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Elevated GSH Level Increases Cadmium Resistance through Down-Regulation of Sp1-Dependent Expression of the Cadmium Transporter ZIP8

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ABSTRACT

Cadmium is a nonessential toxic metal in mammals. Its toxicity is mainly caused by interactions with cellular proteins that result in protein dysfunction and then disturb normal cellular functions. Glutathione (GSH) has been reported to play a role in cadmium resistance by serving as a cofactor for multidrug resistance protein 1/GS-X pump-mediated cadmium elimination. To further investigate the role of GSH in cadmium toxicity, we carried out a comparative study using small-cell lung cancer-derived cell lines, SR3A, and those that were stably transfected with glutamate cysteine ligase catalytic subunit (GCLC), a rate-limiting enzyme in GSH biosynthesis. These GCLC stably transfected cell lines produced higher levels of GSH and were more resistant to cadmium toxicity than the parental cell line was. The rates of cadmium uptake were reduced in these GCLC-transfected cell lines, which were associated with down-regulation of the cadmium transporter ZIP8/SLC39A8. Further analyses demonstrated that Sp1 binding site at the proximal promoter region of ZIP8 was sensitive to the GSH level and that the expression level of transcription factor Sp1 was reduced by increased GSH levels. We also demonstrated that low concentrations of cadmium exposure down-regulated ZIP8 expression with concomitant reduction of Sp1 expression. Taken together, these results demonstrate the importance of Sp1 in the regulation of ZIP8 expression. More important, our results reveal a new mechanism by which elevated GSH levels confer cadmium resistance by down-regulation of ZIP8 expression through the suppression of Sp1.

Cadmium is a toxic metal ion in humans and is considered a type I carcinogen (Waalkes, 2003). Because of its long retention time in the human body and high mutagenicity at low concentrations, cadmium is an important environmental poison to human health. Cadmium toxicity occurs through interactions with proteins that subsequently cause dysfunction of protein complex and organelles (Martelli et al., 2006). Eukaryotic cells develop detoxification mechanism to defend cadmium-mediated toxicity. The cellular cadmium detoxification pathway is mostly dependent on the up-regulation of metallothionein. Metallothionein consists of 61 amino acids and has multiple cysteine residues by which heavy metal ions, including cadmium, are immobilized and lose their toxic effects (Klaassen et al., 1999). However, studies in metallothionein knockout mouse strains showed that overall cadmium sensitivity is not determined solely by the metallothionein phenotype (Liu et al., 2001a,b).

Another important mechanism that affects cadmium toxicity is the cadmium transport system. In eukaryotic cells, several studies have demonstrated that the cadmium efflux system is mediated by the multidrug resistance protein (MRP) family in yeast (Adle et al., 2007) and plants (Kim et al., 2007). MRPs belong to the ATP-binding cassette superfamily of membrane proteins, which are involved in eliminating a wide spectrum of cytotoxic agents from intracellular compartments (Borst et al., 2000). Human MRP1 is induced by cadmium exposure, and its expression levels are associated with cadmium resistance (Ishikawa et al., 1996).

Despite a number of studies on the regulation of the cadmium efflux system, the molecular mechanism of regulation of cadmium uptake has not been well studied. In mammals, cadmium is taken up from the gastrointestinal tract or lung through contaminated food and water or through smoking (Oberdörster, 1992; Bridges and Zalups, 2005). Studies on cadmium uptake using various organisms revealed that cad-

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ABBREVIATIONS: MRP, multidrug resistance protein; GCLC, glutamate-cysteine ligase catalytic subunit; NAC, *N*-acetyl cysteine; BSO, L-buthionine-[*S, R*]-sulfoximine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SRB assay, sulforhodamine B colorimetric assay; ChIP, chromatin immunoprecipitation; ZF; zinc finger; SCLC, small-cell lung cancer; HEK, human embryonic kidney; siRNA, small interfering RNA; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; shRNA, short hairpin RNA; NF-κB, nuclear factor κB; nt, nucleotide(s); WT, wild type; tet, tetracycline.

mium uptake is mainly carried out by a metal transporter with a high affinity for manganese (Martin et al., 2006). At present, several metal transporters have been identified as cadmium transporters. While in the gastrointestinal tract, the divalent metal transporter is an important mediator of cadmium in addition to other divalent metal ions, including Fe^{2+}, Cu^{2+}, and Zn^{2+} (Park et al., 2002). A recent genetic study revealed that ZIP8 plays an important role in cadmium uptake and is known to be expressed in lung rather than the gastrointestinal tract (Begum et al., 2002; Dalton et al., 2005), and a subsequent biochemical study revealed that ZIP8 has a high affinity for cadmium and manganese (He et al., 2006). In addition to these transporters, recent studies have suggested that the calcium channel is involved in cadmium uptake, although the significance of these calcium channels in cellular defense against cadmium toxicity has not been well developed (Leslie et al., 2006).

Glutathione (GSH) is an abundant intracellular thiol-containing compounds (1~10 mM). De novo biosynthesis of GSH is controlled by the rate-limiting enzyme glutamate cysteine ligase, which catalyzes the conjugation of glutamine and cysteine. Glutamate cysteine ligase is a heterodimer consisting of a catalytic subunit (GCLC) and a modifier subunit. We have demonstrated previously that overexpression of GCLC is sufficient to confer increased intracellular GSH content (Yamane et al., 1998). In the present study, we demonstrate that the GCLC-mediated elevated level of GSH confers cadmium resistance through down-regulation of cadmium transporter ZIP8 expression as a result of reduced expression of transcription factor Sp1, which is required for ZIP8 expression. Our results reveal a novel pathway for GSH-mediated reduction of cadmium toxicity in mammalian cells.

Materials and Methods

Cell Culture and Reagents. Small-cell lung cancer cells (SCLC) and their doxorubicin-resistant cell line SR3A (Yamane et al., 1998) were maintained in RPMI medium supplemented with 10% fetal calf serum. We supplemented 200 μg/ml G418 for *GCLC*-transfected SR3A cell lines. SCLC cells and HEK-293 cell lines were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Rabbit anti-Sp1 (PEP-2) and Sp3 (D-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal anti-β-actin (AC-15) and hemagglutinin (HA-7) antibody were from Sigma-Aldrich (St. Louis, MO). Rabbit anti-GCLC antibody was described previously (Tatebe et al., 2002). BSO, *N*-acetyl cysteine (NAC), zinc chloride, manganese chloride, and cadmium chloride were purchased from Sigma.

Plasmid and siRNA Transfection. Synthetic siRNA-targeting ZIP8 was purchased from Sigma. The siRNA sequence used in this study was 5′-GAUUUGAUCCCAAAGUCGA-3′. Control siRNA was purchased from Santa Cruz Biotechnology. The Sp1 full-length or zinc finger domain (encoding amino acids 480–785) sequences were cloned in pcDNA3-HA vector. The siRNA and plasmid DNA were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Total GSH Analysis. Total GSH levels were analyzed using the Colorimetric Microplate Assay for Total glutathione kit (Oxford Biomedical Research, Oxford, MI) following the manufacturer's instruction.

Sulforhodamine B Colorimetric and 3-(4,5-Dimethylthiazol-2-yl)-2–5-disphenyltetrazoliumbromide Assays. We seeded 20,000 cells in a 96-well plate and treated them with various concentrations of cadmium for 72 h. For the 3-(4,5-dimethylthiazol-2-yl)-2–5-disphenyltetrazoliumbromide (MTT) assay, MTT solution (thiazolyl blue tetrazolium bromide; Sigma-Aldrich) was added to the culture after cadmium exposure, and cells were incubated for 4 h. The medium was removed, MTT crystals were dissolved in dimethyl sulfoxide, and the conversion rate of MTT crystal was measured by absorbance at 559 nm. The sulforhodamine B colorimetric (SRB) assay was carried out by following standard protocol (Vichai and Kirtikara, 2006). In brief, cadmium-exposed cells were fixed with 10% trichloroacetic acid for 30 min and were stained by 0.4% SRB solution. After being washed by 1% acetic acid, SRB dye was dissolved in 10 mM Tris buffer, and cell viability was measured by absorbance at 405 nm.

Cadmium Uptake Analysis. Cells (2×10^5) were plated in a six-well plate and exposed with final concentration of 0.3 μM ^{109}Cd (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for 6 h. After cadmium exposure, cells were washed with phosphate-buffered saline containing 0.05 mM EDTA and lysed in 0.2 N sodium hydroxide. Cadmium uptake was analyzed by measuring the radioactivity of ^{109}Cd.

DNA Plasmid Constructs. ZIP8 cDNA was cloned by polymerase chain reaction (PCR) using the forward primer 5′-TTATCTCTGTCCCCCTTTGTCCTC and the reverse primer 5′-GCGATAAGCCTCTAAGCCTGAACT. For the luciferase assay, the promoter region of ZIP8 was cloned using forward primer 5′-CTCGAGCTCCCTTCAGGCATGAATCCTCC and the reverse primer 5′-AAGCTTCGAAAGAACAGCAGCTCGCGACC. For deletion constructs of ZIP8 promoter, DNA fragments were amplified using the reverse primer named above and the following forward primers: F1: 5′-GCTAGCTATGTAGACAATGCAAGGG; F2: 5′-GCTAGCAAGAGGGCATGGCTGATGC; F3: 5′-GCTAGCGGGACAGGGCCCTCCTCC; F4: 5′-GCTAGCTATTTGTAAAGAGCGCCGG; F5: 5′-GCTAGCAGTCTTACGTTGACACGC; and F6: 5′-GCTAGCTCTCACTTCTAAGTTTGC. Amplified DNA fragments were cloned using pGEM easy vector system (Promega, Madison, WI), and the sequence integrity was confirmed. The cloned promoter sequence was digested with NheI/HidIII and ligated into pGL3 Basic vector (Promega). For mutation analysis, mutations were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with following oligonucleotide pairs: MT1, 5′-CACGTGCTACGGTTTCGGGGACAGGGCCCTCCTCC and 5′-GGAGGAGGGCCCTGTCCCCGAAACCGTAGCACGTG; MT2, 5′-CACGTGCTACGGAGGCGGGGACTTTGCCCTCCTCC and 5′-GGAGGAGGGCAAAGTCCCCGCCTCCGTAGCACGTG; and MT3, 5′-CACGTGCTACGGAGGCGTTTACAGGGCCCTCCTCC and 5′-GGAGGAGGGCCCTGTAAACGCCTCCGTAGCACGTG).

Luciferase Assay. The luciferase assay was carried out using a dual luciferase assay kit (Promega), according to the manufacturer's instructions. In brief, cells cultured in 24-well dishes were transfected with 0.3 μg of pGL3 vector and 2 ng of pRLII vector using Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. Forty-eight hours after transfection, cells were lysed with passive lysis buffer. Cell lysates were cleared by centrifuge, and supernatants were used for dual luciferase assay. The promoter activity of ZIP8 was represented as a -fold induction from the empty pGL3 Basic vector to standardize deviation from different cell lines.

Northern Blotting. Northern blotting was carried out using the standard protocol. Total RNA was isolated by TRIzol reagent using the standard protocol (Invitrogen). Fifteen micrograms of total RNA was separated in 1% formaldehyde-denatured agarose gel and transferred to a Hybond N^+ membrane (Amersham). The DNA probe was synthesized with a random prime DNA labeling kit (Roche, Basel, Switzerland) using ZIP8, Sp1, or glyceraldehyde 3-phosphate dehydrogenase cDNA and hybridized to RNA on the membrane at 45°C overnight. The membrane was washed three times with 2× standard saline citrate with 0.1% SDS, and the blots were visualized by X-ray film or the PhosphorImager system.

Electrophoretic Mobility Shift Assay. For electrophoretic mobility shift assay (EMSA) analysis, double-stranded oligonucleotide DNA (5′-CACGTGCTACGGAGGCGGGGACAGGGCCCTCCTCC,

only the top strand sequence is shown) was annealed and radioactively labeled by T4 kinase (New England Biolabs, Ipswich, MA). The labeled DNA oligonucleotide was purified by 8% PAGE. Radiolabeled DNA probe (10,000 cpm) was incubated at ambient temperature with 3 μg of nuclear extract, 3 μg of poly(dI.dC) in binding buffer containing 10 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol, 5% glycerol, 0.1 mg/ml bovine serum albumin, and 1 mM $MgCl_2$. For the supershift assay, 1.0 μg of anti-Sp1 or anti-Sp3 antibody was added to the reaction mixture. The reaction mixture was separated by 4% PAGE. The shift bands were visualized on X-ray film.

Real-Time PCR. The ABI 7900 system was used for real-time PCR analysis. For real-time PCR, the following oligonucleotide DNA was used: human ZIP8: forward, 5′-CCTTATGTGTGATCGAGAGCCATTC, and reverse, 5′-GTAATTCCTGAGATCATTGTTGGGC; human β-actin: forward, 5′-GAGGCCCAGAGCAAGAGAG, and reverse 5′-AGAGGCGTACAGGGATAGCA; and human CacnαG1: forward, 5′-CAAAGATGCACCTCATCTGC, and reverse, 5′-ACTCTAAGCTGCTTCTGGTC. Amplification was monitored by fluorescence of SyberGreen dye (Invitrogen), and expression level was calculated by the cycling time (Ct) value. Expression level of ZIP8 was normalized by the expression level of β-actin.

Western Blotting Analysis. Cells were harvested and washed with phosphate-buffered saline. Cell pellets were suspended in radioimmunoprecipitation assay buffer and incubated on ice for 30 min. Cell lysates were cleared by centrifuge, and protein concentrations were determined by Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). Cell lysates were boiled in SDS sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The membrane was washed with Tris-buffered saline/Tween 20 and blocked with 5% nonfat milk. Target proteins were detected with rabbit anti-Sp1, rabbit anti-Sp3, mouse anti-β-actin, mouse anti-HA epitope, or rabbit anti-GCLC antibodies. Proteins were further labeled with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) and visualized with X-ray film.

Chromatin Immunoprecipitation Assay. The chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's instructions (Millipore, Billerica, MA). The precipitated DNA samples were amplified using oligonucleotide primers specific to the ZIP8 promoter (forward, 5′-AATGGGTCACCACGTGCTAC; reverse, 5′-TTAATCGGAAGCACTCGCTG). PCR products were separated on 2% agarose gel and visualized using ethidium bromide. For quantitative analysis, ChIP samples were analyzed by real-time PCR using the same oligonucleotide primers.

Preparation of Lentiviral Recombinants. Lentiviruses were generated using pLKO.1 vector (Addgene, Cambridge, MA). The shRNA sequences used for targeting Sp1 were 5′-CCAGGTGCAAACCAACAGATT-3′ and 5′-GCTGGTGGTGATGGAATA-3′ for numbers 2 and 3, respectively. The sequences for ZIP8 shRNA 325 and 715 are 5′-AAATTCTCTGTCATCTGTCCA-3′ and 5′-AATGGTCATACCCACTTTGGA-3′, respectively.

Statistical Analysis. Statistical significance was analyzed using Student's t test or one-way analysis of variance and after the Student-Newman-Keuls post hoc test. P values <0.05 were considered to be statistically significant.

Results

Increased GCLC Levels Are Associated with Cadmium Resistance. To study the role of GSH in cellular defense against cadmium toxicity, we first analyzed the effects of cadmium exposure on cellular GSH level. As shown in Fig. 1A, cadmium exposure increased intercellular GSH levels in a dose-dependent manner in SCLC cells. This up-regulation of GSH correlated with the expression level of GCLC (Fig. 1B).

To evaluate the role of up-regulated GSH in cellular defense against cadmium, we modulated the intercellular GSH level by BSO, an inhibitor for GSH biosynthesis, or by NAC. The 24 h of treatment with 100 μM BSO effectively depleted GSH to less than 50% of control and 3 mM NAC treatment increased GSH level approximately three times (data not shown). The SCLC cells were pretreated with BSO or NAC for 24 h, and the effect on cadmium sensitivity was determined. As shown in Fig. 1C, treatment with BSO sensitized cells to cadmium toxicity, whereas treatment with NAC significantly increased cadmium resistance.

SR3A was a doxorubicin-resistant cell line established from SCLC. SR3A cells contain 1.5-fold higher GCLC mRNAs compared with that in SCLC cells (Yamane et al., 1998). No induction of GCLC expression by cadmium at the same concentration range in SR3A cells was found. This is perhaps because the endogenous GCLC levels are already high (Fig. 2A, top). To study how elevated GSH levels affect cadmium toxicity, we used *GCLC*-transfected cell lines SR3A-13, SR3A-14, and SR3A-15. These cells express two to four times higher GCLC and almost equally higher levels of GSH than the parental cell line (Fig. 2A, bottom, and B). We found that *GCLC*-transfected cell lines exhibited significant resistance to cadmium (Fig. 2C). To verify the role of GSH in this increased cadmium resistance, we treated cells with BSO. As shown in Fig. 2D, BSO treatment reversed cadmium resis-

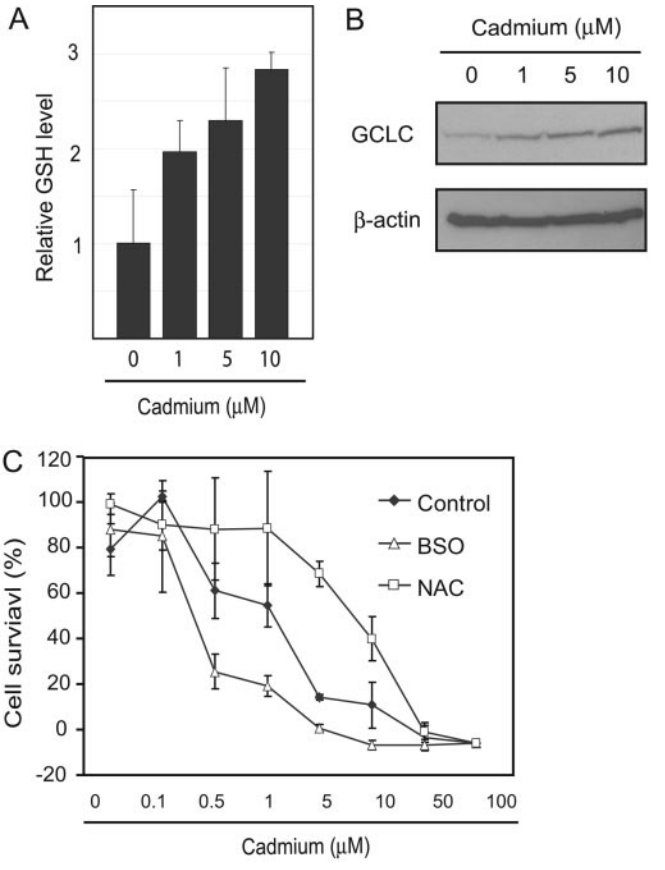

Fig. 1. Protective role of GSH in cadmium toxicity. A and B, SCLC cell lines were treated with 0, 1, 5, and 10 μM cadmium for 20 h, and cellular GSH and GCLC expression levels were analyzed. C, the role of GSH in cadmium toxicity was examined by 24-h pretreatment with 100 μM BSO or 3 mM NAC followed by exposure to various concentrations of cadmium for 72 h. Cadmium toxicity was analyzed by SRB assay. Error bars represent standard deviation from three independent experiments. *, $p < 0.05$.

tance in *GCLC*-transfected cell lines with minimum effect on parental cell line. These results indicate that GCLC-mediated up-regulation of GSH level led to enhanced resistance to cadmium toxicity.

Decreased Cadmium Uptake Is Associated with Down-Regulation of the ZIP8 Transcript in the *GCLC*-Transfected Cell Lines. We next investigated whether the increased cadmium resistance in the *GCLC*-transfected cells were due to reduction in the rates of cadmium uptake. As shown in Fig. 3A, GCLC-overexpressing cells showed reduced cadmium uptake compared with the parental cells. To confirm the contribution of cadmium uptake, we used manganese, which is a known antagonist of cadmium uptake. Cells were exposed to cadmium with or without 100 μM manganese, and cadmium sensitivity was analyzed using the MTT assay. As shown in Fig. 3B, manganese effectively protected cells against cadmium toxicity in parental SR3A but not in *GCLC*-transfected cell lines. The cadmium sensitivity of manganese-treated SR3A cells was similar to that of *GCLC*-transfected cell lines SR3A-13 (Fig. 3B) as well as clones SR3A-14 and SR3A-15 (data not shown). These data indicated that the cadmium transporter is most likely involved in the increased cadmium resistance.

Recently, ZIP8/SLC39A8 was identified as a potent cadmium transporter with a high affinity for manganese (He et al., 2006). Therefore, we determined whether ZIP8 plays a role in cadmium resistance in GCLC-transfected cell lines. By Northern blotting and real-time PCR analysis, we found the parental SR3A cell expressed significantly higher levels of ZIP8 transcripts than the *GCLC*-transfected cell lines (Fig. 3, C and D).

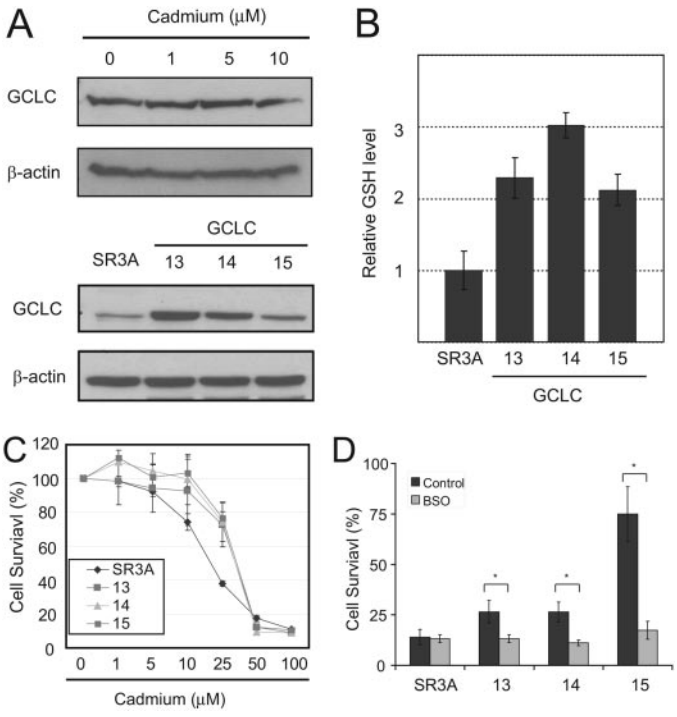

Fig. 2. GCLC overexpression confers cadmium resistance. A, top, Western blotting analysis showing no induction of GCLC expression by the treatment of cadmium at the concentrations indicated. Bottom, Western blotting analysis on GCLC levels in parental and *GCLC*-stably transfected SR3A cell lines. B, GSH levels in the *GCLC*-transfected cell lines. C, cadmium sensitivity in GCLC stably transfected cell lines. Cells were seeded on a 96-well plate and exposed to various concentrations of cadmium. D, cadmium sensitivity was also examined by 72 h of 25 μM cadmium exposure with or without 100 μM BSO. Error bar represents standard deviation from three independent experiments. *, $p < 0.05$.

To validate these results, we used the tetracycline (tet)-off GCLC expression system adenoviral vector AdE1.tTA. GCLC (Savaraj et al., 2005). SCLC cells were transduced with AdE1.tTA. GCLC in the presence (+) or absence (−) of tet for 24, 48, or 72 h. Levels of GCLC expression were increased approximately 11- and 30-fold in cells grown in the absence of tet for 24 and 48 h, respectively, compared with those treated with tet. Comparable levels of elevated GSH were found in the GCLC-overexpressing samples (Savaraj et al., 2005). Levels of ZIP8 mRNA were reduced in the GCLC-overexpressing cells (Fig. 3E) compared correspondingly with the cells cultured under tet(+) conditions. These results, taken together, strongly suggest that the expression of ZIP8 levels was negatively regulated by intracellular GSH contents.

To confirm the contribution of ZIP8 in SR3A cell lines, we transfected ZIP8 siRNA and analyzed the expression of ZIP8 mRNA levels and cadmium uptake rates. As shown in Fig. 3F, transfection of ZIP8 siRNA significantly down-regulated ZIP8 expression to approximately 50% of control levels in SR3A cells and their GCLC-transfected variants. A significant reduction in cadmium uptake and a slight decrease in those of GCLC-transfected cell lines were observed (Fig. 3G). These differences in the effect of ZIP8 siRNA on cadmium uptake may be due to the already low levels of expression of ZIP8 in GCLC-transfected cells and/or to the background levels contributed by other metal transporters.

Analysis of ZIP8 Promoter Activity. Next, we analyzed ZIP8 promoter activity to identify the mechanism that down-regulates ZIP8 expression level in *GCLC*-transfected cell lines. Sequences upstream of the ZIP8 gene were analyzed by the computational method to identify potential promoter region and transcriptional start sites (Fig. 4A). We did not observe the canonical TATA box, but several GC box and several putative nuclear factor κB (NF-κB) binding sites were located in this region. Based on this information, a 620-base pair fragment that included the transcription start site from ZIP8 promoter region was amplified by PCR from human genomic DNA. The resulting fragment was cloned into the pGL3 Basic vector for the reporter expression assay. The reporter construct was transfected into SR3A cells, and *GCLC*-transfected cell lines and promoter activity were analyzed. *GCLC*-transfected cell lines had a drastic reduction in luciferase activity compared with that in the parental cell line. These results suggest that transcription factor(s) interacting with *cis*-acting elements within the −620 nt of the ZIP8 promoter may be down-regulated in *GCLC*-transfected cells.

To determine the locations of the *cis*-acting elements, we generated several *ZIP8* promoter deletion constructs. As shown in Fig. 4B, deletion mapping of ZIP8 promoter activity revealed that removing sequence between −469 nt (construct F2) and −342 (construct F3) resulted in a drastic reduction of reporter activity in SR3A cells. These results suggested that sequences within −469 and −342 nt harbor a *cis*-element that supports basal expression of *ZIP8* and that *trans*-acting factor which interacts with this element may be down-regulated in *GCLC*-transfected cells.

Regulation of ZIP8 Promoter by Sp1. Examining nucleotide sequence between −469 and −342 nt, we found two overlapping GC boxes. To investigate whether these GC

boxes are responsible for the basal transcription activity of the *ZIP8* promoter, we generated three mutants (MT1, MT2, and MT3) containing altered nucleotides in these GC boxes (Fig. 5A) in the F2 reporter construct. These mutant reporter constructs were transfected into SR3A and its *GCLC*-transfected variants. MT2 did not show a reduction in the reporter activity, whereas MT1 and MT3 did (Fig. 5B). These results demonstrated that GC box 1 is involved in the GSH-mediated transcriptional regulation of *ZIP8*.

Next, we performed an electrophoretic EMSA analysis to determine whether any transcription factors can bind to this GC box sequence. Because two GC box sites overlapped, we synthesized oligonucleotides that contain both GC box sequences. A double-stranded DNA oligonucleotide probe was radioactively labeled with ^{32}P, incubated with nuclear extract from SR3A cells, and the mixtures were run on 4% polyacrylamide gel electrophoresis. We found two specific protein-DNA complexes. The slow mobility complex could be

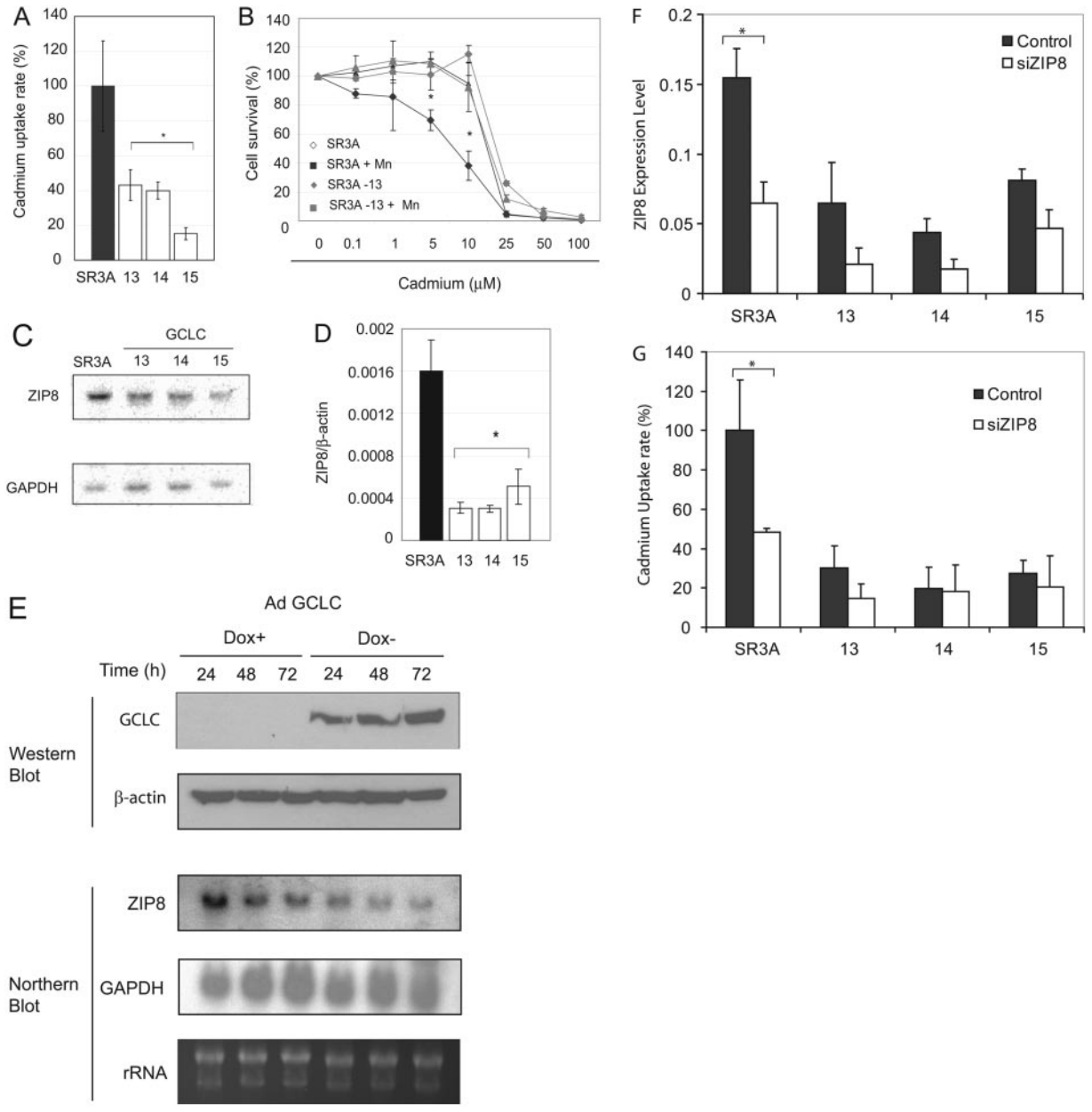

Fig. 3. Down-regulation of cadmium transporter ZIP8 in GCLC-overexpressing cells A, effects of GCLC overexpression on cadmium uptake rate. Cells were treated with 0.3 μM ^{109}Cd for 6 h, and cadmium uptake rates were analyzed. The cadmium uptake rate is represented as a percentage of the cadmium uptake of the parental SR3A cell line. Experiments were done in triplicate, and error bars represent standard deviations. B, protective effect of manganese against cadmium toxicity. SR3A cell and SR3A-13 was exposed to various concentrations of cadmium in the presence or absence of 100 μM manganese for 72 h. Survival cell fraction was assayed by MTT conversion. C and D, down-regulation of ZIP8 in GCLC-transfected cell lines. The expression levels of ZIP8 in SR3A and *GCLC*-transfected cell lines (SR3A-13, -14, and -15) were analyzed by Northern blotting and real-time PCR. E, effect of GCLC on expression level of ZIP8 was also evaluated by transduction with Tet-off adenovirus GCLC expression vector using SCLC cells. Cells were harvested 24, 48, and 72 h after transduction, and the expression level of GCLC and ZIP8 was analyzed by Western and Northern blotting, respectively. F and G, effect of ZIP8 knockdown on cadmium uptake. Parental SR3A and *GCLC*-transfected cell lines were transfected with 100 nM control or ZIP8 siRNA. Seventy-two hours after transfection, cells were harvested and subjected to real-time PCR. The transfected cells were also subjected to a cadmium uptake assay. Error bars represent standard deviation from six independent experiments. $*$, $p < 0.05$.

A

-620

TATGTAGACAATGCAAGGGTCTCCAGGCTCCATTTCAGCAAGTTGGTTTCATCAAGATCACGAAAAG

ATGCATGCGTCCCCGAGACCAGAGAGGAGTTAGGGACCCCGACACGGAGGTGGCATCAGGTAACCCT

CAGGAGTGAGGGTGGGGAAGAGGGCATGGCTGATGCTGGGAATGAGCAGCGCCCGCGGAGGGAAGGC
Sp1

CGGCGGTCCTGCGCCCCGGCGTGGACGCGCGCGGGAGTTCAGGCTGGGGAAATGGGTCACCACGTGC

TACGGAGGCGGGGACAGGGCCCTCCTCCCCTTATTTGTAAAGAGCGCCGGAGCGGATCGCGGAGTTT
Sp1 Sp1

CACCGCGGACCTGTCAGAGATACAGAGGTTGTGGGGGCGGGAGACAGAAAGAGAGAGAGATCCAGAG
Sp1

ACCGAGTCTTACGTTGACACGCAGAGAGAAAGACGCAGAGACAGACAAACAAACAGATAGGAGAGGT

GAGAGGCCAGCGAGTGCTTCCGATTAACTTGCCAAGCTCAGAGAAGATCCGGGGAGAGGGAGGGTCC

CCTTTAAACGCGCAATCCCCAGGACTCCGGAAGAGCCCTTTTCTGCAGCTCCTTGGGGACTGCACGT
NFkB NFkB NFkB

CTCACTTCTAAGTTTGCGGCGGAGAGAATAAAGCAGAAAGACAGGAAGAGGAGGTGGAGTTCTACAG
+1

TTAGTGTGGTTTTAGTTTTTCCTAAGAAGTGGCGTGGTTTGGGGCTTTATATCCGGGAGGAGCATAT

GTACGCAAATCCTGGGGCGTTTGCAAACCCGGATCCGGGGCGTCTGGCCCCATGCCCGGCCGGGCGT

TTGAGGGCTACTGCCAC +372

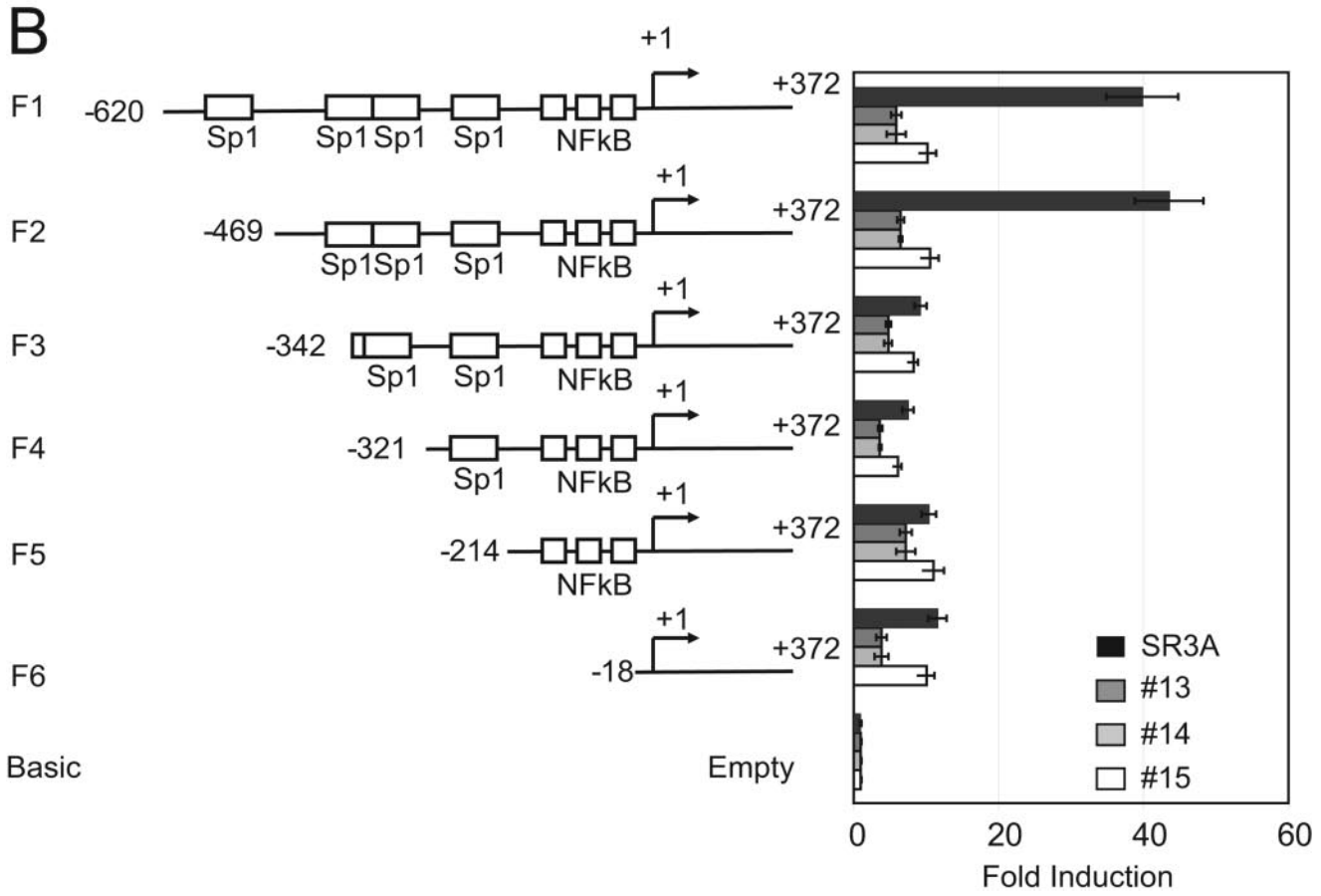

Fig. 4. Analysis of ZIP8 proximal promoter activity. A, ZIP8 promoter sequence. Transcription factor binding sites and the transcription initiation site (+1) are shown. B, ZIP8 promoter deletion and their promoter activity. Parental SR3A and SR3A-13, -14, and -15 cell lines cultured in 24-well plates were transfected with pGL3 vector coding various deletion of ZIP8 promoter (−620/+372, −469/+372, −342/+372, −321/+372, and 214/+372) or empty pGL3 vector and pRLII vector. Seventy-two hours after transfection, the promoter activity of each deletion was analyzed. Promoter activities of each construct were represented by -fold induction from that of the empty vector. Error bars represent standard deviation from three independent experiments. $*$, $p < 0.05$.

reduced partly by anti-Sp1 antibody and yielded a supershift band, whereas the fast mobility complex could be completely diminished by anti-Sp3 antibody, indicating that Sp1 and Sp3 can recognize this GC box in vitro (Fig. 5C). To distinguish two overlapping GC box sites, we labeled WT, MT1, MT2, and MT3 oligonucleotides and performed EMSA. The DNA protein complex was only observed in WT and MT2 (Fig. 5D). Together with the results in Fig. 5C, these results indicate that only GC box 1 is required for the transcriptional activity of ZIP8 expression.

To elucidate whether Sp1, Sp3, or both participated in the transcription regulation of ZIP8 expression, we performed a chromatin ChiP assay using Sp1 and Sp3 antibodies to precipitate the chromatin fragments engaged with Sp1 and Sp3 transcription factors. Figure 5E shows that only Sp1, but not Sp3, bound to ZIP8 promoter in vivo. These results demonstrated that Sp1 is important for the expression of ZIP8.

Sp1 Was Down-Regulated in *GCLC*-Transfected Cell Lines. To investigate the mechanism underlying the reduced promoter activity of ZIP8 in GCLC-transfected cell lines, we measured the Sp1 levels in these cells. Levels of Sp1 mRNA (Fig. 6A) and protein (Fig. 6B) were reduced in the GCLC-transfected cell lines compared with those in untransfected SR3A cells. Moreover, levels of Sp1 were reduced in cultured cells by continuous treatment with NAC (Fig. 6C). These results demonstrated that elevated expression of GSH down-regulated Sp1 expression.

Sp1 function is known to be regulated by post-translational modification, and its protein level does not always reflect its in vivo DNA binding ability. Thus, we performed a ChIP assay using parental and GCLC-transfected cell lines to analyze the capacity of Sp1 DNA binding in vivo. A representative result of ChIP assay, shown in Fig. 6D, clearly demonstrates that Sp1 binding to ZIP8 promoter in parental SR3A cell lines but was almost undetectable in *GCLC*-transfected cell lines. The result of a quantitative analysis using real-time PCR result indicates that Sp1 in GCLC-transfected cell lines occupies less than 0.1% of ZIP8 promoter than that of parental cell lines (Fig. 6E). These results indicate that *GCLC* overexpression reduces both Sp1 protein level and ZIP8 promoter activity.

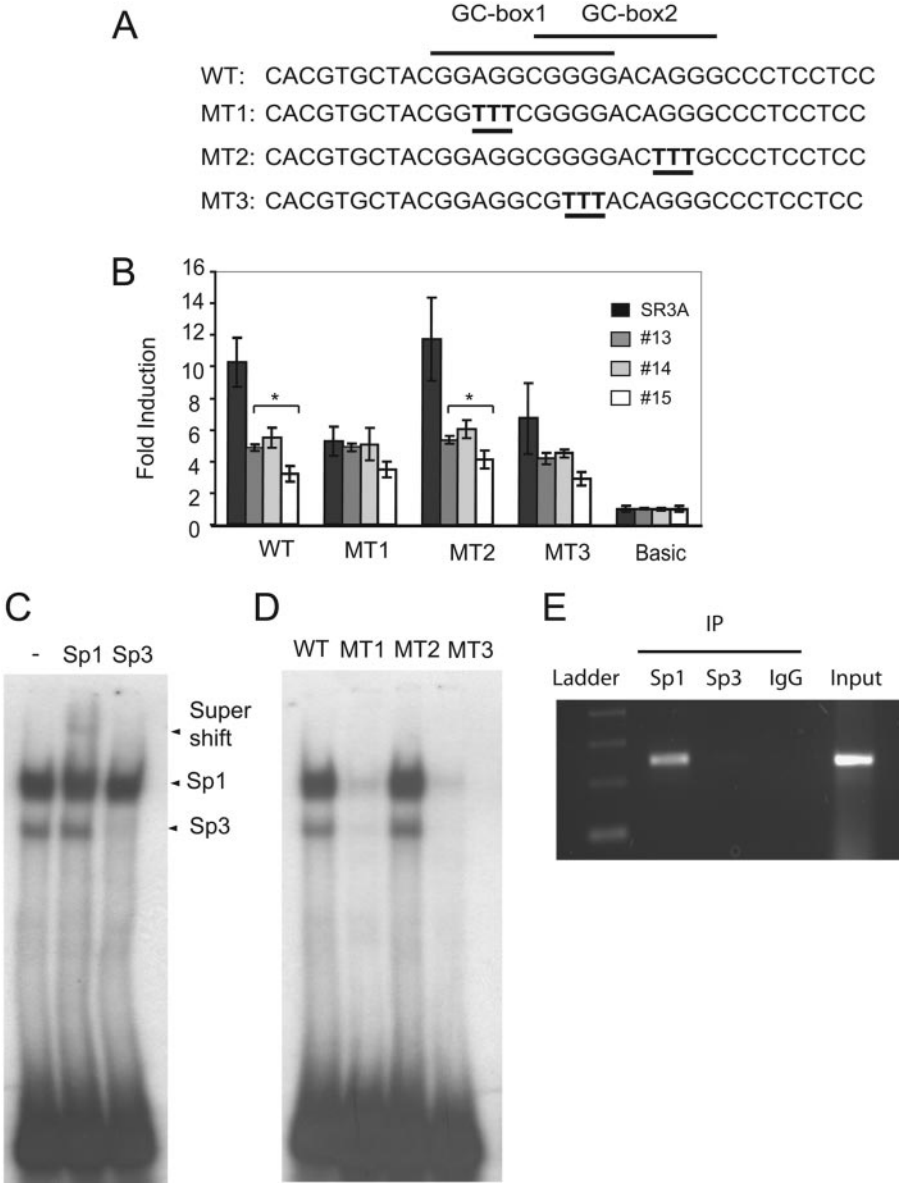

Fig. 5. Analysis of Sp1 binding to the ZIP8 promoter. A, scheme of mutation on GC box. B, effects of mutation on ZIP8 promoter activity. Mutations were introduced into a luciferase construct bearing *ZIP8* promoter sequence (F2: −469 to +372) and transfected into SR3A and SR3A-13, -14, and -15 cell lines. Seventy-two hours after transfection, promoter activity was analyzed. C, EMSA. A radiolabeled oligonucleotide probe was incubated with SR3A nuclear extract, and the DNA protein complex was separated by 4% PAGE. DNA/protein complex were identified by incubation with anti-Sp1 (lane 2) or anti-Sp3 (lane 3) antibody. D, EMSA of mutant oligonucleotides. SR3A nuclear extract was incubated with WT (lane 1), MT1 (lane 2), MT2 (lane 3), or MT3 (lane 4) radioactively labeled DNA probes. Either or both of the overlapping GC boxes were introduced. E, ChIP analysis. SR3A cells were cross-linked by 1% formaldehyde. Cells were lysed, and genomic DNA was sheared by sonication. The DNA protein complex was precipitated with anti-Sp1 or anti-Sp3 antibody and control IgG. DNA was purified and tested by PCR or quantitatively analyzed by real-time PCR. Error bars represent standard deviation from three independent experiments. *, $p < 0.05$.

Regulation of Endogenous ZIP8 by Sp1. To further evaluate the role of Sp1 in ZIP8 promoter, we cotransfected Sp1 expression vector and measured ZIP8 promoter activity. As shown in Fig. 7A, expression of Sp1 up-regulated ZIP8 promoter activity up to 10-fold in all four cell lines. It is noteworthy that the luciferase activity of GCLC-transfected cell lines even exceeded that of the parental cell line when cells were cotransfected with Sp1 expression vector. These results demonstrated that expression of Sp1 enhanced the promoter activity of ZIP8.

Next we determined the role of Sp1 in endogenous ZIP8 promoter activity. HEK-293 cells were transfected with expression vector encoding full-length Sp1 and a deletion mutant encoding the ZF domain. The results showed that full-length Sp1 did not significantly up-regulate endogenous ZIP8 expression, whereas the zinc finger domain of Sp1 down-regulated ZIP8 expression level to 60% of control (Fig. 7B). These results indicate that overexpression of the zinc finger domain has a dominant-negative effect as reported previously (Lee et al., 2006). This dominant-negative effect indicates that Sp1 DNA binding is needed for ZIP8 expression. However, because Sp1 overexpression did not up-regulate endogenous ZIP8 expression level, endogenous Sp1 levels in these cells may have been already high enough that additional Sp1 could not have stimulatory activity.

To further support the role of Sp1 in the regulation of ZIP8, we generated two lentivirus vectors encoding Sp1 shRNAs. HEK-293 cells were transduced with lentivirus, and Sp1 levels were analyzed by Western blotting. Figure 8A shows that both shRNAs dramatically down-regulated Sp1 mRNA and protein levels as analyzed by Northern and Western blotting, respectively. Down-regulation of ZIP8 by Sp1 knockdown was associated with reduced rates of cadmium uptake (Fig. 8B) and slightly enhanced cell resistance to cadmium treatment (Fig. 8C). Taken together, these results strongly support the important roles of Sp1 in the regulation of ZIP8 expression and that down-regulation of Sp1 levels result in down-regulation of ZIP8 expression and cadmium resistance in GCLC-transfected cell lines.

Down-Regulation of Sp1 and ZIP8 by Cadmium Exposure. Sp1 contains three ZF at the C terminus. Each ZF is composed of two cysteine and two histidine residues that are coordinated by zinc in a tetrahedral conformation. Cadmium, like other heavy metal ions, can destabilize the folding of ZF

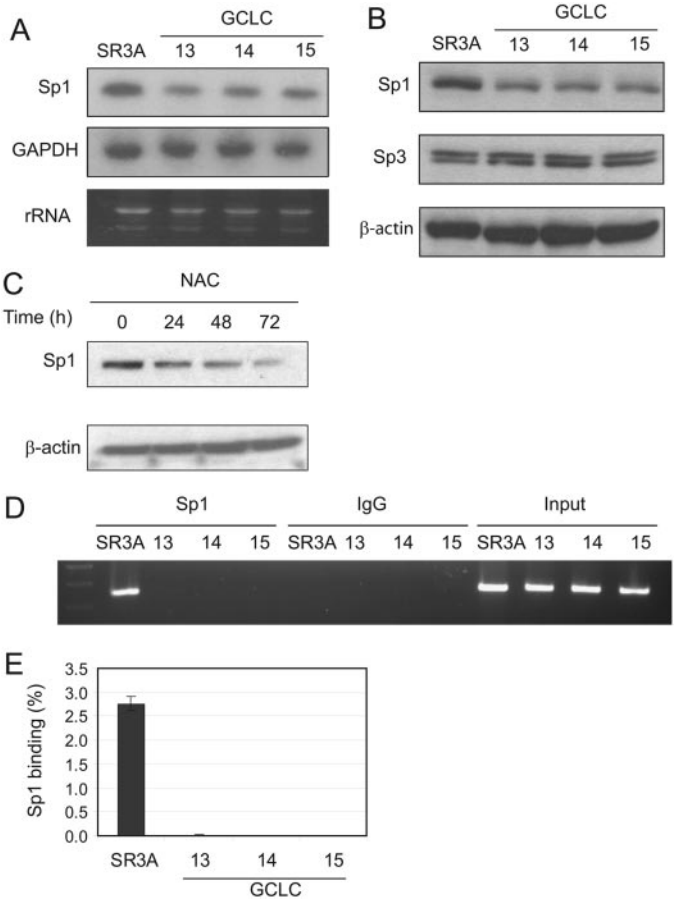

Fig. 6. Down-regulation of protein and DNA binding levels of Sp1 in *GCLC*- transfected cell lines. A and B, exponentially grown SR3A and *GCLC*-transfected cell were harvested, and Sp1 expression level was analyzed by Western or Northern blotting. C, SCLC cells were treated with 5 mM NAC for 24, 48, and 72 h, and Sp1 level was analyzed by Western blotting. D and E, Sp1 DNA binding on ZIP8 promoter was analyzed by ChIP assay. ChIP samples were analyzed by agarose electrophoresis and real-time PCR. Error bars represent standard deviation from three independent experiments.

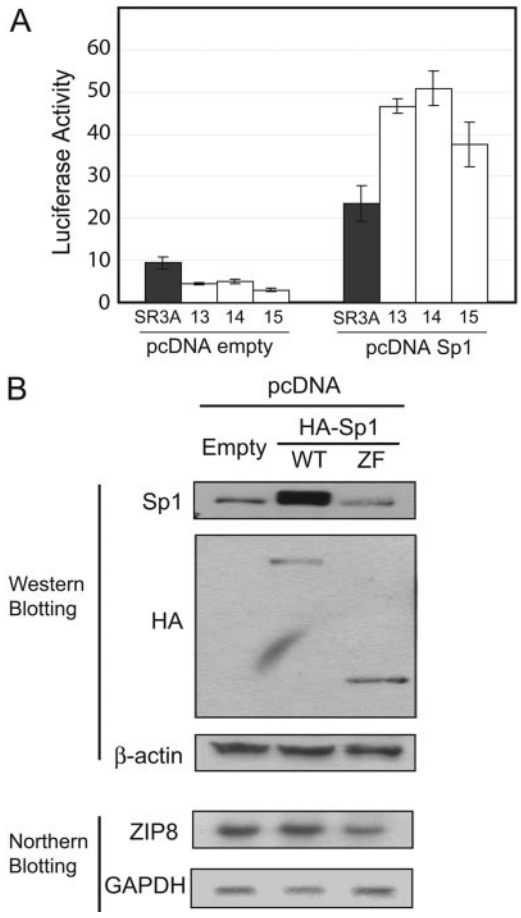

Fig. 7. Effect of Sp1 overexpression on ZIP8 expression. A, SR3A and *GCLC*-transfected cell lines were transfected with the ZIP8 pGL3 vector with or without Sp1 expression vector. Luciferase activity was analyzed 72 h after transfection. B, HEK-293 cell were transfected with empty vector or expression vectors encoding full-length Sp1 or the zinc finger motif of Sp1. Total protein and RNA were extracted 48 h after transfection, and endogenous and exogenous Sp1 expression was confirmed by Western blotting and ZIP8 expression level was analyzed by Northern blotting. Error bars represent standard deviation from three independent experiments. $*$, $p < 0.05$.

by displacement of zinc, formation of mixed complexes, or incomplete coordination of metals (Hartwig, 2001), resulting in loss of DNA binding activity. These observations suggest that expression of ZIP8 may be down-regulated by cadmium exposure through inhibition of Sp1. To test this hypothesis, we treated SR3A cells with various concentrations of cadmium, and expression levels of Sp1 and Sp3 were determined by the Western blotting. Figure 9A shows that the expression levels of Sp1 were reduced in the cells treated with cadmium. Levels of Sp3 were also reduced, but to a lesser extent than that of Sp1. These results were also confirmed by EMSA. Sp1 DNA binding was also more sensitive to cadmium than was Sp3 binding, as analyzed by EMSA (Fig. 9B). We found that expression levels of ZIP8 mRNA were also reduced in the cadmium-treated cells at 1 μM cadmium (Fig. 9C). It is noteworthy that Sp1 DNA binding ability reflects a decrease in ZIP8 level better than Sp1 protein level. These results suggest that inactivation of Sp1 DNA binding by cadmium has a role in ZIP8 down-regulation. It is noteworthy that because the IC_{50} value of cadmium in SR3A cell line is approximately 14 μM, these results indicated that cadmium can down-regulate ZIP8 expression at nonlethal concentrations.

It has been shown that ZIP8 has a high affinity for manganese, in addition to supporting transport activity of zinc, suggesting that these metal ions are also physiological substrates for ZIP8. However, treatment with 1 to 100 μM $MnCl_2$ (Fig. 9D) or $ZnCl_2$ (Fig. 9E) for 20 h did not result in significant changes in Sp1 protein and mRNA. These results suggest that the expression of ZIP8 is not regulated by these metal ions.

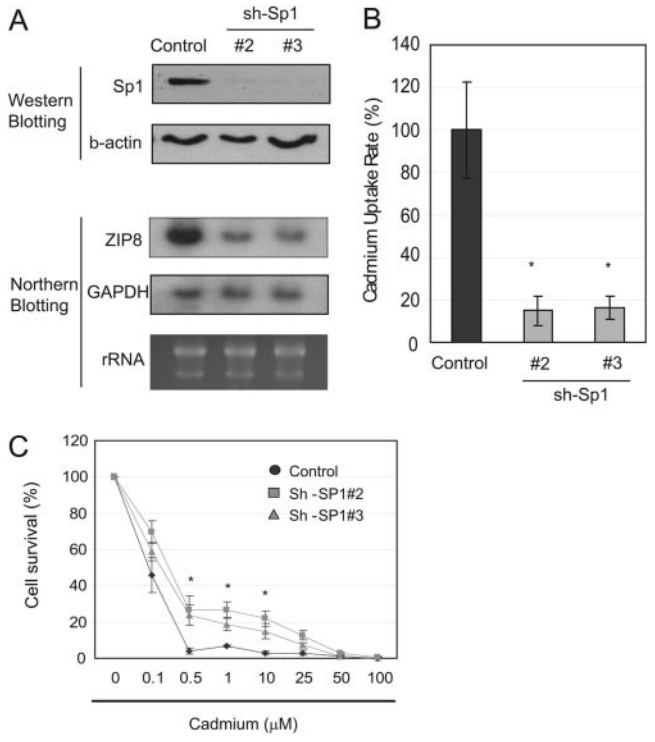

Fig. 8. Effect of Sp1 knockdown on cadmium toxicity A, effects of Sp1 knockdown on ZIP8 expression. HEK-293 cells were transduced with lentivirus bearing control or sh-Sp1. Seventy-two hours after transduction, cells were harvested, and protein and total RNA were extracted. Expression levels of Sp1 and ZIP8 were analyzed by Western or Northern blotting, respectively. B and C, effects of Sp1 knockdown on cadmium sensitivity. HEK-293 cells transduced with control or sh-Sp1 vectors (#2 or #3) were subjected to cadmium uptake and MTT assays. Cadmium uptake rate was calculated and normalized to the control level. For the cadmium sensitivity assay, cells were transduced with lentivirus bearing scrambled or sh-Sp1 sequences. Seventy-two hours after transduction, cells were treated with various concentrations of cadmium for 72 h, and MTT activity was assayed. Error bars represent standard deviation from three independent experiments. $*$, $p < 0.05$.

Discussion

The roles of GSH in regulating cadmium cytotoxicity have been studied in various model organisms, and results showed that treatment with GSH derivative or its precursor NAC can suppress cadmium toxicity (Kaplan et al., 2008). Previous studies have suggested that elevated GSH enhanced cadmium efflux mediated by MRP family from in yeast (Adle et al., 2007) and plant (Kim et al., 2007). In mammalian cells, cadmium exposure up-regulates both GCLC and MRP/GS-X pump expression (Ishikawa et al., 1996). The present communication demonstrated that elevated GSH levels can down-regulate cadmium uptake through down-regulation of

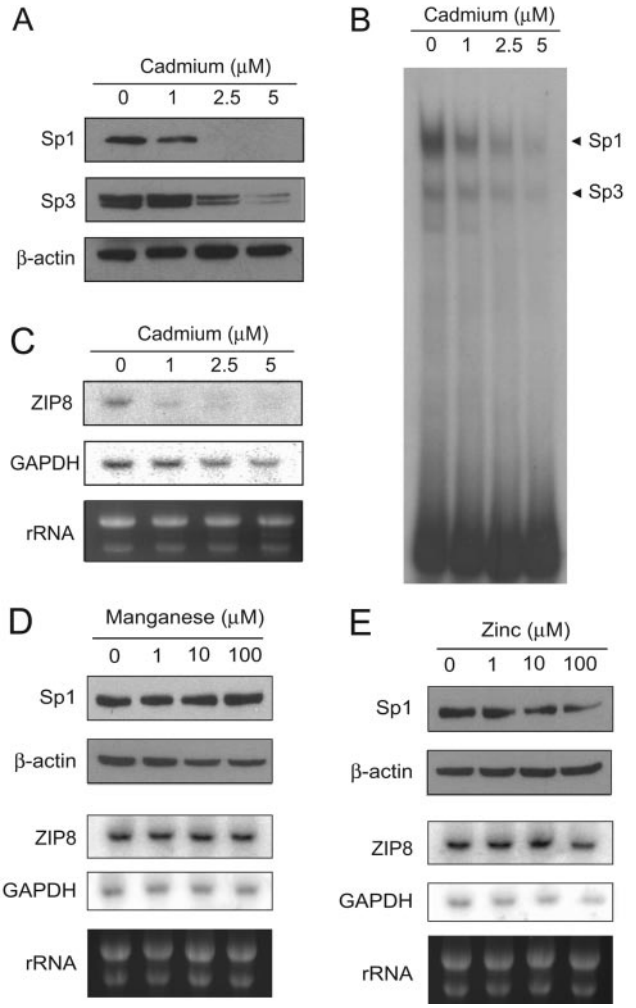

Fig. 9. Effect of metal ions on ZIP8 expression. SR3A cells were treated with various concentrations of manganese, zinc, and cadmium for 20 h. Cells were then harvested, and RNA and protein were extracted. Sp1 protein levels were analyzed by Western blotting (A), and Sp1 DNA binding were analyzed by EMSA (B). The effect of cadmium exposure on ZIP8 expression was analyzed by Northern blotting (C). The effect of manganese (D) and zinc (E) on Sp1 and ZIP8 levels was also examined by Western and Northern blotting, respectively.

the cadmium transporter ZIP8. Together, these results indicate that GSH plays dual roles in regulating the cellular cadmium contents that are responsible for the acquired cadmium resistance.

Recent studies suggest that the calcium channel is involved in cadmium uptake (Leslie et al., 2006). However, siRNA-based ZIP8 knockdown significantly lowered cadmium uptake rate in SR3A cells. In fact, in SCLC and SR3A cell lines, ZIP8 was expressed more than 10 times higher than that of CacnG1α, one of the calcium channels proposed to be involved in cadmium transport (data not shown). This difference may reflect their different tissue distribution, and because ZIP8 expresses highly in lung (Begum et al., 2002), ZIP8 may have potent role in olfactory cadmium intoxication.

ZIP8 was first identified as a lipopolysaccharide-induced gene in monocytes and suggested regulation by NF-κB pathway (Begum et al., 2002). In fact, our promoter sequence analysis identified three putative NF-κB binding sequences. However, our promoter analysis did not show any effect of deletion of these putative transcription factor binding sites. It is important to note that some studies demonstrated that Sp1 is involved in many lipopolysaccharide-induced gene expressions (Lee et al., 2005; Hirasawa et al., 2006; Oh et al., 2008).

In this study, we have demonstrated that elevation of intercellular GSH levels, either by overexpression of GCLC or NAC treatment, down-regulate Sp1 level. Although the precise mechanisms by which elevated GSH levels down-regulate Sp1 expression are not known, several possibilities can be offered. Some studies reported an association of Sp1 with intercellular GSH or reactive oxygen species levels. Oxidative stress activates mitogen-activated protein kinase signaling pathway and up-regulates Sp1 protein and its target gene expression (Schäfer et al., 2003; Dasari et al., 2006). In addition, activation of mitogen-activated protein kinase is prevented by elevated GSH level and in turn inhibits the expression of Sp1 target genes (Vayalil et al., 2007). Sp1 contains three ZF domains that are important for DNA binding. Each ZF is composed of two cysteine and two histidine residues that are coordinated by zinc in a tetrahedral conformation. The structural fold of ZF, particularly the cysteine residues, is very sensitive to redox status, and its stability is affected by intracellular thiol/disulfate pools such as GSH. Indeed, it has been reported that the transcription activity of Sp1 is regulated by intracellular redox conditions in either positive or negative manners, perhaps depending on the contexts of different promoters or cell type (Webster et al., 2001; Schäfer et al., 2003; Dasari et al., 2006). In fact, our results in ChIP assay showed that Sp1 DNA binding was more robustly affected in *GCLC*-transfected cell lines than that of Sp1 expression level. These reports complement our finding that elevated GSH level suppresses ZIP8 expression through down-regulation of Sp1.

The discovery that Sp1 is a transcription regulator for metal transporter ZIP8 supports the important roles of ZF-containing transcription factors in the regulation of metal ion homeostasis (Rutherford and Bird, 2004). In *Sacchromyces cerevisiae*, regulation of high-affinity copper uptake systems encoded by *Ctr1* and *Ctr3* is controlled by the ZF-containing transcription factor Mac1. Likewise, expression of three zinc uptake systems encoded by the *ZRT1*, *ZRT2*, and *FET4* genes is regulated by ZF-containing transcription factor Zap1. Because copper and zinc are essential micronutrients but otherwise toxic at high concentrations, cells use Mac1 and Zap1 to up-regulate the expression of their respective transporters to increase transports of these metal ions under copper and zinc limit conditions. Although cadmium is toxic, cells use down-regulation of Sp1 to reduce the expression of ZIP8 to achieve resistance to cadmium toxicity.

In addition to cadmium, ZIP8 is also known to transport zinc and manganese. Zinc was first reported to be a substrate of ZIP8, and its homeostasis is tightly regulated by its absorption and excretion (Cousins et al., 2006). One of the important pathways for maintaining zinc homeostasis is up-regulation of the transcription factor MTF-1. MTF-1 itself can sense intercellular zinc concentration and up-regulate genes such as metallothionein (Laity and Andrews, 2007). However, we could not find the MTF-1 binding sequence on the proximal region of ZIP8 promoter, and zinc exposure did not change ZIP8 expression level. Based on its high affinity for manganese, ZIP8 is proposed as an Mn^{2+}/HCO_3^- symporter (He et al., 2006). Like zinc ion, manganese is an essential metal in humans. Cells have a sophisticated mechanism to maintain its homeostasis, and disturbance of homeostasis is known to be related to neurotoxicity (Roth, 2006). However, in our study, high concentrations of manganese had no effect on ZIP8 expression. These results demonstrate that transcriptional regulation of ZIP8 does not seem to be involved in manganese or zinc homeostasis and suggest that other transporters may be the major players in this regard.

In summary, our results demonstrate that both GSH and cadmium exposure can suppress ZIP8 expression through the down-regulation of Sp1, thus revealing a new pathway of GSH-mediated enhancement of cadmium resistance. These results bear important implications that it is possible to use antioxidants in combating cadmium toxicity.

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