p53 Phosphorylation at Serine 15 Is Required for Transcriptional Induction of the Plasminogen Activator Inhibitor-1 (PAI-1) Gene by the Alkylation Agent N-Methyl-N'-nitro-N-nitrosoguanidine*

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The alkylation agent N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) is a widely spread environmental carcinogen that causes DNA lesions leading to cell killing. MNNG can also induce a cell-protective response by inducing the expression of DNA repair/transcription-related genes. We recently demonstrated that urokinase-type plasminogen activator, an extracellular protease to which no DNA repair functions have been assigned, was induced by MNNG. Here, we show that the physiological inhibitor of urokinase-type plasminogen activator, PAI-1, is also induced by MNNG in a p53-dependent fashion, because MNNG induced PAI-1 in p53-expressing cells but not in p53−/− cells. MNNG induced p53 phosphorylation at serine 15, resulting in stabilization of the p53 protein, and this phosphorylation event was central for p53-dependent PAI-1 transcription. Finally, we showed that PAI-1 transcriptional induction by MNNG required a p53-responsive element located at −136 base pairs in the PAI-1 promoter, because specific mutation of this site abrogated the event. Because PAI-1 is a prognostic factor in many metastatic cancers, being involved in the control of tumor invasiveness, our finding that a genotoxic agent induces the PAI-1 gene via p53 adds a new feature to the role of the tumor-suppressor p53 protein. Our results also suggest the possibility that genotoxic agents contribute to tumor metastasis by inducing PAI-1 without involving genetic modification.

The plasminogen system is composed of an inactive zymogen, plasminogen, that is converted to its active enzyme, plasmin, by two physiological plasminogen activators (PAs),1 tissue-type plasminogen activator and urokinase-type plasminogen activator (uPA). Their activity is controlled by plasminogen activator inhibitors (PAIs), of which PAI-1 is the predominant physiological inhibitor (reviewed in Ref. 1). The plasminogen/PA system plays a key role in cancer progression, presumably via mediating extracellular matrix degradation and tumor cell invasion. It is accepted that uPA initiates a cascade of proteolysis at the cell surface, which leads to the degradation of the extracellular matrix and thereby promotes cellular migration. This is supported by the fact that uPA and its specific cell surface receptor, uPAR, are highly expressed by invasive tumor cells or by surrounding stromal cells, and they are both independent prognostic indicators in human cancer. PAI-1 is the primary physiological inhibitor of uPA. It regulates both the proteolytic activity of uPA as well as the level of uPA-uPA receptor complex by promoting its endocytosis (2, 3). Alternatively, PAI-1 may inhibit vitronectin-mediated cell adhesion and migration through its avid interaction with vitronectin (4–6). In agreement with this, the transfection of PAI-1 gene in tumor cell lines such as human prostate carcinoma PC-3 cells resulted in a decrease in primary tumor size and decreased metastasis in vivo (7). Surprisingly, however, high levels of PAI-1 are a bad prognostic factor in a number of tumors including carcinomas of the lung, ovary, breast, kidney, gastric system, and colon (8–14). Furthermore, PAI-1-deficient mice have been shown to be resistant to cancer invasion and vascularization (15). In accordance with this observation, the administration of exogenous PAI-1 to these mice was found to promote tumor growth and angiogenesis. Very recently, several reports have shed some light into the apparent paradox that PAI-1 promotes tumor invasion. Bajou et al. (16) have demonstrated that PAI/plasmin proteolysis, although essential, must be tightly controlled by PAI-1 during tumor angiogenesis, probably to allow vessel stabilization and maturation. Other studies have shown that PAI-1 may promote tumor growth through the inhibition of apoptosis, suggesting a novel function for PAI-1 (17). Because it seems that PAI-1 plays multiple roles, either beneficial or deleterious, in tumor progression, the involvement of PAI-1 in cancer deserves further investigation.

Reports from over a decade ago indicated that environmental carcinogens such as the alkylating agents mechlorethamine and N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) could induce the production of plasminogen activator in U-87MG cells, an alkylation repair-deficient (Mer−) human glioblastoma strain, at much higher levels than in alkylation repair-proficient (Mer+) U-178MG cells (18). It was concluded that plasminogen activator induction in alkylation repair-deficient human cells was caused by unrepaird DNA damage and may represent a eukaryotic SOS-like function. In fact, most of the MNNG-inducible genes identified in mammalian cells seem to be involved in DNA repair in a way similar to that of the bacterial SOS response. We have shown recently that DNA-damaging agents such as MNNG induce the expression of uPA, an extracellular protease to which no
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DNA repair functions have been assigned thus far, and determined the mechanisms responsible for this induction (19). However, the involvement of the uPA inhibitor, PAI-1, in the cell-inductive response to DNA-damaging agents has never been investigated.

Monofunctional alkylating agents such as MNNNG and methyl-methanesulfonate (MMS) are widely distributed environmental mutagens and carcinogens that upon activation react with DNA and proteins generating adducts (20–22). Among the adducts, O-6-alkyl guanine (generated by N-alkylation of the DNA base) is the predominant cytotoxic and mutagenic lesion because of its mispairing properties, which eventually leads to chromosomal aberrations, point mutations, and cell killing (23, 24). This lesion also appears to be involved in tumor induction in particular gastric carcinogenesis (25–28). However, monofunctional alkylating agents not only cause cell destruction but also induce the transcription of many genes including genes coding for transcriptions factors such as c-fos, c-jun, junB, and junD (29), for cell cycle regulatory proteins such as p53, p21, and adenomatous polyposis coli (APC) tumor suppressor (30–33), for growth arrest and DNA damage (GADD) proteins (34, 35), and for DNA repair proteins such as O-6-methylguanine-DNA-methyltransferase (MGMT) and DNA polymerase β (β-pol) (36, 37). Recent evidence suggests that the response to DNA-damaging agents may have a protective function other than DNA repair (38, 39). The main effect of genotoxic agents on the cell was believed to be chromosomal DNA damage, which in turn would provide the primary signal triggering the response (40, 41). However, genomic DNA has been ruled out as an absolute prerequisite for the induction of certain cytoplasmic signaling molecules including c-Jun N-terminal kinase activation in response to UV or MMS, because this induction was also detected in enucleated cells (38, 42, 43). Wherever generated, the alkylating signal, similar to the UV-induced signal, seems to activate specific molecules that act as sensors of damage and initiate the cellular response to genotoxic stress (reviewed in Ref. 44).

The tumor suppressor p53 is considered to be a sensor of DNA damage. It plays a central role in preserving genomic integrity by arresting cell cycle progression or activating apoptosis after genotoxic stress (reviewed in Refs. 45–47). The regulation of p53 is complex and includes post-translational events such as phosphorylation and acetylation (48). Phosphorylation at several different serine and threonine residues in p53 has been shown to occur after cells are exposed to DNA-damaging agents. The phosphorylation of serine 392 after UV exposure may be important for p53 oligomerization (48), whereas phosphorylation of N-terminal residues, in particular serine 15, after both ionizing and UV radiation is important for stabilizing p53 (49–53). Chemopreventing agents such as resveratrol and DNA-damaging therapeutic agents such as cisplatin have also been shown to induce serine 15 and serine 33 phosphorylation, and carcinogenic arsenic derivatives can also induce p53 phosphorylation of serine 15 (54, 55). Although the exact functions of specific phosphorylation events remain controversial, evidence indicates that they probably contribute to both the stabilization and activation of p53. N-terminal serine phosphorylations are believed to contribute to p53 stabilization by preventing the binding of its negative regulator MDM2 and rendering p53 more resistant to MDM2 (56, 57). In addition to potentially regulating MDM2 binding, the phosphorylation of N-terminal serines may be important for p53 interactions with the transcriptional coactivators CBP/p300 and PCAF, thus potentiating the transcription of target genes (58–60). Nevertheless, the picture of p53 phosphorylation at N-terminal sites in response to genotoxic stress is incomplete. Certain reports have shown that alkylating agents such as MMS, MNNNG, and mitomycin C, can induce an increase in p53 protein levels (33, 61, 62); however, the mechanisms underlying this effect (including phosphorylation) have not been understood fully. A recent study proposed that MMS and mitomycin C, but not MNNNG, caused down-regulation of MDM2, which resulted in p53 stabilization (63). With this limited understanding, the mechanisms by which specific alkylating agents induce an increase in p53 need further investigation.

In the present study we show that the monofunctional alkylating agent MNNNG induces p53 phosphorylation at serine 15 but not at serine 392, correlating with the MNNNG-induced stabilization of p53. We also demonstrate that MNNNG is a potent inducer of PAI-1 gene expression. Specifically, we show that the PAI-1 gene is induced by MNNNG in p53-expressing cells but not in cells lacking p53. This induction required a p53-responsive element, located at −136 bp in the PAI-1 promoter. Altogether, we provide a mechanism linking external MNNNG stimulation with the induction of the endogenous PAI-1 gene via phosphorylation of p53 at serine 15. Our results suggest that genotoxic agents may contribute to tumor metastasis by inducing PAI-1 without involving genetic modification.

EXPERIMENTAL PROCEDURES

Cell Culture—The murine NIH3T3 cell line was obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium containing 10% FBS. p53−/− and p53+/− 3T3 cell lines were provided kindly by Dr. E. F. Wagner. For MNNNG stimulation, the cells were kept in Dulbecco’s modified Eagle’s medium containing 0.5% FBS for 16 h. The next day, cells were treated with MNNNG (70 μM) for different periods of time. Alternatively, cells were UV-irradiated at 254 nm (30 J/m²). MNNNG was purchased from Sigma and dissolved in a small amount of Me 2SO and diluted with sterile water according to Raina et al. (36); the MNNNG stocks were stored in batches at −80 °C for up to 2 weeks.

Northern Analysis—Total RNA was extracted from cells using the commercial Ultraspec RNA isolation system (Biotec) based on the Chomczynski and Sacchi method (64). RNAs were size-fractionated by electrophoresis on 1% agarose gels, transferred to nylon membranes by capillarity, and UV cross-linked. Membrane prehybridization, probe hybridization, washing, and autoradiography were performed as described (65). cDNA probes used for RNA hybridizations were labeled with [α-32P]dCTP by a standard random oligo-primer reaction using the commercial Megaprime DNA labeling system (Amersham Pharmacia Biotech).

Plasmids—PAI-1 promoter-luciferase reporter plasmids, p5100-Luc, p800-Luc, p187-Luc, and p100-Luc (kindly provided by Drs. D. J. LaBonne and A. J. van Zoelen, respectively) contain 1500, 800, 187, and 100 bp of the PAI-1 promoter region and 72 bp of 5′-untranslated region (+72) as described (66, p800-Luc mt p53), containing a mutated PAI-1 p53-responsive element, was generated using the QuikChange site-directed mutagenesis kit (Stratagene) on p800-Luc according to the manufacturer protocol. The p53-responsive element of the PAI-1 promoter at position −138, was mutated from AGACATCCTGACAAAGTCC to AGACATCCTGACAAAGTCC. The pPG13-Luc plasmid contains 13 copies of a synthetic consensus p53-binding site linked to the poloma virus early promoter (60). pCMV-p53 and pCMVp53/S1A) plasmids, expressing p53 wild type and p53 containing an alanine in position 15, respectively, were provided kindly by Dr. D. W. Mekh. Western Blotting—Cells were cultured in 0.5% FBS, and at the indicated time points post-treatment, whole-cell extracts (WCEs) were prepared by lysing the cells in 20 mM HEPES, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 2.5 mM MgCl2, 2 mM orthovana- 

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a 10% SDS-polyacrylamide gel electrophoresis. Alternatively, phospho-
ylated p53 was detected using anti-phospho-p53 (serine 15) and anti-
phospho-p53 (serine 392) antibodies (Cell Signaling-NEB, 9224S and
9281S, respectively). Immunoblots were developed using the ECL de-
tection system (Amersham Pharmacia Biotech).

Transfections Assays—For transient transfections assays, reporter
plasmids were transfected using the liposome-mediated transfection reagent/N-[(-2,3-dioleoylxyloproplyl)N,N,N-trimethylammonium meth-
ysulfate (Roche Molecular Biochemicals). 1.5 × 10⁵ cells were seeded
overnight on 6-well dishes. The next day, cells were cotransfected with
1 μg of PAI-1-luciferase plasmid and 0.5 μg of RSV-βGal, as internal
controls. After transfection, the cells were cultured in Dulbecco’s modi-
ﬁed Eagle’s medium containing 0.5% FBS for 16 h before MNNG stim-
ulation (70 μM, and reporter activities were analyzed after 8 h of
MNNG treatment. When indicated, the cells were cotransfected with
1 μg of reporter plasmid and 5 or 500 ng of p53 expression plasmids or
empty vector alone together with 0.5 μg of internal control. Firefly
luciferase activities were standardized for β-galactosidase activity used
as internal control. All transfection/reporter assays were repeated at
least three times, showing less than 25% variability. A Student’s t-test
was used to validate the results.

For stable transfection assays, NIH3T3 cells were seeded at 10⁶
cells/100-mm dish in duplicate cultures and cotransfected the following
day with 10 μg of PAI-1-Luc constructs and 1 μg of pRSV-neo plasmid
using N-[(-2,3-dioleoylxyloproplyl)N,N,N-trimethylammonium methyl-
sulfate transfection reagent. Two days later, 6418 (Sigma) was added to
the culture medium, and selection proceeded for 15 days. More than 300
colonies were pooled for each construct, expanded, and analyzed for
luciferase activity, which was standardized to the transfection effi-
ciency of each plasmid. To assess transfection efﬁciency, the gene copy
number was measured by Southern blotting. Brieﬂy, DNA was ex-
tracted, digested with SstI and XhoI, size-fractionated, and blotted.
Hybridization was performed with α-32P-labeled luciferase probe as
described in Ref. 67.

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts
were obtained from NIH3T3 cells before and after MNNG treatment.
The extraction of nuclear proteins was performed as described by
Miralles et al. (68). Brieﬂy, the cells were washed twice in cold PBS and
dissolved and the cellular pellet was resuspended in 10 mM HEPES, pH
7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, and 0.5 mM dithiothreitol
on ice. The cells were passed ﬁve times through a 26-gauge needle and
centrifuged to collect nuclei, which were subsequently resuspended in
an equal volume of 10 mM HEPES, pH 7.9, 0.4 mM NaCl, 1.5 mM MgCl₂,
0.1 mM EGTA, 0.5 mM dithiothreitol, and 5% glycerol to allow the
eution of nuclear proteins by gentle shaking at 4 °C for 30 min. The
nuclei were pelleted at 14,000 rpm for 5 min at 4 °C, and the superna-
tant was aliquoted, snap-frozen in liquid nitrogen, and stored at
−80 °C until use. All solutions contained the protease inhibitors leupeptin
and aprotonin at 1 μg/ml, phenylmethylsulfonyl ﬂuoride (0.5 mM), and
benzamidine (1 mM). A Bio-Rad protein assay was used to determine the
protein concentration. For electrophoretic mobility shift assays, 10 μg of
nuclear extracts were incubated in 50 mM Tris-HCl, pH 7.9, 12.5 mM
MgCl₂, 1 mM EDTA, 1% glycerol, 0.5 mM phenyl-
methylsulfonyl ﬂuoride, and 2 µg of poly(dI-dC) for 10 min at room
temperature to titrate out nonspeciﬁc binding before the addition of
15,000–20,000-cpm labeled oligonucleotide, and the reaction was
further incubated for 20 min at 30 °C. When indicated, nuclear extracts
were incubated with 0.2 μg of anti-p53 antibody pAb421 (Calbiochem,
Ab-1). When unlabeled competing oligonucleotides were added, nuclear
extracts were pre-incubated for 30 min at room temperature before the
addition of the labeled probe. Samples were loaded on a pre-run 5%
polyacrylamide gel (29:1 in 0.25 TBE) and electrophoresed at 200 V.
The gels were dried and autoradiographed at −80 °C.

The sequences of the sense strands of the oligonucleotides used in
the EMSAs are: p53-PAI-1, 5′-ACAACATAGCTGTACAGAAGTCCTGAGA-3′
and IgkB, 5′-CAGAGGGCAGCTTTGCCAGA-3′.

RESULTS

MNNG Induces PAI-1 mRNA Expression—We recently demon-
strated the inducibility of the uPA gene by the alkylating
agent MNNG (19). In this study, we investigated the inducibility
of the uPA physiologic inhibitor, PAI-1, in the NIH3T3
ﬁbroblast cell line. As shown in Fig. 1A, treatment of NIH3T3
cells with MNNG increased PAI-1 transcript levels. PAI-1
mRNA induction was not caused by an unspeciﬁc up-regulation
of RNA synthesis, because MNNG did not signiﬁcantly modify

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PAI-1 promoter. To address this possibility, we generated a PAI-1 promoter construct containing a specific mutant PAI-1 promoter. Because MNNG is a DNA-damaging agent and p53 is activated upon DNA damage, suggesting a functional link between the two, we considered the possibility that the PAI-1 p53-responsive element mediates the effect of MNNG on the PAI-1 promoter. To address this possibility, we generated a PAI-1-promoter-luciferase construct containing a specific mutation in the p53-responsive element (p800-Luc(mt p53)), and the luciferase inducibility by MNNG between this mutation-containing construct and its corresponding wild type was compared. Fig. 2B shows that mutation of the p53 site totally abrogated luciferase induction by MNNG in NIH3T3 cells. This result demonstrated the requirement of the p53 recognition site for PAI-1 transcriptional activation by MNNG.

**p53 Protein Binding to the p53-responsive Element of the PAI-1 Promoter Is Induced by MNNG**—We next investigated whether the p53 site of the PAI-1 promoter showed increased p53 binding activity after treatment of the cells with MNNG (Fig. 3). Nuclear extracts of nontreated and MNNG-treated NIH3T3 cells were prepared at 0, 0.5, 1, 3, and 6 h after treatment, and protein binding to the sequence of the PAI-1 p53 site was analyzed by EMSA. Detection of specific binding of endogenous p53 protein to p53 consensus sites by EMSA has proven to be difficult (70); however, the presence of an antibody against the C terminus of the p53 molecule can enhance and stabilize p53 DNA binding (70, 71). Accordingly, EMSA was performed with NIH3T3 nuclear extracts in the absence or presence of the p53 antibody pAb421 (an antibody raised against the C terminus of p53 amino acids 370–378). As shown in Fig. 3A, in the presence of the anti-p53 antibody, binding of p53 to the PAI-1 p53 site was enhanced and supershifted in MNNG-treated NIH3T3 cells but not in untreated cells. When a cold oligonucleotide of the same sequence was used as a specific competitor, the formation of the complex (Fig. 3B) was competed out, whereas an oligonucleotide of an unrelated sequence (the 3′B site of the Igκ enhancer, IgκB) did not affect the formation of the complex (Fig. 3B), further demonstrating the specificity of the DNA-protein interaction. Altogether, these results demonstrated that MNNG induces p53 binding to the PAI-1 promoter. They also indicated that p53 may influence PAI-1 promoter activity after binding to the PAI-1 p53 site. Therefore, we hypothesized that p53 might be mediating endogenous PAI-1 gene activation by MNNG.
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Fig. 4. Induction of endogenous PAI-1 gene expression by MNNG depends on a functional p53. A, induction of p53 protein levels by MNNG in different fibroblast cell lines. WCEs were prepared as detailed under “Experimental Procedures” from cells differing in p53 content (cells derived from p53-deficient 3T3 mice (p53+/−/3T3) and from parental mice (p53+/+/3T3) as well as from NIH3T3 cells), before and after 2 h of stimulation with MNNG. 10 μg of WCEs were analyzed by Western blotting using an anti-p53 antibody. B, Northern analysis of PAI-1 induction by MNNG in cells containing or lacking the intact p53 site, were not significantly different in the absence of p53. However, in the presence of p53 (by cotransfection of 500 ng of pCMV-p53), a 6–8-fold increase of luciferase expression was observed from the constructs containing the p53 binding site (p187-Luc and p800-Luc) but not from the constructs with a mutated or without the p53 site (p800-Lucmt p53) and p100-Luc, respectively, strongly indicating the p53-dependent transactivation of the PAI-1 promoter (Fig. 5B).

Impact of p53 deletions on PAI-1 promoter activity. A, induction of PAI-1 promoter activity in p53-deficient NIH3T3 fibroblasts, established from NIH3T3 cells, confirming that these cells are p53-deficient. B, p53 protein accumulated in both NIH3T3 and in p53+/−/3T3 fibroblasts after MNNG treatment but not in p53−/−/3T3 cells, clearly confirming that these cells are p53-deficient.

To investigate whether the presence of p53 has any effect on the induction of the endogenous PAI-1 gene product, we analyzed PAI-1 mRNA induction by MNNG in p53+/+ and p53−/−/3T3 cells. As shown in Fig. 4B, in p53+/+/3T3 fibroblasts, PAI-1 mRNA was induced after MNNG treatment, being the induction maximal after 2 h; in contrast, no significant increase in PAI-1 mRNA levels was observed in p53-deficient fibroblasts under identical MNNG treatment. These results clearly demonstrated that p53 expression is required for induction of the PAI-1 gene by MNNG.

Overexpression of wild-type p53 can mimic MNNG-induced PAI-1 promoter activity. We have observed that the levels of p53 protein are increased after MNNG treatment in NIH3T3 and p53+/−/3T3 cells (Fig. 4A). Similarly, PAI-1 promoter activity and gene expression were increased after MNNG treatment in these cells (Figs. 1A, 2A, and 4B). These results are consistent with the idea that p53 levels are limiting in these cells and that increased levels of p53 may be necessary for induced PAI-1 transcriptional activity. If this is the case, then overexpression of p53 should mimic the effect of MNNG treatment on the transcriptional activity of the PAI-1 promoter. To test this idea, p53−/− cells were transiently transfected with p800-Luc with or without two amounts of the p53 expression vector pCMV-p53, and PAI-1 promoter activity was determined by luciferase measurement after MNNG stimulation. As expected, no PAI-1 promoter induction by MNNG was observed in p53−/− cells. However, transfection of 5 ng of pCMV-p53 was sufficient to restore the inducibility of the PAI-1 promoter by MNNG. Moreover, overexpression of p53 (by cotransfection of 500 ng of pCMV-p53) was able to induce PAI-1 promoter activity to levels similar to those obtained after MNNG treatment. These results demonstrated that an increase in p53 protein level is required for PAI-1 transcriptional induction after DNA damage.

Wild-type p53 can induce PAI-1 promoter activity in p53-deficient cells. To evaluate further whether p53 can regulate PAI-1 transcriptional activity in p53-negative cells, p53−/−/3T3 cells were transiently transfected with different PAI-1 promoter-reporter constructs with or without the p53 expression vector pCMV-p53, and the PAI-1 promoter activity was determined by luciferase measurement. As expected, luciferase activities generated by the different PAI-1 promoter constructs, whether containing or lacking the intact p53 site, were not significantly different in the absence of p53. However, in the presence of p53 (by cotransfection of 500 ng of pCMV-p53), a 6–8-fold increase of luciferase expression was observed from the constructs containing the p53 binding site (p187-Luc and p800-Luc) but not from the constructs with a mutated or without the p53 site (p800-Lucmt p53) and p100-Luc, respectively, strongly indicating the p53-dependent transactivation of the PAI-1 promoter (Fig. 5B).

Taken together, these results demonstrate that increased p53 protein levels are sufficient to induce PAI-1 promoter activity, mimicking the MNNG-induced effect. They also show that MNNG induces the PAI-1 gene by augmenting p53 levels.

Induction of p53 phosphorylation at serine 15 by MNNG. We have shown above that MNNG treatment augments the levels of p53 protein in NIH3T3 and p53+/+3T3 cells (Fig. 4A). To unravel the underlying molecular mechanism, we first analyzed the effect of MNNG on p53 mRNA levels in p53+/+3T3 and in p53−/−/3T3 cells. As shown in Fig. 6A (left panel), the levels of full-length p53 mRNA were not modified by MNNG treatment in p53+/+3T3 cells, indicating that MNNG was not affecting the rate of p53 gene transcription or p53 mRNA stability (Fig. 6A, left). Similar results were obtained with NIH3T3 cells (data not shown). Nevertheless, the level of p53 protein increased time-dependently after MNNG treatment in these cells, being maximal at 3 h after treatment (Fig. 6B). As expected, a faster migrating p53 mRNA species corresponding to the neo-p53 transgene was detected in p53−/−/3T3 cells, confirming that these cells are p53-deficient (Fig. 6A, right). These results suggested that the regulation of p53 protein stability, rather than mRNA levels, was the mechanism responsible for the MNNG-induced increase in p53 protein levels (see Fig. 4A).

It has been reported that phosphorylation of p53 is associated with its stabilization (reviewed in Ref. 46). Because serine 15 and serine 392 phosphorylation of p53 has been implicated in cellular responses to several types of DNA damage, including UV irradiation (49, 56), we investigated whether p53 was phosphorylated in vivo at those two residues in NIH3T3 cells treated with MNNG. The phosphorylated status at serine 15 and serine 392 was analyzed in these cells by Western blotting using phospho-specific antibodies that recognized p53 phosphorylated at serine 15 (P-Ser15-p53) and at serine 392 (P-Ser392-p53). As shown in Fig. 6C, MNNG caused phosphorylation of p53 at serine 15 but not at serine 392 in NIH3T3 cells. In
contrast, high levels of phosphorylation of both residues were observed in these cells after 6 h of UV-C treatment (used as control) (Fig. 6C), which were accompanied also by high levels of p53 protein (Fig. 6B). The phosphorylation of p53 at serine 15 was maximal 3 h after MNNG treatment (Fig. 6C), coinciding with the time course of maximal accumulation of the p53 protein after treatment with this alkylating agent. These results strongly suggest that the increase in p53 protein levels that occurs on MNNG treatment in NIH3T3 cells is associated with phosphorylation of serine 15 but not of serine 392.

Mutation of p53 at Serine 15 Reduces PAI-1 Promoter Inducibility by MNNG—To determine whether the modification of serine 15 could influence p53-dependent transactivation of the PAI-1 promoter in response to MNNG, we compared the effects of overexpression of wild-type p53 and mutant p53 (p53S15A), in which serine 15 was converted to alanine, on luciferase expression from transiently transfected p800-Luc. As shown in Fig. 7, in response to MNNG wild-type p53 was more potent than the mutant in activating PAI-1 promoter in p53-deficient cells. As a control of template, we employed the luciferase reporter gene pPG13-Luc (60), which contained 13 copies of a p53-responsive element in the promoter. As shown for p800-
p53-mediated PAI-1 Induction by MNNG

Genotoxic agents such as UV light irradiation and nonfunctional alkylating carcinogens cause cytotoxic and mutagenic lesions, which lead eventually to chromosomal aberrations, point mutations, and cell killing (23, 24). These agents also trigger a rapid, highly regulated adaptive response known as the cellular stress response, which involves coordinate control of signaling events leading to the induction of many genes. The gene-inductive response to UV has been analyzed extensively, and it is known to promote the transcription of genes coding for transcription factors, growth factors, viral proteins, and proteases (reviewed in Ref. 44). However, less is known about the inductive response to alkylating agents such as MMS and MNNG. These agents induce the early expression of several proto-oncogenes including c-fos, c-jun, junB, and junD, although to different levels (29). They also induce the level of cell cycle regulatory/tumor suppressor proteins such as p53, p21, and adenosomatous polyposis coli (30–33) and of DNA repair proteins such as O-6-methylguanine-DNA methyltransferase and DNA polymerase β (36, 37) as well as growth arrest and DNA damage-inducible proteins (34, 35). However, no additional cellular targets of alkylating agents other than those related to gene transcription or DNA repair processes have been identified in mammalian cells. Interestingly, we recently found that the expression of uPA, an extracellular serine protease to which no DNA repair function has been assigned thus far, can be induced both by UV and the alkylating agent MNNG via a mechanism involving c-Jun N-terminal kinase activation of activator protein 1 (19, 68). Here, we have shown that PAI-1, the physiological inhibitor of uPA proteolytic activity, the known activity of which is also exerted extracellularly, is also transcriptionally induced by MNNG. This induction is mediated by an enhanced level of p53 protein, which is brought about by its increased stability caused by the phosphorylation of serine 15. Cotransfection of a mutated form of p53 at serine 15 resulted in reduced PAI-1-promoter activation by MNNG compared with the wild-type p53, confirming the importance of serine 15 phosphorylation in the activation of the PAI-1 gene. Finally, we found that a p53-responsive element located in the proximal promoter region of the PAI-1 promoter is required for transcriptional induction by MNNG, because specific mutation of this element abrogated the induction. Taken together, this is the first functional dissection of a transcription-coupled signal transduction pathway activated by MNNG involving p53.

It has been established that the p53 tumor suppressor protein plays a pivotal role in cellular responses to DNA-damaging events (reviewed in Refs. 46 and 74). Our work showed that this is also the case in MNNG induction of the PAI-1 gene in NIH3T3 cells. Several forms of DNA damage have been shown to activate p53, including those generated by ionizing radiation, chemotherapeutic drugs, UV radiation, and alkylating agents (reviewed in Refs. 46, 54, 55, and 74); however, the effects of these later agents on p53 are much less understood (33, 61, 62). p53 accumulation seems to occur mainly through alterations of p53 protein stability, although changes in p53 gene expression have also been reported (reviewed in Ref. 46). Accordingly, we observed that MNNG treatment did not change the level of p53 mRNA but increased the level of p53 protein in NIH3T3 cells, providing an argument for this being the mechanism operating in the induction of the PAI-1 gene in these cells. Several reports have shown that stabilization of the p53 protein results from specific phosphorylation events; in particular, the phosphorylation of different N-terminal serine and threonine residues located within or very close to the MDM2-binding domain of p53 have been shown to contribute to p53 stabilization by preventing the binding of its negative regulator MDM2 and rendering p53 more resistant to MDM2-mediated degradation (46, 74). Other studies highlight the serine 15 of p53, among several N-terminal phosphorylated residues, as the key phosphorylation target in response to DNA damage (49–53). In this study, we found that MNNG induces p53 phosphorylation at serine 15 in conjunction with MNNG-induced stabilization. Although it had been shown that alkylating agents could induce p53 phosphorylation at serine 392 in a human lymphoblastoid cell line (62), we did not detect any phosphorylation at this position in response to MNNG in NIH3T3 cells, possibly because of cell-specific differences. However, we observed phosphorylation at serine 392 after UV-C irradiation in NIH3T3 cells, as reported previously by other groups (48, 52, 75–77). These data demonstrate that MNNG induces p53 stabilization through the phosphorylation of serine 15 but not serine 392 in NIH3T3 cells.

It has been shown that, besides increasing protein stability, serine 15 phosphorylation of p53 stimulates the transactivation activity of p53 through its increased binding to the p300/CREB coactivator proteins (58–60). In accordance with this, we observed in transient transfection assays that the p53S15A mutant is less potent than wild-type p53 in mediating MNNG-induced PAI-1 promoter activity (or in mediating MNNG-induced activity of a synthetic promoter harboring 13 copies of a p53 binding site). Our results show that the loss of serine 15 in the p53 protein resulted in a significant reduction of PAI-1 induction by MNNG. These results are in agreement with those of Dumaz and Meek (60) showing that serine 15 mutation of the p53 protein resulted in partial inhibition, but not in a complete loss, of transactivation activity. These data demonstrate that the phosphorylation of p53 at serine 15 is clearly relevant for PAI-1 transcriptional induction by p53. Furthermore, the finding that MNNG induces phosphorylation of p53 at serine 15 adds to the emerging idea that serine 15 is a focal point for stress-targeted activation of p53.

Reports from over a decade ago indicate that the alkylating agents mechlorethamine and MNNG could induce the production of plasminogen activator in U-87MG cells, an alkylation repair-deficient (Mer−/) human glioblastoma strain, at much higher levels than in alkylation repair-proficient (Mer+/+) U-178MG cells (18). It was concluded that plasminogen activator induction in alkylation repair-deficient human cells is caused by unrepaired DNA damage and may represent a eukaryotic SOS-like function. In fact, most of the MNNG-inducible genes identified in mammalian cells seem to be involved in DNA repair in a way similar to that of the bacterial SOS response. However, several reports have suggested that the mammalian stress response to genotoxic agents was involved in a protective function other than DNA repair. In particular, c-fos/−/− cells are hypersensitive to MMS and MNNG (39), suggesting that MMS/MNNG-induced c-Fos/activator protein 1 is an essential component of the cellular defense mechanism against the cytotoxic effects of alkylating agents. The results shown in this study indicate that the alkylating carcinogen MNNG induces the expression of the extracellular protease inhibitor PAI-1. Then what is the physiological significance of PAI-1 induction by genotoxic agents? Exposure of cells to MNNG and most other DNA-damaging agents results not only in damage to DNA but also in damage to other cellular components including biomembranes and proteins either directly or after oxidative stress (78). A simple protective mechanism against damage to such components would consist of replacing them with newly synthesized ones. As already mentioned, exposure of cells to DNA-damaging agents can increase DNA repair capacity and activate cell cycle checkpoints, but such exposures may also induce enzymes that metabolize toxins to facilitate their elimina-
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