ABSTRACT

$O^\alpha$-Alkylnitrosoguanine is the major mutagenic and cytotoxic DNA lesion induced by alkylating agents, including 2-chloroethyl-N-nitrosourea-based antitumor drugs. This lesion is repaired by $O^\alpha$-methylguanine-DNA methyltransferase (MGMT), the expression of which is highly variable in both normal tissues and in tumor cells. The promoter of the human MGMT gene was found to contain two putative activator protein (AP)-1 sites. Here, we show that the level of MGMT mRNA in HeLa S3 cells was increased 3.5-fold by phorbol-12-myristate-13-acetate (TPA) and 1,2-diacyl-sn-glycerol (DAG), which are activators of protein kinase C (PKC), as well as by okadaic acid, an inhibitor of protein phosphatases. The PKC inhibitor 1-(5-isoquinoline sulfonyl)-2-methylpiperezine-HCl eliminated MGMT activation by TPA and DAG but not by OA. Prior down-regulation of PKC abolished subsequent effects of TPA or DAG. The results indicate AP-1 to be involved in regulation of MGMT expression. This hypothesis was supported by showing AP-1 binding to two target sequences of the MGMT promoter and transactivation of the MGMT promoter upon cotransfection with c-fos and c-jun in F9 cells. That TPA-mediated induction of MGMT caused increased cellular resistance to 2-chloroethyl-N-nitrosourea suggests a therapeutic significance for PKC-mediated MGMT modulation.

INTRODUCTION

$O^\alpha$-Alkylnitrosoguanine, the major mutagenic base adduct induced in DNA by a variety of alkylating agents, including environmental carcinogens, is repaired by MGMT (EC 2.1.1.63; Refs. 1 and 2). This ubiquitous protein is irreversibly inactivated as a result of the acceptance of the alkyl group in a single-step, stoichiometric reaction. Antitumor alkylating drugs of the CNU type induce $O^\alpha$-chloroethyl-guanine in DNA, ultimately leading to the formation of cytotoxic DNA cross-links (3, 4). Repair of the $O^\alpha$-alkyl-guanine adduct by MGMT, thus, prevents the cytotoxicity of these drugs (5, 6).

MGMT expression is highly variable in normal tissues as well as in tumor cells (7, 8), and the cellular sensitivity of these cells to CNU varies accordingly (9–11). Tumor cells that are resistant to drugs of the CNU type often overexpress MGMT (2, 12). The regulation of MGMT expression, thus, is of significant clinical importance. Earlier studies showed that MGMT expression can be activated by a variety of genotoxic agents in rodent and human cell lines (13–15). However, the molecular mechanisms of MGMT regulation are not well understood. Recent studies have implicated altered chromatin organization and nucleosome positioning and aberrant silencing in the CpG island in the promoter region to altered expression of the gene (16–18), but the relevant cis elements in the promoter have not been explored. To elucidate the signaling pathways that contribute to MGMT overexpression, we cloned and characterized the hMGMT promoter and identified several candidate cis elements (19). An enhancer-like sequence was subsequently identified (20), but its role in MGMT regulation remains to be analyzed. Obvious sequences in the MGMT promoter that are potential targets for involvement in in vivo transcriptional activation of the gene include two each of the glucocorticoid response elements, AP-1 and AP-2. Indeed, the MGMT gene was found to be inducible by the glucocorticoid dexamethasone (21), and the potential function of the glucocorticoid response elements in induced expression of MGMT was explored in detail (22). Because many treatments, including genotoxic exposure, activate AP-1 as an immediate early response (23), it is also important to elucidate the functional role of the AP-1 sequences within the hMGMT promoter.

PKC and cAPK activate the AP-1 and AP-2 transcription factors, respectively, and this activation is required for their binding to the cognate DNA sequences (24). PKC levels and/or activity are altered in many tumor cells, and a change in the expression of PKC isotypes is often associated with a change in the regulation of cell growth, immortalization, and/or malignant transformation (25–27). Changing levels and the functional divergence of PKC family members and their molecular heterogeneity make them attractive targets for anticancer drug development (28–30). We show in this study that: (a) PKC but not cAPK up-regulates hMGMT promoter activity in tumor cells; (b) AP-1 is involved in enhanced MGMT expression; and (c) a TPA-mediated increase in MGMT level enhances cellular resistance against alkylating agents.

MATERIALS AND METHODS

Cell Lines and Reagents. HeLa S3 (ATCC CCL 2.2) cells were maintained in DMEM/F-12 (Life Technologies, Inc.) supplemented with 10% FBS (Life Technologies, Inc.), glutamine (0.292 g/liter), penicillin (100 units/ml), and streptomycin (100 µg/ml). Mouse teratocarcinoma cells (F9) were cultured in DMEM/F10 medium supplemented with 10% FCS, 2 mM L-glutamine, and 0.001% β-mercaptoethanol and antibiotics. All cultures were routinely tested for mycoplasma contamination using Hoechst stain 33258. TPA was obtained from Sigma Chemical Co. DAG, db-cAMP, OA, H-7, and H-89 were purchased from Calbiochem and Immunono. BCNU was obtained from Bristol Laboratories.

Northern Blot Analysis. Total cellular RNA was isolated from HeLa S3 cells using RNAzol (Tel-test, Inc.) and quantitated spectrophotometrically. Northern blots were performed according to standard protocols (31) and probed for hMGMT or 18S rRNA. The MGMT cDNA probe was an EcoRI fragment from pKT100 (32), and the 18S rRNA probe was a 1.15-kb BamHI-EcoRI fragment from pS5 (33). The radioactivity on the membranes were...
subjected to PhosphorImager analysis, and the signals were quantified using Image Quant (Molecular Dynamics) software.

Western Blot Analysis. For Western blot analysis, protein extracts from HeLa S3 cells were resolved by 10% SDS/PAGE and electroblotted to nitrocellulose membranes (Protran, Schleicher & Schuell). Primary antibody (polyclonal rabbit antiserum against bacterially expressed hMGMT) was used at a 1:500 dilution. Immune complexes were visualized using horseradish peroxidase-conjugated secondary antibody (1:3000 dilution) and the enhanced chemiluminescence system (Amersham Life Sciences) using suitable exposure times so that the signals were in the linear range.

Construction of Expression Vectors. A 978-bp BamHI/XbaI fragment containing the AP-1 sites in the promoter and the transcription start site was first subcloned in pUC19. The resultant recombinant was called p1911. Site-directed mutagenesis was carried out using Stratagene's double-stranded mutagenesis kit based on a modification of the unique site elimination mutagenesis procedure (34), to mutate one or both of these AP-1 sites. The AP-1(1) sequence CGAGTCA was changed to CGAGTTG and the AP-1(2) sequence TGAGTCA was changed to TGAGTTG. The mutated plasmid sequences were confirmed by dideoxy sequencing. The KpnI/XbaI fragment from p1911 containing wild-type or mutated AP-1 sites were cloned into KpnI/NheI site of pGL2 basic to generate four different expression plasmids with mutations in none or either one or both of the AP-1 sites and were named p-954/+24 MIIAP2, p-954/+24 MLAPM1, p-954/+24 MLAPM2, and p-954/+24 MLAPM12, respectively.

Assay for Transiently Expressed Reporter Gene. F-9 cells were seeded in 10-cm dishes (10<sup>4</sup> cells/dish) and transfected by calcium phosphate coprecipitation as described previously (11). For each transfection, we used 10 µg of one of the hMGMT luciferase constructs together with 1 µg of each of the RSV-β-gal, RSV-c-jun, RSV-c-fos, or RSV-0 as a control, without RSV-c-jun/RSV-c-fos. The amount of DNA was adjusted to 20 µg with sonicated salmon sperm DNA. Cells were stored for determination of luciferase activity in cell extracts 17 h after transfection. Luciferase activity was measured by chemiluminescence luciferase assay (Promega) according to the manufacturer's protocol. Luciferase activities were normalized for transfection efficiency by measuring in parallel the β-gal activity in the same cell extract using a commercial β-gal assay kit (Tropix).

EMSA. EMSA was performed as described previously (35). Oligonucleotides unique to this assay were MGMT AP-1(1) (agcgccgctgatgctg), MGMT AP-1(2) (agcggctgagggctctg), mutated MGMT AP-1(1) (agcgccgctgatgctg), MGMT AP-1(2) (agcggctgagggctctg), and the mouse collagenase AP-1 oligonucleotide (agcggctgaggtact). We used a random 42-mer as a nonspecific oligonucleotide. Oligonucleotides were synthesized commercially (MWG Biotech, Ebersberg, Germany). In brief, 1–4 µg of nuclear extract from exponentially growing cells were incubated in binding buffer [10% glycerol, 50 mM KCl, 10 mM HEPES-OH (pH 7.9), 4 mM Tris-CI, 0.5 mM DTT, 0.5 mM EDTA, 10 µg BSA, and 1 µg poly(dI-dC)] for 30 min on ice together with 25 fmol of radiolabeled oligonucleotide as indicated. To test the effect of c-Jun antibody (Santa Cruz Biotechnology) on the mobility of the complex, 500 ng of the antibody were added to the nuclear extract 30 min prior to the oligonucleotide binding reaction and incubated at room temperature.

Survival Assay. Exponentially growing HeLa S3 cells were serum-starved for 18 h and then seeded at a density of 200 cells per 60-mm Petri dish in medium containing 0.5% FBS and, 3 h later, were treated with activators/inhibitors of the protein kinase pathway or inhibitors of cellular phosphatases for 1 h. After another 6 h of incubation in serum-free medium, the cells were washed and treated with increasing doses of BCNU for 1 h, by addition of stock solutions directly to the medium. The cells were washed with PBS once and then grown in medium containing 10% FBS. Colonies were allowed to grow to 1 mm in diameter (~10–12 days) and were then fixed and stained. Relative cell survival was calculated from the number of colonies per dish.

RESULTS

Increase in hMGMT mRNA Level by Activation of the PKC Pathway. TPA and DAG activate their intracellular receptor (PKC) by a similar mechanism (36). To investigate the involvement of PKC pathway in hMGMT mRNA expression, cells maintained in 0.5% serum-containing medium for 24 h (to avoid serum stimulation of PKC) were treated with various concentrations of TPA (for 1 h), and total RNA was harvested at various time points. Northern blot analysis was carried out with both hMGMT cDNA and an 18S rRNA probe. Our preliminary studies showed that levels of β-actin or cyclophillin RNA, often used as internal normalization standards, were significantly altered as a result of TPA treatment. The signals on the autoradiograms were quantitated, and changes of hMGMT mRNA levels were normalized to that of 18S rRNA. At doses of >10 ng/ml, TPA increased hMGMT mRNA level in a dose-dependent fashion (Fig. 1A). Higher concentrations of TPA (500–1000 ng/ml) appeared to be cytotoxic (data not shown). Because 100 ng/ml TPA resulted in a consistent induction of hMGMT without being cytotoxic, we used this dose in later studies. Fig. 1B demonstrates the effect of TPA as a function of time after addition of TPA to the medium.

Fig. 1. Modulation of hMGMT expression by PKC activators and inhibitors. Northern blot analyses were carried out with RNA from serum-starved HeLa S3 cultures treated with PKC activators/inhibitors for the indicated time periods. Data points and columns, means of two to four independent experiments. The increases in RNA levels were normalized with respect to the 18S RNA signal. Top, representative blots for each experiment. A, TPA dose-dependent changes in hMGMT mRNA levels. Cells were exposed to 10, 50, and 100 ng/ml TPA or solvent (DMSO) for 1 h and harvested after 6 h of treatment. B and C, time-dependent changes in hMGMT mRNA levels after exposure of cells to TPA and DAG, respectively. hMGMT mRNA levels in cells treated with TPA (8: 100 ng/ml), DAG (C: 100 ng/ml) or DMSO (control; C) were analyzed after 0, 3, 6, and 9 h of treatment. D, effect of TPA pretreatment on TPA-mediated induction of MGMT mRNA. Columns, RNA levels after 6 h in mock-, TPA (10 ng/ml), TPA (100 ng/ml), or TPA (100 ng/ml)-treated cells pretreated with TPA (10 ng/ml) for 24 h. E, effect of H-7 on TPA- or DAG-mediated induction of hMGMT. Columns, RNA levels in mock-, H-7 (20 µM), TPA (100 ng/ml), and H-7 (20 µM) plus TPA (100 ng/ml), plus DAG (100 ng/ml)-treated cells 6 h after addition of the agents.
Phorbol esters activate PKC but may also exhibit other effects besides those on PKC isotypes. To determine whether the effects observed after TPA treatment can be attributed to activation of the PKC pathway, we evaluated the effect of DAG (generated by the hydrolysis of phosphatidylinositol 4,5-diphosphate), another specific activator of PKC (37, 38). In these studies, we used a membrane-permeable synthetic diacyl glycerol. HeLa S3 cells were serum-starved as above and treated with DAG (100 ng/ml), and the total RNA was harvested at different time points and subjected to Northern blot analysis. Fig. 1C shows that DAG mediates a significant increase (~4-fold after 6 h of treatment) in hMGMT mRNA levels. The extent and kinetics of induction were similar to those for TPA. These data suggest that TPA-induced increases in hMGMT mRNA levels are most likely mediated via the PKC pathway.

Prolonged treatment with low concentrations of TPA resulted in down-regulation of PKC. This effect was previously used to investigate the specificity of TPA-mediated cellular responses. Treatment of HeLa S3 cells with 100 ng/ml TPA after a low-dose exposure (10 ng/ml for 24 h) to the same agent prevented the induction of hMGMT mRNA (Fig. 1D). H-7 reversibly inhibits both the holoenzyme and the catalytic subunit of PKC (39). That the increase in hMGMT mRNA was abolished in cells exposed to H-7 (20 μM) before treatment with 100 ng/ml TPA or DAG for 1 h (Fig. 1E) again suggests that PKC plays a critical role in activation of the hMGMT promoter. TPA was removed after 1 h of exposure, but DAG and/or H-7 was present for the entire duration of the experiment. That addition of H-7 to HeLa S3 cells did not substantially decrease basal levels of hMGMT mRNA suggested that the promoter elements responsible for basal expression are not particularly sensitive to PKC-mediated signaling.

Next, Western blot analysis was performed to test whether TPA activation increases hMGMT protein levels. Parallel cell cultures were serum-starved as before and then treated with TPA (100 ng/ml), H-7 (20 μM), or TPA (100 ng/ml) plus H-7 (20 μM); the amount of MGMT protein was quantitated at various times after treatment. Fig. 2 shows an ~4-fold increase of the MGMT protein level after TPA treatment. In agreement with results of Northern blot analysis, H-7 did not substantially affect basal levels; however, it eliminated the TPA-induced increase in hMGMT protein levels. Similar results were obtained after DAG and/or H-7 exposure of cells (data not shown). These results also indicate a concordance of MGMT mRNA and protein levels during regulation of the gene.

Effect of OA on hMGMT mRNA level. In view of the involvement of PKC in regulation of the hMGMT promoter, we asked whether inhibiting the dephosphorylation of transcriptional activators would affect expression of the hMGMT gene. OA, a potent inhibitor of protein phosphatase-1 and protein phosphatase-2A in vitro and in vivo (24), was used to obtain insights into the potential role of phosphorylation. Exposure of HeLa S3 cells to OA (20 nM) resulted in a 3–4-fold increase in hMGMT mRNA level (Fig. 3). Parallel cultures of HeLa S3 cells were also treated with TPA (100 ng/ml) or DAG (100 ng/ml) together with OA (20 ng/ml). TPA was removed after a 1 h exposure, whereas DAG and OA were present for the entire experimental period. No potentiation of TPA- or DAG-mediated activation of hMGMT mRNA was observed with OA.

cAPK Did Not Significantly Affect MGMT mRNA Level in HeLa Cells. db-cAMP (10 μM) and/or theophylline (100 μM; Ref. 40) neither altered hMGMT mRNA expression nor modified the activating effect of TPA on hMGMT expression. Similarly, H-89, an inhibitor of cAPK (41), did not alter the TPA-mediated increase in MGMT RNA levels (data not shown). These results suggest that cAPK, the target of which can be AP-2, is not involved in signaling that modulates hMGMT expression.

AP-1 Binds to MGMT Promoter Sequences. Given the role of PKC in activating MAP kinases and Fos/Jun as downstream targets, our results on the PKC-mediated up-regulation of hMGMT support the view that the observed response was elicited via AP-1-mediated activation of the hMGMT promoter. To confirm this possibility, we measured AP-1 binding to hMGMT promoter sequences, as well as AP-1-mediated promoter activation. Proteins in nuclear extracts of HeLa S3 cells bind to the AP-1 sequences located in the hMGMT promoter as detected by EMSA (Fig. 4A). The binding sites were

![Graph showing effects of TPA and H-7 on hMGMT protein levels.](Image)

Fig. 2. Effects of TPA and H-7 on hMGMT protein levels. Columns, MGMT levels in extracts from mock-, TPA (100 ng/ml), H-7 (20 μM), and H-7 (20 μM)-plus TPA (100 ng/ml)-treated HeLa S3 cells. Top, representative Western blot.

5-fold increase in hMGMT mRNA was observed 6 h after exposure to TPA.

Phorbol esters activate PKC but may also exhibit other effects besides those on PKC isotypes. To determine whether the effects observed after TPA treatment can be attributed to activation of the PKC pathway, we evaluated the effect of DAG (generated by the hydrolysis of phosphatidylinositol 4,5-diphosphate), another specific activator of PKC (37, 38). In these studies, we used a membrane-permeable synthetic diacyl glycerol. HeLa S3 cells were serum-starved as above and treated with DAG (100 ng/ml), and the total RNA was harvested at different time points and subjected to Northern blot analysis. Fig. 1C shows that DAG mediates a significant increase (~4-fold after 6 h of treatment) in hMGMT mRNA levels. The extent and kinetics of induction were similar to those for TPA. These data suggest that TPA-induced increases in hMGMT mRNA levels are most likely mediated via the PKC pathway.

Prolonged treatment with low concentrations of TPA resulted in down-regulation of PKC. This effect was previously used to investigate the specificity of TPA-mediated cellular responses. Treatment of HeLa S3 cells with 100 ng/ml TPA after a low-dose exposure (10 ng/ml for 24 h) to the same agent prevented the induction of hMGMT mRNA (Fig. 1D). H-7 reversibly inhibits both the holoenzyme and the catalytic subunit of PKC (39). That the increase in hMGMT mRNA was abolished in cells exposed to H-7 (20 μM) before treatment with 100 ng/ml TPA or DAG for 1 h (Fig. 1E) again suggests that PKC plays a critical role in activation of the hMGMT promoter. TPA was removed after 1 h of exposure, but DAG and/or H-7 was present for the entire duration of the experiment. That addition of H-7 to HeLa S3 cells did not substantially decrease basal levels of hMGMT mRNA suggested that the promoter elements responsible for basal expression are not particularly sensitive to PKC-mediated signaling.

Next, Western blot analysis was performed to test whether TPA activation increases hMGMT protein levels. Parallel cell cultures were serum-starved as before and then treated with TPA (100 ng/ml), H-7 (20 μM), or TPA (100 ng/ml) plus H-7 (20 μM); the amount of MGMT protein was quantitated at various times after treatment. Fig. 2 shows an ~4-fold increase of the MGMT protein level after TPA treatment. In agreement with results of Northern blot analysis, H-7 did not substantially affect basal levels; however, it eliminated the TPA-induced increase in hMGMT protein levels. Similar results were obtained after DAG and/or H-7 exposure of cells (data not shown). These results also indicate a concordance of MGMT mRNA and protein levels during regulation of the gene.

Effect of OA on hMGMT mRNA level. In view of the involvement of PKC in regulation of the hMGMT promoter, we asked whether inhibiting the dephosphorylation of transcriptional activators would affect expression of the hMGMT gene. OA, a potent inhibitor of protein phosphatase-1 and protein phosphatase-2A in vitro and in vivo (24), was used to obtain insights into the potential role of phosphorylation. Exposure of HeLa S3 cells to OA (20 nM) resulted in a 3–4-fold increase in hMGMT mRNA level (Fig. 3). Parallel cultures of HeLa S3 cells were also treated with TPA (100 ng/ml) or DAG (100 ng/ml) together with OA (20 ng/ml). TPA was removed after a 1 h exposure, whereas DAG and OA were present for the entire experimental period. No potentiation of TPA- or DAG-mediated activation of hMGMT mRNA was observed with OA.

cAPK Did Not Significantly Affect MGMT mRNA Level in HeLa Cells. db-cAMP (10 μM) and/or theophylline (100 μM; Ref. 40) neither altered hMGMT mRNA expression nor modified the activating effect of TPA on hMGMT expression. Similarly, H-89, an inhibitor of cAPK (41), did not alter the TPA-mediated increase in MGMT RNA levels (data not shown). These results suggest that cAPK, the target of which can be AP-2, is not involved in signaling that modulates hMGMT expression.

AP-1 Binds to MGMT Promoter Sequences. Given the role of PKC in activating MAP kinases and Fos/Jun as downstream targets, our results on the PKC-mediated up-regulation of hMGMT support the view that the observed response was elicited via AP-1-mediated activation of the hMGMT promoter. To confirm this possibility, we measured AP-1 binding to hMGMT promoter sequences, as well as AP-1-mediated promoter activation. Proteins in nuclear extracts of HeLa S3 cells bind to the AP-1 sequences located in the hMGMT promoter as detected by EMSA (Fig. 4A). The binding sites were

![Graph showing effects of OA on hMGMT mRNA levels.](Image)

Fig. 3. Effect of OA on hMGMT mRNA levels. Northern blot analysis of total RNA from serum-starved HeLa S3 cells treated with OA (20 nM) alone or in conjunction with TPA (A, 100 ng/ml) or DAG (B, 100 ng/ml) at 0, 3, 6, and 9 h: , mock-treated cells; , TPA- or DAG-treated cells; , OA-treated cells; , OA- plus TPA- or DAG-treated cells.
designated here AP-1(1) and AP-1(2), respectively (for sequences, see Fig. 5A). There was significantly less binding to the AP-1(1) sequence than to the AP-1(2) sequence. In both cases, however, the observed binding was lower than to the AP-1 site in the context of the collagenase promoter sequence (Fig. 4A). Introduction of mutations into either one of the binding sites completely abolished AP-1 binding (Fig. 4A). The factors that bind to the AP-1 site of the collagenase promoter also bind to the AP-1 site in the context of the hMGMT promoter, as shown in competition studies (Fig. 4B). Addition of unlabeled AP-1(1) or AP-1(2) oligodeoxynucleotides of the hMGMT promoter abolished bandshift of the collagenase AP-1. In contrast, the mutated AP-1 sequences of the hMGMT promoter were ineffective in such competition experiments. Interestingly, AP-1(2) of the wild-type hMGMT promoter had a higher ability to compete with the collagenase AP-1 site sequence present in the collagenase promoter. AP-1(1) site sequence in the context of the hMGMT promoter abolished bandshift of the collagenase AP-1. In contrast, the mutated AP-1 sequences of the hMGMT promoter were ineffective in such competition experiments. Interestingly, AP-1(2) of the wild-type hMGMT promoter had a higher ability to compete with the collagenase AP-1 site sequence present in the collagenase promoter.

Activation of MGMT Promoter by Fos and Jun. To demonstrate a functional role for the AP-1 sites in the promoter of the repair gene, hMGMT promoter-luciferase expression plasmids were constructed in which both AP-1 sites were either intact or one or both of these sites were mutated (Fig. 5A); the mutations introduced were the same as those used in EMSA. Because the mouse F9 teratocarcinoma cells were found to express very low levels of c-Fos and c-Jun, these cells provided a sensitive system for directly testing the contribution of AP-1 to activation of hMGMT. Cotransfection of the intact hMGMT promoter-luciferase expression plasmids were constructed in which both AP-1 sites were either intact or one or both of these sites were mutated (Fig. 5A); the mutations introduced were the same as those used in EMSA. Because the mouse F9 teratocarcinoma cells were found to express very low levels of c-Fos and c-Jun, these cells provided a sensitive system for directly testing the contribution of AP-1 to activation of hMGMT. Cotransfection of the intact hMGMT promoter constructs with RSV-c-fos and RSV-c-jun in F9 cells gave rise to a dramatic increase in promoter activity (~30-fold) relative to the control. This induction was significantly reduced although not completely prevented by the hMGMT promoters harboring mutations in either one or both AP-1 sites (Fig. 5B). These data clearly establish that AP-1 is the downstream target of PKC-mediated signaling involved in hMGMT promoter activation.

PKC-induced Increase in hMGMT Level Is Associated with Alkylating Drug Resistance. If stimulation of the PKC pathway increases hMGMT expression in HeLa cells, there should be a resulting increase in cellular resistance to CNU. We tested this possibility by comparing the sensitivity of HeLa S3 cells to toxic doses of BCNU with or without PKC activation, using a clonogenic survival assay. Fig. 6 shows the cell survival determined in cells treated with TPA, H-7, or H-7 plus TPA. TPA mediated a significant increase in cellular resistance to toxic doses of BCNU. H-7, an inhibitor of PKC, elimi-
Fig. 5. Effect of coexpression of c-fos/c-jun on the activity of various hMGMT promoter constructs. A, schematic diagram of the expression plasmid p-954/+24 ML. The 5' and 3' located AP-1 sites within the promoter [with respect to the transcription start site] were designated AP-1(1) and AP-1(2), respectively. The wild-type (wt) and mutated (mut) AP-1 sequences are shown with the mutated bases (boldface letters). B, p-954/+24 ML (Control) and the corresponding derivatives (see "Materials and Methods") harboring mutations in either one of the AP-1 sites or in both sites were cotransfected with RSV-β-gal, RSV-c-fos, RSV-c-jun, or, as a control, RSV-0 into F9 cells. The luciferase and β-gal expression levels were determined, and the luciferase activity was normalized to β-gal activity in each assay. There was no effect of Fos/Jun expression on RSV-β-gal activity. The extent of induction was calculated from relative luciferase activity upon cotransfection of hMGMT promoter constructs with RSV-fos and RSV-jun on the one hand and RSV-0 on the other (background control). Columns, means of three independent transfection experiments; bars, SE. The differences in expression between wild-type and the mutated MGMT promoter constructs are statistically significant.

Fig. 6. Survival curves (determined by clonogenic assay) showing TPA-induced protection of HeLa S3 cells from BCNU cytotoxicity. O, mock-treated cells; ■, TPA-treated cells; □, H-7-treated cells; ▪, H-7- plus TPA-treated cells. Data points, percentage survival, expressed as the mean of three independent experiments; bars, SE. The concentrations of TPA (100 ng/ml) and H-7 (20 μM) used in combination with BCNU were not cytotoxic.

DISCUSSION

DNA repair proteins are generally expressed constitutively, but for some other proteins, a significant variation in its expression between tissues and even within tissues has been observed (1, 2, 42-44). MGMT typifies such cases because a large variation in its level in various normal and tumor tissues is well documented. An understanding of the mechanism of overexpression of MGMT, particularly in tumors, has clinical significance because reversal of such overexpression may lead to sensitization of the tumor to methylating and CNU-type alkylating antineoplastic drugs.

Regulation of MGMT expression in both normal and tumor cells has been widely investigated, but the molecular mechanism(s) have not been elucidated in detail. The levels of hMGMT mRNA and protein are dependent on the cell type, cell cycle stage, and differentiation status (45), as well as on the activity of the signaling network that regulates the promoter. The presence of putative AP-1 and AP-2 response elements in the hMGMT promoter, thus, suggested their involvement in MGMT activation. Here, we have investigated the roles of multiple AP-1 sites in the hMGMT promoter region in HeLa S3 cells, which express various isoforms of PKC. FBS, a regular component of culture medium, often masks effects mediated by activator(s)/inhibitor(s) of PKCs, and we did not observe substantial responses to either TPA or H-7 in terms of mRNA levels for c-fos, c-jun (46), or hMGMT in the presence of 5-10% FBS. However, significant overexpression of hMGMT was observed when cells at 60-70% confluence were conditioned to low serum (0.5%)-containing medium for 24 h prior to TPA treatment. Thus, serum-starved cell cultures were routinely used in this study. There is strong biological rationale for the common procedure of studying gene regulation in cells under starved conditions to down-regulate various signal trans...
duction pathways. In addition to the obvious benefit of inducing a larger signal in gene regulation, the starved cells may be closer to those in vivo in regard to their growth rate and suboptimal conditions of no growth requirements.

Our results suggest that AP-1 sequences within the MGMT promoter are involved in MGMT activation. One of the two AP-1 elements in hMGMT promoter matches the consensus sequence (CGAGTCA) exactly, whereas the other is slightly divergent (TGAGTCA). That TPA, an activator of the PKC family, caused an increase in the steady-state levels of hMGMT mRNA, as well as hMGMT protein, in a time- and dose-dependent fashion argues for an important role of AP-1 factors in regulating hMGMT promoter activity. Moreover, EMSAs and transient expression assays strengthened the idea that the AP-1 sites are involved in affecting transcription. Similar activation of hMGMT by DAG, another specific activator of PKC, provided further evidence of PKC involvement. Although the extent of activation was somewhat less than that observed with TPA, the kinetics of increase in hMGMT mRNA were comparable in the two cases. The effect of H-7, which prevented the increase in MGMT mRNA, also provided evidence for the involvement of PKC in MGMT activation. However, that the basal level of the RNA was not affected suggests that AP-1 contributes only to inducible expression of the gene. Such a possibility is consistent with our earlier observation that the basal level of MGMT is comparable in the wild-type and c-Fos-negative cells (47). An earlier report showed that in vivo dimethyl sulfate footprinting of the hMGMT promoter in a cell line did not detect AP-1 binding to the AP-1 sites in the promoter nor did it provide any evidence of its inaccessibility to transcription factors (18).

There are multiple reasons to explain the inconsistency of our studies versus the footprinting data. It is not clear whether the cell line expressed AP-1 to a significant level. Furthermore, the growth conditions of the cells may affect the promoter binding of trans-acting factors. Our results showing AP-1 expression-dependent activation of MGMT in AP-1-deficient P9 cells provide a strong argument for the in vivo function of AP-1 in the MGMT promoter.

Although not involved in PKC activation, OA, a potent inhibitor of protein phosphatases 1 and 2A both in vivo and in vitro (24), produced a significant (3-fold) increase in hMGMT mRNA level in HeLa cells. This suggests involvement of phosphorylated proteins, possibly AP-1 factors, in MGMT gene activation. No significant enhancement of TPA- or DAG-mediated increase in MGMT expression was apparent upon concomitant OA treatment. These results suggest that both PKC activation and prolonged protein phosphorylation are involved in up-regulation of the hMGMT promoter.

Combined treatment of cells with theophylline (an inhibitor of phosphodiesterase), db-cAMP, or H-89 (an inhibitor of cAPK) did not affect either basal or TPA/DAG-induced hMGMT mRNA/protein levels. In this context, it is also important to recognize that TPA may increase intracellular levels of cAMP sufficiently to mediate a still unidentified interaction between the PKC and cAPK pathways (48, 49). It is interesting to note that, in a different cell type, cAMP did not induce MGMT mRNA levels; however, H-89, an inhibitor of cAPK, did. In any event, cAPK does not appear to contribute to MGMT activation in HeLa S3 cells.

HeLa S3 cells may be ideally suited for these studies involving AP-1 activation because they express various isoforms of PKC. It is known that TPA binds with high affinity to both Ca2+-activated (a, b, and γ; conventional PKC) and Ca2+-insensitive (δ, ε, η, ζ, and μ and novel PKC) PKC isoforms, leading to their activation (24, 25, 27, 50). In addition, TPA forms a stable complex with PKC and mediates a persistent activation of PKC that ultimately leads to depletion (down-regulation) of the enzyme (36). That prolonged treatment of HeLa S3 cells with TPA (10 ng/ml) eliminated hMGMT mRNA inducibility by high doses of TPA (100 ng/ml) or DAG (100 ng/ml) indicated that up-regulation of hMGMT is dependent on conventional and novel PKC isoforms. These data, considered together with the Ca2+-sensitivity of PKC isoforms, may indicate that conventional PKCs (a, b, and γ) are involved in signaling events that lead to AP-1 activation and up-regulation of the MGMT promoter. It is interesting to note that the α, β, and γ isoenzymes of PKC are often found to be associated with maintenance of various human tumor cell types (28, 50).

We found a significant increase in cellular resistance to BCNU after exposure of cells to TPA. This increase in the drug resistance of HeLa S3 cells was reversed by H-7, which inhibits both the holoenzyme and the catalytic subunit of PKC. These results are consistent with the idea that AP-1 sequences in the hMGMT promoter, together with PKC-mediated activation, may enhance the drug resistance of tumor cells. Indeed, biochemical modulation of hMGMT protein activity by use of specific inhibitors in Mer+ (methyltransfer repair proficient) cells has been demonstrated to reverse CNU resistance (51, 52). Thus, pretreatment of Mer+ tumor cells with alkyltransferase substrates (e.g., O6-methyl- or O6-benzylguanine) before exposure to CNUs has demonstrated marked synergism. However, after depletion of hMGMT activity by acute treatment with these biochemical modulators, the level of MGMT activity eventually rebounds, with subsequent reacquisition of the resistant phenotype (52, 53). That this restoration of activity occurs with no concomitant alterations in hMGMT mRNA steady-state levels (54) suggests a direct effect on the enzyme activity.

In conclusion, our results establish a functional role for the PKC signal pathway(s) in regulating hMGMT mRNA levels. Our evidence that TPA-induced AP-1 activation of MGMT expression is associated with increased resistance of HeLa S3 cells to the cytotoxicity of BCNU suggests that MGMT overexpression in certain drug-resistant tumor cells may, in part, reflect transactivation by AP-1. Once this possibility is established for a specific tumor, it may be possible to design an adjuvant chemotherapy approach for sensitizing the tumor to alkylating drugs with specific inhibitor(s) of PKC. This is the first report showing involvement of PKC/AP-1 in regulation of a DNA repair gene having impact on cytotoxic drug resistance.

ACKNOWLEDGMENTS

We thank Dr. D. Konkel for critically reading the manuscript and W. Smith for typing it. We are grateful to Dr. P. Angel (German Cancer Research Center, Heidelberg, Germany) for RSV-c-fos and RSV-c-jun expression plasmids.

REFERENCES


Regulation of Expression of the DNA Repair Gene $O^6$-Methylguanine-DNA Methyltransferase via Protein Kinase C-mediated Signaling

Istvan Boldogh, Chilakamarti V. Ramana, Zhenping Chen, et al.

*Cancer Res* 1998;58:3950-3956.

Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/17/3950

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.