HDAC1 Inhibition by Maspin Abrogates Epigenetic Silencing of Glutathione S-Transferase Pi in Prostate Carcinoma Cells

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Abstract

Both maspin and glutathione S-transferase pi (GSTp) are implicated as tumor suppressors and downregulated in human prostate cancer. It is well established that GSTp downregulation is through DNA methylation–based silencing. We report here that maspin expression in prostate cancer cell line DU145 reversed GSTp DNA methylation, as measured by methylation-specific PCR, MethylLight assay, and bisulfite sequencing. The effect of maspin on GSTp expression was similar to that of the combination of a synthetic histone deacetylase (HDAC) inhibitor and DNA methylation inhibitor 5-aza-2′-deoxycytidine. Maspin expression also led to an increased level of acetylated histone 3, decreased level of methyl transferase, and methyl-CpG–binding domain proteins at the site of demethylated GSTp promoter DNA. Earlier, we have shown that maspin inhibits HDAC1. In PC3 cells, where both maspin and GSTp are expressed at a reduced level, maspin knockdown led to a significant reduction in GSTp expression, whereas dual knockdown of maspin and HDAC1 barely increased the level of GSTp expression. Thus, HDAC1 may play an essential role in cellular response to maspin-mediated GSTp desilencing. Maspin has been shown to increase tumor cell sensitivity to drug-induced apoptosis. Interestingly, GSTp reexpression in the absence of maspin expression perturbation blocked the phosphorylation of histone 2AX, the induction of hypoxia-induced factor 1α (HIF-1α), and cell death of LNCaP cells under oxidative stress. Because DNA hypermethylation–based silencing may couple with and depend on histone deacetylation, our study suggests that endogenous HDAC inhibition by maspin may prevent pathologic gene silencing in prostate tumor progression.

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Introduction

DNA methylation–based pathologic gene silencing is common in carcinogenesis and tumor progression. Mechanistic studies suggest that epigenetic silencing by DNA methylation may be mechanistically coupled to histone deacetylation, whereas maximal desilencing effects require the combination of DNA methylation inhibitor and histone deacetylase (HDAC) inhibitor (1, 2). To date, as efforts are made to target tumor-specific DNA methylation, broad-spectrum pharmacologic HDAC inhibitors have shown promising antitumor activity in clinical trials. It is worth noting that the functional elucidation of HDAC has been conducted almost exclusively through the use of pharmacologic HDAC inhibitors, as the mechanism of endogenous regulation of HDAC activity remains largely unknown. We have recently reported the first evidence that the tumor suppressor maspin specifically inhibits HDAC1, the most abundant nuclear (class I) HDAC in mammalian cells that is commonly upregulated in malignant diseases (3, 4). Maspin is a member of the serine protease inhibitor (serpin) superfamily but lacks inhibitory activity against active serine proteases (5, 6). Nonetheless, structural considerations (7) suggest that maspin may act as a serine protein inhibitor–like molecule to interact with serine protease–like HDAC1. The activity of HDAC1 in hydrolyzing the ε-acetyl lysine residues in its substrate proteins (8, 9) is the function of a catalytic domain, which resembles that of metalloproteinases or serine proteases (10). To our knowledge, maspin is the only endogenous polypeptide HDAC1 inhibitor identified so far. Maspin is expressed in an epithelial-specific manner thus may act as an epithelial-specific regulator of HDAC1 activity.

The biological functions of HDAC1 are thought to derive mostly from its activity in deacetylating nuclear histones (10), leading to chromatin condensation and transcription repression of target genes such as p21, p27, and Bax (11, 12). Consistent with the evidence that maspin inhibits HDAC1, we have shown that maspin expression in prostate epithelial cells increased the expression of p21 and Bax, restored prostate epithelial differentiation, and sensitized tumor cells to drug-induced apoptosis (13, 14). The identification of
HDAC1 as a maspin target (15, 16) could explain, at least in part, the evidence that nuclear maspin correlates with better epithelial differentiation and better prognosis of lung adenocarcinoma (17, 18).

In general, the biological effects of maspin are similar to those of pharmacologic HDAC inhibitors (15). Interestingly, many pharmacologic HDAC inhibitors are also associated with upregulation of detoxifying redox enzymes (19, 20), suggesting that HDAC may naturally repress the expression of these enzymes. Of particular interest is glutathione S-transferase pi isoform (GSTp), commonly downregulated in prostate tumor progression, primarily by DNA methylation (2, 21). We have previously shown that maspin expression increased total cellular GST activity in human cancer cell lines (16). These data are consistent with our earlier observation that maspin is translocated from the nucleus to the cytosol at the step of high-grade prostatic intraepithelial neoplasia (HPIN; ref. 22), coinciding with the GSTp silencing event in prostate tumor progression (23, 24).

The exact biological trigger of GSTp gene silencing in prostate cancer is yet to be further elucidated. On the basis of the emerging evidence that genetic silencing may result from coordinated histone deacetylation and DNA methylation, the current study was aimed at testing whether endogenous inhibition of HDAC1 by maspin may prevent the silencing of GSTp transcription in prostate tumor cells. We present the first evidence that maspin-mediated desilencing of GSTp depends on HDAC1 and is associated with the reduction of GSTp DNA methylation. Our data also suggest that desilencing of GSTp by maspin may block oxidative stress–induced DNA damage and the expression of hypoxia-induced factor 1α (HIF-1α). These data suggest that the loss of endogenous inhibition of HDAC1 by maspin may lead to the silencing of other tumor suppressors such as GSTp thus representing a significant gain of function in tumor progression. Taken together, maspin-mediated GSTp desilencing supports a mechanistic link between endogenous DNA methylation and histone deacetylation.

Materials and Methods

Chemicals, cell lines, and cell culture

Pan HDAC inhibitor M344, an analogue of classic HDAC inhibitor trichostatin A (TSA; refs. 25, 26), was purchased from Calbiochem. Class I HDAC–specific inhibitor LBH589 was provided by Novartis Pharmaceuticals. DNA demethylation agent 5-aza-2'-deoxycytidine (5-Aza) and H2O2 were purchased from Sigma-Aldrich. Protease inhibitor MG132 was purchased from Sigma-Aldrich.

Human prostate carcinoma cell lines DU145, PC3, and LNCaP were obtained from American Type Culture Collection and maintained in RPMI 1640 media (Invitrogen) containing FBS (HyClone; 5% for DU145; 10% for PC3 and LNCaP) at 37°C with 6.5% CO2. DU145-derived, maspin-transfectant clones (M3, M7, and M10) and the mock transfectant clone (Neo) were generated and cultured as described previously (27). Normal human prostate epithelial cell lines CF3, CF91, and MLC8891 were obtained from John Rhim (Uniformed Services University of Health Sciences, Bethesda, MD). For drug treatment, cells were cultured in 6-well plate overnight and treated with the indicated drugs or H2O2 for 24 hours (for subsequent PCR assay) or 48 hours (for subsequent Western blot [WB] assay).

Plasmid construction and stable transfection of LNCaP cells for GSTp expression

GSTp cDNA (NM_000852) in MDA-MB-231 breast cancer cell line was PCR amplified, sequence verified, and used as a template for subcloning. To construct the expression vector, GSTp open reading frame was amplified using the following primers: 5'-CAGGGAATTCATATG-CGGGCCCTACA-3' (forward primer) and 5'-CAGTAC-TCGAGCTCAGTTTCCCGTG-3' (reverse primer). The amplified GSTp coding sequence was inserted in-frame into pcDNA6/His A vector (Invitrogen) via EcoRI and Xhol cloning sites. The ligation product, encoding Xpress-tagged GSTp protein, was used to transform XL-10 Gold Ultra-competent cells (Stratagene). The Escherichia coli clones confirmed positive for GSTp expression plasmid were selected and used to amplify the plasmid DNA that were subsequently sequence verified. The Xpress GSTp encoding plasmid DNA was transfected into LNCaP cells using TransFectin Lipid reagents (Bio-Rad Laboratories), followed by Blasticidin S HCl (BSD; Invitrogen) at a final concentration of 3 μg/mL in RPMI 1640 medium. Control transfection was conducted with the vector that encodes Xpress-tagged β-galactosidase (LacZ). The resulting clonal cell lines were designated as LNCaP/GSTp and LNCaP/LacZ, respectively.

Transient transfection

For maspin knockdown by siRNA, cells cultured in 6-well plates were transfected with vehicle, a maspin-specific siRNA SMARTpool (Mas-siRNA; Dharmacon), or an siRNA with a scrambled sequence (Scr-siRNA) at 15 μmol/L, using the siLentFect Lipid Reagent (Bio-Rad Laboratories), followed by Blasticidin S HCl (BSD; Invitrogen) at a final concentration of 3 μg/mL in RPMI 1640 medium. Control transfection was conducted with the vector that encodes Xpress-tagged β-galactosidase (LacZ). The resulting clonal cell lines were designated as LNCaP/GSTp and LNCaP/LacZ, respectively.
the resulting plasmid DNAs (Mas-shRNA) was used to transfect PC3 cells using Fugene HD transfection reagents (Roche Applied Science). G418-resistant stable clones were selected.

To knockdown HDAC1 expression, a GIPZ lentiviral shRNAmir clone RHS4430-98820597 (Thermo Scientific) was used to infect the cells at 1:1 of MOI (multiplicity of infection) for 48 hours followed by 3 more days of culture with the maintenance medium supplemented with 0.6 mg/mL puromycin. shRNAmir clone RHS4430-98820597 encodes a hairpin sequence of 5'-CCC GAA TCC GCA TGA CTC ATA ATA GTG AAG CCA CAG ATG TAT TAT GAG TCA TGC GGA TTC GG-3', which targets a 158 to 176 sequence at 5'-terminal HDAC1 mRNA. Cells harvested were lysed, and the total cell lysates were subjected to WB.

### Quantification of cell viability

Initially, cells were seeded in 6-well plates at 2 × 10^4 cells per well for vehicle treatment (control) and sublethal dose of M344 (0.1 μmol/L) treatment, and at 5 × 10^5 cells per well for 5-Aza (2 μmol/L) with or without M344 (0.1 μmol/L) treatment. The treatment of 5-Aza lasted for 8 days. During the 8-day treatment period, cell culture medium containing 5-Aza was replenished every 2 days. For combination treatment, cells were treated additionally with M344 in the last 48 hours as described by Okino and colleagues (29). Then, cells were washed gently to remove the floating dead cells. The remaining adherent cells were cultured in the maintenance media for another 24 hours. Cells were evaluated under the microscope and photographed with a SPOT digital camera (Diagnostic Instruments). The cells were then harvested by trypsin digestion, resuspended in an isotonic solution, and counted using a Coulter Particle Counter (Beckman Coulter Inc.).

Cell viability was also determined using the WST-1 Kit (Roche Diagnostics) according to the manufacturer’s instruction. Briefly, cells were seeded at the density of 4 × 10^4/0.1 mL per well in 96-well plate and cultured with or without a cytotoxic agent at the indicated concentrations for 48 hours. Then, the WST assay was conducted. The resulting absorbance at 450 nm (OD_{450}) was considered a function of cell viability. Using this method, the IC_{50} of each cytotoxic drug was determined as the drug concentration that led to 50% of loss of the cell viability as compared with the untreated control.

### Western blot

Protein concentration of cell lysates was determinate by the BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein were loaded on SDS-PAGE for WB as described previously (15). The primary antibodies used for immunoblotting were as follows: rabbit anti-GSTp (Calbiochem), anti-Xpress McAb (Invitrogen), PARP (eBioscience), monoclonal antibody (mAb) against phosphorylated histone H2AX (ser-139; p-H2AX; Cell Signaling Technology), histone 3 (H3) acetylated at Lys9 residue (K9; Upstate cell signaling solution), anti-maspin mAb (BD PharMingen), and mAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Research Diagnostic). Corresponding secondary antibodies were conjugated with horseradish peroxidase and detected by enhanced chemiluminescence (ECL) reaction.

### Northern blotting of mRNA

Total RNA (20 μg) extracted using a method previously described (15) was heat denatured at 68°C for 15 to 20 minutes followed by electrophoresis through 1.0% agarose gel containing 1.7 mol/L formaldehyde. The fractionated RNA was transferred to a Zetaprobe membrane (Bio-Rad). The membrane was baked at 80°C for 2 hours. The prehybridization, hybridization, and X-ray radiography were carried out as described (30, 31).

### Quantitative real-time PCR

Total RNA was extracted from cells using a previously described method (15). One microgram of each RNA sample was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Triplicate quantitative real-time PCR (q-RT-PCR) reactions were routinely conducted in the laboratory as described (15). The primers used were as follows: GAPDH, 5'-ACGGATTTGCTGATTGG-3' and 5'-TGAGTTTTTGAGGAGATCTGCG-3'; GSTp, 5'-ACCTCCGCTGAAAATACATC-3' and 5'-GGGTAG-GACCTCATGGATCA-3'; GSTm3, 5'-GTTGGCAGT-GCTCTATGGTTC-3' and 5'-AGTTGTGTGGCGGAAATCCAT-3'. The generic primers that detect a common sequence in GSTm1, m2, m4, and m5 (GSTm1245) were 5'-GGGACGCTCCTGTATGAC-3' and 5'-GCACATTGGCCTTT-3'. The primers for HIF-1α were 5'-TCAACAGTACGGGAAATGGG-3' and 5'-TTTACACGTITCCTCAGAAGATGATGTA-3'. The q-RT-PCR thermal profile was as follows: 1 cycle of 95°C/10 minutes, 40 cycles of 95°C/30 seconds→55°C/1 minute→72°C/30 seconds, 1 cycle of 95°C/1 minute, and finally 41 cycles of 95°C→40°C/30 sec. Critical threshold cycle numbers (C_t) were calculated using the built-in program of the Stratagene Mx4000 Multiplex Quantitative PCR System. The fold differences in mRNA were calculated by the method (32).

### Methylation-specific PCR

Methylation-specific PCR (MS-PCR) was carried out using an established method with minor modifications (29, 33). Briefly, to amplify the GSTp promoter sequence from 1,001 to 1,302 bp, genomic DNA was isolated from the cultured cells with a FlexiGene DNA kit (QIAGEN). The genomic DNA was then treated by bisulfite using the EpinTech Bisulfite Kit (QIAGEN). After the bisulfite treatment, DNA was purified by using the Wizard Plus Miniprep DNA Purification System (Promega). This DNA, along with untreated genomic DNA (as a control), was subjected to PCR amplification. PCR amplification was conducted using the Phusion PCR Amplification Kit (New England Biolabs). The primers for a 272-bp methylated GSTp promoter fragment were 5'-GAAAGAGGGAAAGGTTTTTTCGG-3' and 5'-CCC GAA TCC GCA TGA CTC ATA ATA GTG AAG CCA CAG ATG TAT TAT GAG TCA TGC GGA TTC GG-3'.
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(forward) and 5′-GAAAACGAAACGGCGGTACT-3′ (reverse). The primers for a 276 bp unmethylated GSTp promoter fragment that could potentially be subjected to methylation were 5′-GGGGAGGGGAAGTGGTTTTT-TG-3′ (forward) and 5′-CCCAAAAAACAAACATAC-3′ (reverse). The primers for total control GSTp promoter fragment were 5′-CCGTGGAAGG-GGAAAGGC-3′ (forward) and 5′-TCAGTGTTGGAG-AAGACTGC-3′ (reverse). The PCR cycling parameters were as follows: 98°C for 2 minutes followed by 35 cycles of 98°C/30 seconds—60°C/30 seconds—72°C/1 minute with a final extension step of 72°C for 5 minutes. The PCR products were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

**MethyLight PCR**

MethyLight PCR was carried according to the specific instruction of Epitect MethyLight PCR kit from QIAGEN. Briefly, methylation specific primers and TaqMan Probes for GSTp (34) and internal reference control MYOD-1 (35) were synthesized by Applied Biosystems. To quantify methylated GSTp DNA (GenBank X08058) in the promoter region (from 894 to 1,116 bp), 2 rounds of real-time PCR were carried out with a genomic GSTp DNA template using the Mastercycler ep reaplex2 (Eppendorf North America Inc.). The PCR cycling program was as follows: 95°C/5 minutes followed by 50 cycles of 95°C/15 seconds—60°C/1 minute (34, 35). The percentage of methylation (POM) was calculated on the basis of 18S and 28S ribosomal RNAs on agarose gel electrophoresis.

**Bisulfite DNA sequencing**

Bisulfite DNA sequencing was carried out using the method of Okino and colleagues (29). Briefly, bisulfite-converted DNA was amplified by 2 rounds of PCR reaction using Phusion High-Fidelity PCR kit (New England Bio-Labs, Inc.) with the cycling conditions described for MS-PCR, except that the annealing step was set at 43°C. The PCR products were resolved by 2% agarose gel electrophoresis, excised from the gel, and sequenced by the Applied Genomics Technology Center of WSU.

**Chromatin immunoprecipitation assay**

The chromatin immunoprecipitation (ChIP) assay was carried out by the EZ-ChIP Kit (Millipore) according to the protocol recommended by the manufacturer. Briefly, cells grown to 80% confluence were fixed by 1% formaldehyde and incubated for 15 minutes to allow protein–DNA crosslinking. Then, the cells were harvested, washed with cold PBS, and resuspended to the density of 5 × 10⁶ cells/500 μL in lysis buffer. Cell suspensions were sonicated (to shear chromatin DNA into 200 to 700 bp fragments), and centrifuged for 10 minutes at 12,000 × g at 4°C. The resulting supernatants were incubated with protein G-agarose beads (Upstate Biotechnology) for 1 hour and centrifuged at 2,000 × g for 30 seconds to remove the agarose slurry. The supernatant of each sample was equally divided into 6 aliquots. One aliquot was used as an input control, whereas the other 5 aliquots were used for immunoprecipitation with HDAC1 McAb, Rabbit anti-Ac-H3 (K9) antibody, rabbit anti-DNMT1 antibody, rabbit anti-MBD2/3 antibody (Upstate Cell Signaling Solution), and normal rabbit immunoglobulin G mixture (Santa Cruz Biotechnology), respectively. The resulting mixtures were gently agitated overnight at 4°C. The immunocomplexes were collected by incubation with protein G-agarose beads (Upstate Biotechnology) for 1 hour at 4°C. After the beads were washed in the sequence of low salt, high salt, LiCl and Tris-EDTA (TE) buffer, the protein–DNA cross-links were reversed by heating at 65°C for 4 hours in 3.5 mol/L NaCl, followed by RNase A treatment for 30 minutes at 37°C and proteinase K treatment overnight. The purified DNA samples were PCR amplified using the Phusion PCR Amplification Kit with the primers designed for a 133-bp fragment in the GSTp promoter region (36). The primer sequences for Gstp1 promoter in the ChIP were previously described (37). The PCR products were visualized by 1.5% agarose gel electrophoresis.

**Miscellaneous**

The quality of RNA was determined on the basis of the integrity of 18S and 28S ribosomal RNAs on agarose gel electrophoresis. For statistical analyses, 1-tailed matched pair Student’s t tests were carried out. Values of P < 0.01 were considered significant.

**Results**

**Maspin downregulates GSTp expression**

Both maspin and GSTp are epigenetically downregulated in human prostate tumor progression (15, 38–40). Interestingly, the level of maspin expression correlated with that of GSTp in a panel of prostate epithelial cell lines (Fig. 1A). Earlier, we also showed that maspin expression in both breast and prostate cancer cell lines increased the total cellular GST enzyme activity, raising the possibility that maspin may positively regulate the expression of GSTp (16). Indeed, when maspin was endogenously expressed in a prostate cancer cell line DU145 via stable transfection, GSTp was significantly and dose dependently upregulated (Fig. 1B). To examine whether maspin expression altered the expression of other GST isoforms, q-RT-PCR was carried out using the primers specific for GSTp, GSTm3, and a consensus sequence shared by GSTm1, m2, m4, and m5 (collectively designated as GSTm1245). As shown in Figure 1C, GSTp mRNA was the only significantly upregulated in M7 cells, as compared with that in the mock-transfected control Neo cells. To verify the specificity of the maspin effect on GSTp expression, prostate cancer cell line PC3 that expresses both maspin and GSTp at a moderate level, as compared with normal immortalized prostate epithelial cells, were subjected to siRNA-mediated maspin knockdown. As shown in Figure 1D and E, either permanent or transient transfection–mediated maspin downregulation

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Maspin Desilences GSTp Transcription

Figure 1. Maspin upregulates the expression of GSTp. A, Northern blotting of GSTp, maspin, and housekeeping gene 36B4 in prostate epithelial cell lines. CF3, CF91, and MLC8891 are immortalized normal human prostate epithelial cell lines, whereas PC3, DU145, and LNCaP are established human prostate carcinoma cell lines. B, WBs of GSTp, maspin, and GAPDH (loading control) in DU145-derived stably transfected clonal cell lines Neo and M7. C, q-RT-PCR detection of different isoforms of GST in DU145-derived stably transfected clonal cell lines Neo, M3, M7, and M10. D, q-RT-PCR detection of GSTm3, GSTm12, and GSTm45. E, WBs of GSTp, maspin, and a scrambled sequence (Scr-siRNA) or of a scrambled sequence (Mas-siRNA) or of a scrambled sequence (Scr-siRNA). F, WBs of GSTp, maspin, and β-tubulin in parental PC3 cells, transiently transfected PC3 cells by siRNA oligonucleotide against maspin (Mas-siRNA) or of a scrambled sequence (Scr-siRNA), or of a scrambled sequence (PC3/Scr), and clonal PC3 cell line 1D5 derived from stable transfection with maspin shRNA.

led to a significant downregulation of GSTp, suggesting that the effect of maspin on GSTp expression was specific. Transcriptional downregulation of GSTp in human prostate tumor and in established prostate cancer cell lines have been shown to result, at least in part, from DNA methylation (39, 41). Thus, the observed GSTp upregulation on maspin transgene expression and GSTp downregulation on maspin knockdown, suggest that maspin may prevent and/or reverse DNA methylation–based transcriptional silencing of GSTp. Indeed, as determined by MS-PCR, the GSTp promoter DNA in maspin-transfected DU145 cells was significantly hypomethylated as compared with that in the Neo control cells (Fig. 2A). The ratio of methylated to unmethylated GSTp promoter DNA was significantly lower in multiple maspin-transfected DU145 clones than in Neo cells as confirmed by quantitative MethyLight analysis (Fig. 2B) and by bisulfite DNA sequencing (Fig. 2C). The extent to which GSTp methylation was reduced in different clonal cell lines varied slightly among the 3 different assays, largely due to the fact that a different set of primers targeting different GSTp genomic DNA sequences were used. Nonetheless, the effect of maspin on GSTp DNA demethylation was dramatic. In fact, all 4 putative CpG methylation sites in the region of −163 bp to −120 bp of GSTp promoter were demethylated in M7 cells. In contrast, these 4 sites remain methylated in Neo cells.

Accumulated evidence suggests that histone acetylation is required for DNA demethylation. Thus, maximal desilencing of many epigenetically silenced genes may be achieved only through simultaneous inhibition of HDAC activity and DNA methylation. As expected, a significant increase of GSTp expression was detected when Neo cells were treated with the combination of 5-Aza (a DNA methyltransferase inhibitor) and M344 (an HDAC inhibitor; Fig. 3A) or the combination of 5-Aza and LBH589 (Fig. 3B). The induction of GSTp on the treatment of Neo cells with either M344 or LBH589 was modest, much less than that observed with maspin-transfected cells. The GSTp induction following the combination treatment of Neo cells with 5-Aza and HDAC inhibitors was comparable as that in maspin-transfected DU145 cells (M7 clonal line). Previously, we showed that tumor cell sensitivity to pharmacologic HDAC inhibitors was enhanced by endogenous expression of maspin (15). We speculated that endogenous inhibition of HDAC1 by maspin may reduce the level of targetable HDACs and generally lower the cytotoxic concentrations of pharmacologic inhibitors. In this study, we found that maspin further enhanced the desilencing effects of pharmacologic inhibitors of DNA methylation and/or HDAC. As shown in Figure 3A and B, when M7 cells were treated with the combination of 5-Aza and LBH589 (or 5-Aza plus M344), GSTp expression was significantly further elevated. To verify the effect of endogenous maspin on the effects of DNA desilencing drugs, PC3 cells that were transiently transfected with maspin siRNA oligonucleotides were treated with 5-Aza. As shown in Figure 3C, maspin knockdown in PC3 cells reduced the desilencing effects of 5-Aza on GSTp.

Because maspin acts as an endogenous HDAC1 inhibitor, we next tested whether HDAC1 was required for the maspin effects of GSTp desilencing. As compared with the effect of maspin knockdown, maspin/HDAC1 double knockdown (1D5/HDAC1-shRNA cells) slightly increased the level of GSTp expression (Fig. 4A). Nonetheless, the level of GSTp expression in 1D5/HDAC1-shRNA cells was...
significantly lower than that in PC3 cell–derived cells that expressed either a scrambled siRNA sequence or the HDAC1 shRNA. These data suggest that maspin and HDAC1 exert opposing effects on GSTp expression and support an antagonistic functional link between the 2 proteins. Earlier, we showed that maspin expression increased the expression of HDAC1 target genes p21, Bax, maspin, and cytokeratin 18 (15). Consistently, using the ChIP condition described by Kobayashi and colleagues (37), the HDAC1 antibody pulled down the promoter sequence of the \( p21 \) gene (Fig. 4B). ChIP was also carried out to test whether maspin was directly involved in the binding of GSTp DNA with enzymes that control DNA methylation and/or histone acetylation. Not surprisingly, M7 cells exhibited a higher level of association between GSTp promoter and acetylated histone 3 (Ac-H3) than Neo cells (Fig. 4B and C). M7 cells also had less GSTp promoter DNA associated with DNA-methylating enzymes, DNA (cytosine-5)-methyltransferase 1 (DNMT1), and methyl-CpG binding domain family of proteins MBD2/3, which has been shown to be directly targeted by HDAC1-mediated gene repression (42, 43). It was noted that the GSTp promoter DNA was not associated with HDAC1 in either Neo or M7 cells. Not surprisingly, maspin and HDAC1 regulate DNA methylation through histone modification, rather than directly participating in the DNA-modifying enzyme complex.

**Maspin regulates GSTp in the balance between apoptosis sensitivity and oxidative stress resistance**

Maspin has been shown to inhibit tumor invasion and metastasis and sensitizes tumor cells to drug-induced apoptosis (13, 31, 44), whereas the biological effects of GSTp are poorly understood except that its glutathione-dependent enzyme activity has been implicated in the reduction of disulfide bonds in JNK (c-\( \text{jun} \) NH, kinase) and the regulation of JNK signaling (45). Although the inhibitory effect of maspin on HDAC1 may potentially desilence other methylated genes, our earlier evidence that maspin increased cellular redox capacity and GST activity (16) suggests an important functional connection between maspin and GSTp transcriptional regulation. To address the potential biological consequences of GSTp desilencing, we stably transfected prostate carcinoma cell line LNCaP with plasmid vector that encodes wither an Xpress-GSTp fusion protein (LNCaP/GSTp cells) or Xpress-LacZ fusion protein (LNCaP/LacZ cells, as control). The LNCaP cell line was of choice for this experiment because it expresses maspin at a low level and does not express GSTp (Fig. 1A). In addition, others have shown that the loss of GSTp expression in LNCaP cells was due to DNA hypermethylation (29). On the basis of the enzymatic activity of GSTp, we speculated that GSTp may confer drug resistance or oxidative stress resistance. Indeed, when treated with LBH589, LNCaP/GSTp cells were more resistant than LNCaP/LacZ
cells (Fig. 5A). The IC_{50} of LNCaP/GSTp and LNCaP/LacZ cell lines were 0.1 and 0.02 µmol/L, respectively. Consistently, as shown in Figure 5B, when treated with LBH589, LNCaP/GSTp cells underwent less apoptosis than LNCaP/LacZ as judged by the extents of PARP cleavage. In both cell lines, LBH589 induced the expression of maspin and increased the level of histone 3 acetylation (Ac-H3). Although this result further supports an earlier notion of the positive feedback loop between maspin and HDAC inhibition (15, 46–48), we also noted that the increase of both maspin and Ac-H3 in response to LBH589 was more significant in LNCaP/GSTp than in LNCaP/LacZ cells. This maspin induction may not be due to the change in protein stability which was not affected by proteasome inhibitor MG132 (at a nontoxic concentration) or by the combination of MG132 and LBH589 in LNCaP/GSTp or M7 cells (Fig. 5C). On the other hand, because the inhibitory effects of maspin on HDAC1 may be further enhanced by a GST, as shown in our earlier report (16), these data raised the possibility that GSTp is not only upregulated by maspin but also enhances the inhibitory effects of maspin on HDAC activity.

LNCaP/GSTp cells were also significantly more resistant than LNCaP/LacZ cells to oxidative stressor H_{2}O_{2} (Fig. 6A), as judged by the level of histone 2A.X phosphorylation (p-H2A.X, a marker for DNA damage). As compared with LNCaP/LacZ cells, LNCaP/GSTp cells treated with H_{2}O_{2} underwent less detachment and rounding (Fig. 6B). In addition, H_{2}O_{2} was less effective in inducing expression of HIF-1α in LNCaP/GSTp cells than in LNCaP/LacZ cells (Fig. 6C). Because HIF-1α is a major transcription activator of VEGF-A, which is known for promoting tumor angiogenesis (49), our data suggest that sustained GSTp expression in the presence of maspin may block angiogenesis in an oxidatively stressed tumor microenvironment. This is consistent with our earlier reports that maspin blocks H_{2}O_{2}-induced VEGF-A expression in vitro (16) and blocks tumor angiogenesis in vivo (13).

Because of the mechanistic link between histone acetylation and DNA methylation, maspin, an endogenous HDAC inhibitor, may regulate the expression of HDAC target genes such as p21 and Bax (12, 50–52), as well as those that are subjected to DNA methylation such as GSTp. While p21 and Bax serve as a rheostat of cell apoptotic sensitivity, GSTp may prevent cellular damage in toxic or stressful tissue microenvironment. To investigate how the proapoptotic effects of maspin and the GSTp-dependent antipapoptotic effects of maspin may be potentially coordinated in response to cancer-targeted chemotherapy, we compared the cytotoxicity of M344, 5-Aza and the combination of the 2 drugs in DU145-derived transfected cells. Because of the potential mixed mechanisms of cell death, we quantified the number of viable cells by the particle counting method. As shown in Figure 7A, the viable number of M7 cells was significantly lower than that of Neo cells by 5-Aza alone, and it was further reduced by the combination of 5-Aza and M344. In parallel, apoptotic-specific PARP cleavage and DNA damage–responsive histone 2A.X...
Figure 4. HDAC1 and histone deacetylation correlate with GSTp DNA methylation. A, WBs of GSTp, HDAC1, maspin, and β-tubulin in lentivirus-transduced PC3/Scr or 1D5 cell lines. Two lentiviruses used encoded shRNA of a scrambled sequence (Scr) or an HDAC1-targeting shRNA (HDAC1-shRNA). B, ethidium bromide–stained, PCR–amplified GSTp promoter fragment that was pulled down by ChIP assay using specific antibodies against Ac-H3, HDAC1, DNMT1, and MBD2. The reaction with preimmune IgG was used as a negative control. The input sheared DNA (input control) of Neo and M7 cells were also PCR amplified for the same region of GSTp promoter fragment. PCR-amplified p21 promoter was carried out as a positive control of the ChIP with HDAC1 antibody. C, densitometric analysis of the PCR products from Neo and M7 cells shown in B, presented as a percentage of the input DNA. All reactions were repeated 3 times. While B showed the representative ethidium bromide–stained agarose gel, data in C represent the average of the 3 repeats and the bars represent the standard errors.

Discussion

Maspin is an epithelial-specific tumor suppressive protein. We have previously shown that maspin reexpression in tumor cells restored epithelial differentiation (13, 15), whereas blocking tumor-induced angiogenesis and sensitizing tumor cells to a variety of apoptosis-inducing drugs (44, 50) including HDAC inhibitor M344 (15). These biological effects may primarily stem from the inhibitory effects of maspin on HDAC1, a major class I HDAC that hydrolyzes the ε-acetyl lysine residues in histone proteins, leading to gene transcription repression. Consistently, maspin expression in prostate tumor cells led to increased expression of several genes normally repressed by the HDAC1 activity including p21, Bax, cytokeratins 8 and 18, and maspin (15, 38, 54, 55). To date, maspin is the only known endogenous polypeptide inhibitor of HDAC1. Our current study is aimed at testing the hypothesis that epigenetic gene silencing is a result of endogenous coordinated histone deacetylation and DNA hypermethylation.

On the basis of the emerging consensus that DNA methylation may depend on histone deacetylation, loss of histone deacetylation inhibition, when maspin is downregulated, may increase pathologic DNA methylation and epigenetic gene silencing. We focused on the effect of maspin on the expression of GSTp because GSTp is a bona fide downregulated gene in prostate cancer by the mechanism of DNA hypermethylation (23, 24). Furthermore, both maspin and GSTp are implicated as tumor suppressors and downregulated in human prostate cancer specimens (13, 23, 41) as well as in established prostate cancer cell lines (Fig. 1). We have previously shown that in normal prostate epithelial (both secretory and basal) cells, maspin is highly expressed and is localized in the nucleus. During the progression of prostate cancer, maspin is differentially regulated both at the level of expression and subcellular localization (13, 56). Maspin is expressed at moderate to a high level but largely translocated from the nucleus to the cytosol in HGPIN. In low-grade prostate carcinoma cells, maspin expression is significantly reduced. In high-grade prostate carcinoma, little or no maspin is detected. On the side of GSTp, hypermethylation of GSTp promoter was found in 6.4% of proliferative inflammatory prostatic atrophy, 70% of HGPIN, and 90% of prostate cancer (23). Findings from the current study provide the first evidence that maspin may be sufficient to reverse the methylation-based GSTp silencing. Consistent with the notion that maspin directly inhibits HDAC1 (15), the effect of maspin on GSTp expression in prostate epithelial cells was similar to that of pharmacologic inhibitors of HDAC and was reduced when HDAC1 was knocked down. Our data suggest that the epigenetic gene silencing of GSTp is most likely a result of the coordinated histone deacetylation and DNA methylation.
M7 cells that were treated with 0.1 μmol/L of LBH589 at the indicated concentrations for 48 hours. C, WBs of PARP, maspin, GSTp, and \( \text{Ac-H3} \), and GAPDH in LNCaP/LacZ and LNCaP/GSTp cells that were treated with HDAC inhibitor LBH589 at the indicated concentrations for 48 hours. Data represent the average of 3 repeats and the bars represent the standard errors. B, WBs of PARP, maspin, Xpress-GSTp, Ac-H3, and GAPDH in LNCaP/LacZ and LNCaP/GSTp cells that were treated with HDAC inhibitor LBH589 at the indicated concentrations for 48 hours. C, WBs of PARP, maspin, GSTp, and β-tubulin in LNCaP/GSTp and M7 cells that were treated with 0.1 μmol/L of LBH589, 0.1 μmol/L of MG132, or the combination of 0.1 μmol/L of LBH589, and 0.1 μmol/L of MG132 for 48 hours.

Figure 5. GSTp increases cellular resistance to HDAC inhibitor LBH589. A, quantification of viable cells by the WST assay after the cells had been treated with LBH589 at the indicated concentrations for 48 hours. Absorbance at 450 nm (OD450) of each sample was normalized by that of the untreated cells. Data represent the average of 3 repeats and the bars represent the standard errors. B, WBs of PARP, maspin, Xpress-GSTp, Ac-H3, and GAPDH in LNCaP/LacZ and LNCaP/GSTp cells that were treated with HDAC inhibitor LBH589 at the indicated concentrations for 48 hours. C, WBs of PARP, maspin, GSTp, and β-tubulin in LNCaP/GSTp and M7 cells that were treated with 0.1 μmol/L of LBH589, 0.1 μmol/L of MG132, or the combination of 0.1 μmol/L of LBH589, and 0.1 μmol/L of MG132 for 48 hours.

Our results show that synthetic HDAC inhibitors, used at sublethal concentrations for a prolonged period of time, were less effective than maspin in restoring GSTp expression. It is possible that the specific mechanism underlying the inhibitory effects of maspin on HDAC1 is not fully recapitulated by pharmacologic HDAC inhibitors. Extensive studies are underway to investigate the structural-functional relationship of maspin in its interaction with HDAC1. In addition, several other possible differences between maspin and synthetic HDAC inhibitors need to be further investigated. First, maspin specifically inhibits HDAC1 (14, 15), as compared with synthetic HDAC inhibitors that generally have a broader spectrum of target specificity. Second, by mechanisms yet to be elucidated, maspin is also tightly regulated at the step of subcellular compartmentalization, as and may underlie the progressive increase of GSTp DNA methylation.

We noticed that the effectiveness of maspin in desilencing GSTp varied in different prostate cancer cell lines. Similarly, pharmacologic HDAC inhibitors exhibited variable degrees of effectiveness in desilencing GSTp. Although more precise epigenetic mechanistic studies need to be conducted in multiple cell lines to address the question whether the findings showed here are universally applicable, we speculate that the effects of gross HDAC inhibition, either by maspin or pharmacologic inhibitors, on gene expression may further depend on other endogenous factors that exert more specific regulations on GSTp DNA methylation and/or histone modifications. To this end, a current "histone code" hypothesis emphasizes on the dynamic interplay between histone modifications (59, 60). In general, methylation at Lys9 (K9), Lys27 (K27), and Lys20 (K20) of histone 3 (H3) are considered hallmarks of a condensed chromatin state that represses gene expression (for a review, see ref. 61), whereas di/tri-methylation of Lys4 (K4) of H3 and elevated levels of H3/H4 acetylation may concomitantly active gene transcription (62, 63). Interestingly, a study with prostate cancer cell line LNCaP suggests that the lack of Lys4 (K4) methylation, rather than a gain in methylation at Lys9 (K9) and Lys27 (K27), of histone 3 (H3) may drive GSTp silencing (29). These data illustrate the difficulty in using the current understanding of "histone code" as a rule, and points to the need to find additional underlying mechanisms that regulate the outcome of histone modifications. As an example, recent studies have shown that lysine-specific demethylase 1 (LSD1) demethylates histone H3 at K9, and release the repression of androgen receptor target genes (64). However, LSD1 complexed with HDAC1 and HDAC2 was shown to remove simultaneously the acetyl groups from lysine residues and the methyl group from Lys4 in H3, leading to transcription repression (65). To date, it is not clear whether maspin also affects histone methylation. Nonetheless, the evidence that maspin and pharmacologic HDAC inhibitors are effective, to various extents, in desilencing genes from a methylated state (1, 66), suggests that histone acetylation may eventually help override other repressive histone modifications.

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shown in the progression of prostate cancer (22, 67) and non–small cell lung cancer (17, 18, 68). Although the inhibitory effects on HDAC1 may be associated with nuclear maspin, the current design of small-molecular-weight HDAC inhibitors does not allow the control of their subcellular localization. Furthermore, endogenous maspin expression in tumor cell lines can be upregulated by synthetic HDAC inhibitors. It is likely that this positive feedback between the maspin effect on HDAC1 and maspin expression is a self-propelling mechanism to sustain effective HDAC1 inhibition.

Studies are underway to examine the extent and efficiency of maspin in desilencing other methylated genes, a role of maspin in the regulation of GSTp may be of

Figure 6. GSTp enhances cellular resistance to oxidative stress by H$_2$O$_2$. A, WBs of p-H2A.X, maspin, Xpress-GSTp, and GAPDH (loading control) after the cells were treated with H$_2$O$_2$ for 48 hours. B, phase contrast microscopic images of LNCaP/LacZ and LNCaP/GSTp cells that were treated with H$_2$O$_2$ for 48 hours. C, q-RT-PCR detection of HIF-1α mRNA in LNCaP/LacZ and LNCaP/GSTp cells that were vehicle treated or treated for 24 hours with 20 µmol/L H$_2$O$_2$ and 100 µmol/L H$_2$O$_2$, respectively. The critical threshold cycle number for HIF-1α mRNA was normalized by that of the internal GAPDH control in the same sample. Data represent the average of 3 repeats and the bars represent the standard errors.

Figure 7. The regulation of prostate tumor cell sensitivity to cytotoxic treatments. A, quantification of remaining live Neo and M7 cells after the treatment with 2 µmol/L 5-Aza with or without additional 0.1 µmol/L M344 as described in Materials and Methods. Cells were counted by a Coulter Partial Counter (Beckman Coulter Inc.). Data represent the average of 3 repeats and the bars represent standard errors. B, WBs of PARP, p-H2A.X, maspin, GSTp, Ac-H3, and GAPDH (loading control) in Neo and M7 cells that were treated with HDAC inhibitor M344. *, 10 µg of total cell lysate protein was loaded in each lane to show the level of GSTp in linear WB detection range. All other WBs were carried out with 30 µg of protein loaded per lane. C, WBs of PARP, p-H2A.X, maspin, GSTp, Ac-H3, and GAPDH (loading control) in Neo and M7 cells that were treated with 5-Aza at the indicated concentrations.
significant biological relevance in prostate cancer. This epigenetic regulation of GSTp by maspin may play an important role in the maintenance of prostate epithelial homeostasis in response to oxidative microenvironmental stress. In stressed or transformed epithelial cells, accumulated electrophilic metabolic intermediates undergo reoxygenation leading to the generation of reactive oxygen species (ROS) such as H$_2$O$_2$ and superoxide (O$_2^-$). ROS can directly cause cell injury by damaging DNA, proteins, and lipids. It is conceivable that normal cells should be equipped with sufficient defense mechanisms to maintain the genomic and epigenetic stability. GSTs, including GSTp, are important components of the cellular defense circuitry against ROS (69). In addition to contributing to cellular detoxification, GSTp has been shown to directly reduce sulfide bridges of proteins such as JNK (70–72), thus it may also act as a regulator of signal transduction in cellular response to oxidative stress and other stress inducers. Of particular interest, our earlier evidence suggested that the interaction between maspin and HDAC1 may require a GST cofactor. Because GSTp expression in LNCaP significantly increased the effectiveness of pharmacologic HDAC inhibitor LBH589 (Fig. 5), it is intriguing to speculate that GSTp may specifically interact with and enhance the activity of maspin in HDAC1 inhibition. A possible direct involvement of GSTp, if proven true, may suggest that the HDAC-dependent epigenetic regulation is connected to the cellular redox circuitry to balance between cell death and survival. As these exciting new possibilities are yet to be further investigated, it is important to note that GSTp is upregulated in several other types of cancer especially as the tumors become multidrug resistant. It is possible that in drug-resistant tumor cells, GSTp may be independently involved in drug-detoxifying mechanisms, such as the upregulation of MDR-1/P-gp170 protein (73, 74).

Taken together, our new evidence suggests that maspin, as an endogenous HDAC1 inhibitor, may play a key role in preventing DNA methylation–based silencing of tumor suppressor genes such as GSTp, and consequently inhibit HDAC1-mediated repression of other proapoptotic genes. Our data support a new paradigm that DNA and histone modifications may be endogenously coupled both in the silencing and the desilencing of gene transcription.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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