DNA Methyltransferase Inhibition Induces the Transcription of the Tumor Suppressor p21<sup>WAF1/CIP1/sdi1</sup>*

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Previous lines of evidence have shown that inhibition of DNA methyltransferase (MeTase) can arrest tumor cell growth; however, the mechanisms involved were not clear. In this manuscript we show that out of 16 known tumor suppressors and cell cycle regulators, the cyclin-dependent kinase inhibitor p21 is the only tumor suppressor induced in the human lung cancer cell line, A549, following inhibition of DNA MeTase by a novel DNA MeTase antagonist or antisense oligonucleotides. The rapid induction of p21 expression points to a mechanism that does not involve demethylation of p21 promoter. Consistent with this hypothesis, we show that part of the CpG island upstream of the endogenous p21 gene is unmethylated and that the expression of unmethylated p21 promoter luciferase reporter constructs is induced following inhibition of DNA MeTase. These results are consistent with the hypothesis that the level of DNA MeTase in a cell can control the expression of a nodal tumor suppressor by a mechanism that does not involve DNA methylation.

DNA methylation of cytosine residues located at the dinucleotide sequence CpG can suppress expression of genes either by directly interfering with the binding of transcription factors (1) or by attracting methylated-DNA binding proteins such as MeCP2 (2–4). To ascertain that the epigenetic information encoded in the methylation pattern is faithfully maintained, the expression of the maintenance DNA MeTase<sup>1</sup> enzyme DNMT1, which catalyzes the transfer of a methyl group from S-adenosyl-methionine to the 5’ position on cytosines residing at the dinucleotide CpG (5), is tightly coordinated with DNA replication and the state of growth of the cell (7). Different protooncogenic pathways can up-regulate <i>dnmt1</i> expression (8), and high levels of <i>dnmt1</i> mRNA have been observed in many cancer cells (9, 10). It has been proposed that increased DNA MeTase levels are a downstream component of oncogenic programs (11) and that they play a causal role in cellular transformation (12, 13). This hypothesis has been supported by the observation that a reduction in DNA MeTase levels by either 5-aza-deoxycytidine (13, 14), DNA MeTase antisense mRNA or antisense oligonucleotides can reverse tumor growth in vivo and in vitro (13–15).

The mechanisms responsible for cellular transformation by DNMT1 and the reversal of transformation by DNA MeTase inhibition are unknown. One attractive hypothesis is that high levels of DNA MeTase lead to ectopic methylation and inactivation of tumor suppressor genes such as p16 (16). Similarly, the inhibition of DNA MeTase could result in demethylation and lead to activation of p16 (17, 18). However, because both the increase in DNA MeTase levels and its inhibition by pharmacological inhibitors are global processes, it is difficult to understand how they could predictably result in a discrete change of the methylation state of specific sites. A more likely explanation is that ectopic methylation of tumor suppressors in tumor cells is a slow and stochastic process, but the aberrant methylation events are selected because they confer a growth advantage. This hypothesis is supported by the observation that in tumors bearing one mutant and one normal allele of p16, only the normal allele is methylated (19). Similarly, partial inhibition of DNA MeTase with pharmacological agents results in a stochastic demethylation of a specific site only in a fraction of the cells at each round of replication.

An alternative possibility is that the DNMT1 protein might have a more direct and immediate effect on the state of cellular growth and transformation (20). To investigate this possibility we inhibited DNA MeTase and examined the expression of genes known to control the cell cycle. In the past, 5-aza-deoxycytidine has been used as the standard inhibitor of DNA MeTase (21). Unfortunately, the incorporation of 5-aza-deoxycytidine into DNA and subsequent trapping of DNA MeTase enzyme unto the replicating DNA (22) results in pleiotropic effects that confound the interpretation of the results (23). Therefore, we have developed two other approaches to inhibit DNA MeTase: antisense oligonucleotides (15) and oligonucleotide-based DNA MeTase antagonists that form a stable complex with DNA MeTase and inhibit its activity at an EC<sub>50</sub> of 60 nm (24, 25). We have previously shown that inhibition of DNA MeTase by DNA MeTase antagonists results in a rapid inhibition of DNA replication that is inconsistent with a stochastic model (25). In this manuscript we demonstrate that inhibition of DNA MeTase results in the rapid induction of the known tumor suppressor and cell cycle regulator p21 by a mechanism that does not involve DNA methylation of the p21 promoter.

**EXPERIMENTAL PROCEDURES**

Cell Culture, Antisense, and DNA MeTase Antagonist Treatment—
A549 cells, a human non-small cell lung carcinoma cell line (26) (ATCC: CCL 185), were grown in Dulbecco’s modified Eagle’s medium (low glucose) supplemented with 10% fetal calf serum and 2 mM glutamine. HEK 293 cells, a human adenovirus type 5 transformed human embryonal kidney cell line (27) (ATCC, CRL 1573), were grown in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum and 2 mM glutamine. For oligonucleotide treatment A549 cells were plated at a concentration of 2.5 × 10<sup>5</sup> cells/100-mm tissue culture dish 18 h prior to treatment. The DNA MeTase antagonist (3118) used in our study is a phosphorothioate modified hemimethylated hairpin of the following sequence: 5’-CTGAA(methyl)CGGAT(methyl)CGTTTCGATCCGTTCAG-3’. The sequence of the inactive analog used in our study (3188) is identical; it is also phosphorothioate modi-
Fig. 1. Inhibition of DNA MeTase induces p21 mRNA. A, a multi-probe RNase protection assay was performed using two sets of probes and 15 μg of total RNA isolated from A549 cells treated for 5 days as follows. In the left two panels the cells were treated with antisense oligonucleotide to dnmt 1 mRNA (as), antisense mismatch control (mm), and the mock treated control (ctrl); in the right two panels the cells were treated with DNA MeTase antagonist (3188), inactive analog of the antagonist (3188), and the mock treated control (ctrl). The RNAs protected with the probes are indicated by the arrows. No signals were detected with the following probes: p16, p18, p57, and p14/15.

B

DNA MeTase

p53

p21

imido black

p130

GAPDH

p18

E2F1

E2F4

L32

time in days

treatment

1

2

5

A

GAPDH

p130

GAPDH

p18

E2F1

E2F4

L32

time in days

treatment

1

2

5

p53

p27

p21

p3

DPI

DPI

p18

L32

Fig. 2. Inhibition of DNA MeTase induces p21 protein. Shown is Western blot analysis of total cell extracts isolated from A549 cells treated with either antisense oligonucleotides to dnmt 1 mRNA (as), antisense mismatch control (mm), DNA MeTase antagonist (3188), inactive analog of the antagonist (3188), or the mock treated controls (ctrl) for 1, 2, or 5 days. The positions of p21 protein (A) and DNA MeTase protein (B) are indicated by arrows.
amplification using the first set of primers described below. PCR products were used as templates for subsequent PCR reactions utilizing nested primers. The PCR products of the second reaction were then subcloned using the Invitrogen TA cloning Kit (as recommended by the manufacturer), and the clones were sequenced using the T7 Sequencing Kit (Amersham Pharmacia Biotech). The primers used for the amplification of the p21 genomic region (GenBank™ accession number U24170) were: 5'-GATAAATAGTTAGTGTG-GG-3' (sense, starting at 4278), 5'-CTCACCTCCTCTAAATACC-3' (reverse, starting at 4640), 5'-AGGGATTGGGGGAGGAG-3' (nested sense, starting at 4342), and 5'-AGGATTGGGGGAGGAG-3' (nested reverse, starting at 4566).

Northern Blot Analysis—RNA was isolated by the guanidinium isothiocyanate method (32). p21 mRNA level was determined by Northern blot analysis using 10 μg of RNA and hybridization with 32P-labeled human p21 probe (positions 205–544, accession number U03106). The levels of expression of p21 mRNA were quantified by densitometric scanning (MasterScan, Scanalytics) and normalized in each lane to the amount of total RNA as determined by hybridization with 32P-labeled 18 S ribosomal RNA oligonucleotide probe (33).

Luciferase Assays—The calcium-phosphate precipitation method was used to transiently co-transfect HEK 293 cells with either 2 μg of promoterless luciferase reporter or luciferase reporter constructs containing 2145 or 94 base pairs of the p21 promoter upstream of the transcriptional start site (34). The cells were co-transfected with 5 μg of either control pCR3.1 DNA (Invitrogen) or a dmntl (base pairs 396–5066) antisense expression vector in pCR3.1 (as). 1 μg of pEGFP-C1 (CLONTECH) was included with all transfections to control for transfection efficiency by fluorescence microscopy. The luciferase activity was assayed as described previously (35).

DNA MeTase Activity Assay—DNA MeTase activity was assayed using 3 μg of HEK 293 nuclear extract protein prepared as described previously (7). The reaction was carried out in a final volume of 30 μl containing 10 mM Tris-HCl, 25% glycerol, 5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 0.15 M NaCl, 1 μl of S'-methyl-3Hadenosyl-L-

Fig. 3. The time course of induction of p21 mRNA levels by the DNA MeTase antagonist. A, total RNA was isolated from control A549 cells (ctrl) and A549 cells treated with DNA MeTase antagonist (3118) or its inactive analog (3188) for 8 h (0.3 days). 1 μg of RNA prepared from control and treated cells was reverse transcribed into cDNA in the presence of radiolabeled nucleotides. Equal counts of labeled cDNA (competitor) and a varying number of competitor molecules were used as templates for competitive PCR reactions with primers targeted to p21 mRNA sequence. PCR reactions with primers targeted to the p21 genomic region (GenBank™ accession number U24170) were: 5'-GTAAAAAAAAGTTAGATTTGTGG-3' (sense, starting at 4278), 5'-CTCACCTCCTCTAAATACC-3' (reverse, starting at 4640), 5'-AGGGATTGGGGGAGGAG-3' (nested sense, starting at 4342), and 5'-AGGATTGGGGGAGGAG-3' (nested reverse, starting at 4566). B, quantification of competitive PCRs performed as in A on the RNA extracted from cells treated for 0.3, 1, 2, and 5 days. The p21 mRNA levels extracted from cells treated with DNA MeTase antagonist (3118) are represented relative to those treated with inactive analog (3188) which were assigned the value of 1.

Fig. 4. Part of the p21 promoter which partially overlaps with the p21 CpG island is completely unmethylated. A, p21 promoter region between +1 and −243 was bisulfite mapped. DNA extracted from control A549 cells was treated with sodium bisulfite, which converts all the cytosines in DNA into thymines but cannot convert methylated cytosines. One of the modified strands was amplified by PCR and cloned into the TA cloning vector as described under “Experimental Procedures.” Depending on the orientation of the cloned insert, either C's or G's will be present only in the methylated positions on the sequencing gel. In this case the presence of a band in the G lane indicates the presence of a methylated cytosine on the complimentary strand. Lollipops indicate unmethylated CpG sites. B, physical map of the partial p21 promoter showing the density of CpG sites (small vertical lines). p53 binding sites and the RNA start site (+1) are indicated with arrows at the bottom. The arrows at the top indicate the primers used to bisulfite map the region shown in A.
methylone (78.9 μM; Amersham Pharmacia Biotech) as a methyl donor, and 0.2 mM of a synthetic hemimethylated double-stranded oligonucleotide as a methyl acceptor as described previously (7). Following a 3-h incubation at 37 °C, the incorporation of methyl groups into the DNA substrate was determined by scintillation counting of tritiated acid precipitable counts. The background counts reflecting methylation of other macromolecules present in the nuclear extract as well as endogenous DNA methylation were determined by incubating the nuclear extract under the same conditions in the absence of the hemimethylated substrate. The background values were subtracted from the values obtained for each point. The results are presented as the averages of three determinations.

RESULTS

Induction of p21 by DNA Methyltransferase Inhibitors—To determine whether inhibition of DNA MeTase in cancer cells results in induction of the expression of tumor suppressors, we treated A549 human lung carcinoma cells with either antisense oligonucleotides (as) directed against dnmt1 mRNA or their mismatched control (mm), or a DNA MeTase antagonist (3118) and its inactive control (3188) for 5 days. The DNA MeTase antagonist was previously shown to inhibit DNA MeTase activity in nuclear extracts prepared from control A549 cells (8, 65 nM; Ref. 25). The antisense oligonucleotide was previously shown to prevent the production of detectable levels of DNMT1 protein at an EC50 of 60 nM (data not shown). Two different methods of inhibition of DNA MeTase were used to exclude the possibility that the changes observed represented a nonspecific side effect. RNA prepared from the treated cells was subjected to an RNase protection assay with two sets of multiple probe templates that revealed the level of expression of 16 known cell cycle regulators and tumor suppressors. As indicated in Fig. 1, p21 was the only known tumor suppressor that was induced by both treatments. p21 protein levels are also induced upon treatment with both DNA MeTase antisense oligonucleotides and antagonists as determined by the Western blot analysis shown in Fig. 2A.

Time Course of p21 Induction by DNA MeTase Antagonists—The kinetics of induction of p21 following inhibition of DNA MeTase can provide an indication as to the mechanism involved. We chose to use the DNA MeTase antagonist (3118) because it directly interacts with DNA MeTase protein. In contrast, the antisense acts indirectly by reducing mRNA levels, which leads to the reduction of protein levels, and the turnover rate of DNA MeTase might confound the interpretation of the results. Any inhibitor of DNA methylation will have its most pronounced effect at the time of DNA replication. Therefore, the fraction of cells that are demethylated at a specific subset of regulatory sites will increase with the number of replication rounds. The level of p21 mRNA following DNA MeTase antagonist treatment was quantified by competitive PCR, which showed that the induction of p21 mRNA was rapid (7-fold after 8 h) and that it decreased over the period from 8 h to 5 days (Fig. 3B). These results are consistent with a mechanism that does not involve demethylation of specific sites. The competitive PCR also verified the induction of p21 following treatment with a DNA MeTase antagonist observed in the RNase protection experiments.

There is a discrepancy between the time course of induction of p21 mRNA and protein levels. The highest level of p21 mRNA induction is observed shortly after treatment (8 h) (Fig. 3), whereas the levels of p21 protein seem to increase up to 5 days following treatment (Fig. 2A). Similarly, although DNMT1 protein is undetectable within 1 day after antisense treatment (Fig. 2B), p21 protein levels continue to increase up to 5 days (Fig. 2A). A possible explanation is that the turnover rate of protein is slower than that of the mRNA, resulting in gradual accumulation of protein over time following induction of p21 transcription. Another possibility is that inhibition of DNA MeTase induces p21 protein levels by an additional post translational mechanism as recently suggested by Fournel et al. (36). Further experiments are required to clarify this question.

Part of the p21 Promoter That Overlaps with the p21 CpG Island Is Not Methylated in Untreated A549 Cells—DNA methylation is unlikely to play a role in the modulation of p21 gene expression because the p21 gene is active even in the untreated cells (Fig. 1). DNA methylation is believed to be an on-off switch in gene expression (37). CpG islands present in the promoter regions have been shown to be susceptible to hypermethylation in many cancer cells (38) and are prime candidates to be involved in regulation of their respective genes. To study the state of methylation of p21 promoter, we performed a bisulfite mapping of the portion of the p21 promoter that also partially overlaps with the p21 CpG island (Fig. 4B). The portion tested (−243 to +1) contains no methylated CpGs in the untreated cells (Fig. 4A). These results are consistent with the hypothesis that DNA MeTase antagonists do not induce p21 mRNA levels by demethylation of its promoter.

Inhibition of DNA MeTase Does Not Detectably Induce p53 Binding to Its Consensus Binding Sequence—p21 plays a central role in the response to DNA damage and has been shown to contain p53 binding sites in its promoter region and to be a downstream effector of p53. Our results do not support the hypothesis that p53 mRNA is induced following treatment with DNA MeTase inhibitors (Fig. 1). An alternative possibility is that either the presence of the oligonucleotide inhibitors or the inhibition of DNA methylation are interpreted as DNA damage resulting in an activation of p53 and an increase in p53 DNA

\[ \text{p53/PAb421 DNA complex} \]

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Binding to p53 recognition sequences (29). To test this hypothesis, we performed the electrophoretic mobility gel shift assay shown in Fig. 5. Treatment of A549 cells with the DNA damaging agent actinomycin D (10 μg/ml) results in activation of p53 and binding to its consensus sequence. This produces a super-shifted complex stabilized by anti-p53 antibody pAb421 as previously shown (29). In contrast, treatment with DNA MeTase inhibitors does not activate p53 consensus sequence binding.

Induction of p21 Is Not Post-transcriptionally Regulated—Several reports have indicated that the stability of the p21 mRNA could be altered by different signals such as differentiational (39) and oxidative stress (40). It has also been shown that p21 mRNA contains a conserved element in its 3′-untranslated region that is bound by the Elav-like mRNA stabilizing proteins (41). To determine whether DNA MeTase antagonists increase the stability of p21 mRNA, we treated A549 cells with either the DNA MeTase antagonist (3118) or the inactive analog (3188). After 4 h, the mRNA transcription inhibitor actinomycin D (10 μg/ml) was added to the treated cultures and an untreated control, and the level of p21 mRNA was determined by a Northern blot analysis at different time points after initiation of actinomycin D treatment. As observed in Fig. 6, DNA MeTase antagonist treatment does not considerably alter the stability of p21 mRNA, suggesting that induction of p21 mRNA occurs at the transcriptional level.

DNA MeTase Inhibition Induces the Expression of p21 Promoter Luciferase Reporter Construct—p21 luciferase reporter constructs were used to confirm that the induction of p21 occurred at the transcriptional level. Plasmids amplified in Escherichia coli, which do not bear a CpG MeTase, are fully unmethylated. Therefore, these experiments directly test the hypothesis that induction of p21 transcription by inhibition of DNMT1 occurs by a mechanism that is independent of methylation of the p21 promoter. HEK 293 cells were used in these experiments because they are easily transfected. The results presented in Fig. 7A show that inhibition of DNMT1 by expression of a full-length antisense dnmt1 mRNA results in induction of both p21 promoter luciferase reporter constructs. An assay of DNA MeTase activity confirmed that the transient transfection of the dnmt1 antisense construct resulted in a significant reduction of DNA MeTase activity in the transfected population of HEK cells (Fig. 7B). To rule out the possibility that the p21 promoter luciferase reporter constructs are de novo methylated in HEK cells and that antisense treatment inhibits this activity, we subjected DNA prepared from transiently transfected HEK cells to digestion with either HpaII, which is sensitive to methylation at the CCmGG sequence, or MspI, which is insensitive to this methylation. The digested DNA was subjected to a Southern blot analysis using the entire p21 promoter luciferase reporter construct as a probe. Methylation in any one of the HpaII sites would have resulted in partially digested fragments. The Southern blot does not reveal partially cleaved HpaII fragments, thus suggesting that no significant de novo methylation of the studied HpaII sites occurs in HEK cells (Fig. 7C).

The induction of p21 by DNMT1 inhibition does not require the presence of sequences upstream to the minimal promoter (−94) and therefore does not require the presence of p53 recognition elements. This also confirms that the induction of p21 promoter activity by DNA MeTase inhibition is independent of the methylation status of the promoter at sequences other than those mapped in the bisulfite sequencing experiment (Fig. 4).

**DISCUSSION**

Inhibition of DNA MeTase has been previously shown to arrest the growth of tumor cells (13–15, 25); however, the mechanisms involved are not clear. An attractive hypothesis is that inhibition of DNA MeTase results in the demethylation of tumor suppressors that were previously inactivated by methylation. In support of this hypothesis it has been shown that the tumor suppressor p16, which is methylated and inactive in many tumor cells (16), could be activated by prolonged treatment with an inhibitor of DNA methylation, 5-aza-deoxycytidine (16–18).

In this manuscript we tested the hypothesis that alternative mechanisms are involved in the arrest of cell growth by DNA MeTase inhibitors. For this reason A549, a NSCLC cell line with a homozygous deletion of p16INK4, was chosen to address...
FIG. 7. Inhibition of DNA MeTase increases p21 promoter activity. A, HEK 293 cells were co-transfected with the luciferase reporter constructs depicted and either a control vector (pcDNA) or a vector expressing dnmt1 mRNA in the antisense orientation (as). The luciferase activity found in whole cell protein extracts isolated 48 h after transfection was measured. B, DNA MeTase activity was measured in nuclear extracts obtained from HEK 293 cells harvested 48 h following transfection with either pcDNA or as. C, a Southern blot of DNA isolated from HEK 293 cells transfected with the full-length p21-luciferase construct (UD), following digestion with MspI (methylated) or HpaII (methylation insensitive) enzymes. The whole plasmid was radiolabeled and used as a probe.

this question (42). Two novel inhibitors of DNA MeTase that inhibit DNA MeTase by different mechanisms were used to increase the confidence that the effects observed were a consequence of DNA MeTase inhibition. Out of 16 genes known to regulate the cell cycle, only one, the housekeeping gene p21, was shown to be induced following inhibition of DNA MeTase.

The negative effects of DNA MeTase inhibitors on the growth of A549 cells, which we have reported previously (25), can be explained by an induction of p21. The ectopic expression of p21 has been shown to arrest the growth of tumor cells (43), and through its inhibition of cyclin dependent kinases p21 is known to block entry into S and G phase progression (44, 45). These functions allow p21 to play a role in mediating stop signals such as those triggered by terminal differentiation and contact inhibition (46, 47). In addition, p21 can directly arrest DNA replication in response to DNA damage by binding to proliferating cell nuclear antigen (48).

Unlike what is expected from the p16 model, the induction of p21 expression is rapid (7-fold induction 8 h after the treatment) and points to a novel mechanism independent of DNA methylation. Our experiments confirm that at least a portion of the p21 promoter is not normally methylated (Fig. 4) and demonstrate the ability of DNA MeTase inhibition to induce expression of a luciferase reporter gene under the control of an unmethylated p21 promoter (Fig. 7).

A recent publication showed that antisense inhibition of dnmt1 mRNA in a bladder carcinoma cell line T24 results in an increase in p21 protein levels (36). Our paper complements these results by (a) showing that the same results are produced when DNA MeTase is directly inhibited by a hemi-methylated hairpin phosphorothioate oligos as well as when inhibited by antisense oligos, (b) demonstrating the induction of p21 in a second, independent cell line, (c) providing strong evidence that the increase in p21 protein after DNA MeTase inhibition is primarily a consequence of p21 mRNA induction, and (d) demonstrating that p21 induction does not require a change in the methylation state (normally unmethylated) of its promoter.

It is still unclear whether p21 induction is triggered by a decline in DNA MeTase activity or DNA MeTase amount. Further experiments are required to address this question. In any case, the ability of DNA MeTase to modulate p21 levels, as well as compete with p21 for its binding site on proliferating cell nuclear antigen (49), suggests that the increased DNA MeTase expression observed in tumor cells may override p21 dependent stop signals and result in unregulated growth.

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