow.

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Received 26 March 2001/17 July 2001; accepted 16 August 2001