

Downregulation of MDM2 stabilizes p53 by inhibiting p53 ubiquitination in response to specific alkylating agents

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Received 27 October 2000; revised 6 December 2000; accepted 6 December 2000

First published online 19 January 2001

Edited by Gianni Cesareni

Abstract p53 is stabilized in response to DNA damaging stress. This stabilization is thought to result from phosphorylation in the N-terminus of p53, which inhibits p53:MDM2 binding, and prevents MDM2 from promoting p53 ubiquitination. In this report, the DNA alkylating agents mitomycin C (MMC) and methylmethane sulfonate (MMS), as well as UV radiation, stabilized p53 in a manner independent of phosphorylation in p53 N-terminus. This stabilization coincided with decreased levels of MDM2 mRNA and protein, and a corresponding decrease in p53 ubiquitination. Importantly, MDM2 overexpression inhibited the stabilization of p53 and decrease in ubiquitination following MMC, MMS, and UV treatment. This indicates that downregulation of MDM2 contributes to the stabilization of p53 in response to these agents. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Alkylating agent; Mitomycin C; Methylmethane sulfonate; p53; Murine double minute 2; Ubiquitination

1. Introduction

The tumor suppressor p53 plays an important role in response to DNA damage by serving as a cell cycle check point determinant [1,2]. Wild-type p53 levels are low in most normal cells due to a short protein half-life [3,4]. However, the p53 protein is stabilized and accumulates in the nucleus upon exposure to various DNA damaging agents [5,6]. The effect of increasing p53 is to stop cell proliferation, through either a G1 cell cycle arrest, or apoptotic cell death [2]. These activities are mediated by proteins such as p21 and bax, whose genes are transcriptionally activated by p53 [7–9].

The mechanism by which p53 is stabilized in response to

DNA damage has not been fully clarified. p53 degradation occurs through the ubiquitin proteolysis pathway, and factors which can promote p53 ubiquitination have been identified. The MDM2 gene is transcriptionally activated by p53, and MDM2 protein can bind p53 and promote its ubiquitination and degradation [10,11]. It has been suggested that MDM2 functions as an E3 ubiquitin ligase that transfers ubiquitin directly to p53 [12,13]. Current models contend that DNA damaging agents stabilize p53 by inhibiting MDM2-mediated p53 ubiquitination (reviewed in [14]). Studies in support of this model demonstrated that ionizing and UV radiation can induce the phosphorylation of p53 N-terminal sites, and that these phosphorylations can inhibit p53:MDM2 binding [15,16]. In particular, phosphorylation at serine 15 (S15) or 20 (S20) diminished p53:MDM2 binding [16,17]. Further studies demonstrated that high doses of UV radiation could inhibit p53:MDM2 binding and p53 ubiquitination in transiently transfected cells [18,19]. While phosphorylation is likely to play a key role in p53 stability, other studies reported that p53 proteins mutated in all potential N- and C-terminal phosphorylation sites could be stabilized in response to certain DNA damaging stresses [20–22]. These findings suggest that DNA damaging agents can stabilize p53 through alternative mechanisms that do not involve p53 phosphorylation.

DNA alkylating agents promote monoadduct formation on ring nitrogens or extra-cyclic oxygens of nucleotide bases, which result in chromosomal loss or single base mutation [23]. The toxic nature of DNA alkylating agents has resulted in their use in various chemotherapy treatment regimens [24]. However, these agents are also carcinogenic and their use in chemotherapy has been linked to the development of secondary leukemias and lymphomas [25]. Certain specific alkylating agents have been shown to signal an increase in p53 protein levels [5,26], and to stimulate p53-dependent and p53-independent apoptosis pathways [27,28]. The mechanisms by which DNA alkylating agents signal an increase in p53 have not been determined.

The purpose of the current study was to examine the effect of multiple DNA damaging agents, including DNA alkylating agents, on the ubiquitination and stability of p53. p53 was stabilized in response to each of the DNA damaging agents tested. In contrast, the DNA alkylating agents mitomycin C (MMC) and methylmethane sulfonate (MMS), as well as UV radiation, signaled a marked decrease in the level of MDM2 mRNA and protein. This downregulation of MDM2 coincided with decreased levels of ubiquitinated p53 and p53:MDM2 binding complexes. Further, MMC, MMS and UV radiation stabilized both wild-type p53 and a p53 mutant

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Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CDDP, cisplatin; DNA, deoxyribonucleic acid; cDNA, complementary DNA; EMS, ethylmethane sulfonate; EDTA, ethylenediaminetetraacetic acid; IR, ionizing radiation; MDM, murine double minute; MMC, mitomycin C; MMS, methylmethane sulfonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 4NQO, 4-nitroquinoline-1-oxide; PMSF, phenylmethylsulfonyl fluoride; p53 N-term, mutant p53 in which S6, S9, S15, T18, S20, S33 and S37 are converted to alanine; RIPA, radioimmunoprecipitation assay; RNA, ribonucleic acid; mRNA, messenger RNA; SDS, sodium dodecyl sulfate; SSC, NaCl, sodium citrate; UV, ultraviolet rays

lacking all potential N-terminal phosphorylation sites in transiently transfected cells, and this coincided with decreased MDM2 protein levels. Importantly, p53 was not stabilized in cells transiently overexpressing MDM2 that were treated with MMC, MMS, or UV radiation. These results indicate that the inhibition of ubiquitination and consequent stabilization of p53 in response to MMC, MMS, and UV radiation results at least in part from a downregulation of MDM2.

2. Materials and methods

2.1. Cell strains and tissue culture

RKO, MCF7, and SaOS-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg/ml penicillin and streptomycin. Radiation treatments were carried out in the laboratory of radiobiology, Harvard School of Public Health. Ionizing radiation (IR) was carried out using a ^{60}Co gamma ray source (US Nuclear) at a dose rate of 8.1 cGy/s. The UV source was a specially constructed apparatus consisting of five UV bulbs which delivered 254 nm light at a dose of 0.35 J/m²/s. 4-Nitroquinoline-1-oxide (4NQO; 0.8 µg/ml), cisplatin (CDDP; 50 µM), etoposide (20 µM), MMC (100 µM), actinomycin D (30 ng/ml), MMS (100 µg/ml), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; 0.5–4.0 µg/ml), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; 50–200 µM) and ethylmethane sulfonate (EMS; 50–200 µg/ml) were added to the growth medium where indicated. All these chemicals were from Sigma Chemical.

2.2. SDS-PAGE, immunoprecipitations and immunoblots

Cells were rinsed with phosphate buffered saline and scraped into either radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris (pH 7.5), 2 mM EDTA, 150 mM NaCl, 1.0% Nonidet P-40, 1.0% deoxycholate, 0.25% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml aprotinin, 5 µg/ml leupeptin), or lysis buffer (50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, 2 µg/ml aprotinin, 5 µg/ml leupeptin). After harvest with RIPA buffer, cells were sonicated for 10 pulses at setting 5, 50% output, with a Branson 450 sonifier, and spun at 15000×*g* for 16 min to remove cellular debris. Lysates prepared in lysis buffer were incubated on ice for 30 min with occasional light vortexing, and spun at 15000×*g* for 15 min to remove cellular debris. Protein extracts were examined by immunoblotting with the p53 monoclonal antibody DO-1 (Ab-6, Oncogene Science) or phospho-p53 (at S15) antibody (New England Biolabs). The p53 blot was reprobed with the MDM2 antibody SMP-14 (Santa Cruz Biotechnology). For detection of ubiquitinated p53, lysates were immunoprecipitated using DO-1, and examined by immunoblotting with DO-1. For coimmunoprecipitation analysis, p53 was immunoprecipitated using the p53 antibody PAb421 (Ab-1, Oncogene Science) followed by immunoblotting with SMP-14.

2.3. Transfections

MCF7 cells were transfected using the calcium-phosphate method [29] when the cells were approximately 80% confluent. MCF7 cells were transfected with either wild-type p53 or p53 N-term (S6, S9, S15, T18, S20, S33 and S37 converted to alanine) alone, or cotransfected with MDM2. 16 h after addition of the DNA precipitate, cells were rinsed and refed. 8 h later, the cells were UV radiated (20 J/m²), or treated with either MMS (100 µg/ml) or MMC (100 µM), and then cell lysates were prepared at 5 h after treatments. p53 levels were monitored in the transfected cells by immunoblotting with DO-1 or PAb1801 (Ab-2, Oncogene Science). MDM2 levels were detected by immunoblotting with SMP-14. Detection of p53:ubiquitin conjugates was carried out by immunoprecipitation of p53 with DO-1 followed by immunoblot analysis with DO-1.

2.4. Northern blots

RNA was isolated using RNazol B as described by the manufacturer (Tel-test, Inc.). 20 µg (RKO) or 10 µg (SaOS-2) of total RNA was resolved in a formaldehyde-agarose gel and transferred to a nylon membrane (Nytan, Schleicher and Schuell). Filters were hybridized with ³²P-labeled human MDM2 cDNA, followed by two 15 min washes at room temperature with 2×SSC (150 mM NaCl, 15 mM

sodium citrate), 0.1% SDS, and one 30 min wash at 60°C in 0.1×SSC, 0.1% SDS.

3. Results

p53 is stabilized in response to various stresses, including DNA damage, hypoxia, ribonucleotide depletion, and oncogene expression [14]. The wide variety of stresses which can induce p53 raises the question as to the nature of the inducing signal, and whether different agents stabilize p53 through common mechanistic pathways. Current models suggest that the stabilization of p53 in DNA damaged cells results from phosphorylation of p53 N-terminal sites, which inhibits

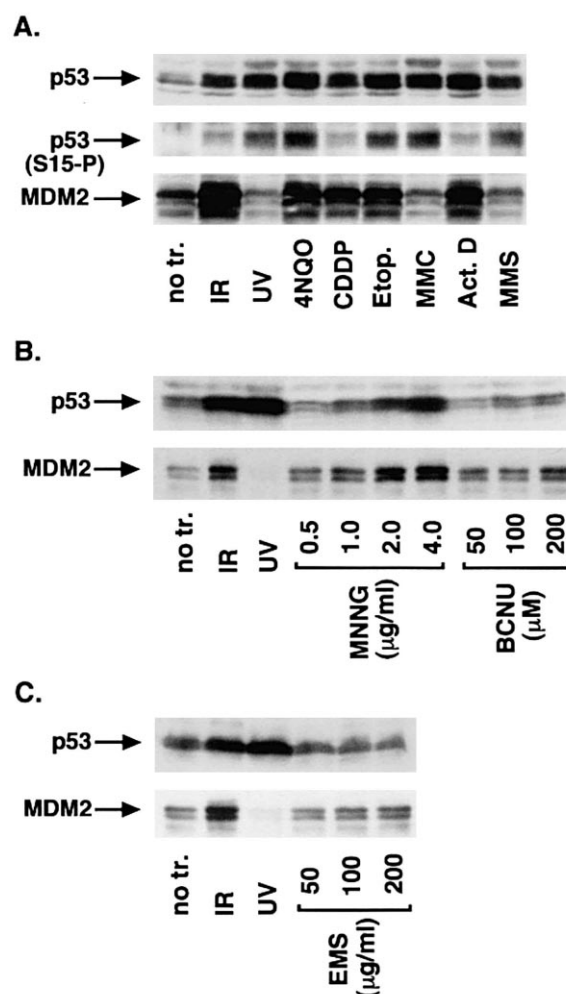


Fig. 1. Levels of p53, p53 phosphorylated at serine 15 and MDM2 in RKO cells treated with various DNA damaging agents. A: Cells were either untreated (no tr.), irradiated with 6.8 Gy ionizing radiation (IR) or 20 J/m² UV light for 5 h, or exposed to 4-nitroquinoline-1-oxide (4NQO; 0.8 µg/ml), cisplatin (CDDP; 50 µM), etoposide (Etop.; 20 µM), mitomycin C (MMC; 100 µM), actinomycin D (Act. D; 30 ng/ml) or methylmethane sulfonate (MMS; 100 µg/ml) for 6 h. Cell extracts were then prepared and examined by immunoblot analysis with the p53-specific antibody DO-1 (Ab-6), phospho-p53 at serine 15 (S15-P) antibody, and MDM2-specific antibody SMP-14. B, C: Cells were either untreated (no tr.), or treated with IR or UV radiation as in A, or exposed to the indicated concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), or ethylmethane sulfonate (EMS) for 6 h. Cell lysates were then examined by immunoblot analysis for p53 and MDM2.

p53:MDM2 binding, preventing MDM2 from promoting p53 ubiquitination (reviewed in [14]). In particular, DNA damage-induced phosphorylation of p53 at S15 or S20 has been reported to partially inhibit p53:MDM2 binding, suggesting that phosphorylation at these sites may contribute to p53 stabilization [16,17]. In the current study, we examined p53 and MDM2 levels, as well as p53 ubiquitination and phosphorylation at S15 following exposure to various DNA damaging agents.

RKO cells (p53 wild-type) were exposed to IR, UV radiation, the UV mimetic 4NQO, CDDP, etoposide, actinomycin D, or the DNA alkylating agents MMC and MMS. As shown in Fig. 1A, p53 levels were increased in response to each agent, indicating that the p53 protein was stabilized. Treated cell lysates were also probed with an antibody directed against p53 phosphorylated at serine 15. As shown in Fig. 1A, p53 was phosphorylated at S15 in response to each agent, though to different extents. In these experiments, the extent of phosphorylation at S15 was not correlated with p53 levels. For example, p53 was induced to a relatively high level following IR and actinomycin D treatment, but phosphorylated at S15 relatively little, whereas p53 was both induced and phosphorylated at S15 to relatively high levels following treatment with 4NQO, etoposide, and MMC.

MDM2 protein levels were also examined in the DNA damaged cells (Fig. 1A). MDM2 levels were increased in cells treated with IR, 4NQO, CDDP, etoposide and actinomycin D. Interestingly, however, MDM2 levels were decreased in response to UV radiation and the DNA alkylating agents MMC and MMS compared to non-treated control cells. De-

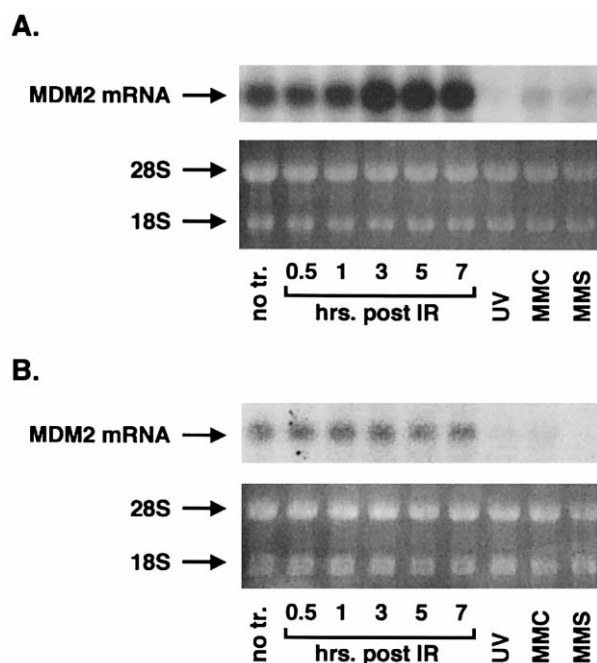


Fig. 2. Northern blot analysis of MDM2 mRNA levels in RKO (A) or SaOS-2 (B) cells treated with IR, UV light, MMC or MMS. RKO cells or SaOS-2 cells, which lack wild-type p53, were either untreated (no tr.), irradiated with 6.8 Gy IR for 0.5–7 h or 20 J/m² UV light for 5 h, or exposed to 100 μ M MMC or 100 μ g/ml MMS for 6 h. Total RNA was then isolated and the Northern blot membranes were probed with cDNA comprising MDM2 coding sequence. Levels of 18S and 28S ribosomal RNA were also indicated.

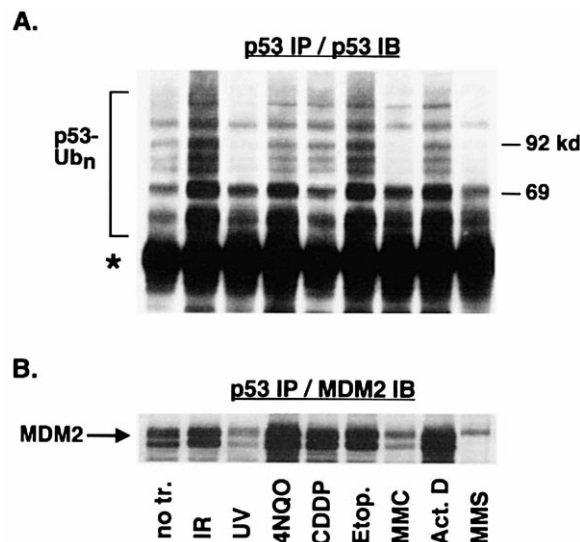


Fig. 3. Levels of ubiquitinated p53 and p53:MDM2 binding complexes. A: RKO cells were either untreated (no tr.), or treated with the indicated DNA damaging agents as in Fig. 1A. Cell lysates were then prepared, and p53 was immunoprecipitated from 2.4 mg cell lysates with the p53-specific antibody DO-1 (Ab-6). The immunoprecipitates were examined for ubiquitinated p53 (p53-Ubn) by immunoblot analysis with DO-1. Asterisk indicates the position of the Ab heavy chain used in the immunoprecipitation. B: RKO cells were either untreated (no tr.), or treated with the indicated DNA damaging agents as above. p53 was immunoprecipitated from 6.4 mg cell lysates using the p53-specific antibody PAb421 (Ab-1). The immunoprecipitates were then examined by immunoblot analysis with the MDM2-specific antibody SMP-14.

creased MDM2 levels following UV treatment is consistent with a previous report showing that UV radiation can inhibit MDM2 gene transcription [30]. However, this represents the first indication that MMC and MMS can also signal a downregulation of MDM2. We next wished to determine whether the downregulation of MDM2 is specific to MMC and MMS, or is also observed with other alkylating agents. Towards this end, p53 and MDM2 levels were determined in cells treated with the alkylating agents MNNG, BCNU (Fig. 1B) and EMS (Fig. 1C). p53 and MDM2 levels were unchanged in response to BCNU and EMS treatment. In contrast, p53 and MDM2 levels were both increased in cells treated with increasing amounts of MNNG. These results suggest that the downregulation of MDM2 protein levels is specific to MMC and MMS, and is not observed for all alkylating agents.

MDM2 mRNA levels were assessed to determine whether the decreased MDM2 protein levels reflected a decreased MDM2 gene transcription. As shown in Fig. 2A, MDM2 mRNA levels were increased 6 h after exposure to IR, consistent with p53 activating the MDM2 gene. In contrast, MDM2 mRNA levels were decreased in UV-treated cells, consistent with previous studies [30]. Importantly, MMC and MMS also downregulated MDM2 mRNA levels, indicating that the decrease in MDM2 protein levels resulted from a decrease in MDM2 gene transcription. MDM2 mRNA levels were also decreased in SaOS-2 cells (p53 null) following MMC or MMS treatment (Fig. 2B), indicating that this decrease can occur in a p53-independent fashion.

Ubiquitin:p53 conjugates are detected as a ladder of p53 bands that are recognized by multiple p53 antibodies, and by antibodies against ubiquitin [3,29]. Because MDM2 levels

were decreased by specific DNA damaging agents, we wished to determine whether ubiquitinated p53 levels were also decreased in response to these agents. p53 was immunoprecipitated from RKO cells treated with various DNA damaging agents and examined for ubiquitinated p53 with a p53 antibody (Fig. 3A). A characteristic ladder of ubiquitin:p53 conjugates was detected in untreated RKO cells. The levels of ubiquitinated p53 were increased following treatment with IR, 4NQO, CDDP, etoposide and actinomycin D (Fig. 3A), consistent with the increased MDM2 protein levels in response to these treatments (Fig. 1A). In contrast, ubiquitinated p53 levels were decreased in response to UV, MMC and MMS (Fig. 3A), consistent with the decreased MDM2 levels in cells treated with these agents. As shown in Fig. 3B, the level of p53:MDM2 binding complexes was similarly increased in response to IR, 4NQO, CDDP, etoposide and

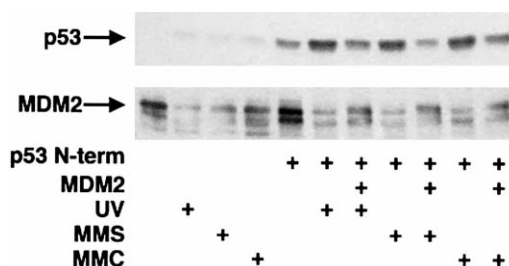


Fig. 5. Levels of p53 and MDM2 in cells transiently transfected with N-terminal phosphorylation mutant (N-term) of p53 with or without MDM2. MCF7 cells were mock-transfected, or transfected with p53 N-term (10 µg) only, or p53 N-term (10 µg) and MDM2 (10 µg) DNA. 24 h later, the cells were treated with UV light (20 J/m²), MMS (100 µg/ml) or MMC (100 µM). 5 h later, cell lysates were prepared. p53 levels were monitored by immunoblot analysis with PAb1801 (Ab-2). MDM2 levels were monitored by stripping the p53 blot and reprobing with the MDM2 antibody SMP-14.

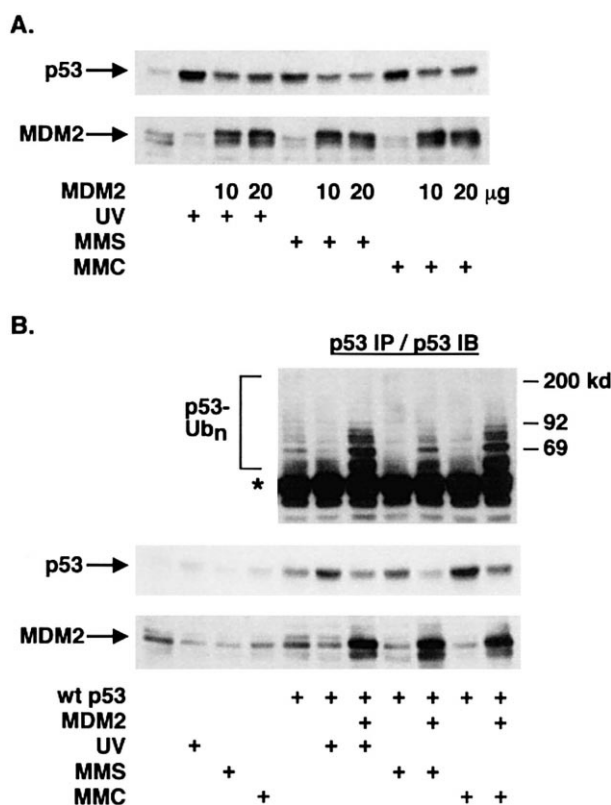


Fig. 4. A: Levels of p53 and MDM2 in cells mock-transfected or overexpressed with MDM2. MCF7 cells were mock-transfected or transfected with either 10 or 20 µg of MDM2 DNA. 24 h later, the cells were irradiated with 20 J/m² UV light, or treated with either 100 µg/ml MMS or 100 µM MMC. 5 h later, cell lysates were prepared. p53 levels were monitored by immunoblot analysis with DO-1 (Ab-6). MDM2 levels were monitored by stripping the p53 blot and reprobing with the MDM2 antibody SMP-14. B: Levels of p53, MDM2 and ubiquitinated p53 in cells transiently transfected with wild-type (wt) p53 with or without MDM2. MCF7 cells were mock-transfected, or transfected with 4 µg of wt p53 alone, or wt p53 (4 µg) and MDM2 (10 µg). 24 h later, the cells were treated with UV light (20 J/m²), MMS (100 µg/ml) or MMC (100 µM), and cell lysates were prepared 5 h later. p53 and MDM2 levels were determined by immunoblot analysis with the DO-1 and SMP-14 antibodies. Levels of p53:ubiquitin conjugates (p53-Ubn) were determined by immunoprecipitation of p53 with p53-specific antibody DO-1 followed by immunoblot analysis with DO-1. Asterisk indicates the position of the Ab heavy chain.

actinomycin D, and decreased in response to UV radiation, MMC and MMS (Fig. 3B).

Our results suggested that MMC, MMS, and UV radiation stabilize p53 by signaling a decrease in MDM2 protein levels, and a subsequent loss of p53 ubiquitination. In this case, maintaining MDM2 expression would be expected to block the stabilization of p53 in MMC-, MMS-, and UV-treated cells. To test this, cells were mock-transfected or transfected with increasing amounts of an MDM2 expression DNA, and subsequently exposed to UV radiation, MMC or MMS. As shown in Fig. 4A, each of these agents caused an increase in p53 levels in mock-transfected cells, coincident with a decrease in MDM2. In contrast, MDM2 was maintained at high levels in MMC-, MMS-, or UV-treated cells transfected with MDM2 expression DNA, and in this case the induction of p53 was partially inhibited. These results are consistent with the downregulation of MDM2 contributing to the stabilization of p53 in the MMC-, MMS-, and UV-treated cells.

Cells were next transfected with either wild-type p53 alone, or cotransfected with MDM2, and then treated with UV, MMC or MMS (Fig. 4B). The transfected p53 protein was stabilized and its levels increased following treatment of cells transfected with p53 alone, and this was coincident with decreased MDM2 expression. In contrast, overexpression of MDM2 inhibited stabilization of the transfected p53 protein (Fig. 4B). Further, ubiquitinated p53 levels were decreased in the UV-, MMC-, and MMS-treated cells transfected with p53 alone, but remained elevated in cells coexpressing p53 and MDM2 (Fig. 4B). These results indicate that MMC, MMS and UV radiation stabilized the transfected p53 protein by reducing MDM2 protein levels, and causing a consequent decrease in p53 ubiquitination.

Finally, we wished to determine whether MMC and MMS could stabilize p53 in a manner independent of phosphorylation in p53 N-terminus. Towards this end, cells were transfected with an expression DNA encoding a p53 mutant (referred to as p53 N-term) which lacks all potential N-terminal phosphorylation sites (S6, S9, S15, T18, S20, S33 and S37). Levels of p53 N-term were assessed in either untreated cells, or in cells exposed to MMC, MMS, or UV radiation. As shown in Fig. 5, levels of p53 N-term were increased in transfected cells following treatment with these agents, coincident with a decrease in MDM2 levels. These results indicate that

the ability of MMC, MMS, and UV radiation to stabilize p53 does not require phosphorylation of p53 N-terminal sites. Importantly, cotransfection of MDM2 inhibited the stabilization of p53 N-term in response to MMC, MMS, and UV treatment, indicating that the stabilization of p53 N-term resulted from a downregulation of MDM2.

4. Discussion

The mechanism by which DNA damaging agents stabilize p53 has not been fully clarified. Two proteolytic pathways have been implicated in the degradation of p53: the calpain proteolytic pathway [31,32], and the ubiquitin-dependent proteolytic pathway [10,11,18,29]. Given that p53 can be degraded through these pathways, one possibility is that DNA damaging agents signal an inhibition of p53 degradation through either the calpain pathway, the ubiquitin pathway, or both. The effect of DNA damaging agents on the calpain-mediated degradation of p53 has not been assessed. However, several studies have suggested that DNA damaging agents can inhibit p53:MDM2 binding and MDM2-dependent p53 ubiquitination. For example, IR and UV radiation can induce the phosphorylation of p53 N-terminal sites, and this phosphorylation can inhibit p53:MDM2 binding [17,33]. These findings and others have led to a model in which MDM2 binds p53 under normal conditions and promotes its ubiquitination and subsequent degradation. In response to DNA damage, phosphorylation of p53 leads to an inhibition of MDM2 binding, and thus stabilization of the p53 protein. Further support for this model comes from studies in which high doses of UV radiation inhibited p53 ubiquitination and p53:MDM2 binding in transfected and non-transfected cells [18,19].

While phosphorylation of p53 is likely to be an important determinant of p53 stability, the involvement of specific phosphorylation events has not been fully determined. In fact, phosphorylation of N- and C-terminal sites in p53 is not absolutely required for p53 to be stabilized in response to certain DNA damaging agents. For example, Ashcroft et al. [21] reported that a p53 protein mutated in all potential N- as well as C-terminal phosphorylation sites remained susceptible to stabilization induced by UV radiation and actinomycin D. Similar results were obtained using IR, UV and actinomycin D by Blattner et al. [22]. These studies indicate that certain DNA damaging agents can stabilize p53 through alternative pathways that are independent of p53 phosphorylation. While the identity of such alternative pathways has not yet been fully determined, downregulation of MDM2 expression has been suggested as a likely secondary mechanism by which UV radiation can stabilize p53 [3,19].

The current report demonstrates that the DNA alkylating agents MMC and MMS can stabilize p53 while simultaneously signaling a decrease in MDM2 mRNA and protein levels. Recent reports indicate that MDM2 is phosphorylated in response to certain DNA damaging treatments, and that this phosphorylation inhibits recognition by specific monoclonal antibodies, leading to the impression that MDM2 levels are decreased [34]. It is unknown at present if MDM2 is phosphorylated in response to MMC and MMS. However, the fact that MDM2 mRNA levels are decreased in response to these agents indicates that the decrease in MDM2 observed reflects a true decrease in MDM2 protein levels. This report repre-

sents the first demonstration that MMC and MMS have a specific effect on MDM2 gene transcription, and indicates that the stabilization of p53 in response to these agents is likely to involve the downregulation of MDM2 mRNA and protein levels. Further, the experiment using p53 N-term indicates for the first time that stabilization of p53 by MMC and MMS does not require p53 phosphorylation at N-terminus.

It is perhaps interesting that MDM2 protein levels, as well as levels of ubiquitinated p53, were increased in response to certain DNA damaging agents but decreased in response to others. These findings indicate that the stabilization of p53 in response to various DNA damaging agents is not likely to occur through a single pathway, but instead may involve multiple mechanisms that are unique to the individual DNA damaging agent being tested. The degradation of p53 by MDM2 involves several steps which could potentially be regulated in response to DNA damage, including MDM2 expression, the interaction of MDM2 with ubiquitin system enzymes, p53:MDM2 complex formation, the export of p53 from the nucleus to the cytoplasm, and the degradation of ubiquitinated p53 by the proteasome. It remains to be seen whether any or all of these steps are regulated in a DNA damaged cell.

Clinically, MDM2 appears to be a promising target of cancer therapy, since overexpression of MDM2 has been correlated with poor prognosis or advanced stage in several cancers [35,36]. Therapy aimed at inactivating MDM2 could be used to reestablish p53 activity in cancers that have a high percentage of cases where p53 is wild-type, including leukemia, neuroblastoma, and breast cancer [37]. In these cases the chemotherapeutic alkylating agents such as MMC may be useful treatment adjuvants due to the fact that they mediate a down-regulation of MDM2 protein.

Acknowledgements: This work was supported by Public Health Service grant 1R01CA80918, and by a breast cancer research grant from the Massachusetts Department of Public Health (both to C.G.M.).

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