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Glucocorticoid Receptor (GR)-Associated SMRT Binding to C/EBPβ TAD and Nrf2 Neh4/5: Role of SMRT Recruited to GR in GSTA2 Gene Repression

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The expression of the glutathione *S*-transferase gene (*GST*), whose induction accounts for cancer chemoprevention, is regulated by activation of CCAAT/enhancer binding protein β (C/EBPβ) and NF-E2-related factor 2 (Nrf2). The present study investigated the repressing effects of activating glucocorticoid receptor (GR) on C/EBPβ- and Nrf2-mediated *GSTA2* gene induction and the mechanism. Dexamethasone that activates GR inhibited constitutive and oltipraz- or *tert*-butylhydroquinone (t-BHQ)-inducible GSTA2 expression in H4IIE cells. Also, dexamethasone repressed *GSTA2* promoter-luciferase gene activity. Dexamethasone-GR activation did not inhibit nuclear translocation of C/EBPβ or Nrf2 nor their DNA binding activities induced by oltipraz or t-BHQ. Deletion of the glucocorticoid response element (GRE) in the *GSTA2* promoter abolished dexamethasone inhibition of the gene induction. Immunoprecipitation-immunoblotting, chromatin immunoprecipitation, and GST pull-down assays revealed that silencing mediator for retinoid and thyroid hormone receptors (SMRT), a corepressor recruited to steroid-GR complex for histone deacetylation, bound to TAD domain of C/EBPβ and Neh4/5 domain of Nrf2. The *GSTA2* promoter-luciferase activities were decreased by SMRT but not by truncated SMRTs. The small interference RNA (siRNA) against SMRT abolished SMRT repression of the gene induction by C/EBPβ or Nrf2. The plasmid transfection and siRNA experiments directly evidenced the functional role of SMRT in GSTA2 repression. In conclusion, dexamethasone antagonizes C/EBPβ- and Nrf2-mediated *GSTA2* gene induction via ligand-GR binding to the GRE, and steroid-mediated GSTA2 repression involves inactivation of C/EBPβ and Nrf2 by SMRT recruited to steroid-GR complex.

The induction of glutathione (GSH) *S*-transferase (GST), a phase II detoxifying enzyme, accounts for cytoprotective and cancer chemopreventive effects (2, 19). Previously, we have shown that nuclear translocation of CCAAT/enhancer binding protein β (C/EBPβ) and its binding to the C/EBP binding site in the *GSTA2* gene by oltipraz, a representative cancer chemopreventive agent, lead to strong and persistent induction of the gene (13). We showed that phosphatidylinositol 3-kinase (PI3-kinase)-mediated nuclear translocation of C/EBPβ, which is promoted by oltipraz, leads to the induction of the *GST* gene via activating C/EBPβ binding to the C/EBP binding site present in the *GSTA2* gene. Oxidative stress including sulfur amino acid deprivation and *tert*-butylhydroquinone (t-BHQ), a representative prooxidant, induces GSTA2 primarily through nuclear translocation of NF-E2-related factor 2 (Nrf2) and Nrf2's binding to the antioxidant response element (ARE) in the gene promoter (14, 15, 17, 31). Oxidative stress by decreased glutathione or t-BHQ activates PI3-kinase, and the pathway involving PI3-kinase plays an essential role in Nrf2/ARE-mediated GSTA2 induction (14, 15, 17).

The glucocorticoids are predominantly used for their anti-inflammatory and immunosuppressive activities. The glucocorticoids exert nonspecific anti-inflammatory effects regardless of the etiology of inflammation. Glucocorticoids show differential relative anti-inflammatory potencies, with that of dexamethasone (Dex) being 30 times that of hydrocortisone. The tissue concentrations of Dex in clinical use are proportional to its clinical effects. Because of the relatively high potency, the tissue concentrations of Dex are in the nanomolar range during the glucocorticoid therapy of chronic inflammatory diseases. Glucocorticoids modulate transcription of the responsible genes and are also necessary for the changes in the expression of some acute-phase response proteins (1). The mechanisms by which the expression of acute-phase response is altered include activation of the transcription factors such as C/EBPβ, nuclear factor κB, signal transducers and activators of transcription, and activator protein 1.

Glucocorticoids act as ligands for the glucocorticoid receptor (GR) to enhance or repress the expression of target genes. In particular, glucocorticoids via GR activation repress the constitutive expression of GSTA2. Falkner et al. (5) showed that low concentrations of Dex suppressed GSTA2 gene expression via a GR-dependent mechanism, whereas high concentrations of Dex induced the gene in a pregnane X receptor (PXR)-dependent manner. Hence, the effects of Dex at low (i.e., nanomolar) concentrations are mediated with GR activation. In view of the importance of GR and glucocorticoid response element (GRE) in the repression of gene transactivation and of the potential interaction of C/EBPβ with the modulator(s) recruited to ligand-bound GR, we determined whether Dex repressed C/EBPβ- and Nrf2-mediated induction

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of the *GSTA2* gene via ligand-bound GR and its binding to the GRE.

Evidence suggests that GR-steroid complex binding to the hormone response element recruits silencing mediator for retinoid and thyroid hormone receptors (SMRT) (35) or nuclear receptor corepressor (NCoR) (11) as a corepressor, whereas gene activation by ligand-bound GR results from recruitment of the coactivators such as GR-interacting protein 1 (GRIP1) (4), transcriptional intermediary factor 2, steroid receptor coactivator 1, and amplified in breast cancer 1 to target promoters (20, 23). Beyond these findings, nothing is known about the functional role of coregulators of glucocorticoid-bound GR in the expression of phase II enzymes or physical association of the coregulators with other activating transcriptional factors. Given GSTA2 repression by glucocorticoid-bound GR and the essential role of C/EBPβ and Nrf2 in the constitutive and inducible phase II enzyme expression, we were tempted to study the role of SMRT or NcoR recruitment as a corepressor of GR-steroid complex in GSTA2 repression by Dex and the potential interaction of the corepressors with activating transcription factors C/EBPβ and Nrf2.

Specifically, we raised the questions including (i) whether Dex at the nanomolar concentrations is capable of inhibiting C/EBPβ- and Nrf2-mediated *GSTA2* gene expression by chemical inducers; (ii) whether ligand-bound GR alters C/EBPβ and Nrf2 activation; (iii) whether ligand-GR binding to the GRE represses C/EBP- and Nrf2-mediated *GSTA2* gene induction and, if so, what site of GRE is responsible for the gene repression; and (iv) what the role of SMRT or NcoR is in *GSTA2* gene repression by glucocorticoid. We were particularly interested in the physical and functional interactions of SMRT, or the receptor-interacting domain (RID) and silencing domain (SD) of SMRT, with C/EBPβ and Nrf2 in the gene repression. Finally, we identified the SMRT-interacting regions within Nrf2 and C/EBPβ using deletion mutants for each molecule.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (3,000 mCi/mmol) and [^{35}S]methionine (>1,000 Ci/mmol) were purchased from New England Nuclear (Arlington Heights, IL). Oltipraz was provided from Aventis Pharma France (Vitry-sur-Seine, France). Dithiothreitol, RU-486, Dex, t-BHQ, and trichostatin A (TSA) were purchased from Sigma Chemicals (St. Louis, MO). Anti-C/EBPβ, anti-heme oxygenase 1, anti-c-*myc*, anti-NcoR, anti-NQO1, anti-Nrf2, anti-SMRT, and antiactin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GSTα antibody specific for GSTA2 was provided from Detroit R&D (Detroit, MI).

Cell culture. H4IIE (or NIH 3T3) cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Oltipraz or t-BHQ was dissolved in dimethyl sulfoxide. Cells were incubated in the presence or absence of oltipraz (10 μM) or t-BHQ (30 μM) for the indicated time period.

Immunoblot analysis. Cell lysates and subcellular fractions were prepared according to the previously published methods (13, 16, 33). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses were performed as previously described (16, 19). Immunoreactive proteins were visualized by an ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, United Kingdom). Specificity of the antibody directed against GSTA2 or microsomal epoxide hydrolase was previously determined (13, 16). Equal loading of proteins was verified by Coomassie blue staining of gels and actin immunoblottings. At least four separate experiments were performed with different samples to confirm changes in protein levels.

Scanning densitometry. Scanning densitometry of the immunoblots was performed with Image Scan & Analysis System (Alpha-Innotech Corporation, San Leandro, CA). The area of each lane was integrated using the software AlphaEase version 5.5, followed by background subtraction.

RT-PCR and real-time RT-PCR. Total RNA was prepared from untreated H4IIE cells or the cells treated with oltipraz or t-BHQ in the presence or absence of 100 nM Dex, as described previously (13, 28). Reverse transcription-PCR (RT-PCR) was performed using the selective primers for GSTA2 (sense primer, 5′-GAAGGACATGAAGGAGAGAGC-3′; antisense primer, 5′-TTCTTCGATTTGTTTTGCATC-3′) (398-bp) and glyceraldehyde-3-phosphate dehydrogenase (sense, 5′-TCGTGGAGTCTACTGGCGT-3′; antisense, 5′-GCCTGCTTCACCACCTTCT-3′) (510-bp) genes. Real-time RT-PCRs (50 cycles) were carried out according to the manufacturer's instruction (Light-Cycler 2.0; Roche, Mannheim, Germany).

Gel shift assay. Double-stranded DNA probes (C/EBP response element, 5′-TGCAGATTGCGCAATCTGCA-3′; ARE, 5′-GATCATGGCATTGCACTAGGTGACAAAGCA-3′; and GRE, 5′-AGAGGATCTGTACAGGATGTTCTAGAT-3′ [core sequences are underlined]) end labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase were used for gel shift analyses, as previously described (13, 28). In some analyses, specificity of binding was determined by competition experiments, which were carried out by adding a 20-fold molar excess of an unlabeled oligonucleotide to the reaction mixture before the labeled probe was added. SP-1 oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) was used as a negative control for competition experiments. In other analyses, known as supershift or immunoinhibition assays, antibody to C/EBPβ, Nrf2, or GR (2 μg each) was added to the reaction mixture 20 min after the labeled probe was added, and the reaction was continued for another hour at 25°C. Samples were separated on 4% polyacrylamide gels at 100 V. The gels were fixed with 40% methanol-10% acetic acid, dried, and subjected to autoradiography.

Immunocytochemistry. H4IIE cells were grown on Lab-TEK chamber slides (Nalge Nunc International Corp., Rochester, NY) and incubated in serum-free Dulbecco's modified Eagle's medium for 6 h at 37°C. Standard immunocytochemical methods were used for immunostaining of C/EBPβ or Nrf2, as previously described (14, 27). Counterstaining with propidium iodide (PI, 2 μg/ml) was used to verify the location and integrity of nuclei. Stained cells were washed with phosphate-buffered saline (PBS) and examined using a laser-scanning confocal microscope (Leica TCS NT; Leica Microsystems, Wetzlar, Germany).

Plasmid construction. The pCDNA-Nrf2, pCDNA-mNrf2(#Neh4/5), and pCMV5-mGR plasmids, which contain the full-length coding regions of mouse Nrf2, Nrf2 lacking Neh4/5, and GR, respectively, were gifts from M. Yamamoto (Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan) and J. E. Bodwell (Department of Physiology, Dartmouth Medical School, Lebanon, NH). The pGTB-1.65 plasmid construct containing the rat GSTA2 promoter region (−1651/+66 bp) was kindly provided by C. B. Pickett (Schering-Plough Corp. Inst., Kenilworth, NJ). The pGL-1651 reporter gene construct was generated by ligating the region 1.65 kb upstream of the transcription start site of the rat *GSTA2* gene to the firefly luciferase reporter gene coding sequence. A series of 5′-deleted chimeric gene constructs, pGL-1585, pGL-1128, pGL-797, and pGL-197, were also prepared for reporter gene analysis. pGL-1585 misses the distal GRE site. pGL-1128 comprises the half-GRE, the C/EBP binding site, and the ARE. The pGL-797 gene construct, in which the C/EBP binding sequence was deleted, contains only the ARE. The pGL-197 was used as a vector containing the minimal promoter region. The ΔGRE-pGL-1651 construct, whose distal GRE site was specifically deleted, was prepared by the overlapping PCR method using pGL-1651 as a template. The pCMX-SMRT and pGEX-2tk-GST-RID plasmids were gifts from R. M. Evans (Howard Hughes Medical Institute, La Jolla, CA). pET-GST-SD, which encodes the schistosomal GST-fused SD of SMRT, was produced by PCR amplification of DNA using pCMX-SMRT as a template and ligation of the amplified DNA into pET-42b(+) (Novagen, Darmstadt, Germany). The pCDNA-SMRT-RID and pCDNA-SMRT-SD constructs were generated by PCR amplification. The DNA fragments that are flanked by start and stop codons were then inserted into pCDNA (Invitrogen, Carlsbad, CA). pCDNA-C/EBPβ, which encodes C/EBPβ, was generated by PCR amplification of the coding region of the *C/EBP*β gene using rat genomic DNA as a template (12), and the amplified DNA was cloned into pCDNA. Similarly, the pCDNA-C/EBPβ-TAD (N-terminal transactivation domain), pCDNA-C/EBPβ-bZIP (C-terminal basic leucine zipper domain), and pCDNA-Neh4/5 (encoding Neh4/5 domain of Nrf2) constructs were prepared by PCR amplifications and cloning. DNA sequencing (ABI 7700 DNA cycle sequencer) verified authenticity of all constructs.

Transient transfection. H4IIE cells were transiently transfected with the pCMX-SMRT, pCMV5-mGR, or *GSTA2*-promoter chimeric constructs using Lipofectamine Plus reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), as previously described (13). In some experiments, cells were transiently transfected with the pCMX-SMRT, pCDNA-SMRT-RID, or

pCDNA-SMRT-SD plasmid. Briefly, cells were replated 24 h before transfection at a density of 7×10^5 cells in six-well plates. Cells were transfected by addition of 1 ml minimal essential medium containing 1 μg of each plasmid and 4 μl of Lipofectamine and then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 h. After addition of 1 ml minimal essential medium containing 1% fetal calf serum, cells were incubated for an additional 13 h at 37°C. Control cells were transfected with an equal amount of the respective empty plasmid (i.e., mock transfection).

GSTA2 promoter-luciferase assay. To determine the activities of the deleted GSTA2 promoter constructs, we used the dual luciferase reporter assay system (Promega, Madison, WI) as described previously (13, 29). For some experiments, cells were cotransfected with pCMX-SMRT, pCDNA-SMRT-RID, or pCDNA-SMRT-SD plasmid (1 μg each) in addition to GSTA2 promoter-luciferase construct (1 μg). Control cells were mock transfected with the pCMX or pCDNA empty vector (1 μg each).

Immunoprecipitation. To determine the physical interaction of SMRT or NCoR with C/EBPβ or Nrf2, a fraction of total cell lysates (250 μg protein in 1 ml) was incubated with anti-SMRT antibody or anti-NCoR antibody (Santa Cruz, CA) overnight at 4°C. The antigen-antibody complex was immunoprecipitated following incubation for 2 h at 4°C with protein G-agarose. Immune complexes were solubilized in 2× Laemmli buffer and boiled for 5 min. Samples were separated and analyzed using 7.5% SDS-PAGE and then transferred to nitrocellulose membranes. The samples were then immunoblotted with an antibody directed against GR, C/EBPβ, or Nrf2. Blots were developed using an ECL chemiluminescence detection kit to visualize immunoblots.

ChIP assays. H4IIE cells were treated with Dex alone or in combination with oltipraz or t-BHQ for 6 h, and then formaldehyde was added to the cells to a final concentration of 1%. SMRT was cross-linked to chromatin by incubating the cells for 10 min at 37°C. The cells were washed with ice-cold PBS and lysed in the Tris-HCl buffer (50 mM, pH 8.1) containing 1% SDS and 10 mM EDTA. The lysates were sonicated and centrifuged at 10,000 × *g* for 10 min to remove debris. The supernatants containing chromatin were diluted with 10 volumes of the chromatin immunoprecipitation (ChIP) dilution buffer (16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, and 1.1% Triton X-100). One-fourth of the chromatin solution was reserved for total input. The remaining solution was precleared with protein G-agarose; subsequently incubated with anti-SMRT antibody, anti-acetylated histone H3 antibody, or preimmune immunoglobulin G (IgG) (2 μg each) for 12 h at 4°C with shaking; and then further incubated with protein G-agarose for 2 h. The immunoprecipitates were washed and reverse cross-linked by adding 5 M NaCl to a final concentration of 200 mM and incubating for 4 h at 65°C, as described previously (34). DNA was phenol-chloroform extracted. PCR was performed with the specific primers flanking the C/EBP binding element and the ARE in the *GSTA2* promoter (sense, 5′-GGACAACACACTCAGCTTTG-3′; antisense, 5′-TCAGTGCAGCCTGTGAGTC-3′, −989/−643 bp) or the primers amplifying the irrelevant region of the promoter (sense, 5′-GAATGGATCAAGCTCTTCAC-3′; antisense, 5′-AGCTCCAGAGGAGTGTGCA-3′, −197/−1 bp). Amplified fragments from the *GSTA2* gene were analyzed on a 1.5% agarose gel.

GST pull-down assays. Association of SMRT with C/EBPβ or Nrf2 was analyzed using agarose-immobilized GST fusion proteins, as described previously (32). *Escherichia coli* BL21(DE3) cells harboring the plasmid encoding GST-SD (or GST) or DH5α cells harboring the GST-RID plasmid were cultured at 37°C with shaking. GST fusion proteins were induced by incubating the cells with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Bacteria expressing GST, GST-RID, or GST-SD were precipitated and resuspended with 1/10 volume of PBS containing 0.1% Tween 20, 2 mM EDTA, 0.1% β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Aliquots of cell suspension were stored at −80°C until use. Bacterial cell lysate was mixed with GSH-agarose beads (100 μl, 50% slurry) preswollen with PBS containing 0.1% Tween 20. GST fusion protein was immobilized to GSH-agarose by incubating at 4°C for 20 min and washed three times with PBS containing 0.1% Tween 20 and 0.2 mM PMSF and once with 20 mM HEPES buffer (pH 7.8, buffer A) containing 50 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 6% glycerol, 0.05% Triton X-100, 0.1 mM PMSF, 1 mM dithiothreitol, and 0.05 mg/ml bovine serum albumin. Radiolabeled protein was produced with the TNT system (Promega, Madison, WI) using the pCDNA-C/EBPβ, pCDNA-Nrf2, pCDNA-C/EBPβ-TAD, pCDNA-C/EBPβ-bZIP, pCDNA-mNrf2(#Neh4/5), or pCDNA-Neh4/5 plasmid (1 μg). For binding reaction, GSH-agarose immobilized with GST fusion protein (or GST as control) (25 μl) was mixed with ^{35}S-C/EBPβ, ^{35}S-Nrf2, ^{35}S-C/EBPβ-TAD, ^{35}S-C/EBPβ-bZIP, ^{35}S-Neh4/5, or ^{35}S-mNrf2(#Neh4/5) (10 μl) in buffer A (500 μl) for 2 h. The protein-bound agarose was washed three times with 20 mM HEPES buffer (pH 7.8) containing 50 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 6% glycerol, 0.05% Triton X-100, and 0.1 mM PMSF. Bound proteins were eluted with 20 μl of 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced GSH and 10% glycerol. Eluate was resolved by SDS-PAGE. Labeled proteins were visualized by autoradiography.

Knockdown experiment using siRNA. Stealth small interference RNA (siRNA) against SMRT (5′-CAUGAAGGGUAUCAUCACCGCUGUG-3′) and RNAi negative control duplex (scRNA) were provided from Invitrogen (Carlsbad, CA). NIH 3T3 cells were used for the knockdown experiment with siRNA. Cells were transfected with the siRNA against SMRT or nonspecific RNA (scRNA) (100 pmol/ml) using Lipofectamine 2000 according to the manufacturer's instructions. Knockdown of SMRT was confirmed by RT-PCR analysis (sense primer, 5′-GAAGGATCCATCCTCACGTC-3′; antisense primer, 5′-TCCATGAGGGTAGGGTAGAC-3′) (364 bp). After transfection of the cells with pGL-1651 and pCMX-SMRT in combination with the plasmid encoding C/EBPβ or Nrf2, the whole lysates were used in the luciferase assay.

Statistical analysis. One-way analysis of variance was used to assess statistical significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman-Keuls test was used for comparisons between multiple group means. The data were expressed as means ± standard deviations or means ± standard errors (SE).

RESULTS

Effects of Dex on the constitutive or inducible expression of GSTA2. Dex activates GR at nanomolar concentrations and also stimulates PXR at the micromolar concentrations (5). First, we treated H4IIE cells with a variety of concentrations of Dex to assess changes in the expression of GSTA2. The level of GSTA2 was 55 to 65% suppressed in the cells treated with 10 to 100 nM of Dex for 48 h (Fig. 1A, left). By contrast, Dex at the concentrations of 1 μM or above induced GSTA2 by two- to threefold, compared to control. We determined the time course of the GSTA2 expression in response to 100 nM Dex (Fig. 1A, right). The expression of GSTA2 was 68 to 80% suppressed 48 to 72 h after Dex treatment. Dex at the concentration of 10 μM induced GSTA2 at least up to 72 h.

Previous studies from this laboratory have shown that oltipraz induces GSTA2 through activation of C/EBPβ and its binding to the C/EBP response element in the gene (13), whereas t-BHQ increases GSTA2 expression primarily through activation of Nrf2 and binding of activating Nrf2 to the ARE (14, 15). We were interested in whether oltipraz (10 μM)- or t-BHQ (30 μM)-inducible GSTA2 expression was affected by Dex treatment. Dex at the concentration of 10 or 100 nM substantially inhibited GSTA2 induction by oltipraz and further down-regulated the protein expression below the constitutive level (Fig. 1B, left). Suppression of GSTA2 expression by Dex was persistent at least up to 72 h (Fig. 1B, right). We next assessed the effects of Dex on GSTA2 induction by t-BHQ, which induces GSTA2 primarily via Nrf2 activation (14). The induction of GSTA2 by t-BHQ was significantly, but not completely, inhibited by treatment with 100 nM Dex (48 h) (Fig. 1C, left). Treatment of cells with t-BHQ resulted in a maximal increase in the level of GSTA2 at 48 h, followed by a gradual decrease towards control at 72 h (Fig. 1C, right). Dex inhibited t-BHQ induction of GSTA2 throughout the times examined. Previously, others and we have shown that GSTA2 expression was transcriptionally regulated (14, 15, 28). To verify changes in GSTA2 mRNA by Dex, RT-PCR and real-time RT-PCR were additionally performed (data not shown). Real-time RT-PCR analyses confirmed that Dex (24 h, 100 nM) decreased the GSTA2 mRNA levels in untreated cells (56% ± 4% of control) or the cells treated with oltipraz (24 h, 48% ± 9% of oltipraz alone) or t-BHQ (12 h, 41% ± 11% of t-BHQ

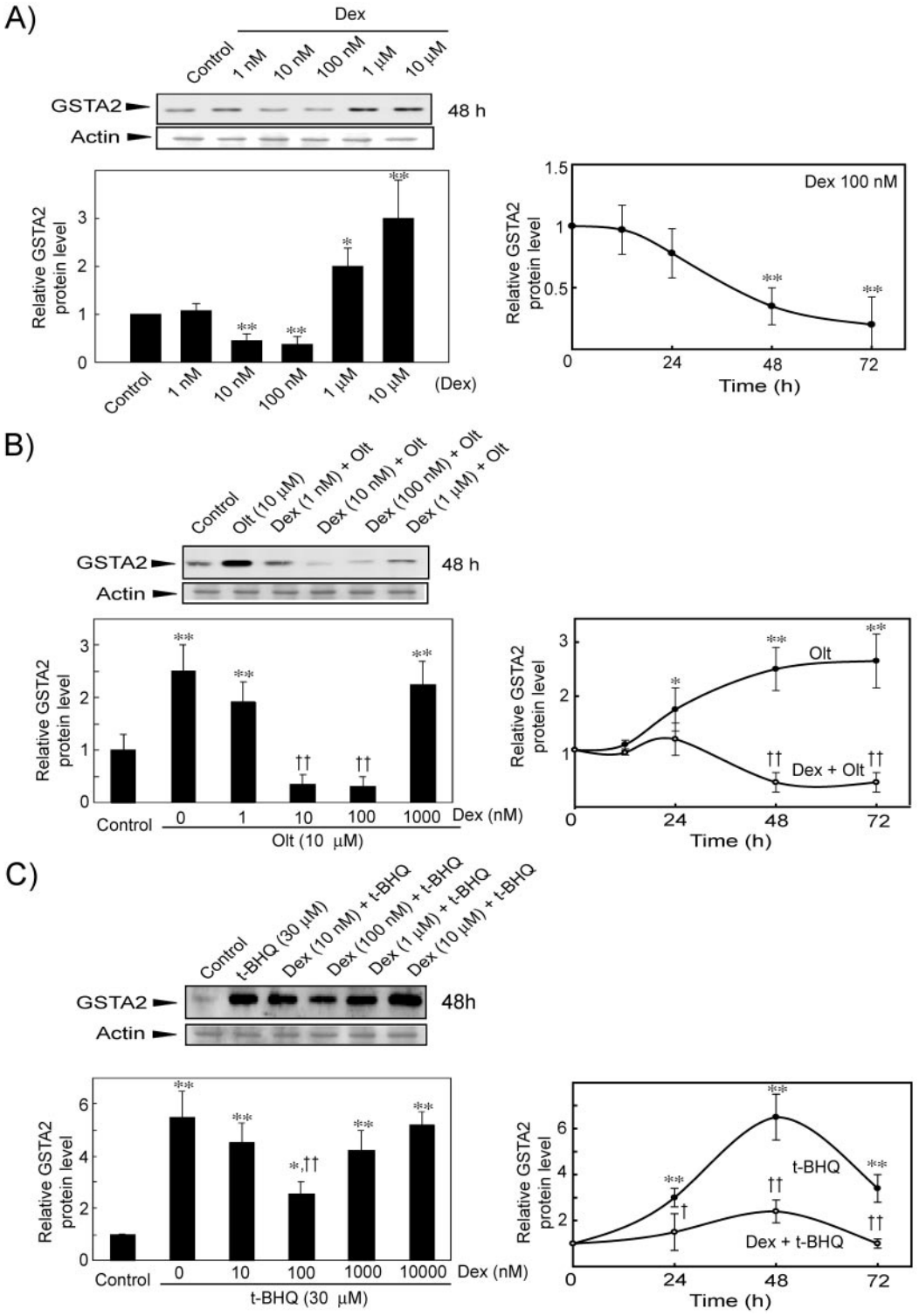


FIG. 1. Suppression of the constitutive or inducible expression of GSTA2 by Dex. (A) Representative immunoblot analysis of the GSTA2 protein in H4IIE cells incubated with Dex for 48 h (left). Each lane was loaded with 10 μg of cytosolic proteins. The relative levels of GSTA2 in the cells treated with 100 nM Dex for 12 to 72 h were assessed by immunoblot analyses (right). Data represent the mean ± standard deviation with four separate experiments. (B) Suppression of oltipraz (Olt)-inducible GSTA2 expression by Dex. H4IIE cells were treated with Olt or Dex plus Olt for 48 h (left). Time courses of GSTA2 expression were assessed in cells treated with Olt (10 μM) or Dex plus Olt for 12 to 72 h (right). (C) Suppression of t-BHQ-inducible GSTA2 expression by Dex. H4IIE cells were treated with t-BHQ or Dex plus t-BHQ for 48 h (left). Time courses of GSTA2 expression were assessed in cells treated with t-BHQ (30 μM) or Dex plus t-BHQ for 24 to 72 h (right) (significant compared to control, * [$P < 0.05$] and ** [$P < 0.01$]; significant compared to Olt or t-BHQ alone, *; significant compared to Olt or t-BHQ at the respective time point, † [$P < 0.05$] and †† [$P < 0.01$]; control = 1).

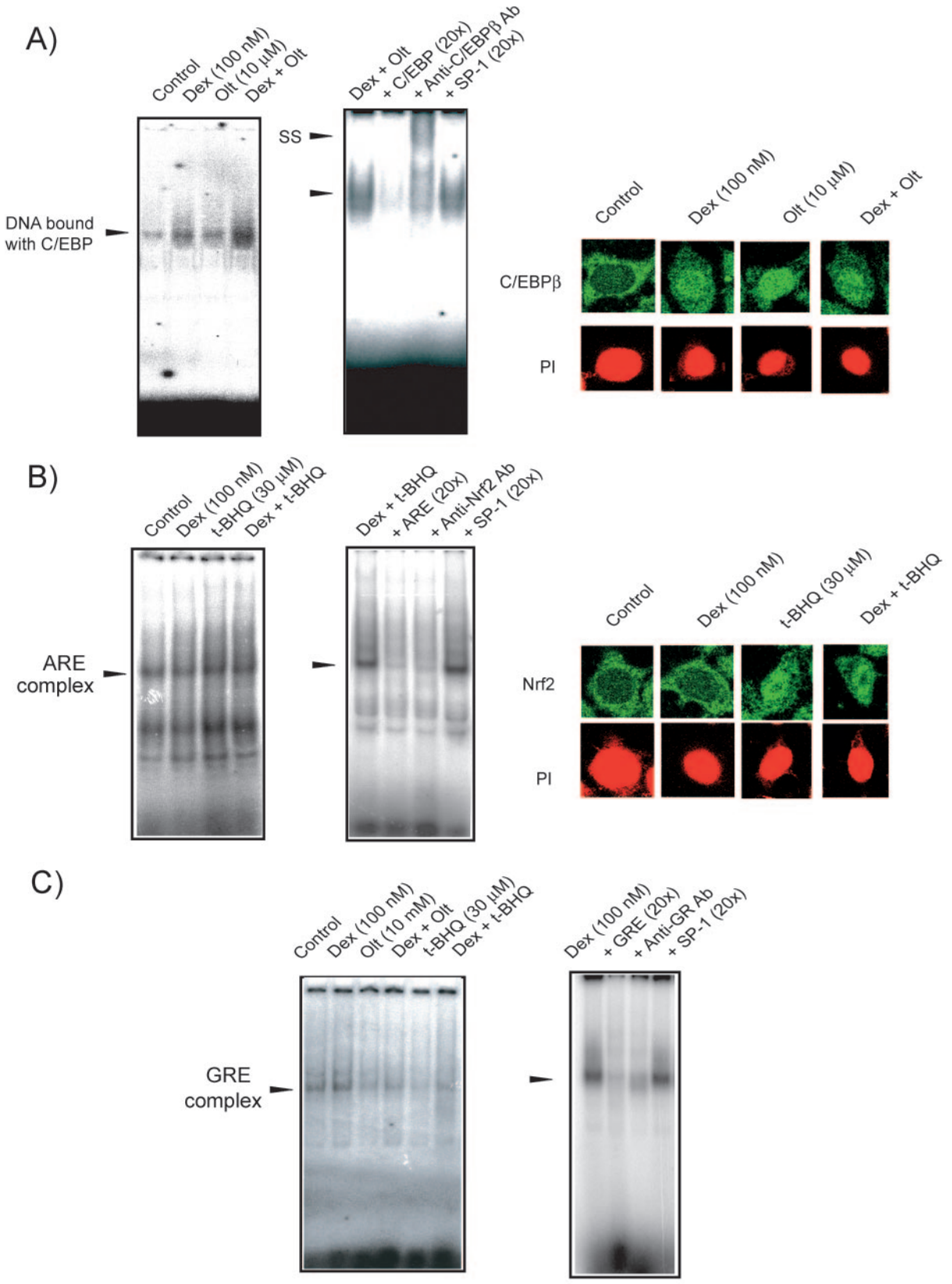


FIG. 2. The effects of Dex on the activation of C/EBPβ, Nrf2, or GR. (A) Gel shift analysis of protein binding to the C/EBP binding site (left). H4IIE cells were treated with Dex in the presence or absence of oltipraz (Olt) for 6 h. For competition assays, a 20-fold molar excess of unlabeled C/EBP or SP-1 oligonucleotide was added to the nuclear extracts from the cells treated with Dex plus Olt for 6 h (middle). The nuclear extract incubated with anti-C/EBPβ antibody (2 μg) was then mixed with a labeled probe for the C/EBP binding site. SS indicates the supershift of C/EBP

alone). These data suggested that activation of GR by Dex might antagonize C/EBPβ- and Nrf2-mediated GSTA2 induction.

Effects of Dex on C/EBPβ, Nrf2, or GR activation. To investigate this hypothesis, we first asked whether Dex affected C/EBPβ activation and, if so, whether Dex changed the extent of C/EBPβ activation by oltipraz. Gel shift analysis of protein binding to the C/EBP binding site was performed with nuclear extracts of H4IIE cells using a radiolabeled C/EBP binding oligonucleotide. Treatment of H4IIE cells with 100 nM Dex for 6 h resulted in an increase in C/EBPβ binding to the DNA compared with control (Fig. 2A, left). Oltipraz also increased C/EBP DNA binding activity. The band intensity of protein binding to the C/EBP binding oligonucleotide was further enhanced by treatment with Dex plus oltipraz. Similar enhancement of C/EBP binding to DNA was observed 12 h after treatment, which returned to the control level at 24 to 48 h (data not shown). Addition of a 20-fold excess of an unlabeled C/EBP binding oligonucleotide to the nuclear extract (Dex plus oltipraz, 6 h) completely abolished the binding activity, whereas excess unlabeled SP-1 oligonucleotide failed to inhibit binding, suggesting that the binding protein is C/EBP (Fig. 2A, middle). Supershift experiments indicated that increase in C/EBP DNA binding activity by Dex plus oltipraz is dependent on C/EBPβ. To determine whether increases in band intensities obtained in gel shift assays occurred as a result of nuclear translocation of C/EBPβ, the subcellular localization of C/EBPβ was determined by immunocytochemical analysis (Fig. 2A, right). Whereas C/EBPβ was located predominantly in the cytoplasm of untreated cells, C/EBPβ showed nuclear localization at 6 h in the cells treated with Dex or oltipraz. C/EBPβ was also localized in the nucleus at 6 h in the cells treated with Dex plus oltipraz. These data provided evidence that either Dex or oltipraz activated C/EBPβ at 6 to 12 h and that suppression of GSTA2 induction by Dex did not result from inhibition of C/EBPβ nuclear translocation or its binding to DNA.

In the previous reports, we have shown that the ARE protein complex consists of Nrf2 and small Maf (14, 15, 17). To determine whether Dex changed the binding activity of Nrf2 to the ARE, the nuclear extracts isolated from cells treated with t-BHQ or Dex plus t-BHQ were probed with a radiolabeled ARE oligonucleotide. Gel shift analysis revealed that Dex treatment did not alter the basal or t-BHQ-inducible Nrf2 binding to the ARE (Fig. 2B, left). Competition experiments using excess oligonucleotide or immunoinhibition experiments confirmed the specificity of Nrf2 DNA binding (Fig. 2B, middle). Immunocytochemistry verified that nuclear translocation of Nrf2 by t-BHQ was not affected by concomitant Dex treatment (Fig. 2B, right). Thus, suppression of t-BHQ-inducible GSTA2 expression by Dex was not associated with inhibition of Nrf2 activation (i.e., nuclear translocation or DNA binding).

To verify steroid-GR binding to the GRE, gel shift analysis was performed with the nuclear extracts prepared from the cells treated with Dex in the presence or absence of oltipraz (10 μM) or t-BHQ (30 μM). The band intensity of GR binding activity to DNA was increased by Dex treatment (6 h) (Fig. 2C, left). Oltipraz or t-BHQ treatment slightly, if any, decreased the activity of steroid-GR binding to DNA. GR DNA binding activity in the cells treated with Dex, Dex plus oltipraz, or Dex plus t-BHQ returned to that of control at 12 h or later times (data not shown). Addition of a 20-fold excess of unlabeled GRE completely abolished the binding activity (Dex, 6 h), whereas excess of SP-1 oligonucleotide failed to inhibit steroid-GR binding to the GRE, suggesting that the binding protein is GR (Fig. 2C, right). Competition experiments with unlabeled oligonucleotides and anti-GR antibody confirmed the specificity of GR DNA binding.

Analysis of GRE in the GSTA2 promoter. To examine the functional role of GRE in the *GSTA2* gene repression, we used *GSTA2* promoter deletion analyses. Reporter gene assays were performed using the H4IIE cells transfected with pGL-1651, which contained the luciferase structural gene downstream of the −1.65-kb *GSTA2* promoter region (Fig. 3A). The cells were cotransfected with the plasmid encoding GR because the expression of *GSTA2* promoter-luciferase plasmid was not activated by glucocorticoid without GR cotransfection (30). Treatment of the transiently transfected cells with either oltipraz (10 μM) or t-BHQ (30 μM) caused six- to eightfold increases in luciferase activity compared to control (Fig. 3B). Exposure of the cells transfected with pGL-1651 to oltipraz or t-BHQ in combination with Dex resulted in significant decreases (50 to 63%) in the luciferase inducibility compared to either oltipraz or t-BHQ alone.

To define the DNA sequence required for GSTA2 repression by Dex, a series of chimeric gene constructs with promoter deletions pGL-1585, pGL-1128, pGL-797, and pGL-197 were used (Fig. 3A). pGL-1651 contains one GRE site and four GRE half-sites, whereas pGL-1585 misses the most upstream GRE site. pGL-1128 missed the upstream GRE site and two GRE half sites. In the cells transfected with pGL-1585, Dex failed to decrease the luciferase inducibility. Treatment of the cells transfected with pGL-1128 with oltipraz or t-BHQ resulted in 33 to 37% decreases, compared to those observed with pGL-1651. The luciferase inducible activities of pGL-1128-transfected cells in response to Dex plus oltipraz or Dex plus t-BHQ were comparable to those of cells treated with oltipraz or t-BHQ alone. Exposure of the cells transfected with pGL-797 to oltipraz or t-BHQ resulted in only 31 to 38% of the luciferase inducibility that was observed with pGL-1651 (Fig.

DNA complex. C/EBPβ was immunochemically localized using anti-C/EBPβ antibody and in H4IIE cells treated with Dex, Olt, or Dex plus Olt for 6 h (right). The same fields were counterstained with PI to verify the location and integrity of nuclei. (B) Gel shift analysis of Nrf2 binding to the ARE. Nuclear extracts were prepared from H4IIE cells incubated with Dex (100 nM), t-BHQ (30 μM), or Dex plus t-BHQ for 6 h (left). Immunocompetition assay of Nrf2 binding to the ARE binding site was performed with anti-Nrf2 antibody (2 μg). Immunocytochemistry of Nrf2 was performed as described in panel A in cells treated with Dex with or without t-BHQ for 6 h (right). (C) Gel shift analysis of GR. Nuclear extracts were prepared from H4IIE cells incubated with Dex, Olt, Dex plus Olt, t-BHQ, or Dex plus t-BHQ for 6 h (left). Arrowhead indicates the GR DNA binding complex. The specificity of GR binding to DNA was also determined with unlabeled GRE or SP-1 oligonucleotide or anti-GR antibody (2 μg). Results were confirmed by repeated experiments.

3B), which was not affected by Dex treatment. In addition, the pGL-197 gene construct, which lacked nucleotides −1651 bp to −198 bp of the *GSTA2* gene flanking region and contained one downstream GRE half site, showed only minimal inducibility with no repression by Dex. The promoter deletion experiments demonstrated that the position of the GSTA2 regulatory region that confers Dex responsiveness included the upstream GRE site present in the region between −1651 bp and −1585 bp from the transcriptional start site. We finally determined if a specific disruption of the GRE in pGL-1651 abolished the ability of Dex to repress luciferase-reporter activity. H4IIE cells that had been transfected with ΔGRE-pGL-1651, in which the GRE consensus sequence was specifically deleted (Fig. 3A), were treated with Dex in the presence or absence of oltipraz or t-BHQ. ΔGRE-pGL-1651 showed inducibility of luciferase by oltipraz or t-BHQ, but the activity of luciferase was not repressed by concomitant Dex treatment (Fig. 3B).

SMRT binding to C/EBPβ or Nrf2. Activation of C/EBPβ contributes to GSTA2 induction by oltipraz (13). The studies from our laboratories and others showed that Dex activates C/EBPβ (8, 24). Given the repression of GSTA2 by Dex in spite of C/EBPβ activation, we raised the hypothesis that C/EBPβ activation by Dex recruits a protein that inhibits C/EBPβ-mediated *GSTA2* gene expression. SMRT represses GR-mediated gene transcription, whereas CBP/p300 and GRIP1 increase gene expression through enhanced interaction with the transcription factors bound to the consensus DNA elements (e.g., HNF4α) (4).

To understand the mechanisms governing the regulation of GR repression in the inducible GSTA2 expression, we determined whether SMRT associated with steroid-GR complex binds to the transcription factors responsible for transactivation of the *GSTA2* gene. To verify that GR-steroid complex binds to SMRT, SMRT was immunoprecipitated with anti-SMRT antibody in lysates obtained from the GR-transfected H4IIE cells exposed to Dex (100 nM), and the immunoprecipitates were immunoblotted with an anti-GR antibody. GR was immunochemically detected as a major band in the immunoprecipitates from GR-transfected cells treated with Dex, but not in those without Dex treatment (Fig. 4A).

To explore whether SMRT in the GR-transfected cells treated with Dex physically interacts with activating C/EBPβ or Nrf2, we immunoprecipitated the lysates of oltipraz- or t-BHQ-treated (6 h) cells with anti-SMRT antibody and the immunoprecipitates were analyzed by immunoblotting using anti-C/EBPβ antibody or anti-Nrf2 antibody. SMRT coprecipitated with C/EBPβ in lysates of the GR-transfected cells exposed to Dex plus oltipraz (Fig. 4B). C/EBPβ immunoprecipitable with SMRT was not detected in the GR-transfected cells exposed to Dex or Dex plus t-BHQ. Our results demonstrate that SMRT, which is recruited to GR-steroid complex, may physically interact with activating C/EBPβ. Immunoprecipitation and immunoblot analyses revealed that SMRT also bound to Nrf2 in GR-transfected cells exposed to Dex plus t-BHQ (Fig. 4B). Without GR activation by Dex, SMRT failed to interact with activating C/EBPβ or Nrf2. An additional immunoprecipitation and immunoblot study showed that NCoR, another corepressor recruited to steroid-GR (11), failed to bind to C/EBPβ or Nrf2 (Fig. 4C, left). A positive control study confirmed that NcoR bound to peroxisome proliferator-activated receptor α (PPARα) in untreated cells, while clofibrate treatment (20 μM, 6 h) caused NcoR to dissociate from PPARα (3) (Fig. 4C, right). Thus, NCoR was not responsible for GSTA2 repression by Dex. These results suggested that GSTA2 repression by Dex might be mediated through binding of SMRT, which was recruited to GR-steroid complex, to activating C/EBPβ or Nrf2.

Next, to verify recruitment of SMRT to the GST promoter region in vivo, we performed ChIP analysis in H4IIE cells. The DNA-protein complexes were immunoprecipitated with anti-SMRT antibody, followed by reversal of cross-linking and PCR amplification using primers flanking the proximal and distal regions of the DNA comprising the C/EBP binding site and the ARE in the GSTA2 promoter (−989/−643 bp) (Fig. 4D). In the cells treated with Dex, the intensity of the PCR product was significantly higher compared with untreated cells or the cells treated with oltipraz or t-BHQ, confirming that the SMRT recruited by ligand-activated GR increases its binding to the GSTA2 promoter through its interactions with C/EBPβ and Nrf2. Exposure of Dex-treated cells to either oltipraz or t-BHQ also showed increases in the band intensity compared to control. To show that SMRT specifically interacts with the region spanning the C/EBP binding site and ARE, ChIP assay was additionally performed with the primers which amplify the region between −197 bp and −1 bp of the GSTA2 promoter. No DNA amplification from the region verified the specificity of protein binding to the C/EBP binding site and ARE (Fig. 4D).

Reduction of histone acetylation by Dex. TSA inhibits the activity of histone deacetylase (21). We determined whether treatment of cells with TSA reversed the inhibitory effect of Dex. Inhibition of constitutive and oltipraz- or t-BHQ-inducible GSTA2 expression by 100 nM Dex was all reversed to certain extents by concomitant TSA treatment (200 nM, 48 h) (Fig. 5A), which suggested that histone deacetylase was recruited to the *GSTA2* gene by Dex treatment.

To further show that Dex treatment led to reduction in histone acetylation in the *GSTA2* promoter region, we performed ChIP/PCR assays using an antibody directed against acetylated histone H3 in combination with primers amplifying a *GSTA2* promoter region. Dex decreased the intensities of PCR-amplified DNA bands in the samples immunoprecipitated with anti-acetylated histone H3 in untreated cells or cells treated with oltipraz or t-BHQ (6 h), compared to respective controls (Fig. 5B). The extent of acetylated H3 binding to the β-*actin* gene was not affected by Dex treatment. These data in association with the results in Fig. 4D showed that Dex reduced histone acetylation in the *GSTA2* promoter region via recruitment of SMRT by GR activation.

Functional analysis of SMRT overexpression in GSTA2 repression. SMRT contains two independent RIDs that display different affinities for the members of the nuclear receptor family and two SDs responsible for transcriptional repression (22, 37). RIDs are contained within the region spanning amino acids 1055 to 1495 (37). To test the ability of C/EBPβ or Nrf2 to associate with SMRT directly, we determined the ability of in vitro-translated ^{35}S-C/EBPβ or ^{35}S-Nrf2 to interact with the RIDs and SDs of SMRT expressed in vitro. ^{35}S-C/EBPβ or

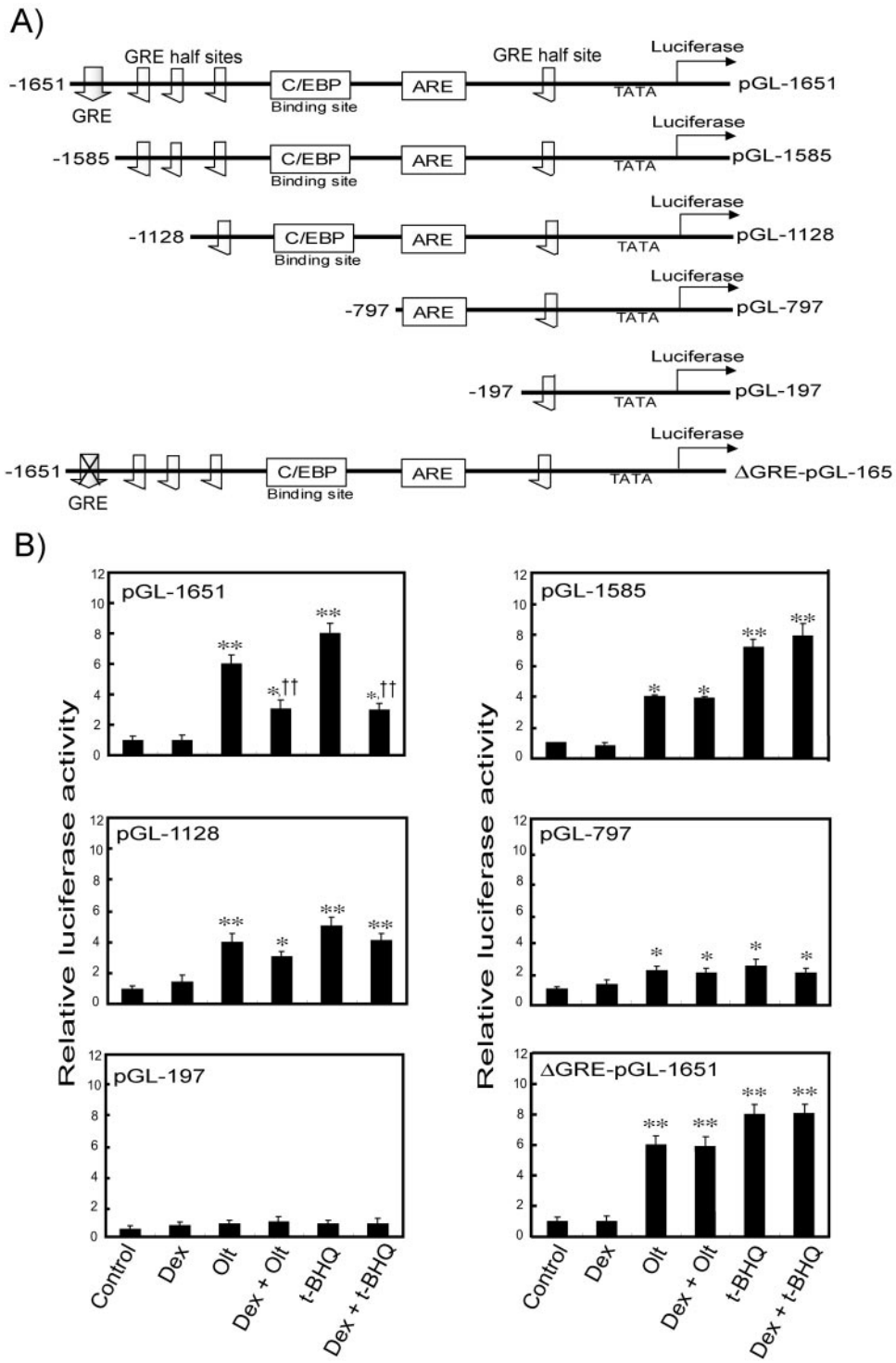


FIG. 3. The effects of Dex on the *GSTA2* promoter reporter gene expression. (A) The size of the flanking insert in each *GSTA2* chimeric gene construct. The open arrows indicate GRE and GRE half-sites. The distal GRE site was specifically deleted in ΔGRE-pGL-1651. (B) Suppression of oltipraz (Olt)- or t-BHQ-inducible luciferase activity by Dex in H4IIE cells transiently transfected with GSTA2 chimeric gene construct pGL-1651, which contains the GREs, C/EBP, and ARE of the *GSTA2* promoter. Dual luciferase reporter assays were performed on the lysates of cells that had been cotransfected with the *GSTA2*-luciferase gene construct pGL-1651 (firefly luciferase) and pRL-SV (*Renilla* luciferase) at a ratio of 200:1 and treated with Olt (10 μM) or t-BHQ (30 μM) in the absence or presence of Dex (100 nM) for 18 h. Activation of the reporter gene was calculated as a change in the ratio of firefly luciferase activity to *Renilla* luciferase activity. The basal and inducible luciferase activities were also measured in the cells transfected with 5′ deletion mutants or the specific GRE deletion mutant of the *GSTA2* gene. The experimental value for luciferase activity was expressed as the relative luciferase unit of cell lysates and represented the mean ± SE with four separate experiments (significant compared to control, * [$P < 0.05$] and ** [$P < 0.01$]; significant compared to cells treated with either Olt or t-BHQ alone, †† [$P < 0.01$]).

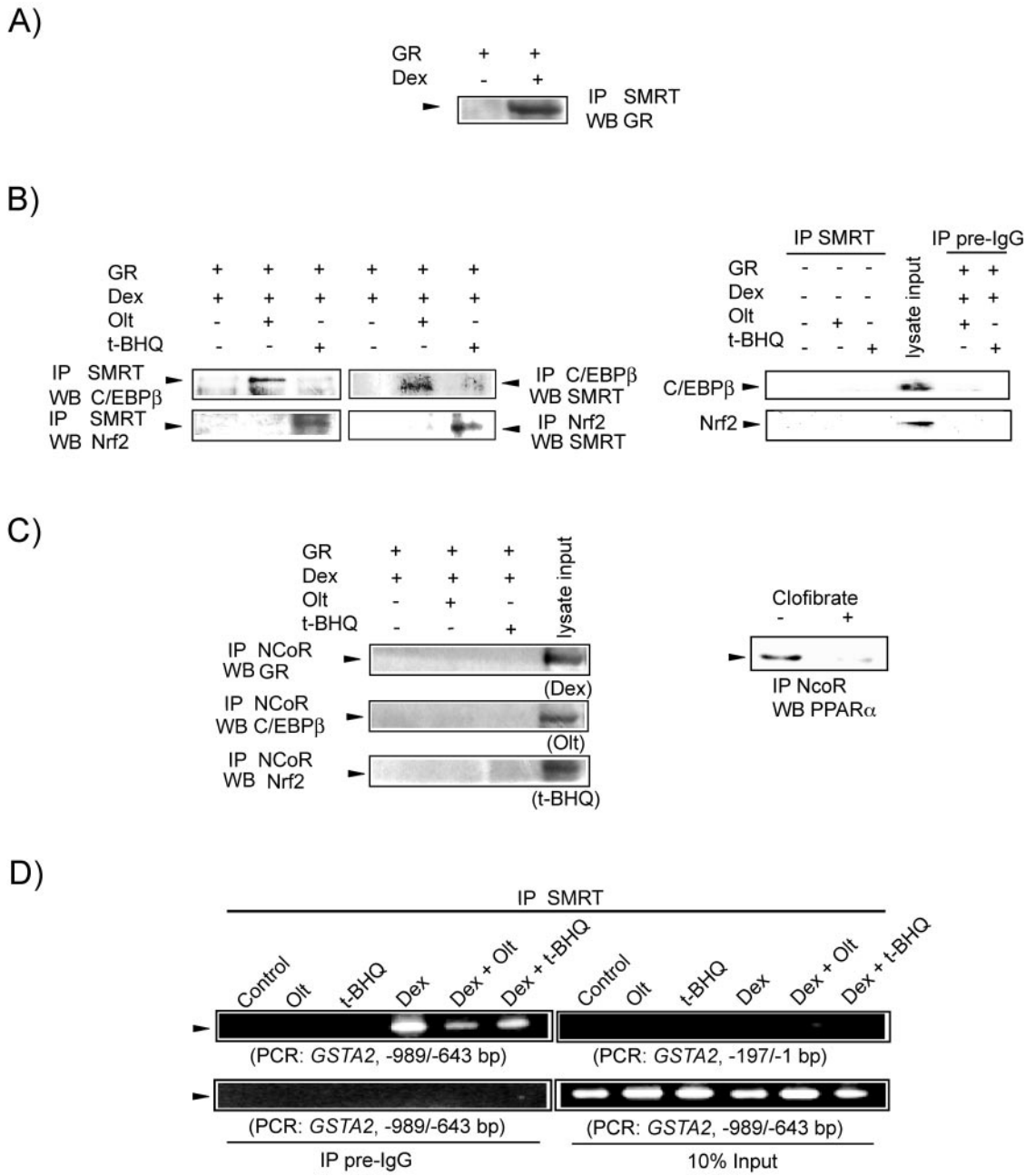


FIG. 4. Association of SMRT with activating C/EBPβ or Nrf2. (A) Recruitment of SMRT by steroid-GR complex. GR-transfected H4IIE cells (12 h) treated with 100 nM Dex (6 h) were immunoprecipitated with anti-SMRT antibody followed by immunoblotting with anti-GR antibody. (B) SMRT binding to activating C/EBPβ or Nrf2. H4IIE cells were transfected with GR (12 h) and incubated with 100 nM Dex in combination with oltipraz (Olt, 10 μM) or t-BHQ (30 μM) for 6 h. Whole-cell lysates were precipitated with anti-SMRT antibody, and immunocomplexes were immunoblotted with anti-C/EBPβ or anti-Nrf2 antibody. SMRT was also immunoblotted with immunoprecipitates of C/EBPβ or Nrf2. To assess the effects of Olt or t-BHQ without GR activation, immunoblot analyses were also performed with the immunoprecipitates with anti-SMRT antibody or preimmune IgG (pre-IgG). One-tenth of the total input was loaded as control. (C) Immunoprecipitation analysis of NCoR. Cell lysates were immunoprecipitated with anti-NCoR antibody, and the immune complexes and crude lysates (equivalent to 1/10 of the volume used for each immunoprecipitation reaction) were immunoblotted as described in panel B. Results were confirmed by repeated experiments. NcoR binding to PPARα in untreated H4IIE cells, but not in cells treated with clofibrate (20 μM, 6 h), was shown as positive control. (D) Chromatin immunoprecipitation assays. The DNA-protein complexes prepared from untreated cells or cells treated with Olt, t-BHQ, Dex, Dex plus Olt, or Dex plus t-BHQ were immunoprecipitated with anti-SMRT antibody or pre-IgG. The samples were PCR amplified using primers flanking the proximal and distal regions of the DNA comprising the C/EBP binding site and the ARE in the *GSTA2* promoter. The irrelevant region (−197/−1 bp) of the GSTA2 promoter was also PCR amplified as negative control. One-tenth of the total input was loaded as control. Results were confirmed by repeated experiments. IP, immunoprecipitation; WB, Western blot.

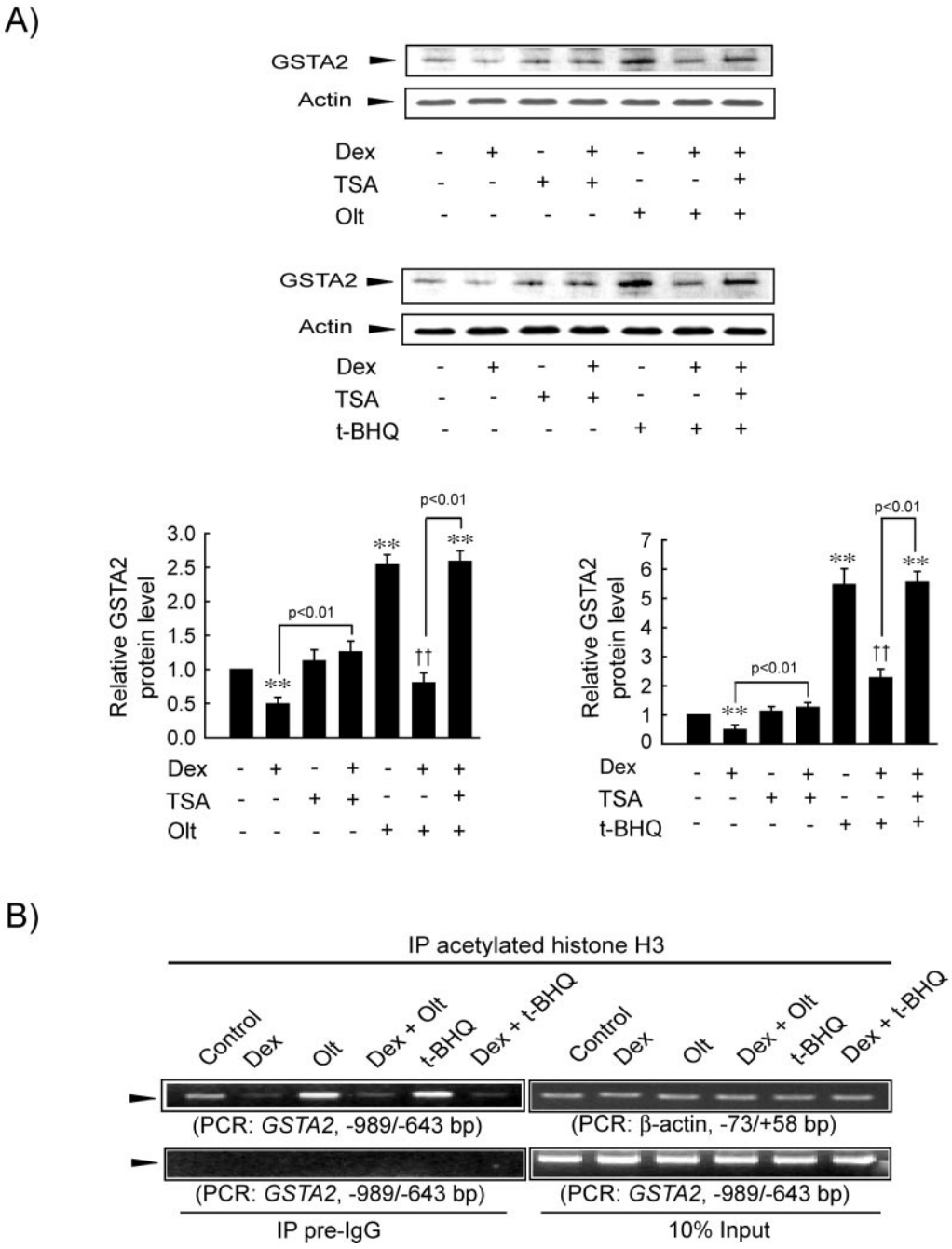


FIG. 5. The effects of Dex on histone acetylation. (A) TSA reversal of GSTA2 repression by Dex. H4IIE cells were treated with Dex in the presence or absence of 200 nM TSA for 48 h. The effects of TSA were also determined in cells treated with oltipraz (Olt) or t-BHQ with or without Dex for 48 h. Equal loading of proteins was verified by probing the replicate blots for actin. The relative protein levels were determined by scanning densitometry of the immunoblots. Data represent the mean ± standard deviation with three separate experiments (significant compared to control, ** [$P < 0.01$]; significant compared to Olt or t-BHQ alone, †† [$P < 0.01$]; control = 1). (B) Chromatin immunoprecipitation assay using anti-acetylated histone H3 antibody. The DNA-protein complexes prepared from control cells or the cells that had been treated with Dex with or without Olt or t-BHQ were immunoprecipitated with anti-acetylated histone H3 antibody or preimmune IgG (pre-IgG). The acetylated histone H3 immunoprecipitates were PCR amplified using the primers flanking the proximal and distal regions of the DNA comprising the C/EBP binding site and the ARE in the *GSTA2* promoter (−989/−643 bp) or flanking the region of the β-*actin* gene (−73/+58 bp). Pre-IgG samples were PCR amplified using the primers flanking the *GSTA2* promoter. One-tenth of the total input was loaded as control. Results were confirmed by repeated experiments. IP, immunoprecipitation.

^{35}S-Nrf2 protein was expressed in vitro from the pCDNA-C/EBPβ or pCDNA-Nrf2 plasmid using the in vitro translation kit (Fig. 6A, left). The ability of C/EBPβ or Nrf2 to bind to GST-RID or GST-SD was tested by GST pull-down assays. GST-RID or GST-SD protein was bacterially produced, purified, and incubated with in vitro-translated ^{35}S-C/EBPβ or ^{35}S-Nrf2. Either C/EBPβ or Nrf2 bound to the RIDs or SDs of SMRT to comparable extents (Fig. 6A, middle and right). The

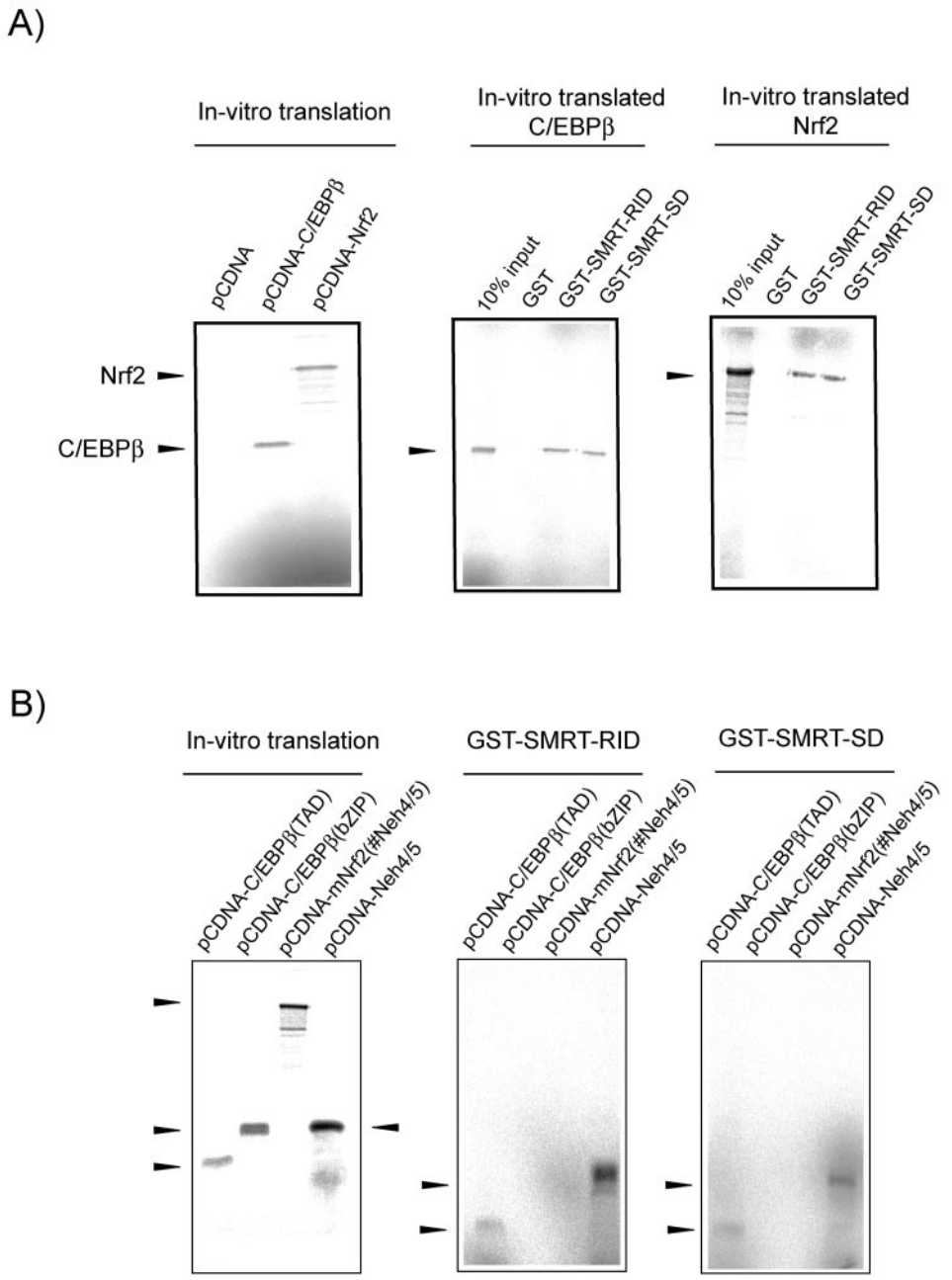


FIG. 6. Direct interaction of C/EBPβ or Nrf2 with the RID or SD of SMRT. (A) In vitro translation of ^{35}S-C/EBPβ or ^{35}S-Nrf2. ^{35}S-C/EBPβ or ^{35}S-Nrf2 was expressed by in vitro translation using the pCDNA, pCDNA-C/EBPβ, or pCDNA-Nrf2 plasmid. The representative SDS-PAGE gel shows autoradiography of ^{35}S-C/EBPβ or ^{35}S-Nrf2 (left). Middle and right panels show in vitro interaction of C/EBPβ or Nrf2, respectively, with the RID or SD of SMRT. GST pull-down assays were performed using in vitro-translated ^{35}S-C/EBPβ or ^{35}S-Nrf2 and bacterially purified GST-RID or GST-SD protein. A bacterially expressed GST protein was used as a negative control. Results were confirmed by repeated experiments. (B) Interactions of the N-terminal transactivation (TAD) domain of C/EBPβ or the Neh4/5 domain of Nrf2 with SMRT. The representative SDS-PAGE gel shows autoradiography of TAD and bZIP domains of C/EBPβ or the Neh4/5 domain of Nrf2 and the Nrf2 lacking Neh4/5 expressed by in vitro translation (left). Middle panel shows in vitro interaction of GST-SMRT-RID with ^{35}S-C/EBPβ-TAD or ^{35}S-C/EBPβ-bZIP or with ^{35}S-Nrf2 lacking Neh4/5 [mNrf2(#Neh4/5)] or ^{35}S-Nrf2-Neh4/5 (Neh4/5). Similarly, in vitro binding of GST-SMRT-SD to the ^{35}S-labeled truncated products of C/EBPβ or Nrf2 was shown (right). GST pull-down assays were performed as described in panel A.

GST pull-down assays strongly support that C/EBPβ and Nrf2 directly bind to the RID and the SD of SMRT.

To further determine the SMRT-interacting region(s) within C/EBPβ or Nrf2, we truncated C/EBPβ and Nrf2 into the fragments containing major domains and then determined potential interactions of the in vitro-translated fragments with GST-SMRT-SD or GST-SMRT-RID by GST pull-down assays (Fig. 6B, left). It has been shown that the N-terminal transac-

tivation (TAD) domain of C/EBPβ binds to CBP/p300 (10, 25). Therefore, we prepared the truncated mutant plasmids that encode the TAD and bZIP domains of C/EBPβ. Also, we made the constructs that encode the Neh4/5 domain of Nrf2 and the Nrf2 lacking Neh4/5 because the Neh4/5 domain had been shown to interact with CBP/p300 (18). GST pull-down assays revealed that either SMRT-RID or SMRT-SD bound to C/EBPβ-TAD but not C/EBPβ-bZIP domain (Fig. 6B, middle and right). Similarly, SMRT-RID or SMRT-SD interacted with the Neh4/5 domain of Nrf2 but not the Nrf2 lacking Neh4/5 (Fig. 6B, middle and right). Hence, the TAD domain of C/EBPβ or the Neh4/5 domain of Nrf2 directly binds to SMRT.

Repression of GSTA2 by SMRT. To obtain the evidence of the functional role of SMRT in responsiveness, we treated cells with oltipraz or t-BHQ following transfection with both pCMX-SMRT and pGL-1651. Transfection of untreated control cells with pCMX-SMRT significantly decreased the luciferase inducible activity from pGL-1651 (Fig. 7A). Mock transfection indicates transfection of pCMX empty vector in combination with pGL-1651. The luciferase activities of the pGL-1651 plasmid inducible by oltipraz (10 μM) or t-BHQ (30 μM) were also significantly decreased by pCMX-SMRT transfection. We verified that overexpression of SMRT also repressed the constitutive GSTA2 expression and GSTA2 induction by oltipraz or t-BHQ in H4IIE cells (Fig. 7B). Our results demonstrate that SMRT, which interacts with both C/EBPβ and Nrf2, inhibits the constitutive and inducible expression of the *GSTA2* gene.

We also assessed the effects of transfection with the plasmid encoding SMRT-RID or SMRT-SD on luciferase reporter activity and GSTA2 expression to verify that the truncated SMRT proteins were functionally inactive in blocking the *GSTA2* gene expression. In contrast to the significant inhibition of luciferase expression from pGL-1651 by full-length SMRT overexpression (Fig. 7A), neither SMRT-RID nor SMRT-SD inhibited the induction of luciferase by oltipraz or t-BHQ (Fig. 7C). Lack of GSTA2 repression by the truncated SMRT proteins was also confirmed by immunoblot analysis of GSTA2 in cells treated with oltipraz or t-BHQ (Fig. 7D). These results confirmed that the full-length SMRT, but not the truncated SMRTs, was functionally active in repressing the *GSTA2* gene expression.

The role of SMRT in transcriptional repression of pGL-1651 was additionally determined using siRNA. A recent study has shown that 2,584/2,604-bp nucleotide double-stranded RNA (siRNA) acted as a guide sequence to target the mRNA of human SMRT and that the siRNA had the expected knockdown effect and specificity (40). Luciferase expression assays were conducted in NIH 3T3 cells that were transfected with the plasmid encoding SMRT and the siRNA against SMRT. The effect of the siRNA was verified by RT-PCR analysis, which clearly had a knockdown effect on SMRT expression (Fig. 7E, inset). We next tested the effect of the siRNA on SMRT repression of the pGL-1651 gene (Fig. 7E). While transfection of control RNA (scRNA) with SMRT completely repressed C/EBPβ- or Nrf2-inducible gene expression, treatment of cells with the SMRT siRNA effectively relieved repression. Overexpression of C/EBPβ or Nrf2 in the cells was analyzed by Western blotting (Fig. 7E, lower). The knockdown experiment corroborates the essential role of SMRT in repressing C/EBPβ- or Nrf2-mediated gene expression.

Repression of other phase II enzymes by Dex. To extend the physiological impact of the ligand-bound GR/GRE-mediated mechanism, we confirmed whether Dex influences the expression of the phase II enzymes whose genes contain GREs in their 5′-regulatory regions. As expected, treatment of H4IIE cells with 100 nM Dex (48 h) substantially down-regulated the expression of microsomal epoxide hydrolase, NADPH quinone oxidoreductase, and heme oxygenase 1 (Fig. 8).

DISCUSSION

The expression of phase II detoxification enzymes is transcriptionally activated partly through activation of C/EBPβ and Nrf2 via their binding to the respective response elements in the promoter regions of the target genes (13, 36). The phase II detoxification genes that contained C/EBP and Nrf2 as core sequences include class α GSTs, human γ-glutamylcysteine synthetase, NAD(P)H:quinone reductase, and human heme oxygenase 1 (13, 36). Among the GST genes, the *GSTA2* gene is a representative member of class alpha GSTs that contains GREs as well as C/EBP response element and ARE. The regulation of the *GSTA2* gene expression is complex and occurs at multiple levels, orchestrated by multiple transcription factors. Other GST genes, which are involved in regulation of the genes in similar pathways, also contain multiple DNA response elements. Therefore, the studies of the regulation of the *GSTA2* gene bring insights into the transcriptional control mechanism of the phase II enzymes involved in carcinogen detoxification. In particular, the information on the GR-mediated regulatory mechanism for the *GSTA2* gene would allow us to extend the physiological impact of GR activation to other phase II enzymes, whose genes contain GREs in their promoter regions.

Studies have shown that Dex at nanomolar concentrations inhibits the constitutive expression of GSTA2 in hepatocytes, whereas micromolar Dex induces GSTA2 (5, 6, 38). The GSTA2-repressing effect of Dex at the nanomolar range is mediated with GR activation and activating GR binding to the GRE in the gene promoter. The PXR that complexes with retinoid X receptor binds to and transactivates several DNA response elements of the target genes. The GSTA2-repressing and -inducing responses of Dex are considered to be mediated with GR and PXR, respectively. GSTA2 induction by high concentrations of Dex is not relevant under normal conditions because the tissue concentrations of Dex in most clinical applications are in the nanomolar range. In our previous study, the radioprotective effect of oltipraz, which correlated with GST induction, was abolished by treatment of animals with low doses of Dex (26). GSTA2 repression by Dex at the physiologically relevant concentrations in the present study is in line with our previous in vivo report (26).

In this report, we showed that repression of GSTA2 (constitutive or inducible) is mediated by steroid-GR activation of the GRE. It has been proposed that the GRE site present at the distal region of pGL-1651 and the half-site GREs of the *GSTA2* gene may contribute to repression of the constitutive GSTA2 expression (6). The promoter deletion analysis of the *GSTA2* gene in the present study revealed that the distal GRE

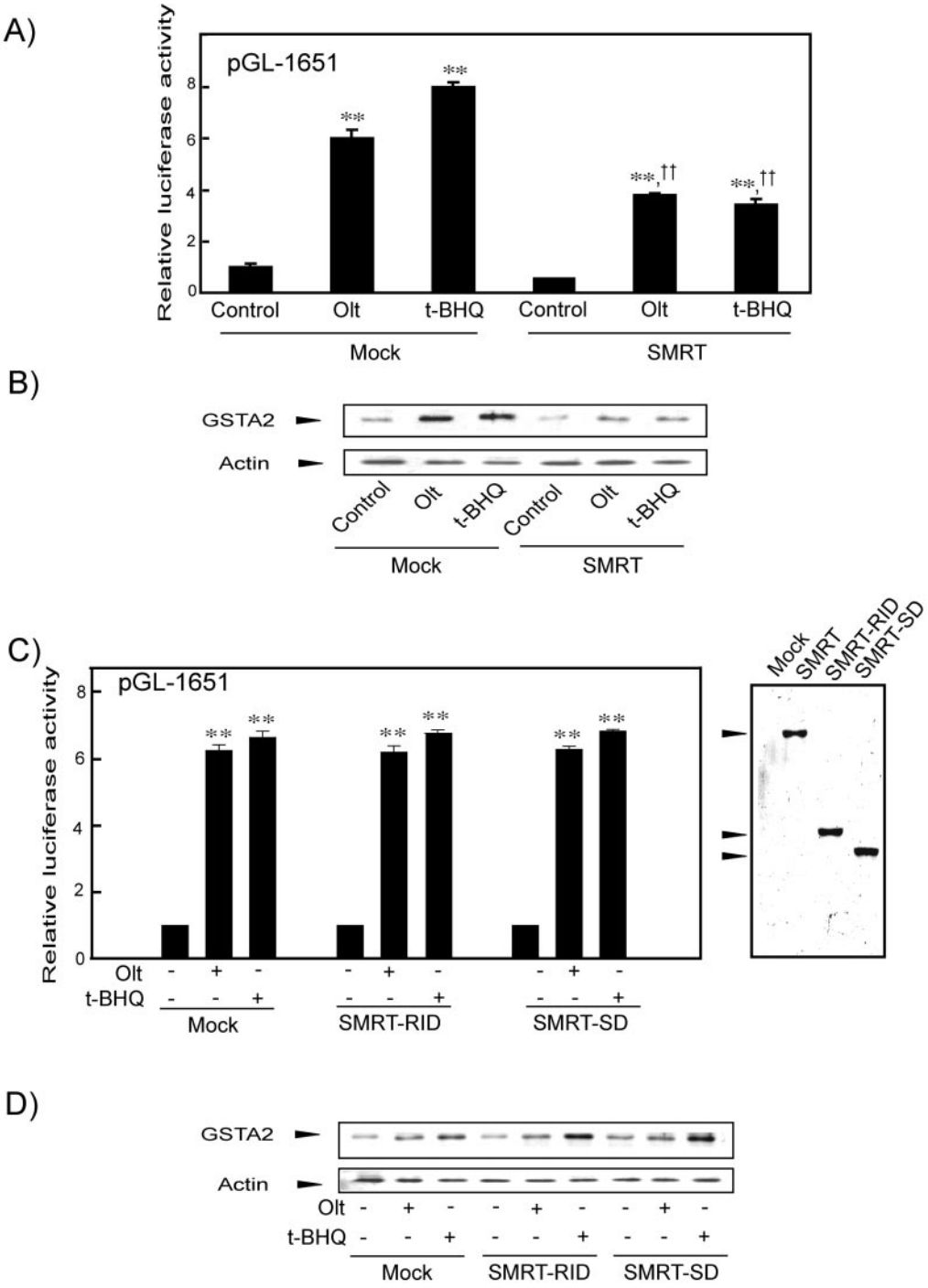
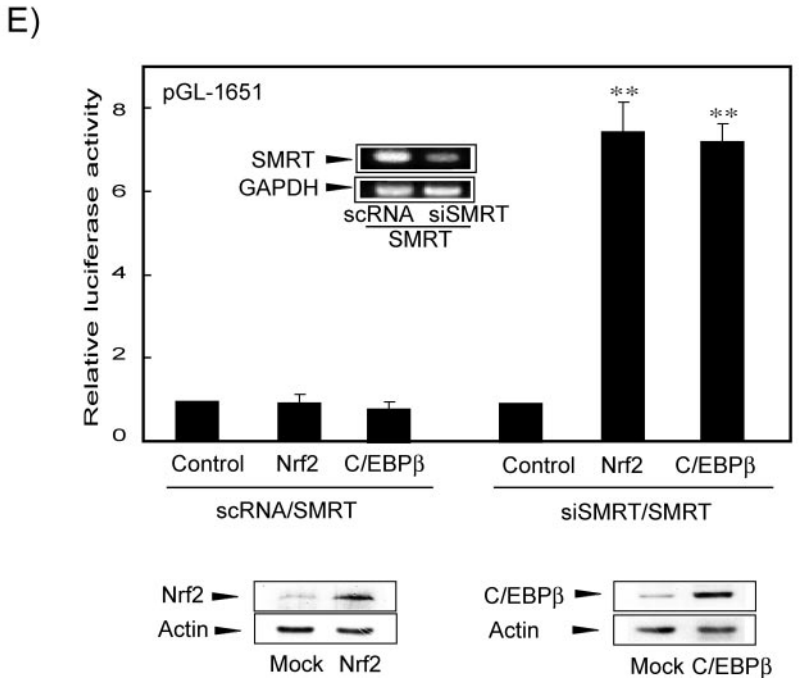


FIG. 7. Functional analyses of SMRT. (A) Repression of the pGL-1651 *GSTA2* gene by SMRT. H4IIE cells were transfected with the pGL-1651 luciferase reporter plasmid (1 μg), pCMX-SMRT vector (1 μg), and *Renilla* luciferase (5 ng). At 16 h after transfection, cells were treated with vehicle, oltipraz (Olt, 10 μM), or t-BHQ (30 μM) for 18 h, and then the cell extracts were assayed for firefly and *Renilla* luciferase activities. The fold inductions of the normalized luciferase activity were compared with that in control cells transfected with the pGL-1651 and pCMX empty plasmids. Data represented the mean ± SE with four separate experiments (significant compared to mock-transfected control, ** [$P < 0.01$]; significant compared to the respective mock-transfected cells, †† [$P < 0.01$]). (B) The effects of SMRT overexpression on GSTA2 expression. GSTA2 was immunoblotted in the lysates of untreated H4IIE cells or cells treated with Olt or t-BHQ (24 h) following transfection with pCMX empty plasmid (Mock) or the plasmid encoding SMRT (12 h). Results were confirmed by repeated experiments. (C) The effects of transfection of H4IIE cells with the plasmid encoding SMRT-RID or SMRT-SD on luciferase reporter activity from pGL-1651. H4IIE cells were transfected as described in panel A with the pGL-1651 luciferase reporter plasmid (1 μg), pCDNA-SMRT-RID (or pCDNA-SMRT-SD) vector (1 μg), and *Renilla* luciferase (5 ng). The fold inductions of the normalized luciferase activity were compared with that in control cells transfected with the pGL-1651 and pCDNA empty plasmids (Mock). Data represented the mean ± SE with four separate experiments (significant compared to mock-transfected control, ** [$P < 0.01$]). Immunoblot analysis for c-*myc* in the right panel shows the expression levels of SMRT, SMRT-RID, or SMRT-SD from the *myc*-tagged expression plasmids. (D) The effects of SMRT-RID or SMRT-SD overexpression on GSTA2 expression in



H4IIE cells. GSTA2 was immunoblotted in the lysates of untreated cells or cells treated with oltipraz or t-BHQ (24 h) following transfection with the pCDNA empty plasmid (Mock) or the plasmid encoding SMRT-RID or SMRT-SD (12 h). Results were confirmed by repeated experiments. (E) Functional analysis of SMRT using siRNA. Luciferase expression was determined in NIH 3T3 cells transfected with siRNA or scRNA, and 2 days after transfection, the level of target gene in the cells was analyzed by RT-PCR analysis (inset). Similarly, cells were transfected with the siRNA (or scRNA), pGL-1651, and pCMX-SMRT in combination with the plasmid encoding C/EBPβ or Nrf2 and incubated for 2 days. The lysates from the cells were used in the luciferase assay and Western blot analyses. Data represented the mean ± SE with three separate experiments (significant compared to the respective scRNA transfection, ** [$P < 0.01$]).

site of pGL-1651 (−1651/−1585 bp) was responsible for the suppression of C/EBPβ- or Nrf2-mediated *GSTA2* gene induction by Dex. To verify the role of GR in the cells treated with Dex, we determined whether RU-486, a GR antagonist, reversed GSTA2 repression by Dex. RU-486 treatment abolished repression of constitutive or inducible GSTA2 expression by Dex (data not shown), which provided evidence that the observed gene repression by Dex was due to GR activation. The results of the reporter gene analyses and RU-486 experiment strongly support the conclusion that the ability of Dex to suppress GSTA2 induction results from activation of the distal GRE site in the promoter (i.e., via ligand-GR binding to the GRE). We observed that deletion of the promoter region containing the C/EBP response element or the ARE had a significant inhibitory effect on both oltipraz- and t-BHQ-inducible gene expression (13, 28). The constitutive expression of GSTA2 is regulated by the basal activation of the transcription factors including C/EBPβ, Nrf2, and HNF1 (13, 15, 29). Ineffectiveness of the mutant promoters, in which the region comprising either the C/EBP response element or the ARE was deleted, in mediating gene expression strongly suggests that C/EBPβ and Nrf2 are both required as transcription factors for the *GSTA2* gene transactivation. Hence, oltipraz induction of GSTA2 requires the basal Nrf2-DNA binding, as t-BHQ induction needs the basal C/EBPβ-DNA activation.

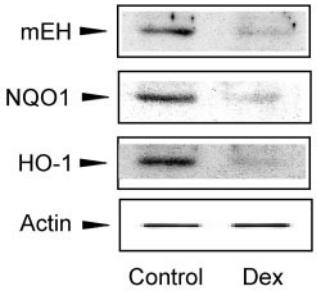


FIG. 8. The effects of Dex on the expression of representative phase II enzymes. Representative immunoblots show the levels of microsomal epoxide hydrolase (mEH), NADPH quinone oxidoreductase (NQO1), and heme oxygenase 1 (HO-1) in H4IIE cells incubated with 100 nM Dex for 48 h. Western blot analyses were performed as described in the legend to Fig. 1. Each lane was loaded with 20 μg of microsomal (mEH) or lysate proteins. Immunoreactive proteins were visualized through incubation with horseradish peroxidase-conjugated secondary antibody and an ECL chemiluminescence detection kit. Equal loading was verified by probing the replicate blots for actin (one of the actin blots is shown).

C/EBPβ is enriched in the liver and plays a role in specific transcription of several genes (39). It has been shown that glucocorticoids induce C/EBPβ by transcriptional activation of the *C/EBP*β gene that leads to the secondary glucocorticoid response of the gene expression (e.g., arginase) (24). Glucocorticoid induces the liver-specific arginase gene through binding of the proteins including C/EBPβ to the enhancer elements (8). In the present study, we found that Dex activated C/EBPβ, as evidenced by nuclear translocation of C/EBPβ and its bind-

ing to the C/EBP consensus oligonucleotide. Dex suppresses the expression of GSTA2 in spite of activating C/EBPβ binding to DNA, which suggests that the protein(s) recruited by steroid-GR complex on the GRE site in the *GSTA2* promoter antagonize(s) C/EBPβ-mediated *GSTA2* transactivation (e.g., coactivator repression).

The results in the present study demonstrate that GR activation by Dex does not inhibit nuclear translocation of C/EBPβ or Nrf2 and binding of the proteins to the respective response elements, as supported by C/EBPβ or Nrf2 translocation and their DNA binding in the cells treated with chemical inducer plus Dex. Rather, the protein(s) recruited to the ligand-GR-bound GRE complex may prevent activating C/EBPβ and Nrf2 from the gene transactivation. Dex persistently inhibited the constitutive and inducible expression of GSTA2. Activation of C/EBPβ and Nrf2 by chemical inducers occurred at 6 to 12 h, whereas repression of GSTA2 by Dex occurred maximally at 48 h or later time points in H4IIE cells. Prolonged GSTA2 repression by Dex may result from its lipophilicity or the inhibitory effect of GR-recruited protein GRE complex, which is capable of stalling the essential transcription factors (i.e., C/EBPβ and Nrf2) required for the basal and inducible gene transactivation. The endogenous *GSTA2* gene was significantly repressed by ligand-GR activation without GR transfection. We could also observe suppression of the GSTA2 pGL-1651 gene expression in cells treated with Dex for 18 h (an optimal time period for reporter gene transfection assay). The extent of pGL-1651 luciferase repression by Dex was less than that of endogenous GSTA2. Also, we had to transfect the GR plasmid to observe repression in inducible promoter-luciferase activity. It is likely that luciferase expression requires the higher concentration of GR for the full responsiveness to Dex. The observed absence of significant inhibition by Dex in the constitutive luciferase activity from pGL-1651 may have resulted from the low limit of sensitivity.

The hormone-dependent translocation of the homodimeric GR was demonstrated. The GR in the absence of ligand cannot interact with the target gene, and hence, no transcriptional activation occurs. Once the receptor bound with hormone interacts with a response element, it then activates transcription by directing histone hyperacetylation and assembly of an initiation complex. Conversely, the GR interacts with the corepressors such as SMRT and NCoR that have receptor-interacting domains (RID1 and RID2) (22, 37). Gene repression mediated by interactions of ligand-bound GR with the corepressors results from recruitment of histone deacetylases that remove acetyl groups from histones and induce the recondensation of chromatin (7, 9). This was supported by the present findings that TSA, a histone deacetylase inhibitor, prevented repression of GSTA2 by Dex and that Dex treatment reduced histone acetylation in the GSTA2 promoter region.

In the present study, we revealed that steroid-GR complex recruited SMRT, but not NcoR, in H4IIE cells. NCoR possessed no measurable interaction with ligand-bound GR, C/EBPβ, or Nrf2. The present study demonstrates for the first time that Dex inhibition of GSTA2 induction by oltipraz results from the ability of SMRT to repress C/EBPβ-mediated transactivation and that SMRT recruited to hormone-bound GR binds to activating C/EBPβ. Dex activates both GR and C/EBPβ. Concomitant activation of GR and C/EBPβ by Dex would lead to C/EBPβ inactivation as a consequence of GR-mediated SMRT recruitment, which explains the lack of C/EBPβ-mediated *GSTA2* gene induction by Dex alone in spite of its increase in the C/EBPβ DNA binding activity. Immunoprecipitation and immunoblot analyses revealed that SMRT also binds to Nrf2, inhibiting Nrf2-mediated gene expression. In the ChIP analyses, we were able to obtain the amplified DNA fragments derived from the C/EBP binding element and the ARE that specifically interacted with activating C/EBPβ and Nrf2. The results support the conclusion that SMRT interacts with C/EBPβ and Nrf2. Association of C/EBPβ or Nrf2 with ligand-bound GR-SMRT complexes was further verified by GST pull-down assays showing direct interactions of in vitro-translated C/EBPβ or Nrf2 with the RID and/or SD of SMRT. We demonstrated in additional experiments that both C/EBPβ and Nrf2 indeed directly bind to the RID and SD of SMRT and that the C/EBPβ TAD and the Nrf2 Neh4/5 interact with SMRT-RID or SMRT-SD. These observations strongly support the conclusion that binding of SMRT, recruited to hormone-bound GR complex on the GRE, to activating C/EBPβ and Nrf2 negatively regulates *GSTA2* gene transcription because the two transcription factors are required for the formation of transactivation complex and thus mutually stimulate inducible gene expression. Because C/EBPβ and Nrf2 interact with CBP coactivator (18, 25), the inactivation of C/EBPβ or Nrf2 upon the interaction with SMRT antagonizes CBP-mediated gene transcription activated by the transcription factors. Our present findings that overexpression of SMRT by transfection with pCMX-SMRT, but not that of the truncated SMRT proteins (presumably due to their inability in recruiting histone deacetylases), downregulated the constitutive and inducible expression of the pGL-1651 *GSTA2* gene and that the SMRT siRNA completely abolished SMRT repression of C/EBPβ or Nrf2 induction of the gene strengthen this view. As additional evidence, the endogenous *GSTA2* gene in H4IIE cells was also suppressed by overexpression of SMRT, but not that of truncated SMRTs.

In conclusion, we provide evidence that (i) the physiologically relevant concentrations of Dex suppress C/EBPβ- and Nrf2-mediated GST induction by oltipraz and t-BHQ; (ii) steroid-GR complex exerts repressive effect through the distal GRE site in the GSTA2 promoter; (iii) repression of the gene expression by Dex is mediated by interactions of SMRT, recruited to steroid-GR complex on the GRE, with activating C/EBPβ and Nrf2; and (iv) the TAD domain of C/EBPβ or the Neh4/5 domain of Nrf2 directly binds to SMRT-RID or SMRT-SD.

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REFERENCES

1. **Alonzi, T., D. Maritano, B. Gorgoni, G. Rizzuto, C. Libert, and V. Poli.** 2001. Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation in the liver. Mol. Cell. Biol. **21:**1621–1632.

2. **Bolton, M. G., A. Munoz, L. P. Jacobson, J. D. Groopman, Y. Y. Maxuitenko, B. D. Roebuck, and T. W. Kensler.** 1993. Transient intervention with oltipraz protects against aflatoxin-induced hepatic tumorigenesis. Cancer Res. **53:** 3499–3504.
3. **Dowell, P., J. E. Ishmael, D. Avram, V. J. Peterson, D. J. Nevrivy, and M. Leid.** 1999. Identification of nuclear receptor corepressor as a peroxisome proliferator-activated receptor alpha interacting protein. J. Biol. Chem. **274:** 15901–15907.
4. **Eggert, M., C. C. Mows, D. Tripier, R. Arnold, J. Michel, J. Nickel, S. Schmidt, M. Beato, and R. Renkawitz.** 1995. A fraction enriched in a novel glucocorticoid receptor-interacting protein stimulates receptor-dependent transcription *in vitro*. J. Biol. Chem. **270:**30755–30759.
5. **Falkner, K. C., J. A. Pinaire, G. H. Xiao, T. E. Geoghegan, and R. A. Prough.** 2001. Regulation of the rat glutathione S-transferase A2 gene by glucocorticoids: involvement of both the glucocorticoid and pregnane X receptors. Mol. Pharmacol. **60:**611–619.
6. **Falkner, K. C., T. H. Rushmore, M. W. Linder, and R. A. Prough.** 1998. Negative regulation of the rat glutathione S-transferase A2 gene by glucocorticoids involves a canonical glucocorticoid consensus sequence. Mol. Pharmacol. **53:**1016–1026.
7. **Fischle, W., F. Dequiedt, M. J. Hendzel, M. G. Guenther, M. A. Lazar, W. Voelter, and E. Verdin.** 2002. Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol. Cell **9:**45–57.
8. **Gotoh, T., S. Chowdhury, M. Takiguchi, and M. Mori.** 1997. The glucocorticoid-responsive gene cascade. Activation of the rat arginase gene through induction of C/EBPβ. J. Biol. Chem. **272:**3694–3698.
9. **Guenther, M. G., O. Barak, and M. A. Lazar.** 2001. The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. Mol. Cell. Biol. **21:**6091–6101.
10. **Guo, S., S. B. Cichy, He, X., Q. Yang, M. Ragland, A. K. Ghosh, P. F. Johnson, and T. G. Unterman.** 2001. Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPβ). Signaling to p300/CREB-binding protein by protein kinase B disrupts interaction with the major activation domain of C/EBPβ. J. Biol. Chem. **276:**8516–8523.
11. **Horlein, A. J., A. M. Naar, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Soderstrom, C. K. Glass, and M. G. Rosenfeld.** 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature **37:**397–404.
12. **Jover, R., R. Bort, M. J. Gomez-Lechon, and J. V. Castell.** 2002. Down-regulation of human CYP3A4 by the inflammatory signal interleukin 6: molecular mechanism and transcription factor involved. FASEB J. **16:**1799–1801.
13. **Kang, K. W., I. J. Cho, C. H. Lee, and S. G. Kim.** 2003. Essential role of phosphatidylinositol 3-kinase-dependent CCAAT/enhancer binding protein β activation in the induction of glutathione S-transferase by oltipraz. J. Natl. Cancer Inst. **95:**53–66.
14. **Kang, K. W., S. J. Lee, J. W. Park, and S. G. Kim.** 2002. Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. Mol. Pharmacol. **62:** 1001–1010.
15. **Kang, K. W., M. K. Cho, C. H. Lee, and S. G. Kim.** 2001. Activation of phosphatidylinositol 3-kinase and Akt by *tert*-butylhydroquinone is responsible for antioxidant response element-mediated GSTA2 induction in H4IIE cells. Mol. Pharmacol. **59:**1147–1156.
16. **Kang, K. W., J. H. Ryu, and S. G. Kim.** 2001. Activation of phosphatidylinositol 3-kinase by oxidative stress leads to the induction of microsomal epoxide hydrolase in H4IIE cells. Toxicol. Lett. **121:**191–197.
17. **Kang, K. W., J. H. Ryu, and S. G. Kim.** 2000. The essential role of phosphatidylinositol 3-kinase and of p38 mitogen-activated protein kinase activation in the antioxidant response element-mediated GSTA2 induction by decreased glutathione in H4IIE hepatoma cells. Mol. Pharmacol. **58:**1017–1025.
18. **Katoh, Y., K. Itoh, E. Yoshida, M. Miyagishi, A. Fukamizu, and M. Yamamoto.** 2001. Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. Genes Cells **6:**857–868.
19. **Kensler, T. W.** 1997. Chemoprevention by inducers of carcinogen detoxication enzymes. Environ. Health Perspect. **105**(S4)**:**965–970.
20. **Kurihara, I., H. Shibata, T. Suzuki, T. Ando, S. Kobayashi, M. Hayashi, I. Saito, and T. Saruta.** 2002. Expression and regulation of nuclear receptor coactivators in glucocorticoid action. Mol. Cell. Endocrinol. **189:**181–189.
21. **Laherty, C. D., W. M. Yang, J. M. Sun, J. R. Davie, E. Seto, and R. N. Eisenman.** 1997. Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell **89:**349–356.
22. **Li, H., C. Leo, D. J. Schroen, and J. D. Chen.** 1997. Characterization of receptor interaction and transcriptional repression by the corepressor SMRT. Mol. Endocrinol. **11:**2025–2037.
23. **Ma, H., H. Hong, S. M. Huang, R. A. Irvine, P. Webb, P. J. Kushner, G. A. Coetzee, and M. R. Stallcup.** 1999. Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. Mol. Cell. Biol. **19:**6164–6173.
24. **Matsuno, F., S. Chowdhury, T. Gotoh, K. Iwase, H. Matsuzaki, K. Takatsuki, M. Mori, and M. Takiguchi.** 1996. Induction of the C/EBPβ gene by dexamethasone and glucagon in primary-cultured rat hepatocytes. J. Biochem. (Tokyo) **119:**524–532.
25. **Mink, S., B. Haenig, and K. H. Klempnauer.** 1997. Interaction and functional collaboration of p300 and C/EBPβ. Mol. Cell. Biol. **17:**6609–6617.
26. **Nam, S. Y., C. K. Cho, and S. G. Kim.** 1998. Correlation of increased mortality with the suppression of radiation-inducible microsomal epoxide hydrolase and glutathione S-transferase gene expression by dexamethasone: effects on vitamin C and E-induced radioprotection. Biochem. Pharmacol. **56:**1295–1304.
27. **Nancy, V., R. M. Wolthuis, M. F. De Tand, I. Janoueix-Lerosey, J. L. Bos, and J. De Gunzburg.** 1999. Identification and characterization of potential effector molecules of the Ras-related GTPase Rap2. J. Biol. Chem. **274:** 8737–8745.
28. **Park, E. Y., I. J. Cho, and S. G. Kim.** 2004. Transactivation of the PPAR-responsive enhancer module in chemopreventive glutathione S-transferase gene by the peroxisome proliferator-activated receptor-gamma and retinoid X receptor heterodimer. Cancer Res. **64:**3701–3713.
29. **Park, I. N., I. J. Cho, and S. G. Kim.** 2004. Ceramide negatively regulates glutathione S-transferase gene transactivation via repression of hepatic nuclear factor-1 that is degraded by the ubiquitin proteasome system. Mol. Pharmacol. **65:**1475–1484.
30. **Pearce, D., and K. R. Yamamoto.** 1993. Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. Science **259:**1161–1165.
31. **Ramos-Gomez, M., P. M. Dolan, K. Itoh, M. Yamamoto, and T. W. Kensler.** 2003. Interactive effects of Nrf2 genotype and oltipraz on benzo[a]pyrene-DNA adducts and tumor yield in mice. Carcinogenesis **24:**461–467.
32. **Rushing, S. R., and M. S. Denison.** 2002. The silencing mediator of retinoic acid and thyroid hormone receptors can interact with the aryl hydrocarbon (Ah) receptor but fails to repress Ah receptor-dependent gene expression. Arch. Biochem. Biophys. **403:**189–201.
33. **Schreiber, E., K. Harshman, I. Kemler, U. Malipiero, W. Schaffner, and A. Fontana.** 1990. Astrocytes and glioblastoma cells express novel octamer-DNA binding proteins distinct from the ubiquitous Oct-1 and B cell type Oct-2 proteins. Nucleic Acids Res. **18:**5495–5503.
34. **Seo, J. B., M. J. Noh, E. J. Yoo, S. Y. Park, J. Park, I. K. Lee, S. D. Park, and J. B. Kim.** 2003. Functional characterization of the human resistin promoter with adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element binding protein 1c and CCAAT enhancer binding protein-α. Mol. Endocrinol. **17:**1522–1533.
35. **Szapary, D., Y. Huang, and S. S. Simons, Jr.** 1999. Opposing effects of corepressor and coactivators in determining the dose-response curve of agonists, and residual agonist activity of antagonists, for glucocorticoid receptor-regulated gene expression. Mol. Endocrinol. **13:**2108–2121.
36. **Thimmulappa, R. K., K. H. Mai, S. Srisuma, T. W. Kensler, M. Yamamoto, and S. Biswal.** 2002. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res. **62:**5196–5203.
37. **Wong, C. W., and M. L. Privalsky.** 1998. Transcriptional repression by the SMRT-mSin3 corepressor: multiple interactions, multiple mechanisms, and a potential role for TFIIB. Mol. Cell. Biol. **18:**5500–5510.
38. **Xiao, G. H., J. A. Pinaire, A. D. Rodrigues, and R. A. Prough.** 1995. Regulation of the Ah gene battery via Ah receptor-dependent and independent processes in cultured adult rat hepatocytes. Drug Metab. Dispos. **23:**642–650.
39. **Yeh, W. C., Z. Cao, M. Classon, and S. L. McKnight.** 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. **9:**168–181.
40. **Yoon, H. G., D. W. Chan, Z. Q. Huang, Li, J., J. D. Fondell, J. Qin, and J. Wong.** 2003. Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1. EMBO J. **22:**1336–1346.