The Methyl Donor S-Adenosylmethionine Inhibits Active Demethylation of DNA

A CANDIDATE NOVEL MECHANISM FOR THE PHARMACOLOGICAL EFFECTS OF S-ADENOSYLMETHIONINE

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S-Adenosylmethionine (AdoMet) is the methyl donor of numerous methylation reactions. The current model is that an increased concentration of AdoMet stimulates DNA methyltransferase reactions, triggering hypermethylation and protecting the genome against global hypomethylation, a hallmark of cancer. Using an assay of active demethylation in HEK 293 cells, we show that AdoMet inhibits active demethylation and expression of an ectopically methylated CMV-GFP (green fluorescent protein) plasmid in a dose-dependent manner. The inhibition of GFP expression is specific to methylated GFP; AdoMet does not inhibit an identical but unmethylated CMV-GFP plasmid. S-Adenosylhomocysteine (AdoHcy), the product of methyltransferase reactions utilizing AdoMet does not inhibit demethylation or expression of CMV-GFP. In vitro, AdoMet but not AdoHcy inhibits methylated DNA-binding protein 2/DNA demethylase as well as endogenous demethylase activity extracted from HEK 293, suggesting that AdoMet directly inhibits demethylase activity, and that the methyl residue on AdoMet is required for its interaction with demethylase. Taken together, our data support an alternative mechanism of action for AdoMet as an inhibitor of intracellular demethylase activity, which results in hypermethylation of DNA.

S-Adenosylmethionine (AdoMet) is the main methyl donor in numerous methyltransferase reactions in all organisms (1). The reduced derivative of 5,10-methylenetetrahydrofolate, 5-methyltetrahydrofolate, provides the methyl group for methionine and AdoMet synthesis (2). A series of rodent experiments as well as epidemiological data have suggested a correlation between diets deficient in folate or in sources of methyl groups (i.e. foods containing methionine, one-carbon compounds, and choline) and the risk for colorectal adenomas and cancer (3). Such diets, referred to collectively as methyl-deficient, have been shown to promote liver cancer in rodents (4, 5), and AdoMet treatment was shown to prevent the development of liver cancer in rat (6).

In light of the clinical and epidemiological data suggesting a link between AdoMet levels and cancer, it is important to understand the tumor protective mechanism of action of AdoMet, as well as the tumor promoting action of methyl-deficient diets. This is of importance not only for realizing the therapeutic potential of AdoMet, but also for unraveling basic mechanisms of tumorigenesis, especially the role of methyl group metabolism. AdoMet is the cofactor for transmethylation reactions including DNA methylation (7, 8), whereas S-adenosylhomocysteine (AdoHcy) is the product of transmethylation reactions and an inhibitor of DNMTs (9). A current model is that exogenous administration of AdoMet increases the intracellular ratio of AdoMet to AdoHcy, thus stimulating DNMT activity resulting in increased DNA methylation (6, 10). An increase in AdoHcy concentrations, even without a concomitant reduction in AdoMet results in inhibition of DNMT and DNA hypomethylation (11). Methyl-deficient diets decrease intracellular AdoMet concentration, increase AdoHcy concentrations, and trigger DNA hypomethylation (5, 12, 13). A genetic link was established between polymorphisms in the *methyltetrahydrofolate reductase* gene encoding the enzyme catalyzing the synthesis of 5-methyltetrahydrofolate, and DNA hypomethylation (14, 15). Global hypomethylation of DNA is a hallmark of cancer (16, 17). If the mechanism of action of methyl-rich diets in cancer chemoprevention and methyl-deficient diets in cancer promotion is through changing genomic methylation status, then it implies that global hypomethylation plays a causal role in cancer. This hypothesis is supported by the observation that 5-azacytidine, a DNMT inhibitor (18) can reverse AdoMet-mediated chemoprevention of liver carcino genesis (19).

Although it has been controversial, there is now little doubt (1) that exogenous AdoMet increases the intracellular AdoMet levels. AdoMet uptake into cells has also been verified through a high performance liquid chromatography analysis (20). A number of data support the notion that exogenous AdoMet causes hypermethylation of DNA (10, 21).

Whereas this model provides an attractively simple explanation as to the possible relationship between exogenous AdoMet administration and DNA methylation, there are a number of unresolved issues. First, increased AdoMet should increase DNMT activity only if the normal intracellular concentration of...
AdoMet is below the $K_m$ for the enzyme, this has not been demonstrated as of yet. It is, however, possible that the main mechanism by which elevating AdoMet levels increases DNMT activity is by competing with AdoHcy, an inhibitor of DNMT. Such an indirect mechanism of activation might be relevant even if the basal level of AdoMet is above the $K_m$. Second, even if exogenous administration of AdoMet increases the activity of DNMT, it is not clear whether the normal level of enzyme is limiting, or whether the specificity of DNA methylation patterns is determined by the molecular activity of DNMT. There is no evidence to suggest that specific sites remain unmethylated in vertebrate genomes simply for the reason that the molecular activity of DNMT is limiting. Third, methyl-deficient diets cause hypomethylation in the liver whose cells are mostly postmitotic and do not replicate (5). If the mechanism of this hypomethylation involves only inhibition of DNMT, it could take effect only in cells that actively synthesize DNA. Because measurable demethylation is seen with these diets, this could only occur if a significant fraction of liver cells proliferate during the treatment. Whereas an increase in proliferation in the liver is seen as a consequence of methyl-deficient diets, it is not clear whether proliferation precedes or follows global demethylation.

The current hypothesis on the mechanism of action of AdoMet is based on the assumption that DNA methylation is a unidirectional and irreversible reaction, which is catalyzed by DNMT exclusively. However, an increasing list of data suggests that inhibiting histone acetylation inhibits active demethylation activity. We have recently used this assay to illustrate that a protein that inhibits histone acetylation inhibits active demethylation in living cells (31).

In this paper we took advantage of this assay to test the hypothesis that exogenous administration of AdoMet inhibits demethylase activity in living cells. Using an in vitro demethylase assay, we then tested whether AdoMet inhibits both recombinant MB2/dMTase activity extracted from infected SF9 insect cells, as well as endogenous demethylase activity from HEK 293 cells. Taken together, our results support a new alternative hypothesis for the mechanism of action of AdoMet as a DNA hypermethylating agent.

**MATERIALS AND METHODS**

**In Vitro Methylation of Substrates**

CMV-GFP (pEGFP-C1 from Clontech; GenBank™ accession number U55763) was methylated in vitro by incubating 10 μg of plasmid DNA with 12 units of Sss1 CpG methyltransferase (New England Biolabs) in the recommended buffer containing 800 μM AdoMet for 3 h at 37 °C. Twelve units of Sss1 and 0.16 amol of AdoMet were then added and the reaction was further incubated for 3 additional hours. The methylated plasmid was recovered by phenol/chloroform extraction and ethanol precipitation, and complete methylation was confirmed by observing full protection from HpaII digestion.

**Cell Culture and Transient Transfections**

Human embryonic kidney HEK 293 cells (ATCC CRL 1573) were plated at a density of 7.5 × 10^5/well in a 6-well dish and transiently transfected with 80 ng of CMV-GFP (methylated or mock methylated) using the calcium phosphate precipitation method as described previously (32). 0.3 μM TSA was added 24 h post-transfection. After an additional 24 h, cells were treated with or without various concentrations (0–5 μM) of AdoMet or AdoHcy. Cells were harvested 72 h post-transfection. Each experiment was performed in triplicate, and experiments were performed several times using different cultures of HEK 293 cells.

**Western Blot Analysis**

Whole cell extracts were prepared using radioimmunoprecipitation assay buffer according to the Santa Cruz Biotechnology protocol, and protein concentrations were determined using the Bradford reagent (Bio-Rad). 2.5 μg of protein were resolved on a 12.5% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (Amersham Biosciences). After blocking the nonspecific binding with 5% skim milk, GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-3534) at 1:500 dilution, followed by peroxidase-conjugated anti-rabbit IgG (Sigma) at 1:5000, and enhanced chemiluminescence detection kit (Amersham Biosciences). Membranes were stained with 0.2% Ponceau S (Sigma) to determine loading of total protein in each lane. Both the Western blots and Ponceau-stained membranes were quantified using NIH Image 1.62 software, and the GFP signal was normalized to the total protein (which varied only slightly) in each lane.

**Southern Blot Analysis**

DNA was extracted from HEK 293 cells using the DNeasy Tissue Kit (Qiagen). DNA was first digested with 50 units of EcoRI, followed by digestion with 20 units of either HpaII or MspI restriction enzymes. Samples were subjected to electrophoresis on a 1.5% agarose gel and then transferred to Hybond-N membrane (Amersham Biosciences). Blots were probed with a 32P-labeled CMV-GFP cDNA probe (AvaiI-Cfr101 fragment) synthesized using a random priming labeling kit (Roche Diagnostics). Membranes were hybridized at 68 °C for 4–6 h in a buffer containing 0.5 M sodium phosphate, pH 6.8, 1 mM EDTA, 0.75 SDS, and 0.2 mg/ml herring sperm DNA. Following hybridization, the membranes were washed twice for 10 min in a 5% SDS, 0.04 M sodium phosphate, pH 6.8, 1 mM EDTA solution, and then four times for 10 min in the same solution containing 1% SDS. The demethylation assay measures the fraction of GFP molecules that were demethylated using HpaII restriction enzyme, which cleaves unmethylated CCGG but does not cleave methylated CCGG sequences. The methylated GFP DNA remains intact following HpaII digestion and is identical to the fragment obtained following EcoRI digestion (indicated by M in Fig. 2B), whereas the unmethylated GFP DNA is cleaved by HpaII resulting in a 0.5-kb fragment (indicated by U in Fig. 2B). We scanned each HpaII digested lane and measured the intensity of the total signal hybridizing with the GFP probe in the same HpaII lane (including the unmethylated U and methylated M fragments), this value is equal to 100% of GFP molecules in the lane. We then determined the intensity of the unmethylated signal per HpaII lane, and divided this value (U) by the total signal for GFP (U + M) in the same HpaII lane. To exclude the
possibly that the HpaII digestion was skewed by differences in loading, we used the ethidium bromide-stained gels as loading controls for the corresponding Southern blots. We normalized the values obtained upon the calculation [M(U + M) + 100] to the amount of DNA in each HpaII lane as determined by quantification of the ethidium bromide-stained gels by NIH Image 1.62. The results of three independent experiments were quantified by densitometry (NIH Image 1.62).

AdoMet Preparations for in Vitro Studies
AdoMet was prepared as a 50 mM solution in distilled water by dissolving lyophilized powder (Sigma) in distilled water. AdoHcy was purchased from Sigma and dissolved in distilled water at a 50 mM concentration.

Purification of Recombinant MBD2/dMTase from Sf9 Cells
A fragment containing human MBD2/dMTase was excised from pCB2.1-dMTase (24) with BamHI and XhoI and transferred to the Baculovirus expression transfer vector pBlueBacHis2 C (Invitrogen). PBlueBacHis2 C-MBD2/dMTase and Bac-N-Blue viral DNA were co-transfected into the Sf9 insect cell line, and recombinant viruses were isolated, identified, and amplified according to the manufacturer’s protocol (Invitrogen) with no modifications. High titer SF viral stocks were used to infect 500 ml of Sf9 insect cells cultured in spinner flasks to a density of 2.5 x 10^6 cells/ml. Insect cell homogenates were harvested by scraping in cold phosphate-buffered saline. Cell pellets from 10 liters of culture were frozen and kept at -70°C until they were used for enzyme purification. Frozen pellets were thawed in 5 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 500 mM NaCl, 0.05% Tween 20, 10% glycerol, and 10 mM imidazole) containing 1 μg/ml of the following protease inhibitors: aprotinin, leupeptin, and Pefabloc. Protease inhibitors were added to all the solutions used in the purification. The homogenates were subjected to two cycles of freezing and thawing (5 min per step). DNA in the homogenate was sheared by passing through an 18.5-gauge needle 10 times. The extracts were then subjected to 15 cycles of sonication (10 s burst, 10 s gap per cycle at 20% of maximal output). The extracts were centrifuged at 10,000 x g for 35 min. The supernatant was transferred into a fresh tube and was recentrifuged for additional 25 min at 15,000 x g. The extract was filtered through a 5-micron filter to remove any particulate matter and the buffer was exchanged on a PD-10 buffer exchange column. DNA containing fractions were again concentrated as described above and concentrated on a Microcon 10, and subjected to a second Nap5 desalting column. DNA containing fractions were again concentrated as described above.

S-Adenosylmethionine Inhibits Active DNA Demethylation
In Vitro Demethylation Assay
A typical reaction mixture (50 μl) consisted of 25 ng of 32P-labeled DNA (prepared as described above) incubated in demethylation buffer (10 mM Tris-HCl, 5 mM MgCl₂, pH 7.0) with either the purified MBD2/dMTase (5 μl, ~5 ng) or the purified demethylase activity from HEK 293 cells (5 μl) for 24 h at 37 °C. The reaction mixture was boiled for 5 min in the presence of AdoMet and AdoHcy, respectively. The DNA was extracted from the enzyme by incubation in 2 volumes of DNA extraction buffer (10 mM Tris-HCl, 0.5 mM MgCl₂, 1% SDS), containing 0.1 unit of proteinase K (Roche Diagnostics) at 50 °C for 2 h. Subsequent phenol-chloroform extraction (one part of each phenol or chloroform per three parts of reaction volume) in the presence of RNA (50 μg) as a carrier and ethanol precipitation with salt and 95% ethanol resulted in almost quantitative recovery of the input DNA. The DNA pellets were resuspended in distilled water (8 μl) and digested with micrococcal nuclease to 32P-labeled 3′ mononucleotides as described elsewhere (27, 33). The labeled mononucleotides were separated by thin layer chromatography and visualized by autoradiography on a phosphorimaging plate. The levels of cytosine (C) and 5-methylcytosine (mC) were quantified by the MCID-M software (Imaging Research Inc.). The percent demethylation (C/(C + mC)) was calculated per each sample and then normalized to the value obtained for the demethylase reaction in the absence of inhibitor (0 μM AdoMet/AdoHcy).
S-Adenosylmethionine Inhibits Active DNA Demethylation

RESULTS

AdoMet Inhibits TSA-induced Active Demethylation of Ectopically Methylated and Transiently Transfected CMV-GFP in a Dose-dependent Manner—There have been several reports demonstrating that exogenous administration of AdoMet leads to DNA hypermethylation (19, 21, 35). Similarly, other studies have shown that a decrease in dietary folate, or a depletion of intracellular AdoMet, results in DNA hypomethylation (6, 12, 36, 37). However, it is not known whether the effects of AdoMet on methylation are because of changes in DNMT or DNA demethylase activities.

We utilized a previously described transient transfection-based assay system (Fig. 1 and Ref. 30) to study the effects of AdoMet on active demethylation of ectopically methylated DNA. In prior studies we have shown that the in vitro methylated CMV-GFP reporter plasmid is actively demethylated 72 h following transfection into HEK 293 cells when histone hyperacetylation is induced with TSA (30). Because CMV-GFP does not replicate nor is it de novo methylated in HEK 293 cells (30), this assay specifically measures active demethylation in a living cell.

We first determined the effects of increasing doses of AdoMet, or the product of AdoMet-dependent methyltransferase reactions, AdoHcy, on the demethylation of methylated CMV-GFP (Fig. 2). DNA was isolated from HEK 293 cells transfected with methylated CMV-GFP DNA and treated with either TSA and AdoMet, or TSA and AdoHcy. DNA was first linearized with the EcoRI restriction enzyme, followed by digestion with MspI (which cleaves the sequence CCGG) or HpaII (which cleaves the sequence CCGG only when it is not methylated). The demethylated HpaII-digested 529-bp fragment (U) and the methylated HpaII-undigested DNA (M) were quantified within the same lanes, and the percent methylation for CMV-GFP DNA in each lane was determined as $\frac{\text{M} - \text{U}}{\text{M}} \times 100$. Values were then normalized to the total DNA per lane as determined by ethidium bromide staining. As can be seen in Fig. 2, A and C, the addition of TSA results in nearly complete demethylation of CMV-GFP by endogenous demethylase activity, as indicated by the complete HpaII digestion of CMV-GFP to the 529-bp fragment (U). Upon the addition of increasing concentrations of AdoMet (Fig. 2, B and D), the percentage of methylated GFP remaining increases in a dose-dependent manner, illustrated by the decrease in the ratio of the 529-bp HpaII fragment (U) to the undigested DNA (M). AdoHcy has an insignificant effect on the demethylation of CMV-GFP (Fig. 2, B and D), indicating that the methyl moiety of AdoMet is required for inhibition of demethylation.

We then determined whether AdoMet stimulates de novo methylation of an identical unmethylated CMV-GFP substrate. Fig. 2E illustrates that unmethylated CMV-GFP, transfected under identical conditions, does not get de novo methylated, even in the presence of 8 mM AdoMet. This indicates that AdoMet does not cause an increase in DNMT activity on ectopic CMV-GFP. Thus, the likely mechanism by which AdoMet causes hypermethylation of CMV-GFP in comparison with the TSA-treated control is by inhibiting its active demethylation by resident demethylases.

AdoMet Reduces TSA-induced Expression of Methylated CMV-GFP in a Dose-dependent Manner, but Has No Effect on Unmethylated CMV-GFP—A number of studies have shown that an increase in AdoMet inhibits gene expression (20, 21), however, it is not clear whether AdoMet specifically affects genes whose methylation state it alters exclusively, or whether it has a nonspecific effect on gene expression. We took advantage of the CMV-GFP system described above to address this question. We determined whether AdoMet influences the expression of either methylated CMV-GFP, whose methylation state is affected by AdoMet, or unmethylated CMV-GFP, whose methylation state is not affected by AdoMet. HEK 293 cells were transiently transfected and treated with TSA and either AdoMet or AdoHcy, as described in the previous section. Extracts were then prepared and subjected to a Western blot analysis using an antibody directed against GFP protein.

Fig. 3, A and C, illustrates that methylated CMV-GFP is completely repressed in untreated HEK 293 cells. This is as expected, because it is well documented that DNA methylation leads to gene silencing. The addition of TSA leads to a dramatic induction of GFP expression as expected from the complete demethylation following TSA treatment. Upon the addition of increasing amounts of AdoMet, GFP expression is decreased in a dose-dependent fashion. AdoHcy has no significant effect on the expression of methylated GFP, consistent with its lack of effect on DNA demethylation.

Because our system measures expression and demethylation that is dependent on histone hyperacetylation, there are two possible mechanisms whereby AdoMet exerts its effects on demethylation (Fig. 1). AdoMet could directly inhibit a demethylase activity, or it could inhibit histone acetylation, which we have previously shown leads to an inhibition of demethylation (30). If the latter were true, then AdoMet should also inhibit the TSA-induced expression of unmethylated GFP, whose expression is induced by histone acetylation as well. Fig. 3, B and D, indicates that this is not the case, because AdoMet has no significant effect on the induction of unmethylated GFP by TSA. The fact that AdoMet specifically affects the expression of a methylated copy of CMV-GFP, and not an unmethylated copy, supports the model that AdoMet inhibits gene expression by directly inhibiting the active demethylation of methylated CMV-GFP.

AdoMet but Not AdoHcy Inhibits Demethylation Activity in Vitro—To further confirm that the observed effect of AdoMet and AdoHcy is because of inhibition of active demethylation and not an indirect effect, in vitro studies with a recombinant MBD2/dMTase (the only demethylase characterized thus far) were performed (24). Because it is not certain whether MBD2/dMTase is responsible for the demethylation seen in HEK 293 cells, we also performed these studies with purified endogenous demethylase activity from HEK 293 cells. Together, these in
vitro experiments should test whether AdoMet can act as an inhibitor of one or more demethylase activities.

First, His-tagged MBD2/dMTase was partially purified by chromatography on Q-Sepharose from cell extracts of Sf9 cells infected with the recombinant MBD2/dMTase construct as described under “Materials and Methods.” Fractions were eluted with a stepwise gradient of NaCl and assayed for demethylation activity with a32P-prelabeled methylated DNA from *M. lysodeikticus* (Fig. 4, A, left panel). Conversion of methyl-dCMP to dCMP, whereas not extensive, was almost exclusively detected with the 0.4 M NaCl fraction (16% demethylation). This correlates with the peak presence of the His-tagged recombinant MBD2/dMTase protein in this fraction as demonstrated by Western blot analysis (Fig. 4B) using an anti-Xpress antibody. To confirm that the 0.4 M NaCl fraction contained demethylase activity, the fraction was concentrated 10-fold on a Microcon concentrator. As expected, the demethylase activity in the 0.4 M NaCl fraction increased accordingly (Fig. 4A, right panel); 5 μl of the concentrated fraction completely converted methyl-dCMP to dCMP for the same amount of DNA as used before (Fig. 4A, left panel).

Next, we determined whether AdoMet inhibits the demethylation activity of MBD2/dMTase. The aforementioned DNA was incubated with MBD2/dMTase from Sf9 cells in the presence of increasing AdoMet concentrations, and conversion of methyl-dCMP (mC) to dCMP (C) was assessed as above. Fig. 5 presents the autoradiography and quantification of one representative experiment. Because of the numerous steps involved in this assay, it is impossible to avoid small loading differences between samples. To determine percent activity, the percent demethylation, (C/(C + mC)), was thus calculated within each sample to control for these differences, and then normalized to

Fig. 2. *AdoMet inhibits active demethylation of CMV-GFP*. A, B, and E, either in vitro methylated CMV-GFP plasmid (m-GFP) (A and B), or unmethylated CMV-GFP (GFP) (E) were transiently transfected into HEK 293 cells. Cells were treated with a final concentration of 0.3 μM TSA (+TSA), or left untreated (-TSA), and increasing concentrations of either AdoMet or AdoHcy (2, 4, and 8 mM) were added. Cells were harvested 72 h post-transfection, and the methylation status of CMV-GFP was determined by *MspI/HpaII* restriction digestion and Southern blot analysis as outlined under “Materials and Methods” and in the legend to Fig. 1. M, methylated and *HpaII* undigested GFP; U, unmethylated and *HpaII* fully digested GFP (529 bp); B, *lower panel*, ethidium bromide-stained gels. C, the results of three independent experiments as shown in A were quantified by densitometry, and the average percent methylation remaining for each sample was calculated as outlined under “Materials and Methods” and charted ± S.D. D, the results of three independent experiments as shown in B were quantified as in C and the averages ± S.D. are presented. O, AdoMet; □, AdoHcy.
the value obtained for the demethylase reaction in the absence of inhibitor (0 mM AdoMet/AdoHcy). Conversion of methyl-dCMP to CMP was greatly reduced at 0.5 mM AdoMet and abolished completely at concentrations higher than 0.7 mM.

In contrast to AdoMet, no inhibition of demethylation occurred in the presence of increasing concentrations of AdoHcy (Fig. 5B). These results indicate that the small differences in the chemical structure (methyl group and positive charge on the sulfur) between AdoMet and AdoHcy are responsible for their different interactions with the MBD2/dMTase.

To test whether AdoMet inhibits endogenous HEK 293 demethylase activity, we extracted demethylase from HEK 293 cells using Q-Sepharose fractionation as previously described (27) and incubated it with increasing concentrations of AdoMet. The results shown in Fig. 5C indicate that, similar to recombinant MBD2/dMTase, the demethylase activity extracted from HEK 293 cells is inhibited by 50% at 0.5 mM AdoMet. Taken together, the above experiments demonstrate that AdoMet can inhibit the in vitro demethylase activity of recombinant MBD2/dMTase as well as endogenous demethylase extracted from HEK 293 cells.

AdoMet and AdoHcy compete for binding to the catalytic site on DNMTs. It was therefore proposed that the ratio of AdoHcy to AdoMet determines DNMT activity as discussed in the Introduction. AdoHcy inhibits DNMTs whereas increased AdoMet offsets this inhibition. We therefore determined whether a similar relationship applies to MBD2/dMTase. A competition experiment between AdoMet and AdoHcy is presented in Fig. 5D. Increasing concentrations of AdoHcy were added in the presence of an inhibitory concentration of AdoMet (10 mM), in a series of demethylation reactions. The results of this experiment illustrate that even a 10-fold concentration excess of AdoHcy to AdoMet does not diminish inhibition of the demethylase reaction by AdoMet. This is consistent with the hypothesis that AdoMet has a higher affinity for MBD2/dMTase as compared with AdoHcy. Further studies are necessary to elucidate the mode of inhibition: whether AdoMet is a competitive inhibitor with the substrate DNA or an allosteric
inhibitor as was demonstrated for methylene tetrahydrofolate reductase (39). Furthermore, we do not know how MBD2/dMTase recognizes AdoMet on a structural basis.

DISCUSSION

The currently accepted mechanism for the effects of the methyl donor AdoMet on DNA methylation and tumorigenesis is founded on the assumption that the DNA methylation reaction is irreversible and defined exclusively by the DNMT. Taking advantage of our previously developed assay of demethylase activity in living cells (Fig. 1), we tested an alternative hypothesis: that AdoMet inhibits demethylase activity. If the steady state methylation status of DNA is maintained by an equilibrium of DNMT and demethylase activities (29), then inhibition of the demethylase side of the equilibrium should result in hypermethylation. Therefore, the reported DNA hypermethylation effects of exogenous AdoMet might be caused in part by inhibiting the level of demethylase activity in tumor cells. The main advantage of the system used in this paper is that it studies active demethylation exclusively, without interference from either replication-dependent passive demethylation or de novo DNMT activities (30).

We show here that exogenous AdoMet inhibits TSA-stimulated demethylation of ectopically methylated and transiently

FIG. 5. AdoMet, but not AdoHcy, inhibits demethylation activity in vitro. A and C, 32P-prelabeled methylated DNA from M. leisodeikticus was incubated with either MBD2/dMTase (A) or HEK 293 cells' extracted demethylase (C) and increasing concentrations of AdoMet. The autoradiography and quantification of one representative TLC plate is shown. Percent activity was calculated as described under “Materials and Methods.” B, increasing concentrations of AdoHcy were added to the demethylase reaction; a representative experiment is shown. D, increasing concentrations of AdoHcy were added to reaction mixtures containing 10 mM AdoMet and demethylase activity was determined. NM, unmethylated control; M, methylated control; 5mC, 5-methyldeoxycytidine 3′-monophosphate; C, deoxycytidine 3′-monophosphate.

FIG. 6. Possible model depicting how AdoMet may alter DNA methylation patterns and exert a chemoprotective effect. The steady state methylation pattern of a gene is determined by an equilibrium of DNMTs and DNA demethylases acting upon it. In cells where DNA demethylase is overexpressed, certain genes may have a tendency to become hypomethylated, and some of these genes may promote anchorage independent growth and tumorigenesis. In this case, the administration of AdoMet would have a tumor protective effect by inhibiting demethylation and shifting the equilibrium to the normally methylated state.
transfected CMV-GFP DNA (Fig. 2, B and E). Because methylation inhibits the expression of CMV-GFP (30), inhibition of demethylation of CMV-GFP results in reduction of GFP protein expression (Fig. 3, A and C), illustrating that AdoMet affects both demethylation of DNA and gene expression. This association of inhibition of demethylation and silencing of gene expression prompted us to rule out the possibility that AdoMet has a general, methylation-independent inhibitory effect on gene expression, or a general toxic effect, which also might result in inhibition of expression.

It is possible that AdoMet increases histone methyltransferase activity, resulting in hypermethylation of Lys-9 on H3 histones, which has been shown to correlate with inhibition of acetylation (40, 41). Inhibition of acetylation was shown to inhibit expression and demethylation of CMV-GFP (31). To address this alternative possibility, we measured in parallel the effects that AdoMet might have on methylated as well as unmethylated CMV-GFP plasmid, both transfected and treated with exogenous AdoMet under equivalent conditions. We first show that AdoMet treatment does not result in de novo methylation of unmethylated CMV-GFP (Fig. 2E). Thus, exogenous AdoMet does not stimulate DNA methylation as might be predicted by the current hypothesis of the mechanism of action of AdoMet. Second, we show that exogenous AdoMet does not inhibit expression of unmethylated CMV-GFP under conditions where a clear inhibition of expression of methylated CMV-GFP is observed (Fig. 3, B and D). Thus AdoMet specifically affects the expression of methylated genes. To our knowledge, this is the first demonstration that AdoMet specifically targets methylated DNA. This result also rules out the possibility that AdoMet exerts a general toxic effect on the cell. Our data therefore demonstrate that exogenous AdoMet specifically affects methylated DNA and prevents its expression. This most probably occurs by inhibiting an endogenous demethylase activity, resulting in hypermethylation of CMV-GFP and methylation-dependent repression.

We used the product of AdoMet-dependent methyltransferase reactions, AdoHcy, as a control. AdoHcy differs from AdoMet by a single methyl group. We show that AdoHcy has no effect on either gene expression (Fig. 3, A and C) or demethylation (Fig. 2, B and E). Taken together, these results indicate that both activities of AdoMet, inhibition of demethylation and inhibition of gene expression, are tightly associated and that they are both dependent on the methyl moiety in AdoMet.

In addition, we show that AdoMet directly inhibits recombinant MBD2/dMTase as well as demethylase activity extracted from HEK 293 cells (Fig. 4) in a dose-dependent manner using an in vitro assay (Fig. 5). AdoHcy does not inhibit MBD2/dMTase at the same concentrations (Fig. 5). Because an increase in intracellular AdoHcy was previously shown to be associated with hypomethylation (11), we tested the possibilities that AdoHcy (a) stimulates MBD2/dMTase activity, and (b) competes with AdoMet binding to MBD2/dMTase and relieves AdoMet inhibition. Our results suggest that AdoHcy does not interact with MBD2/dMTase and that it has no effect on AdoMet inhibition of this enzyme in vitro. Our results support the conclusion that the methyl group in AdoMet is required for its interaction with MBD2/dMTase. Although our results demonstrate that exogenous AdoMet inhibits demethylase activity in vitro and in living cells, there is no evidence that the intact AdoMet is the inhibitor. Because AdoMet is not intrinsically stable, particularly at physiological pH, it is difficult to assess whether AdoMet or a breakdown product is the inhibitory compound. At the AdoMet concentrations (mM) used in our studies, micromolar or even nanomolar concentrations of breakdown products of AdoMet may be present. Further experiments are required to test this possibility. Nevertheless, our experiments demonstrate that pharmacological administration of AdoMet inhibits active demethylation and alters gene expression.

Further experiments are also required to determine whether MBD2/dMTase is responsible for demethylation of our methylated plasmid in HEK 293 cells. Nevertheless, the fact that AdoMet inhibits both recombinant MBD2/dMTase and endogenous demethylase activities (Fig. 5) provides support for the hypothesis that demethylase(s) is inhibited by AdoMet. Thus, in addition to its role as a cofactor of transmethylation reactions, AdoMet can also act as a regulator of DNA methylation metabolism by inhibiting demethylase activity.

Our data further emphasize that the demethylase side of the methylation equilibrium has to be taken into account when dissecting the mechanism of action of drugs that modify the DNA methylation pattern. Based on our data, we suggest that AdoMet can alter DNA methylation patterns by inhibiting demethylase, which is expressed in some or most cells (Fig. 6). In this case, a reduction in the intracellular levels of AdoMet by methyl-deficient diets removes this inhibition and increases the demethylase tone, resulting in active demethylation of DNA that could take place even in postmitotic tissue. Interestingly, AdoMet has recently been shown to inhibit the overall demethylation of a CG site in the 5′ of the myogenin gene during C2C12 differentiation (21). However, this report did not determine whether AdoMet stimulated DNMT or inhibited DNA demethylase.

What are the potential implications of the inhibition of demethylation by AdoMet? It is well documented that a correlation exists between reduced intracellular AdoMet (either as a consequence of decreased folate intake or pharmacological intervention) and an increase in cell proliferation and tumorigenesis (6, 35, 36, 38). In addition, other studies have shown that a decrease in dietary folate, or a depletion of intracellular AdoMet, results in DNA hypomethylation (6, 12, 36, 37). This is consistent with the well documented observations of global hypomethylation in cancer cells (42). There is evidence that the tumor protective mechanism of AdoMet involves DNA methylation because this protection is removed when the animals are co-treated with 5-azacytidine and AdoMet (19). In accordance with this hypothesis, we have recently shown that antisense inhibition of MBD2/demethylase inhibits tumorigenesis (43). It is tempting to speculate that certain genes that are required for anchorage independent growth might be inhibited by methylation and activated by demethylase activity. Inhibition of the demethylase tone by AdoMet is proposed to result in silencing of these genes. If the mechanism of action of AdoMet in inhibiting tumorigenesis involves inhibition of demethylation, it would support the hypothesis that demethylase plays a causal role in tumorigenesis, and serve as a warning against using inhibitors of DNA methylation as anticancer agents.

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The Methyl Donor S-Adenosylmethionine Inhibits Active Demethylation of DNA: A CANDIDATE NOVEL MECHANISM FOR THE PHARMACOLOGICAL EFFECTS OF S-ADENOSYLMETHIONINE

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