Sp1 activates the transcription of many cellular and viral genes with the GC-box in either the proximal promoter or the enhancer. Sp1 is composed of several functional domains, such as the inhibitory domain (ID), two serine/threonine-rich domains, two glutamine-rich domains, three C2H2-type zinc finger DNA binding domains (ZFDBD), and a C-terminal D domain. The ZDDBD is the most highly conserved domain among the Sp-family transcription factors and plays a critical role in GC-box recognition. In this study, we investigated the protein-protein interactions occurring at the Sp1ZFDBD and the Sp1ID, and the molecular mechanisms controlling the interaction. Our results found that Sp1ZFDBD and Sp1ID repressed transcription once they were targeted to the proximal promoter of the pGal4 UAS reporter fusion gene system, suggesting molecular interaction with the repressor molecules. Indeed, mammalian two-hybrid assays, GST fusion protein pull-down assays, and co-immunoprecipitation assays showed that Sp1ZFDBD and Sp1ID are able to interact with corepressor proteins such as SMRT, NcoR, and BCoR. The molecular interactions appear to be regulated by MAP kinase/Erk kinase (MEK). The molecular interactions between Sp1ID and the corepressor might explain the role of Sp1 as a repressor under certain circumstances. The siRNA-induced degradation of the corepressors resulted in an up-regulation of Sp1-dependent transcription. The cellular context of the corepressors and the regulation of molecular interaction between corepressors and Sp1ZFDBD or Sp1ID might be important in controlling Sp1 activity.

Transcriptional regulation of the eukaryotic gene is a complicated process, involving a series of complex molecular inter-

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1 The abbreviations used are: Sp1, specificity protein 1; BCoR, BCL-6 interacting corepressor; CV-1, African green monkey kidney cell; DBD, DNA binding domain; GST, glutathione S-transferase; ID, inhibitory domain of Sp1; MAP kinase, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; MEK, MAP kinase/Erk kinase kinase; NCoR, nuclear receptor corepressor; TBP, TATA-box-binding protein; TAF, TBP-associated factors; UAS, upstream activator Gal4 binding sequence; SMRT, silencing mediator for retinoid and thyroid receptors; SUMO, small ubiquitin-related modifier; ZFDBD, zinc finger DNA binding domain; DMEM, Dulbecco's modified Eagle's medium; EMSSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation assay; EGF, epidermal growth factor; MEKDN, dominant negative form of MEK; MEKCA, constitutive activator form of MEK; siRNA, small interfering RNA; Luc, luciferase; CMV, cytomegalovirus.
which has the essential characteristics of an acid-blob, has been implicated in transcriptional activation. The ZFDBD of Sp1 is the most highly conserved region among the Sp-family proteins. The D domain also plays an important role in the synergistic activation of Sp1 (11).

Sp1 binds to the GC boxes located in the proximal promoters or enhancers found in almost all genes (3–5). In general, it acts as a transcriptional activator (and as a repressor in some cases), and the cellular context of the Sp-family is important with regard to the regulation of transcription (12–14). For example, Sp1 activates many different types of genes, including simian virus 40 (SV40), thymidine kinase (TK), and many mammalian housekeeping and tissue-specific genes (3–5). To activate transcription, Sp1 also interacts with nuclear proteins such as the TATA-box-binding protein TBP, and the TBP-associated factors tTAFII130, hTAFII130, hTAFII55, and CRSP (a cofactor required for Sp1 activation). Sp1 also interacts with a variety of other transcription factors, such as the retinoblastoma-related proteins p107, p130, and p135 (Invitrogen) (9, 15–22). Currently, available data suggest that Sp1 acts mainly as a transcription activator, but also, in some cases, as a transcription repressor (Refs. 1, 3–5 and references therein, Refs. 20–24). Indeed, many reports have implied that Sp1 and the Sp-like transcription factors exhibit a bipolar character: i.e. they function as either transcription activators or repressors, depending on the promoter to which they bind and the coregulators with which they interact (1, 2, 9, 12–14, 23–27).

The Sp-family exhibits several highly conserved domains, as stated above. Accordingly, it would appear that a certain mechanism exists for the regulation of Sp-family activities, functioning via these highly conserved domains, regardless of their properties as either transcription activators or repressors. Particular attention has been currently focused on the most highly conserved domain, the ZFDBD. It has been hypothesized that there is a reason, other than target recognition, for the high degree of conservation seen in the amino acid sequences of the ZFDBDs in the Sp-family transcription factors. It may interact with other regulatory proteins, and the protein involved in the interaction might function as a negative or positive regulator by controlling the GC-box target recognition of the Sp-family ZFDBDs.

The molecular mechanism underlying the repressor functions of Sp1 in the expression of some genes remains uncertain. The molecular role of the N-terminal ID (inhibitory domain) present in full-length Sp1 cDNA has not yet been characterized, due both to the lack of full-length Sp1 cDNA (GenBank accession number NM_138473 XM_028606) and to the widely circulating truncated version of Sp1 originally cloned by the Tjian group (2). Full-length cDNA, containing the N-terminal ID, has been cloned only very recently (28; GenBank accession number NM_138473 XM_028606), and its ID may well play a role in the Sp1-mediated repression of transcription.

In this study, we discovered that the ZFDBD and ID domains of Sp1 are involved in protein-protein interactions with the other transcription regulatory molecules: the corepressors SMRT, NCoR, and BCoR (29–31). We also determined that these interactions are important in the transcriptional regulation of Sp1. Our results indicate that these interactions could be regulated by MEK, a component of the MAP kinase/Erk kinase signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The mammalian expression plasmids for the Gal4-zinc finger domain (amino acids 622–720) fusion proteins or the inhibitory domain (amino acids 1–82) fusion proteins of Sp1 were constructed by subcloning the ZFDBD or ID cDNA into a pCMX-Gal4 fusion expression plasmid. The expression plasmids for the VP16-corepressors, the NCoR (amino acids 1709–2215), SMRT (amino acids 194–657), and BCoR (amino acids 112–753) fusion proteins (pKHT3/110EF-NCoR, pKH135EF-BCoR), were kindly provided by Drs. Ronald Evans (The Salk Institute), Vivian Hardwick (University of Minnesota), and Dominique Leprince (Institut Pasteur de Lille) (29–31). To generate the GST fusion protein expression plasmids, the ZFDBD and the ID domains of Sp1 were subcloned into pGEX4T3 (Amersham Biosciences). The mammalian expression plasmids were prepared by subcloning the cDNA fragments encoding for SMRT (amino acids 194–657), NCoR (amino acids 1709–2215), and BCoR (amino acids 112–753) into pGEXNA3.0 (Invitrogen) (29–31).

Interactions between the Sp-family and its corepressors and reporter assays were assessed via the cloning of five tandem Sp1 binding sites (sense: 5′-CTAGCTGATCGGGGCGGCGGAGC-3′; antisense: 5′-CTAGCCGCTCCGCCCGCGGTAGCAG-3′) upstream of the adenovirus major late promoter in the vector, pG5-Luc (Promega), to generate gpo5-5x(GC)-Luc. All constructs were verified by sequencing.

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**Cell Culture and Mammalian Two-hybrid Reporter Assays**—African green monkey kidney cells (CV-1) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml of streptomycin, and 100 units/ml of penicillin (Invitrogen). Cells were inoculated on 6-well tissue culture plates at a density of 1×10⁵ cells/well in 2 ml of DMEM. After the cells had grown for 24 h, they were transiently transfected with the Gal4 fusion bait expression plasmid, the VP16 fusion effector plasmid, and the reporter plasmid, using Lipofectamine Plus reagent (Invitrogen) in serum-free DMEM, according to the manufacturer's recommendations. After 3 h, the cells were supplied with fresh, complete DMEM, and allowed to grow for additional hours. The cells were then harvested and lysed in 150 μl of reporter lysis buffer (Promega), vortexed for 1 min, and then centrifuged at 12,000 rpm for 3 min at 4°C. The luciferase reporter assays were performed with 5 μl of cell extracts, using 50 μl of luciferase assay reagent (Promega) on a luminometer (Microplate Luminometer LB 96V, EG&G Berthold). Luciferase activities were normalized to either the protein concentration or to β-galactosidase activity. The protein concentration of the cell extracts was determined by the Bradford method (32).

**Recombinant Sp1 Zinc Finger DNA Binding Domain Expression**—A recombinant Sp1 zinc finger DNA binding domain vector was constructed by cloning the cDNA fragment encoding from amino acids 624 to 718 of Sp1 into pTrHisB/Bam H1 (Invitrogen) to generate pTrHisB-Sp1ZF. pTrHisB-Sp1ZF was introduced into Escherichia coli BL21(DE3) by transformation and cultured at 18°C in the presence of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. E. coli were harvested and then lysed by sonication. Recombinant Sp1ZFWT polypeptide was purified by Ni-nitrotriacetic (NTA) acid bead column affinity chromatography.

**Site-directed Mutagenesis of pTrHisB-Sp1ZF and pGal4-Sp1ZF**—To investigate the role of each zinc finger of Sp1, mutations were introduced into the Sp1 zinc finger sequence of pTrHisB-Sp1ZF or pGal4-Sp1ZF using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To introduce a mutation at the zinc fingers, the following oligonucleotides were used (only top strands are shown): zinc finger 1, amino acids 628 cysteine to serine, 5′-TTGCTGATCCAAAAC-TATATTGACCATTCAAGGGCTG-3′; zinc finger 2, amino acids 658 cysteine to serine, 5′-GCGAGAGGGCTTATTGCCATGAACCTCTG-3′; zinc finger 3, amino acids 688 cysteine to serine, GGTGAGAAGATAATGCAGCTCCTG-3′; zinc finger 4, amino acids 718 cysteine to serine, GGGTGAAGAAGATAATGCAGCTCCTG-3′; zinc finger 5, amino acids 748 cysteine to serine, GGGTGAAGAAGATAATGCAGCTCCTG-3′; zinc finger 6, amino acids 778 cysteine to serine, GGGTGAAGAAGATAATGCAGCTCCTG-3′. Each binding reaction was carried out in 20 μl and contained 10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 7% glycerol, and recombinant Sp1 zinc finger wild-type or mutant proteins (0.1 μg). Where indicated, an antibody against Express epitope peptide (Invitrogen) was added to the EMSA binding mixture.

**GST Fusion Protein Purification, in Vitro Transcription and Translation of Corepressors**—GST and GST fusion protein expression were induced in E. coli ER2566 via the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and with 5 μM of induction. The cells were lysed and purified with glutathione-agarose 4 bead affinity chromatography (Peptron, Daejeon, Korea). The purified proteins were then resolved...
with 10% SDS-PAGE, and the protein concentrations were determined by the Bradford method (26). The *in vitro* translated corepressor polypeptides were prepared by incubating the pcDNA3.0 corepressor expression plasmids (1 μg) with TnT Quick-coupled Transcription/Translation Extract (Promega), containing 40 μl of TnT Quick Master Mix, 4 μl of [35S]methionine (1175.0 Ci/mmol, PerkinElmer Life Sciences, Inc.), and 4μl of amino acid mixture minus the methionine, at 30 °C for 90 min. Polypeptide levels were then analyzed by running 2 μl of the total mixture with 10% SDS-PAGE.

**GST Fusion Protein Pull-down Assays**—The purified GST fusion proteins (5 μg) were incubated with GSH-agarose (Sigma) for 1 h in HEM buffer (40 mM HEPES, pH 7.9; 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl2, 0.1% Nonidet P-40, 10% glycerol, 1.5 mM diithiothreitol; and protease inhibitor mixture, 1 tablet/50 ml of a protease inhibitor mixture, Roche Applied Science) at 4 °C. After the agarose-GST protein, 1 tablet/50 ml of a protease inhibitor mixture, Roche Applied Science) at 4 °C. After the agarose-GST protein was incubated with GSH-agarose (Sigma) for 1 h in HEM buffer at 4 °C. The reaction mixtures were centrifuged at 3,000 × g at 4 °C, and the supernatants were removed and washed five times with cold HEM buffer. The bound proteins were separated by 10% SDS-PAGE, and the SDS-PAGE gels were dried and exposed to x-ray film using image-intensifying screens (Kodak).

**Chromatin Immunoprecipitation (ChIP) Assays**—We investigated whether *in vivo* molecular interaction between Sp1ID and corepressors on the pG5-UAS-Luc reporter could affect transcription. Polynucleosomes (20 μg) were incubated further with M2-anti-FLAG antibody-agarose beads (Sigma) for 2 h. Protein-antibody-agarose complexes were precipitated, washed, and analyzed by SDS-PAGE and autoradiography. *In vitro* translated [35S]methionine-labeled luciferase was used as a control.

**Regulation of Sp1 Transcription Activity by Corepressors**—We examined whether the regulation of Sp1 transcription activity by corepressors is involved in transcription repression. The interaction of the Sp1-ZFDBD or Sp1ID with corepressors could affect the transcription of the three Sp1-dependent promoter reporter fusion constructs. The pG5-5x(GC)-Luc reporter plasmid (0.2 μg), the p21WAF1/CIP1-Luc (0.2 μg), and the ADH5/FDH-Luc reporter plasmid (0.2 μg) were cotransfected into CV-1 cells, either with or without the expression plasmids of the constitutive activator (MEKCA), the dominant negative form (MEKDN) of MEK (0.8 μg), and pCMV-β-galactosidase (0.2 μg). The transfection and reporter assays were performed as described above.

In addition, we also tested whether the ERK activator, EGF (50 ng/ml), or the U0126 MEK inhibitor (10 μM, Cell Signaling Technology) could affect the transcription of pG5-5x(GC)-Luc, p21WAF1/CIP1-Luc, and ADH5/FDH-Luc. The CV-1 cells were transfected with pG5-5x(GC)-Luc or pGL2-ADH5/FDH-Luc (0.8 μg) and pCMV-β-gal (0.2 μg), according to the procedures described above, and the cells were treated with EGF or U0126 for 24 h before harvesting.

**Knockdown of Corepressor mRNA and Expression by siRNA**—The siRNA pool of the SMRT, NCoR, and BCoR corepressors was purchased from Dharmacon (catalog numbers M-020145, M-004584, and M-003518; West Lafayette, IN). The siRNA for glyceraldehyde-3-phosphate dehydrogenase was purchased from Ambion Inc. (TX) (catalog number 4605). A mixture of the siRNA pool (20 ng of each) and the plasmids (0.3 μg pG5-5x(GC)-Luc promoter and 0.1 μg pCMV-β-gal) was transfected into HeLa cells (6 × 104 cells) by electroporation, as described (25). The cells were harvested, and the cells and extracts were prepared. Reverse transcription-PCR was performed with one-third of the cellular extracts to confirm corepressor mRNA knockdown, and the other two-thirds were used to analyze the luciferase and β-galactosidase activities.

**RESULTS**

**Sp1ZFDBD and Sp1ID Can Repress Transcription Once Targeted to the Proximal Promoter of the Yeast GAL4-UAS Reporter System**—Previous reports revealed that Sp1ZFDBD interacts with the HDAC1 and POZ domains (21, 22). It was suspected that the Sp1ZFDBD and the Sp1ID might also interact with the other polypeptides involved in transcription repression, and that these interactions may be important in the regulation of Sp1 activity. To investigate this possibility, Gal4-Sp1ZFDBD fusion protein expression plasmids were prepared by subcloning cDNA fragments coding for Sp1ZFDBD (amino acids 622–720) and Sp1ID (amino acids 1–82) into a pCMX-Gal4 fusion expression vector. The CV-1 cells were transfected with pGal4-UAS luciferase plasmids (pG5-Luc) and Gal4-Sp1ZFDBD or -Sp1ID fusion expression plasmids, and after 48 h, luciferase activity was analyzed. The Gal4-Sp1ZFDBD or Gal4-Sp1ID was targeted to the proximal UAS and exhibited strong inhibitory effects on the transcription of the reporter gene compared with the Gal4DBD control (Fig. 1). Both Sp1ZFDBD and Sp1ID repressed transcription by more than 95%, which indicates that Sp1ZFDBD or Sp1ID might be interacting with the regulatory proteins involved in transcription repression.

**The Mammalian Two-hybrid Assays Showed That the ZFDBD and ID of Sp1 Interact with Corepressors in Vivo**—Our data suggest that both Sp1ZFDBD and ID might interact with the repressor or corepressor proteins that can inhibit transcription in the test promoters. Accordingly, we investigated whether the Sp1ZFDBD or Sp1ID could interact with the corepressors such as SMRT, NCoR, and BCoR, all of which have been shown to repress transcription (20–31), using mammalian two-hybrid assays.

Transient transfection assays in CV-1 cells indicated that the Sp1ZFDBD or Sp1ID both interacted strongly with SMRT, NCoR, and BCoR (Fig. 2B). The interaction between the Sp1ZFDBD and the VP16 corepressors resulted in an increase in transcription by 2–7-fold, depending on the corepressors, and the Sp1ZFDBD was found to interact most vigorously with BCoR. Also, the N-terminal Sp1ID interacted robustly with the corepressors, increasing transcription by 3.5–8.5-fold over the control (Fig. 2C). SMRT and BCoR both exhibited robust interactions with the Sp1ID.
These results indicate that either the Sp1ZFDBD or the Sp1ID interacts with the corepressors in vivo. This suggests that Sp1ZFDBD is probably not in its naked state and may be forming a complex with the corepressors. In addition, these molecular interactions with the corepressors may constitute a key factor in the transcriptional regulation of the Sp-family, via the modulation of the target GC-box binding of Sp1ZFDBD.

Moreover, the molecular interactions occurring between Sp1ID and the corepressors may explain the transcription repression by the Sp1 bound to the enhancer or to the proximal promoter GC-box (23–27).

The Mutation of Sp1ZFDBD Does Not Affect Interaction with Corepressors in Vivo—The above data indicate that the Sp1ZFDBD probably interacts with the corepressor proteins that inhibit transcription on the test pUAS-Luc promoter. However, one can argue that the Sp1ZFDBD targeted to the proximal promoter of pUAS-Luc via Gal4DBD can repress transcription by binding to the promoter GC-box of other genes. Such interactions with other promoters can deplete the general transcription available to pUAS-Luc and can also repress transcription in trans by the repressors bound to the other GC-box-containing promoters.

Therefore, we prepared three recombinant mutant Sp1 zinc fingers by changing each one of the three cysteine residues critical in their formation into serine, by site-directed mutagenesis (Fig. 3A). EMSA showed that mutation at the first zinc finger resulted in a markedly decreased binding activity of the Sp1 zinc finger to the GC-box probes. In contrast, mutations introduced at the second and third zinc fingers of Sp1 abolished the binding of recombinant mutant zinc finger proteins to the GC box (Fig. 3B). Having generated the mutant Sp1ZF polypeptides lacking the ability to bind to the GC-box, we used mammalian two-hybrid assays to test whether the Sp1 zinc finger to the GC-box probes. In contrast, mutations introduced at the second and third zinc fingers of Sp1 abolished the binding of recombinant mutant zinc finger proteins to the GC box (Fig. 3B). Having generated the mutant Sp1ZF polypeptides lacking the ability to bind to the GC-box, we used mammalian two-hybrid assays to test whether the Sp1 zinc finger could still interact with the corepressors in vivo. The mutant Sp1ZF DBD targeted by Gal4DBD to UAS also interacted strongly with SMRT and BcoR, and moderately with NcoR, just like the Sp1ZF (Fig. 3C). Accordingly, our data suggest that transcription repression by Gal4Sp1ZF occurs via interaction with corepressor proteins, and not by the binding of Sp1ZF to the GC-box of other promoters with repressor or corepressors bound nearby, or by the competitive depletion of general transcription factors by other promoters.

The Sp1ZFDBD and Sp1ID of Sp1 Directly Interact with the Corepressors in Vitro—To investigate whether the molecular interaction between the Sp1ZFDBD or Sp1ID and the corepressors is direct, a GST fusion protein pull-down assay was performed using recombinant GST-Sp1ZFDBD, GST-Sp1ID, and in vitro translated corepressor proteins. The GST and GST-Sp1ZFDBD or -Sp1ID were immobilized onto agarose and incubated with [35S]methionine-labeled corepressor polypeptides prepared with the TNT Quick-coupled Transcription/Translation system. After the precipitation and extensive washing of...
the complex, the precipitants were analyzed via SDS-PAGE and autoradiography. Our results indicated that both Sp1ZFDBD and Sp1ID interact directly with the corepressor polypeptides (Fig. 4), which suggests that the molecular interactions evidenced by the mammalian two-hybrid assays do indeed exist. In addition, we determined that the interaction involved the following modules of corepressor molecules: amino acids 194–657 of SMRT, amino acids 1709–2215 of NCoR, and amino acids 112–753 of BCoR. These domains were involved in interaction with nuclear receptors. Domains of corepressors used in in vitro pull-down assays are indicated below by light gray filled bars. ZF, zinc finger domain; A and B, two glutamine and serine/threonine-rich domains; D, D-domain; *, two Sp1 domains (ID and ZF) used in pull-down of [35S]methionine-labeled corepressors polypeptides. B, recombinant GST, GST-Sp1ZFDBD, and GST-Sp1ID fusion proteins were incubated with the in vitro synthesized [35S]methionine-labeled corepressor polypeptides, and then were pulled down. The precipitated samples were resolved by 10% SDS-PAGE and exposed to x-ray film. Input, 10% of the corepressors added in the binding reactions. C, co-immunoprecipitation showed that the full-length FLAG-tagged Sp1 can also interact with corepressors (SMRT, BcoR, NCoR), whereas the control luciferase cannot. Control, [35S]methionine-labeled luciferase; WB, Western blot.

ChiP Assays Show That Sp1ID Binds to the UAS Promoter Region of pG5-UAS-Luc and Deacetylates Histone in Vivo—The interaction between the cis-regulatory UAS proximal promoter element of the pG5-Luc and Gal4-Sp1ID in vitro and resulting histone modification was investigated by ChiP assay. After cross-linking of the chromatin with protein using formaldehyde, sheared chromatin was immunoprecipitated with purified polyclonal anti-acetylated histone antibody and Sepharose A. PCR amplifications of unsheared input genomic DNA and anti-acetylated histone 4 antibody-mediated immunoprecipitated of the cells transfected with Gal4 expression plasmid gave PCR products of the expected size (246 bp), but the same reaction with precipitant prepared from the cells transfected only with Gal4-Sp1ID expression plasmid did not give amplified product (Fig. 5B). The data suggested that Gal4-Sp1ID interacts with the UAS of pG5-Luc in vivo and interacted with corepressors, which deacetylated histone and repressed transcription (Fig. 5C).

The Molecular Interactions between the Sp1ZFDBD and the Corepressors Are Controlled by MEK of the MAP Kinase Signaling Pathway in Vivo—The molecular interactions...
Regulation of Sp1 Transcription Activity by Corepressors

The Sp1ZFDBD and the BCoR showed a particularly strong molecular interaction. However, BCoR exhibited different results when exposed to ectopic MEKCA and MEKDN. Rather than weakening the interactions occurring between Sp1ZFDBD and BCoR, MEKCA increased the interaction (Fig. 6C), and MEKDN weakly increased the interaction between Sp1ZFDBD and BCoR. The molecular interaction between Sp1ZFDBD and BCoR can be regulated by MEK of the MAP kinase/Erk kinase signaling pathway, but in the opposite way compared with the SMRT/NCoR corepressor interaction.

Among the other components of the MAP kinase/Erk kinase signaling pathway tested, the molecular interactions were not significantly affected by the presence of ERK1, the ERK1 dominant negative form MKP3, the RAF wild type, or the RAF mutant form RAFS259A, and they appeared not to modulate the interaction (data not shown).

These results suggest that the molecular context of the corepressors in a certain cell type might be critical in the regulation of the transcription of many genes controlled by Sp1, because of the modulation caused by the interactions between Sp1ZFDBD and the corepressors.

The Molecular Interaction between the Sp1ID and the Corepressors Is Also Controlled by MEK in Vivo—The molecular interactions occurring between the Sp1ID and the corepressors were tested in order to determine whether they could also be regulated by a component of the MAP kinase/Erk kinase signaling pathway (Fig. 6, D–F). VP16-SMRT, -NCoR, and -BCoR increased reporter activity strongly, suggesting a positive interaction between the corepressors and Sp1ID. Most notably, MEKCA drastically reduced molecular interactions, whereas the addition of MEKDN increased interactions by 7-fold (Fig. 6D). Similar findings were also observed with the Sp1ZFDBD and the corepressor NCoR (Fig. 6E). These results suggest that the molecular interactions between Sp1ID and the corepressors SMRT or NCoR can be weakened by MEK, and that Sp1ID could be freed from the corepressor-bound state by MEK, just as for Sp1ZFDBD. MEK may allow the Sp1 bound on the regulatory sequence to activate transcription by freeing the corepressors. As observed in the Sp1ZFDBD, MEK showed different effects on the molecular interactions between Sp1ID and BCoR. MEKCA strongly increased the interaction between Sp1ID and BCoR, but MEKDN showed very weak or no significant effects (Fig. 6F).

Our data suggest that the interactions between Sp1ID and the corepressors can be modulated by MEK, and that the molecular context of the corepressors in a certain cell type is important for transcriptional regulation in many genes controlled by Sp1, as the interactions are differentially regulated depending on the corepressors involved.

MAP Kinase Activator EGF and U0126 MEK Inhibitor of the MAP Kinase/Erk Kinase Signaling Pathway Control the Transcription of the pG5–5x(GC)-Luc Promoter—We tested whether the MAP kinase/Erk kinase signaling pathway was able to regulate the molecular interactions involving Sp1ZFDBD, using the Sp1-regulated test promoter reporter fusion construct, pG5–5x(GC)-Luc, in the presence of the MAP kinase activator EGF and the U0126 MEK inhibitor. The reporter pG5–5x(GC)-Luc responds well to both EGF and U0126. EGF-activated transcription and the MEK inhibitor also repressed transcription of the pG5–5x(GC)-Luc promoter—

We tested whether EGF and U0126 could regulate the transcription of the two well characterized Sp1-dependent human gene promoters, p21^Waf/Cip1 and ADH5/FDH. The treatment of transfected cells with EGF and U0126 resulted in
FIG. 6. The interactions between the Sp1ZFDBD or Sp1ID and the corepressors can be modulated by the MEK of MAP kinase/Erk kinase signaling pathway. A–C, mammalian two-hybrid assays demonstrate that the molecular interactions between the Sp1ZFDBD and the corepressors (SMRT, NCoR, and BCOR) can be modulated by MEK, a component of the MAP kinase pathway. The presence of MEKCA attenuates the interaction and MEKDN augments the interaction, suggesting that MEK disrupts the interaction between Sp1ZFDBD and the corepressors SMRT and NCoR, renders more Sp1ZFDBD available for promoter binding, and activates transcription. However, upon MEKCA expression, BCOR exhibited an opposite effect compared with SMRT/NCOR. D–F, molecular interactions between the Sp1ID and corepressors (SMRT, NCoR) can be also modulated by MEK. The presence of the constitutive activator form of MEK attenuates the interaction, and the MEKDN form increases interaction, suggesting that MEK disrupts the interaction. However, BCOR showed an opposite effect compared with SMRT/NCOR, and MEKCA increased interactions between the Sp1ID and the corepressors. The cell extracts were analyzed for their reporter luciferase activities and were normalized with β-galactosidase activity. Data are an average of three independent assays. Bar represents S.D.

FIG. 7. Transcription on the pG5–5x(GC)-Luc, p21Waf/Cip1-Luc, and ADH5/FDH-Luc can be increased by MAP kinase activator EGF and repressed by U0126 MEK inhibitor. CV-1 cells were transfected with the various promoter-luciferase fusion plasmids (0.8 μg) and pCMV-β-gal (0.2 μg), and the cells were treated with EGF (50 ng/ml) or U0126 (10 nM) for 24 h prior to harvest. The cell extracts were analyzed for their reporter luciferase activities and normalized with β-galactosidase activity or protein concentration. Data are an average of four independent assays. Bar represents S.D.
modulations of transcription similar to what was observed with pG5–5x(GC)-Luc (Fig. 7, B and C).

The MEK Constitutively Active MEKCA and Dominant Negative Form MEKDN of the MAP Kinase/Erk Kinase Signaling Pathway Control the Transcription of Sp1-dependent pG5–5x(GC)-Luc and p21Waf/Cip1-Luc—We also tested whether the transcription of the two Sp1-dependent promoters could be modulated by MEK. The mammalian two-hybrid assay data suggested that activated MEK could release the Sp1ZFDBD or Sp1ID from the corepressor (SMRT/NCoR) complex and activate transcription. We tested this possibility by co-transfecting CV-1 cells with pG5–5x(GC)-Luc or p21Waf/Cip1-Luc, with the plasmid expressing MEKCA or MEKDN. MEKCA was able to activate transcription, and MEKDN repressed MEKCA-induced transcription activation (Fig. 8). Overall, these data (Figs. 2–7) suggest that molecular interactions exist between Sp1ZFDBD or Sp1ID and the corepressors (SMRT, NcoR, and BcoR), and that the interactions are regulated by MEK.

Knockdown of the Corepressors by siRNA Treatment Increases the Transcription of the Sp1-dependent Promoter—The interactions between the Sp1ZFDBD or Sp1ID and the corepressors might repress transcription by inhibiting the GC-box target recognition of Sp1. Another possibility is that the corepressor proteins repress transcription by being recruited via the Sp1ID domain onto the promoter-bound Sp1. To characterize the functional significance of this molecular interaction on Sp1-controlled transcription, we knocked down the expression of the endogenous corepressors by treating human HeLa cells with an siRNA pool designed to destroy the mRNA of SMRT, NcoR, and BcoR. The Sp1-dependent reporter plasmid, pG5–5x(GC)-Luc, was cotransfected with the siRNA pool (SMRT, NcoR, and BcoR) into HeLa cells, and the luciferase reporter gene expression was analyzed. Treatment with the siRNA pool significantly reduced the mRNA levels of NcoR, SMRT, and the glyceraldehyde-3-phosphate dehydrogenase control, while β-actin mRNA remained unchanged (Fig. 9A). The BcoR mRNA was detected in neither the control nor the siRNA-treated HeLa cells (Fig. 9A). The disruption of corepressor expression resulted in a 1.7-fold increase in the transcription of the Sp1-dependent test promoter (Fig. 9B). This suggests that knocking down the mRNA of the corepressors via siRNA treatment might result in the liberation of Sp1ZFDBD from the corepressors, thus enhancing Sp1 target recognition and activating transcription. Also, it is possible that the siRNA treatment resulted in a depletion of the corepressors that could be recruited to the Sp1ID of the proximal promoter-bound Sp1, and thereby activating transcription.
DISCUSSION

The molecular mechanisms underlying biological processes can be understood by investigating the network of protein-protein interactions involved. The Sp1 transcription factor regulates gene expression by binding to the GC boxes located in the proximal promoters of many cellular and viral genes (3–5). Sp1 was initially purified and isolated as a transcription factor that could recognize the SV40 early promoter (1, 2), also forming a family with factors such as Sp2–8 (3–5). Thus far, Sp1 has been demonstrated to interact with various proteins (e.g. TBP, dTAF110, TAFII130, TAFII55, Rb, YY1, E2F, CRSP, FBI-1, p300, HAT, etc.), and these interactions have proved to be crucial in the regulation of many cellular functions by transcription-level control of gene expression. We, along with other investigators (9, 21, 22), determined that the most highly conserved region of Sp1, the Sp1ZFDBD, is involved in protein-protein interactions with other proteins, including the POZ domains of FBI-1, p300, and HDAC1.

We showed in this report that the Sp1ZFDBD interacts with corepressors (SMRT, NCoR, and BCoR), and that the interactions are important in the regulation of Sp1 activity and the transcription of Sp1-dependent promoters. Our data suggest that the Sp1ZFDBD might not be in a naked state and that instead, Sp1ZF is associated with the corepressors. The molecular interactions provide an intriguing and novel mechanism for regulating Sp1 activity at the level of GC-box target recognition by the Sp1ZF (Fig. 10).

In addition, the molecular interactions between Sp1ID and the corepressors can explain how Sp1 can function as a transcription repressor in certain Sp1-bound promoters (23–27), even though Sp1 is known to be a transcription activator, in general (Refs. 3–5 and references therein) (Fig. 10).

Considering the fact that the ZFDBD is the most highly conserved domain in the Sp-family transcription factors, with 84–95% amino acid sequence homology among Sp1–6 (3), the interactions might also exist in the ZFDBDs of the other members of the Sp-family, and the interactions may be important in the regulation of transcription by the Sp-family. Preliminary studies with the other Sp-family members indicate that the interactions are indeed present with Sp3 and Sp4. Because few studies have been done with Sp2, Sp5, or Sp6 in the transcriptional regulation of GC-box-containing promoters, we have scant evidence of how they might contribute to transcription regulation. However, it appears that Sp1 has the most potent transcription activation potential, while Sp4 activates transcription rather weakly and Sp3 mostly represses transcription (14). Accordingly, the transcription of a particular

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GC-box-containing promoter is more likely to be controlled by Sp1. Indeed, the molecular interactions between Sp4 and the corepressors are at least 3–5 times stronger, as revealed by mammalian two-hybrid assays (data not shown). This suggests that Sp4 is most likely still associated with corepressors in the situation where most Sp1 molecules have been freed to bind the target GC-box and activate transcription.

Previously, it has been reported that members of the Sp-family could clearly be regulated by phosphorylation, glycosylation, acetylation, and SUMOylation (6–10). In addition, the Sp1 binding GC-box sites in the p21Waf/Cip1 promoter and urokinase are the targets of the MAP kinase/Erk kinase signaling pathway, suggesting that Sp1 recognition of the GC-box sites might be the target of MAP kinase/Erk kinase signaling (36, 37). Because controlling DNA binding and protein interactions with the corepressors via ZFDBD or ID could be important functions in regulating Sp1 activity, it was assumed that the molecular interactions between Sp1 and the corepressors might be the target of the MAP kinase signaling pathway, which could thereby regulate the transcription of the Sp1-dependent promoters. We tested whether the MAP kinase signaling pathway could regulate molecular interaction between Sp1ZFDBD and the corepressors, using the Sp1-dependent pG5–5×(GC)-Luc reporter and mammalian two-hybrid assay. Treatment of the transfected cells with EGF and U0126 MEK inhibitor, or the ectopic expressions of MEKCA and MEKDN, modulated the molecular interactions between Sp1ZFDBD or Sp1ID and the corepressors. The Sp1 reporter pG5–5×(GC)-Luc itself and native human gene promoters responded well to EGF and U0126, and MEKCA activated transcription. Overall, these results suggest that molecular interactions do occur between Sp1ZFDBD or Sp1ID and the corepressors, and that these interactions are modulated by MEK. These results are in line with previous reports that the Sp1-dependent promoter was controlled by the MAP kinase signaling pathway, as has been reported for the p21Waf/Cip1 promoter (36–38, 40, 41).

It has been suggested that MEK, from the MAP kinase pathway, phosphorylates Sp1 at amino acids 453 and 739 (36, 38, 40). The phosphorylation of these two sites results in an increase in Sp1 binding to the target sites and in increased transcription activation. However, the Sp1ZFDBD (amino acids 622–720) and Sp1ID (amino acids 1–82) used in this assay do not contain the two phosphorylation sites. Therefore, MEK may more likely functions by phosphorylating the corepressor molecules, thus controlling the molecular interactions between Sp1ZFDBD or Sp1ID and the corepressors. Previously, the ability of SMRT to associate with a variety of transcription factors such as nuclear hormone receptors, PLZF, and Bcl-6, and thereby to mediate repression, was shown to be strongly repressed by the phosphorylation of SMRT by MEKK1 or MEK-1 of the MAP kinase pathway (42, 43). More recently, it was demonstrated that the protein-protein interactions between the C-terminal nuclear receptor interaction domains of SMRT (S1, S2 domains; amino acids 1733–2471) or NCoR (N1–3 domains; amino acids 1946–2435) and TRα or RARα are differentially regulated by MEKK1, depending on the repressors (43). The domain of the corepressors used in our assays is the domain that has been implicated in the repression of transcription via interaction with the POZ domains, PLZF or Bcl-6. This is a different domain from those (C-terminal) investigated by Privalsky and co-workers (42, 43).

The molecular interactions occurring between Sp1 and the corepressors do not appear to be unique to Sp1 and can be found in the other members of the Sp-family or the Sp-like transcription factors. Although different corepressors are involved, Sp1-like transcription repressors like the BTEB-family and TIEG1–2 have been demonstrated to interact with another corepressor molecule, the mammalian Sin3A (mSin3A), via the Sin3-interacting domain (SID) (39, 44, 45). This interaction is important in the repression of transcription. Intriguingly, the interaction is inhibited by the EGFR-Ras-MEK1-ERK2 signaling pathway, which disrupts SID-mSin3A interactions, thereby inhibiting repression activity (39).

Our data suggest that the molecular interactions occurring between the Sp1ZFDBD or Sp1ID and the corepressors NcoR and SMRT may constitute an important regulatory aspect of many cellular and viral genes controlled by Sp1. In contrast, MEK regulates the interaction between Sp1ZFDBD or Sp1ID and SMRT or NcoR. In summary, the activity of the Sp1 transcription factor can be regulated by a novel protein-protein interaction that involves Sp1ZFDBD or Sp1ID and the corepressors. The mechanism by which the interaction is modulated by the MAP kinase signaling pathway, and the cellular context of the corepressors, may be crucial to the regulation of the expression of many other cellular and viral genes controlled by Sp1, the Sp-family, and perhaps other more extended Krüppel-like zinc finger transcription factors harboring highly conserved C2H2-type zinc fingers.

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