Cytoine Methylation Represses Glutathione S-Transferase P1 (GSTP1) Gene Expression in Human Prostate Cancer Cells

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ABSTRACT

Methylation of the glutathione S-transferase P1 (GSTP1) gene has been described as a highly specific and sensitive biomarker for prostate cancer. However, at present, it is not known whether methylation represses GSTP1 gene expression in human prostate cancer. We found the GSTP1 gene promoter to be completely methylated in the LNCaP prostate cancer cell line, where this gene is transcriptionally inactive. In contrast, Du145 and PC3 prostate cancer cells express the GSTP1 gene and exhibit methylated and unmethylated GSTP1 alleles. In a transient transfection assay using LNCaP cells, methylation of the GSTP1 promoter-driven luciferase reporter vector (GSTP1-pGL3) resulted in a >20-fold inhibition of transcription, and this repression was not relieved by the presence of a histone deacetylase inhibitor, trichostatin A (TSA). Treatment of LNCaP cells with a DNA methyltransferase inhibitor, 5-Aza-2′-deoxycytidine, resulted in demethylation and activation of the GSTP1 gene. In contrast, TSA treatment failed to demethylate or activate the GSTP1 gene. Fully methylated but not unmethylated GSTP1 promoter fragment was shown to bind to a complex similar to methyl cytosine-binding protein complex 1 that contains methyl-CpG-binding domain 2 protein (MBD2) in electrophoretic mobility shift assays using LNCaP cell nuclear extracts. These data demonstrate that cytosine methylation can repress GSTP1 gene expression in LNCaP prostate cancer cells and that this effect is possibly mediated by a methyl cytosine-binding protein complex 1-like complex. Furthermore, these data also support the notion of the dominance of methylation over TSA-sensitive histone deacetylation in silencing genes with a high CpG density in the promoter region.

INTRODUCTION

GSTs are a group of isoenzymes that catalyze intracellular detoxification reactions by conjugating glutathione with electrophilic compounds including carcinogens and exogenous drugs. Among the isoenzymes, the role of class GSTP1 in cancer has been studied extensively. A genetic variant of GSTP1 has been associated with cancer susceptibility, and increased skin tumorigenesis has been observed in mice lacking GSTP1. Aberrant cytosine methylation at CpG dinucleotides within 5′ CpG islands has been associated with transcriptional repression and is an important and common mechanism for inactivation of tumor suppressor genes. The 5′ region of the GSTP1 gene contains a CpG island. Promoter region CpG islands are usually unmethylated in normal tissues, regardless of the transcriptional activity of the gene. Cytosine methylation in GSTP1 regulatory sequences associated with the loss of GSTP1 expression has been observed in a majority of human prostate carcinoma tissues.

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3 The abbreviations used are: GST, glutathione S-transferase; TSA, trichostatin A; 5-Aza-CdR, 5-aza-2′-deoxycytidine; MeCPC, methyl cytosine-binding protein complex; MBD, methyl-CpG-binding domain; PIN, prostatic intraepithelial neoplasia; EMSA, electrophoretic mobility shift assay; MS-PCR, methylation-sensitive PCR; HDAC, histone deacetylase complex; RT-PCR, reverse transcription-PCR; 4820

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CAGTCGCTTCATACATGTCATCC-3'). GSTP1 cDNA was then amplified from cDNA using the R3 primer and the custom primer F3 (5'-TCACT-CAAAGGCTCCTGCGTATACT-3') as described previously (12). For RNA integrity and loading control, β-actin DNA was amplified from cDNA using the custom primers F2 (5'-ACCATTGATTGATGATCGC-3') and R2 (5'-ACAGGCTGGGGAAGGTCAG-3') as described previously (13). Custom primers were provided by Sigma Genosys (The Woodlands, TX). PCR was performed in a PTC-200 thermal cycler from MJ Research (Waltham, MA) using the following parameters: (a) one cycle of 5 min at 95°C after which 2.5 units of RedTaq (Sigma Chemical Co.) were added; (b) 15 cycles of 94°C for 30 s, 65°C for 1 min (decreasing 0.5°C/cycle), and 72°C for 1 min; (c) 15 cycles of 94°C for 30 s, 58°C for 1 min and 72°C for 1 min; and (d) a final 7-min extension at 72°C.

Bisulfite Genomic Sequencing. DNA was obtained from LNCaP, DU145, and PC3 prostate cancer cell lines, and bisulfite reaction was carried out on 5 µg of genomic DNA as described previously (14). A PCR-amplified product of the GSTP1 promoter was obtained using the following primer pair, which was constructed after taking into account the bisulfite conversion reaction (see Fig. 2B): (a) forward primer (primer F), 5'-TTTATTGAGGTGG-GGATTGGG-3' derived from the wild-type sequence 5'-TCTTACGC-CCCCCTGGGACCTG-3'; and (b) reverse primer (primer R), 5'-CCCCATAAACTTAAACCCC-3' derived from the wild-type sequence 5'-CCCCATCTGGGACTCTGACCCCG-3'. Sequencing of the PCR-amplified product was performed using the forward and reverse primers. The [α-32P]-dideoxyadenosine triphosphate terminator kit (USB Corp., Cleveland, OH) was used for sequencing. The sequencing gel was dried, and radioactive bands were analyzed using a Cyclone phosphorimager (Packard Instrument Co., Meriden, CT).

MS-PCR. Five µl of the bisulfite-treated genomic DNA from 100 µl of the final resuspended product solution were amplified with a set of primers for the unmethylated reaction and with another set of primers for the methylated reaction (see Fig. 2B): (a) unmethylated forward primer (GSTP-UF) 5'-AAAGGGGAAAAGGTTTGGATGTTGTTGGT-3' and unmethylated reverse primer (GSTP-UR) 5'-AAACTTCACAAACACCTTCA-3'; and (b) methylated forward primer (GSTP-MF) 5'-GGTTTTTTTGTTCAGGGCGG-3' and methylated reverse primer (GSTP-MR) 5'-CCACCGAAAACTCCGACCTCCG-3'. PCR was performed in a PTC-200 thermal cycler (MJ Research) for 35 cycles. Twenty µl of the PCR product were loaded on 2% agarose gel for analysis.

Drug treatment of LNCaP cells was accomplished by adding reagents to the culture medium to final concentrations as follows: (a) 5-Aza-CdR, 3, 5, and 10 µM; and (b) TSA, 100 and 300 nM. Cells were treated with 5-Aza-CdR on a daily basis and with TSA on alternate days. The medium was replaced each time, and treatment was continued for 1 week.

Plasmids. Methods for construction of recombinant DNA followed standard procedures. For the construction of the GSTP1-promoted luciferase reporter plasmid (GSTP1-pGL3), SacI (5'-AGGGAGCTTCCAGCCGGCGGCGGCGGCGG-3') and HindIII (5'-GGTAAGCTTGGCCGCGGCTACTCCT-3')-linked primers were used to amplify a 179-bp (~134 to +45) GSTP1 gene fragment using human placental DNA as template. Plasmid DNA was harvested from cultures of Escherichia coli strain DH5α (Life Technologies, Inc.) using a Qiagen (Valencia, CA) kit. Both ends were sequenced to confirm the orientation. Plasmid DNA was quantitated by reading absorbance at 260 nm on a Beckman DU 64 spectrophotometer and by comparison of ethidium bromide-stained plasmid DNA restriction fragments with DNA standards of known concentration electophoresed on agarose gels. In the transient transfection experiment, one plasmid sample for transfection was unmethylated, and one plasmid sample was methylated. Methylation was accomplished using SsII methylase (New England BioLabs, Beverly, MA), and the extent of methylation was determined by digestion with a mixture of the methylation-sensitive restriction enzymes AcII, HhaI, and HpaII (Fig. 3A).

Transient Transfections. Cells were transfected using LipofectAMINE (Life Technologies, Inc.) as per the supplier’s protocol. Cells were plated on 60-mm dishes and grown to a density of 40–60% before transfection. Transfections were performed in triplicate to help control for variation in cell number and culture conditions. Cells in serum-free medium were cotransfected with 2 µg of a specific plasmid construct plus 200 ng of Renilla luciferase plasmid pRL-TK (as a control for transfection efficiency) complexed with 20 µl of LipofectAMINE per dish. The plasmid used was GSTP1-promoted luciferase reporter plasmid GSTP1-pGL3, which was either unmethylated or fully methylated by SsII methylase. The LipofectAMINE DNA complexes were incubated with cells 5 h before feeding with an equal volume of DMEM containing 20% fetal bovine serum. Lysates were made from transfected cells 48 h after feeding.

Luciferase Assays. Luciferase assays were performed using the Dual-Luciferase reporter assay system protocols manual as supplied by Promega. After transfection, the growth medium was removed, and 4 ml of PBS were added to each 60-mm dish of cells. The dishes were swirled gently to wash the cell surfaces, followed by removal of the rinse solution and the addition of 400 µl of 1× Passive Lysis Buffer (Promega) to each dish. The dishes were incubated for 15 min at room temperature before harvesting the cell lysates by scraping the bottom of the dishes with disposable plastic scrapers. Each lysate was pipetted several times to obtain a homogenous solution and transferred to a microfuge tube. The lysates were cleared by centrifugation in the microfuge at 4°C for 1–2 min, transferred to fresh tubes, and stored at −70°C.

Luciferase assays were performed in a Beckman LS6000SC scintillation counter with the coincidence counter disabled. Firefly luciferase and the Renilla luciferase assays were performed manually in one reaction tube. Firefly and Renilla luciferase activities were sequentially measured for 2 min as directed by the protocol set forth for manual luminometers in the Dual-Luciferase reporter assay system technical manual. Results obtained were normalized for Renilla luciferase activity. Protein concentrations were measured spectrophotometrically using a program contained in a Soft-Pac module on a Beckman DU series 64 spectrophotometer.

EMSAs. HeLa cell and LNCaP cell nuclear extracts were prepared according to a modified Dignam procedure as described previously (14). The probes used were CG11 (14) and GSTP1, a 180-bp fragment from the promoter region of the GSTP1 gene. Each probe was obtained by restriction enzyme digestion of the parent plasmid. In each case, half of the DNA was methylated with SsII methylase (New England BioLabs), and in a parallel reaction, the other half was incubated with methylase in the absence of S-adenosylmethionine (methylation treated). The extent of methylation after each reaction was determined by digestion with a methylation-sensitive restriction enzyme. Probes were labeled using the Klenow fragment of DNA polymerase I and [α-32P]dATP. Assay conditions were as described previously (14), except that a 2% agarose gel was used. Two µg of Micrococcus lysodeikticus DNA-digested with SsII were used as a nonspecific competitor in all reactions. The agarose gel was dried, and radioactive bands were analyzed using a Cyclone phosphorimager.

For antibody supershift/ablation experiments, EMSAs was performed as described above, with or without the addition of one of the following antibodies before the addition of probe to LNCaP cell nuclear extracts: (a) goat polyclonal MBD1, 0.4 and 0.6 µg; or (b) MBD2, 0.4 and 0.6 µg (Santa Cruz Biotechnology, Santa Cruz, CA). These antibodies were raised against the NH2 terminus of MBD1 or MBD2 proteins (Santa Cruz Biotechnology).

RESULTS

For this study, we have used the LNCaP prostate cancer cell line as the model system. Similar to human prostate cancer cells, LNCaP cells have a high affinity nuclear androgen receptor and respond to androgens that stimulate their growth as well as prostatic acid phosphatase and prostate-specific antigen expression at different concentrations (15, 16). Furthermore, as in human prostate cancer, the GSTP1 gene is highly methylated and repressed in LNCaP cells (9).

GSTP1 Gene Expression in LNCaP, DU145, and PC3 Prostate Cancer Cell Lines. Previous studies have examined the expression of the GSTP1 gene by immunohistochemistry or by Northern blot analysis. We studied the expression of the GSTP1 gene in LNCaP, DU145, and PC3 prostate cancer cells by RT-PCR (12). Consistent with a previous report (7), GSTP1 mRNA was not detected in LNCaP cells but was easily detected in DU145 and PC3 prostate cancer cells (Fig. 1). We used RT-PCR for the β-actin gene for RNA integrity and loading control.

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Methylation Pattern of the \textit{GSTP1} Gene Promoter in LNCaP, Du145, and PC3 Prostate Cancer Cell Lines. We determined the methylation pattern of the \textit{GSTP1} gene promoter using MS-PCR techniques as well as bisulfite genomic sequencing (6, 17, 18). Genomic DNA is treated with bisulfite under conditions that convert unmethylated cytosine to uracil, whereas the 5-methylcytosine remains unchanged (17).

For the MS-PCR assay (18), the amplification of the modified DNA is carried out with a set of primers designed to distinguish methylated and unmethylated alleles in the bisulfite-modified DNA, taking advantage of the sequence differences resulting from the bisulfite modification. These primers were designed for genomic sequences containing several cytosines, which allows discrimination between modified and unmodified DNA, and CpG pairs near the 3' end of the primers to provide maximal discrimination in PCR between methylated and unmethylated DNA. In addition, primers for methylated DNA were shorter than those for unmethylated DNA, so that the resultant PCR product from unmethylated and methylated alleles could be easily distinguished on gel electrophoresis.

For the bisulfite genomic sequencing, the PCR amplification of the modified DNA was carried out with a set of primers that can amplify both methylated and unmethylated sequences. These primer sequences include cytosines that allow discrimination between modified and unmodified DNA, and they are devoid of CpG dinucleotides.

We obtained consistent results with both techniques. The \textit{GSTP1} promoter was completely methylated in non-\textit{GSTP1}-expressing LNCaP cells (Fig. 2, C and D), and this result is consistent with the previous reports (7, 9). In Du145 and PC3 cells, the \textit{GSTP1} promoter was partially methylated (Fig. 2, C and D), consistent with the cell populations containing a mixture of unmethylated (\textit{GSTP1}-expressing) and methylated (non-\textit{GSTP1}-expressing) alleles.

Methylation of a \textit{GSTP1} Promoter-driven Reporter Plasmid Represses Expression in Transient Transfection Assays. Because \textit{GSTP1} has an important role in the conjugation and detoxification of potential carcinogens, it was proposed that early loss of its expression could lead to increased susceptibility to carcinogens, promoting mutations and cancer development (7). It is possible that repression of
transcriptional repression may involve direct binding of specific transcriptional repressors to methylated DNA (6, 20). Methylation-mediated repression is a major mechanism of gene repression. We tested whether the GSTP1 proximal promoter (−130 to mRNA start site) has necessary elements for optimal expression (19). We used SacI- and HindIII-linked primers to amplify the GSTP1 promoter from −134 to +45, and cloned it in the pGL3 basic vector. This GSTP1-pGL3 construct shows good activity in a transient transfection assay system. The plasmid GSTP1-pGL3 was methylated using SssI methylase, which is capable of de novo methylation of every CpG in a double-stranded DNA template. The extent of methylation was determined by digestion with methylation-sensitive restriction enzymes (Fig. 3A).

We transfected LNCaP cells with unmethylated or completely methylated GSTP1-pGL3 construct with a pRL-TK plasmid as a control for transfection efficiency. Methylation of the GSTP1-pGL3 plasmid resulted in a >20-fold inhibition of reporter activity (Fig. 3B). Similar results were obtained when Du145 cells were transfected (data not shown). The absolute level of luciferase activity from the mock-methylated GSTP1-pGL3 construct was similar in both LNCaP and Du145 cell lines (data not shown), suggesting that lack of a transcription factor is unlikely to account for GSTP1 gene repression in LNCaP cells.

Methylation-mediated Repression of the GSTP1-promoted Reporter Plasmid Is Not Relieved by the Histone Deacetylase Inhibitor TSA. Direct binding of specific transcriptional repressors to methylated DNA appears to be a major mechanism of methylation-mediated transcriptional repression (6, 20). Methylation-mediated transcriptional repression may involve direct binding of specific transcriptional repressors MeCP1 and MeCP2 to methylated DNA (6). A region of MeCP2 associates with a corepressor complex containing a transcriptional repressor, mSin3A, and histone deacetylases. Transcriptional repression in vivo was relieved by TSA (21). MBD2 is a component of the MeCP1 complex, together with histone deacetylases HDAC1 and HDAC2 (22). It is possible that methylation-mediated repression of the GSTP1 gene promoter may involve interaction of MeCP1 or MeCP2 with a HDAC. If so, TSA might alleviate this repression. To determine whether histone deacetylase activity is central to the methylation-mediated repression of the GSTP1 gene, transcription of methylated and mock-methylated GSTP1-pGL3 constructs in LNCaP cells was carried out in the presence or absence of TSA. Although reporter activity from both mock-methylated and methylated constructs increased 2–3-fold in the presence of TSA (Fig. 3C), the relative difference in the expression remained the same, suggesting that TSA-sensitive histone deacetylase activity is not central to the methylation-mediated repression of the GSTP1 gene.

5-Aza-CdR, but Not TSA, Demethylates the GSTP1 Promoter and Induces GSTP1 Expression in LNCaP Cells. 5-Aza-CdR inhibits DNA methylation by reducing the biochemical activity of DNA methyltransferase via the formation of a covalent complex with this enzyme (23, 24). This is believed to deplete methyltransferase activity and to result in DNA demethylation. We treated LNCaP cells with 5-Aza-CdR in varying concentrations in an attempt to induce GSTP1 gene expression. Consistent with our methylation analysis, induction of GSTP1 mRNA was seen (Fig. 4A). To determine whether this activation was associated with a change in the methylation pattern of the GSTP1 promoter, DNA was isolated from drug-treated LNCaP cells and subjected to MS-PCR assay. As shown in Fig. 4B, 5-Aza-CdR treatment results in at least partial demethylation of the GSTP1 promoter.

We also investigated whether TSA can induce expression of the GSTP1 gene in LNCaP cells. TSA treatment failed to induce GSTP1 expression in the presence of TSA.
MBD1 is a component of MeCP1 (30), subsequent histone deacetylases HDAC1 and HDAC2 (22). Although a previous study (26, 29) has identified four new proteins with closely related MBDs, the mediators involved in the MeCP1 complex (22). Antisera raised against the NH2-terminal of MBD2 depleted the MeCP1 complex from the HeLa extracts (22). We examined the ability of NH2-terminal MBD1 and MBD2 antibodies (Santa Cruz Biotechnology) to deplete the complex formed with methylated GSTP1 probe and LNCaP cell nuclear extract in a bandshift assay. This complex was competed by NH2-terminal MBD2 but not by MBD1 antibody (Fig. 5, B and D), suggesting that this complex is similar to the previously reported MeCP1 complex in HeLa cells (22).

**Discussion**

The principal findings in this study are as follows. The GSTP1 gene promoter is completely methylated in non-GSTP1-expressing LNCaP cells. In contrast, Du145 and PC3 prostate cancer cells, which express the GSTP1 gene, contain unmethylated and methylated GSTP1 alleles. In vitro methylation of the GSTP1 promoter-driven reporter plasmid markedly reduces the expression in a transient transfection assay in LNCaP cells, and this repression is not relieved by the presence of a histone deacetylase inhibitor, TSA. The GSTP1 gene expression associated with demethylation is induced in LNCaP cells by a DNA methyltransferase inhibitor, 5-Aza-CdR, but not by TSA. A methyl CpG-binding protein complex that behaves like MeCP1 binds to the fully methylated GSTP1 promoter fragment but not to the unmethylated GSTP1 promoter fragment in EMSAs using LNCaP cell nuclear extracts.

Glutathione plays an important role in scavenging reactive oxygen species and their metabolites, which, in turn, may be direct downstream mediators of growth factor receptor signaling. Overexpression of a superoxide-producing enzyme in fibroblasts appears to be sufficient for transformation (31). Glutathione and GST could act as tumor suppressors by down-regulating this pathway. A direct role for GSTP1 in oncogenic signal transduction pathways has recently been elucidated, and GSTP1 has been identified as an inhibitor of the protein kinase c-jun NH2-terminal kinase (32). The GSTP1 gene methylation is an early event in prostate carcinogenesis and is detected in a high percentage of high-grade PIN (8). It is possible that failure of physiological production of GSTP1 renders prostatic cells susceptible to the carcinogenic effect of electrophilic substances. Several studies have shown the association of GSTP1 methylation and lack of expression in human prostate cancer (7–10). Methylation of the GSTP1 gene may be important in its repression in human prostate cancer. Alternatively, it is possible that GSTP1 gene repression may be due to the loss of a transcription factor necessary for maintenance of GSTP1 expression and that methylation is a secondary event. Because the unmethylated GSTP1 promoter-driven reporter plasmid has similar activity in non-GSTP1-expressing LNCaP cells and GSTP1-expressing Du145 cells, it is unlikely that lack of a transcription factor is responsible for GSTP1 repression in LNCaP cells. Our transfection data, together with demethylation and activation of the GSTP1 gene with 5-Aza-CdR treatment, support the view that cytosine methylation represses GSTP1 gene expression in the LNCaP prostate cancer cell line.

Many transcription factors have CpG sites in their DNA recognition site, and binding of several of these factors is abolished by CpG methylation. Because several densely methylated genes continue to be transcribed in the absence of chromatin and/or methyl-CpG-binding proteins, direct interference with transcription factors is likely to play only a minor role in methylation-mediated transcriptional repression (20). Direct binding of specific transcriptional repressors to methyl-

mRNA expression in LNCaP cells (Fig. 4A). In Neurospora crassa, TSA treatment resulted in derepression associated with demethylation of the hph gene (25). We examined the methylation pattern of the GSTP1 promoter in TSA-treated cells. As shown in Fig. 4B, the GSTP1 promoter remained completely methylated in these cells.

**Methylated GSTP1 Promoter Binds to a MeCP1-like Complex in Nuclear Extracts from LNCaP Cells.** Methylation-mediated transcriptional repression may involve direct binding of specific transcriptional repressors to methylated DNA (6). MeCP1 and MeCP2 have been identified and shown to bind to methylated CpG residues in any sequence context (6). MeCP1 binds to at least 10 symmetrically methylated CpGs, in contrast to MeCP2, which is more abundant than MeCP1 in the cell and is able to bind to DNA containing a single methylated CpG pair (22, 26, 27). MeCP1 has been implicated in methylated transcription-mediated transcriptional repression of several genes including the human α-globin gene in heterologous cultured cells (28, 29). F9 cells, which contain low MeCP1 levels, cannot efficiently repress methylated gene promoters (29).

We have previously shown that a MeCPC with electrophoretic mobility similar to that of MeCP1 forms efficiently with the methylated but not with the unmethylated avian embryonic α-globin gene promoter sequence (14). Despite numerous examples of methylation-mediated gene silencing in cancer, little is known about the mediators involved. The GSTP1 promoter region that was cloned in the expression construct, GSTP1-pGL3, contains more than 20 CpG dinucleotides. This 180-bp fragment could potentially bind to MeCP1. We investigated whether the methylated GSTP1 promoter fragment binds to MeCP1 in an EMSA as described previously (14, 26), using LNCaP nuclear extract. Fully methylated but not unmethylated GSTP1 probe binds to a complex similar in mobility to MeCP1 (Fig. 5; data not shown). This complex is effectively competed by an excess of methylated GSTP1 promoter fragment but not by unmethylated GSTP1 promoter fragment (Fig. 5, A and C).

A search of the expressed sequence tag database with the MBD of MeCP2 has identified four new proteins with closely related MBDs 1–4. MBD2 is a component of the MeCP1 complex, together with histone deacetylases HDAC1 and HDAC2 (22). Although a previous report claimed that MBD2 is a component of MeCP1 (30), subsequent experiments with antibodies highly specific for MBD1 failed to supershift or immunodeplete MeCP1 activity and argued against its involvement in the MeCP1 complex (22). Antisera raised against the NH2-terminal of MBD2 depleted the MeCP1 complex from the HeLa extracts (22).
ated DNA appears to be a major mechanism of transcriptional repression (6, 20). MeCP1 binds to DNA containing multiple symmetrically methylated CpGs and migrates as a large complex on EMSA (22, 26). MeCP1 can mediate repression of transcription from densely methylated genes, and cells deficient in MeCP1 show greatly reduced repression of methylated genes (28). We have shown that a MeCPC with electrophoretic mobility similar to that of MeCP1 forms efficiently with methylated but not unmethylated avian embryonic \(\alpha\)-\text{globin} gene promoter sequence (14). This complex can be detected in nuclear extracts from the primary avian erythroid cells in which methylation-mediated transcriptional inhibition was demonstrated, suggesting that MeCP1 or a similar complex is involved in developmental silencing of embryonic globin genes in normal erythropoiesis (14). Although there are numerous examples of cancer-associated gene hypermethylation (6, 33), little is known regarding the mediators involved in methylation-mediated repression. The present study indicates that \(\text{GSTP1}\) repression in the LNCaP prostate cancer cells may be mediated by a MeCP1-like complex.

Recent work has indicated that underlying patterns of methylated cytosines are important in guiding histone deacetylation to certain residues (21, 34). A region of MeCP2 that localizes with the transcriptional repressor domain was shown to associate with a co-repressor complex containing the transcriptional repressor mSin3A and histone deacetylases. Transcriptional repression \textit{in vivo} was relieved by the deacetylation inhibitor TSA, suggesting that two global mechanisms of gene regulation, DNA methylation and histone deacetylation, can be linked by MeCP2 (21, 34). Recently, histone deacetylases HDAC1 and HDAC2 have also been shown to be components of MeCP1 (22). However, another study has shown that the hypermethylated genes \(\text{MLH1}, \text{TIMP3}, \text{CDKN2B} (\text{INK4B}, p15),\) and \(\text{CDKN2A} (\text{INK4}, p16)\) cannot be transcriptionally reactivated with TSA alone in tumor cells in which TSA alone can up-regulate the expression of nonmethylated genes (35). Our data also support the notion of the dominance of methylation over histone deacetylation in silencing genes with a high CpG density in the promoter region. However, certain recently described deacetylases such as yeast SIR2 (36) and yeast HOS3 (37) are not inhibited by TSA. It remains to be determined whether methylation-mediated repression involves TSA-insensitive HDAC activity.

In summary, cytosine methylation in \(\text{GSTP1}\)-regulatory sequences associated with the loss of \(\text{GSTP1}\) expression has been observed in a majority of human prostate carcinomas (7) and PIN (8). In this study, we demonstrated that cytosine methylation represses expression of the \(\text{GSTP1}\) gene in LNCaP cells and has a dominant role over TSA-sensitive histone deacetylase activity. Understanding the molecular mechanism for the methylation of the \(\text{GSTP1}\) gene promoter may provide insights into the development of prostate cancer.
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