Regulation of Sp-family Transcription Factor Activity by Novel Protein-Protein Interaction with Corepressors

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Regulation of Sp-family Transcription Factor Activity by Novel Protein-Protein Interaction with Corepressors

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A master's thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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December, 2002

This certifies that the master's thesis of

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December, 2002

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저를 너무나 아끼고 사랑을 주신 우리 부모님과 하나밖에 없는 우리 오빠에게도 이 마음을 전하고 싶습니다.

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Regulation of Sp-family Transcription Factor Activity by Novel Protein-Protein Interaction with Corepressors

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(Directed by Professor **Man-Wook Hur)**

Sp1 is an important transcription factor involved in the expression of many cellular and viral genes. Sp1 activates transcription by binding to the GC-box in proximal promoter region. Sp-family proteins are characterized by three C_2H_2 zinc fingers at their C-termini. Sp1 can bind to target promoter through zinc fingers. So far, the zinc finger DNA binding domain (ZFDBD) was considered to be important only in the recognition of the GC-Box. However, we found that the DBD is an motif which regulates transcriptional activity of Sp-family transcription factors by novel protein-protein interaction. The ZFDBDs of Sp1, Sp3, and Sp4 can interact with corepressor proteins such as SMRT, NCoR, BCoR by mammalian two hybrid and GST pull down assay.

We also found that the inhibitory domains (IDs) of Sp-family interact with corepressors. The protein-protein interaction of Sp1 protein is critical in the transcriptional regulation by Sp-family transcription factors. Our data also suggest that the interaction between the ZFDBD and corepressors may be regulated by MAP kinase signaling pathway.

Key Words : Sp1, zinc finger DNA binding domain (ZFDBD), inhibitory domain (ID), corepressor, MAP kinase

Regulation of Sp-family Transcription Factor Activity by Novel Protein-Protein Interaction with Corepressors

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I. Introduction

The transcription is an essential process by which a large number of genes are regulated for development, growth, and survival of eukaryotic organisms. The organisms make use of a variety of mechanisms for the expression of a specific set of genes.[1] The key step of transcription in the complex of genome of eukaryotes is binding of RNA polymerases to the promoter region. Eukariotic RNA polymerases are classified into RNA polymerase I, II and III according to the genes they transcribed. RNA polymerase I transcribes large, tandem repeated, ribosomal RNA genes and RNA polymerase II transcribes the protein-coding genes (mRNA genes) as well as some small nuclear RNA (snRNA) genes. RNA polymerase III is engaged in the transcription of an eclectic

collection of genes whose main common features are encoding structural or catalytic RNAs.[2]

The transcription of a gene that involves RNA polymerase II is controlled by regulatory elements such as proximal promoter, enhancers, silencers, and boundary/insulator elements around a core promoter.[3-5] Transcriptional regulation is achieved by combinatorial action of regulators that bind to distinct promoter and enhancer elements. Among the *cis*-acting DNA elements, G-rich element such as the GC-box (KRGGMGKRRY) is important in the expression of many ubiquitous, and tissue-specific genes.[6] Sp1 is a well characterized sequence-specific DNA binding protein that plays a role in the transcription of many cellular and viral genes that contain GC boxes in their promoters.[7-10] Additional human and rodent transcription factors (Sp2, Sp3, Sp4) similar in structural and transcriptional properties to Sp1 were cloned, and form a Sp-multigene family.[6] Sp1, Sp3, and Sp4 bind to the same recognition sequence (GC boxes) with nearly identical affinity.[11,12] Sp1 and Sp4 generally act as transcriptional activators. In contrast, Sp3, in most case, acts as a repressor and rarely as an activator.[6,12-16] Sp2 has a DNA binding specificity different from those of Sp1, Sp3, and Sp4.[17]

The Sp-multigene family is an important regulator of cell cycle, differentiation, and development.[6] Sp1 acivates many different type genes such as simian virus 40 (SV40) and thmidine kinase (TK), mouse dihydrofolate reductase (DHFR), and many housekeeping and tissue-specific genes.[12-16] Sp1 transcription factor forms multimeric complex through phosphorylation and glycosylation.[17-21] Sp1 interacts with nuclear proteins such as TATA-box binding protein TBP and TBP associated factors dTAFII130, hTAFII130, and hTAFII55.[22-25] Also, Sp1 interacts with retinoblastoma related protein p107, transcription

factors such as YY1, E2F, and CRSP (cofactor required for Sp1 activation).[26-32] The Sp1 knock-out mice exhibit severe developmental retardation. Therefore, Sp1 is an essential transcription factor in differentiated cells.[6] Sp2 was shown to regulate T-cell antigen receptor α (TCRα) gene but little is known about other function of Sp2.[6,33] Sp3 exists in three different isoforms and the structure and the arrangement of the recognition sites appear to determine whether Sp3 is in transcriptionally inactive or active state.[6] In contrast to other ubiquitous factors, the expression of Sp4 is limited to a few tissues. Sp4 is expressed predominantly in brain and knock-out of mouse Sp4 gene leads to behavioral defects.[16,34]

Four Sp-family proteins have similar domain structures (Fig. 1) and are evolutionally closely related.[6] All proteins have highly conserved C_2H_2 type zinc finger DNA binding domain at the C-terminal and belong to Krüppel-like zinc finger superfamily. The proteins consist of several domains, i.e. N terminus-inhibitory domain (a.a. 1-82, ID), serine/threonine-rich domains (a.a. 87-143; a.a. 243-350, S/T-rich region), glutamine-rich domains (a.a. 138-232; a.a. 351-500, Q-rich region), zinc finger DNA binding domain (a.a. 622-720, ZFDBD), and C terminus D domain (a.a. 721-788).[6,35] The S/T-rich region is regulated by phosphorylation and is important in the regulation of Sp1. The Q-rich region with characteristics of acid-blob are important in transcriptional activation.[36] Recently, it has been reported that Sp-family proteins are post-translationally modified by various mechanisms. For example, Sp1 is phosphorylated by Erk2, PKC, casein kinase II, PKA, and Sp3 is SUMOylated by PIAS1.[37-41] The zinc finger DNA binding domain (ZFDBD) of Sp-family is the most highly conserved part of the proteins.[6] D domain has critical role in the synergistic activation of Sp1.[20]

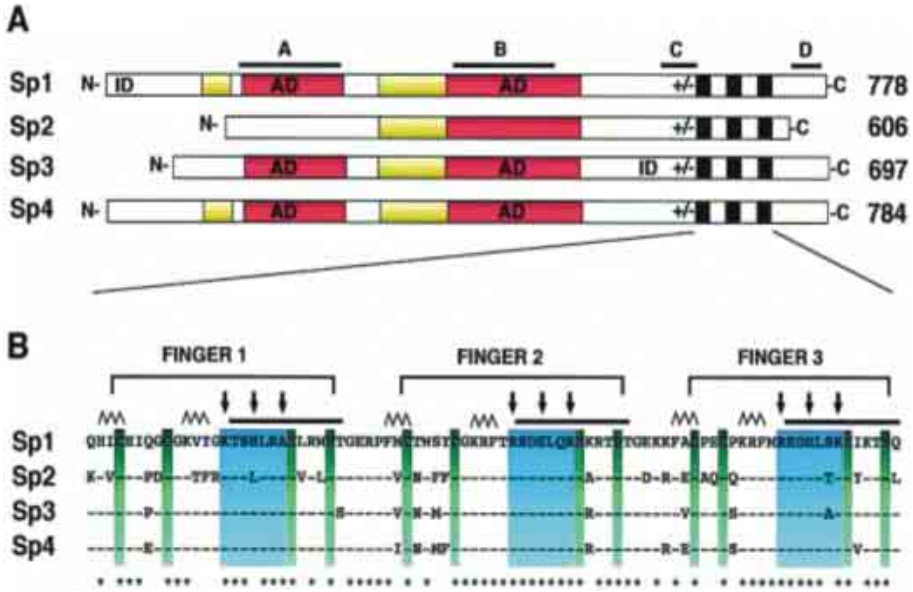

Figure 1. Schematic representation of four Sp-family members, Sp1, Sp2, Sp3 and **Sp4 (Suske G., Gene ;237:291-300).**

Currently available data suggest that Sp1 and Sp4 act as transcription activators, and Sp3 acts as a transcription repressor in general.[6] However, we came up with an idea that there must be a certain mechanism of regulating the activities of Sp-family transcription factors regardless of their properties as transcription activator or repressor. We initially suspected that the inhibitory domains (IDs) of Sp1 and Sp3 might serve as negative regulators of their activities by interacting with the proteins that can inhibit transcription. We also suspected that there must be a reason on the high conservation of amino acid sequence of ZFDBD other than target recognition. It may interact with other proteins, and the interacting protein might act as negative regulator by controlling the recognition of target GC-box by ZFDBD .

Our research hypothesis is that the ZFDBD of Sp-family is not be in freely accessible naked state. Our investigation indeed show that the activities of Sp-family transcription factors may be regulated by novel protein-protein interaction that involves corepressors (SMRT, NCoR, BCoR), IDs and ZFDBDs.

II. Materials and Methods

1. Construction of mammalian two hybrid plasmid vectors expressing Sp-family ZFDBDs and IDs

The ZFDBDs of Sp1, Sp3 and Sp4 were amplified from their cDNAs by polymerase chain reaction (PCR) (PCR condition: 94℃ 5 min, 94℃ 30 sec, 55℃ 30 sec, 72℃ 1 min. 30 cycles, followed by 72℃ 5 min. Sp1 ZF PCR forward primer MWH 562: 5'-GAT CGT CGA CCC GGA TCC GAA GGA AGG GGC TCG GGG GAT CCT-3', Sp1 ZF reverse primer MWH 563: 5'-GAT CTC TAG AGA ATT CCT AAC TCA GAG CTA CAC CTG GGC CTC-3', Sp3 ZF forward primer MWH 571: 5'-GAT CGT CGA CCC GGA TCC AGG GTA GCT TGC ACC TGT CCC AAC-3', Sp3 ZF reverse primer MWH 572: 5'-GAT CTC TAG AGA ATT CCT ATC GCG CAG CTT CCA CAG ATG CCA-3', Sp 4ZF forward primer MWH 560: 5'-GAT CGT CGA CCC GGA TCC AGG AGA AGG AAG AGG CAG TAA TGA-3', Sp4 ZF reverse primer MWH 561: 5'-GAT CTC TAG AGA ATT CCT AGT AAC AGA TGA GTC CAG TTC TCC-3') and cloned into pBIND vector (*Sal*I/*Xba*I) (Promega, Madison, WI, USA) for GAL4 fusion Sp-family ZFDBDs fusion constructs.

Also, the IDs of Sp1 and Sp3 transcription factors were amplified from Matchmaker® human liver cDNA library (Clontech, Palo Alto, CA, USA) or cDNA using PCR (PCR condition: Sp1 ID: 94℃ 5 min, 94℃ 30 sec, 50℃ 30 sec, 72℃ 1 min. 30 cycles followed by 72℃ 5 min, Sp3 ID: 94℃ 5 min, 94℃ 30 sec, 55℃ 30 sec, 72℃ 1

min. 30 cycles, followed by 72℃ 5 min). The PCR products were cloned into pBIND (*Sal*I/*Xba*I) and pBIND (*BamH*I/*Not*I) (Clontech), respectively. The sequences of the constructs were confirmed by dideoxy chain termination DNA sequencing using DNA sequencing kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). VP16-corepressors, NCoR (a.a. 1007-2043), and BCoR (a.a. 112-753) fusion constructs (pKH 73/110 EF-NCoR, pKH 135 EF-BCoR) were kindly provided by Drs. Ronald Evans (The Salk Institute, CA, USA), and Vivian Bardwell (University of Minnesota, MN, USA), and VP16-SMRT fusion protein was kindly offered by Dr. Dominique Leprince (Institut Pasteur de lille, France).

2. Preparation of recombinant Sp-family ZFDBDs and IDs

a. Construction of bacterial over-expression plasmids for Sp-family ZFDBDs and IDs

DNA fragments encoding the ZFDBD regions of Sp1,Sp3 and Sp4 were obtained from the cDNA templates by polymerase chain reaction (PCR condition: 94℃ 5 min, 94℃ 30 sec, 55℃ 30 sec, 72℃ 1 min. 30 cycles, followed by 72℃ 5 min. Sp1 ZF forward primer MWH 532: 5'-CGA TCG GAT CCC GCT TCA TGA GGA GTG ACC ACC-3', Sp1 ZF reverse primer MWH 533: 5'-CGA TCT CTA GAC TCG AGT CAC TTG TCA TCG TCG TCC TTG TAG TCC AGA GCT ACA CCT GGG CCT CC-3', Sp3 ZF forward primer MWH 571: 5'-GAT CGT CGA CCC GGA TCC AGG GTA GCT TGC

ACC TGT CCC AAC-3', Sp3 ZF reverse primer MWH 572: 5'-GAT CTC TAG AGA ATT CCT ATC GCG CAG CTT CCA CAG ATG CCA-3', Sp4 ZF forward primer MWH 560: 5'-GAT CGT CGA CCC GGA TCC AGG AGA AGG AAG AGG CAG TAA TGA-3', Sp4 ZF reverse primer MWH 561: 5'-GAT CTC TAG AGA ATT CCT AGT AAC AGA TGA GTC CAG TTC TCC-3') and cloned into *EcoRI/BamH*I sites of pGEX4T3 (Amersham Pharmacia Biotech Inc). Also, the IDs of Sp1 and Sp3 were obtained from the Matchmaker® human liver cDNA library and cDNA respectively by PCR (PCR condition: Sp1 ID: 94℃ 5min, 94℃ 30 sec, 50℃ 30 sec, 72℃ 1 min. 30 cycles followed by 72℃ 5 min, Sp3 ID: 94℃ 5 min, 94℃ 30 sec, 55℃ 30 sec, 72℃ 1 min. 30 cycles, followed by 72℃ 5 min). The PCR products were cloned into the *EcoRI/BamH*I sites of pGEX4T3 and *BamH*I/NotI sites of pGEX4T3 (Amersham Pharmacia Biotech Inc), respectively. The DNA sequences of constructs were confirmed by dideoxy chain termination DNA sequencing using DNA sequencing kit.

b. Bacterial over-expression of Sp-family ZFDBDs and IDs

GST fusion protein expression plasmids, pGEX4T3-Sp1 ZFDBD, Sp3 ZFDBD, Sp4 ZFDBD, pGEX4T3-Sp1 ID and pGEX4T3-Sp3 ID were transformed into the expression host *E. coli* ER2566 by heat shock method.[42] Transformed single colony was inoculated into 2×YT medium (Bacto-tryptone 16g, Bacto-yeast extract 10g, NaCl 5g per 1 liter Biobasic, Canada) containing 100 μg/ml ampicillin and cultured until $O.D^{600nm}$ reaches 0.5 at 37℃. *E. coli* were induced to express the fusion proteins with IPTG (isopropyl-1-

thio-β-D-galactopyranoside, 0.5 mM) for 5 hrs. The bacteria were collected by centrifugation at 1,2000 rpm and lysed by sonication for 40 sec at power 35 (Fisher, Dismembrator, model 300) in *E. coli* lysis buffer 300 μl (1% Triton X-100, 0.1% β-mercaptoethanol, 2 mM EDTA, pH 8.0, 1 mg/ml lysozyme, and 0.2 mM PMSF in PBS). Over-expressed proteins were resolved by a 10% SDS-PAGE and the gel was stained with Coomassie Brillant Blue.

c. Purification of recombinant GST fusion proteins

GST fusion Sp-family proteins were over-expressed in large scale (200 ml) with 2×YT containing 100 μg/ml ampicillin. After bacterial cells were pelleted by centrifugation at 1,2000 rpm, and bateria were lysed in *E. coli* lysis buffer and the supernatant was collected from lysed bacterial cell debris by centrifugation at 1,2000 rpm, for 30 min at 4℃. GST fusion proteins were purified using glutathione-agaroge 4 Beads (Peptron, Taejeon, Korea). Purified proteins were resolved by 10% SDS-PAGE and protein concentration was determined according to Bradford method.[43]

3. Cell culture and transient transfection

African green monkey kidney cells (CV-1) cell were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 μg/ml) (Invitrogen, CA, USA). Plasmids were transformed into *E. coli* DH5α and prepared a by alkaline lysis methods in large scale (250 ml) and

purified by CsCl/EtBr ultra centrifugation.

Cells were inoculated on a six-well tissue culture plates at a density of 1×10^5 cells/well in 2 ml of DMEM medium. After growing the cells for 24 hrs, cells were transiently transfected with LipofectAmine Plus reagent (Invitrogen) according to the manufacturer's recommended protocol in serum free DMEM medium. After 3 hrs, cells were supplied with fresh complete DMEM and allowed to grow for 48 hrs. Cells were harvested and lysed in 150 μl of reporter lysis buffer (Promega) and vortexed for 1 min and centrifuged at 1,2000 rpm, for 3 min at 4℃. Cellular extracts (20 μl) were analyzed for β-galactosidase activity by mixing with 180 μl of substrate solution (o-nitrophenyl-β-D-galactopytanoside 4 mg/ml in 0.1 M sodium phosphate, pH7.5; 0.1 M sodium phosphate buffer, pH 7.5; 100× Mg^{2+} (0.1 M $MgCl_2$; 4.5 M β-mercaptoethanol)). Luciferase reporter assays were performed with 5 μl of cell extracts using 50 μl Luciferase Assay System assay reagent (Promega) on Luminometer (Microplate Luminometer LB 96V, EG & G Berthold). Luciferase activities were normalized with β-galactosidase activity.

4. *In vitro* protein-protein interaction assays between ZFDBDs or IDs with corepressors

a. Construction of corepressors expression plasmids for *in vitro* translation

DNA fragments encoding SMRT (a.a. 194-657) and NCoR (a.a. 1709-2215) were amplified from their cDNA templates by PCR (SMRT PCR condition: 94℃ 5 min, 94℃

30 sec, 60℃ 1 min , 72℃ 1 min. 30 cycles, followed by 72 ℃ 5 min. SMRT forward primer MWH 454: 5'-GAT CGA ATT CGG TAC CAT GGC CTC GGA CAG CGC CAT CAC ATA CCG-3', SMRT reverse primer MWH 455: 5'-GAT CTC TAG AGC TAG CTC ACA GCG CCG CCG TGT CGG GGT AGC CGC GG-3', NCoR PCR condition: 94℃ 5 min, 94℃ 30 sec, 52℃ 1 min , 72℃ 2 min. 30 cycles, followed by 72 ℃ 5 min. NCoR forward primer MWH 691: 5'-GGA TCG GTA CCA TGG CAA GTG TGA GAG GGA ACG GGA ACG-3', NCoR reverse primer MWH 692: 5'-GGA TCT CTA GAT CAC TTA CGA AAA ATC TCC TGC TTC TTT GAT TTA-3') and cloned into the *Kpn*I/*Xba*I sites of the pcDNA 3.0 (Invitrogen). BCoR (a.a.112–753) was amplified from their cDNA templates by PCR (BCoR PCR condition: 94℃ 5 min, 94℃ 30 sec, 60℃ 1 min , 72℃ 1 min. 30 cycles, followed by 72℃ 5 min. BCoR forward primer MWH 687: 5'-GGA TCA AGC TTA CCA TGG GGA TTT CTT CGG AAA GAA ATC CAG-3', BCoR reverse primer MWH 688: 5'-GGA TCT CTA GAT CAG GCT CTC TCA TGG GAC CGG GAT CTC C-3') and cloned into the *Hind*III/*Xba* I sites of the pcDNA 3.0 (Invitrogen).

b. Preparation of [^{35}S]-methionine labelled corepressor peptides *in vitro*

pcDNA 3.0-corepressors expression plasmids (1μg) were incubated with TNT Quick Coupled Transcription/Translation extracts (Promega) containig 40 μl TNT Quick Master Mix, 4 μl [^{35}S]-methionine (1175.0 Ci/mol, PerkinElmer Life Sciences, Inc. Boston, MA, USA), 4 μl amino acid mixture minus methionine at 30℃, for 90 min. Expression of polypeptides was analyzed by running 2 μl out of total mixture on a 10% SDS-PAGE.

c. *In vitro* GST-pull down assay

Purified GST fusion proteins (5μg) were incubated with GSH-agarose (Sigma, St. Louis, MO, USA) for 1 hr in HEMG buffer (40 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 1.5 mM DTT, protease inhibitor cocktail 1 tablet/50 ml of protease inhibitor cocktail, Roche, Germany) at 4℃. After the agarose-GST-protein complexes were washed with 1 ml cold HEMG buffer 3 times, 20 μl [^{35}S]-methionine labelled corepressors were incubated in HEMG buffer at 4℃ for 4 hrs. The reaction mixtures were centrifuged at 3,000g, 4℃ and removed supernatant and washed 5 times with cold HEMG buffer. The bound proteins were resolved by 10% SDS-PAGE and the SDS-PAGE gels were dried and exposed to X-ray film using image intensifying screens (Kodak, CT, USA).

5. Transient transfection using MAP kinase signaling pathway regulatory chemicals

CV-1 cells were inoculated on a six-well tissue culture plates at a density of 1×10^5 cells/well in 2 ml of DMEM medium. Reporter plasmid pG5-Luc, GAL4-Sp1 ZF and VP16-corepressors were prepared by alkaline lysis methods in large scale (250 ml) and CsCl/EtBr ultra centrifugation. After growing the cells for 24 hrs, cells were transiently transfected with LipofectAmine Plus reagent (Invitrogen) according to the manufacturer's recommended protocol in the serum free DMEM medium. After 3 hrs, cells were supplied with fresh complete DMEM. After 24 hrs, cells were treated with MAP kinase regulatory

chemicals (EGF, U0126) and were allowed to grow for 24 hrs. Cells were harvested and lysed in 150 μl of reporter lysis buffer (Promega). Cellular extracts (20 μl) were analyzed for β-galactosidase activity. Luciferase reporter assays were performed with 5 μl of cell extracts supernatant using Luciferase Assay System (Promega). Luciferase activities were normalized with coexpressed β-galactosidase activity.

III. Results

1. The ZFDBDs and IDs of Sp-family were successfully over-expressed in *E. coli* ER2566

For protein-protein interaction studies, we prepared various recombinant proteins. GST-Sp1 ZFDBD (a.a. 622-720), GST-Sp3 ZFDBD (a.a. 584-711), GST-Sp4 ZFDBD (a.a. 632-751) were expressed in *E. coli* ER2566. Also, we also over-expressed GST-Sp1 ID (a.a. 1-82) and GST-Sp3 ID (a.a 500-568) in *E. coli* ER2566. Each of the proteins were resolved by SDS-PAGE and analyzed for expression level. The recombinant proteins were successfully over-expressed and purified (Fig. 2). The size of GST fusion proteins are GST-Sp1 ZFDBD, 37.78 kDa; GST-Sp3 ZFDBD, 40.97 kDa; GST-Sp4 ZFDBD, 40.09 kDa; GST-Sp1 ID, 36.02 kDa; GST-Sp3 ID, 34.4 kDa. The IDs were expressed abundantly, but the expression level of ZFDBDs relatively low compared with IDs.

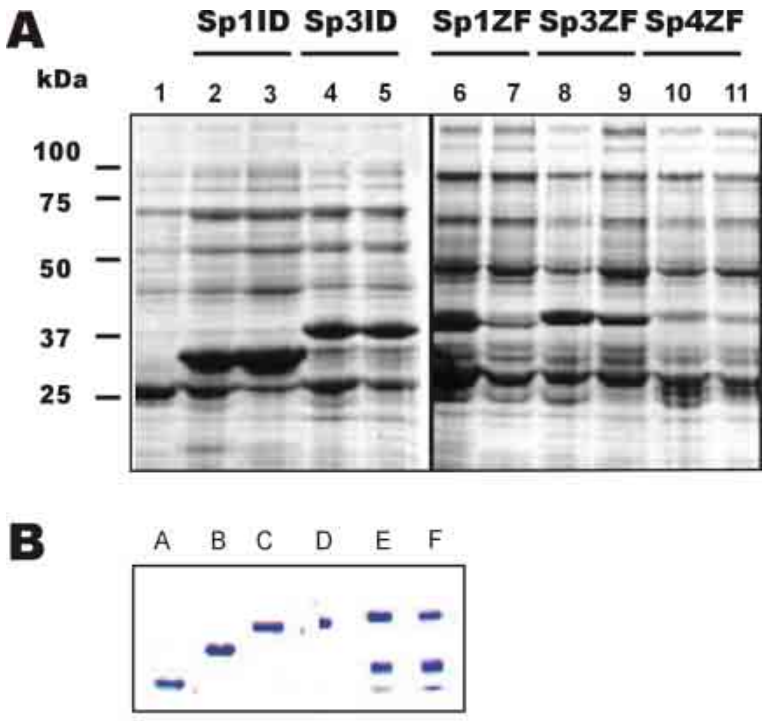

Figure 2. Bacterial over-expression of recombinant ZFDBDs and IDs. (A) GST-Sp1 ZFDBD (a.a. 622-720), GST-Sp3 ZFDBD (a.a. 584-711), GST-Sp4 ZFDBD (a.a. 632-751) were over-expressed in *E. coli* ER2566. Each of the proteins are resolved by SDS-PAGE and analyzed for expression level. The size of GST (lane 1) is 37.8 kDa, GST-Sp1 ID (lane 2, T; lane 3, S) is 36.02 kDa, GST-Sp3 ID (lane 4, T; lane 5, S) is 34.4 kDa, GST-Sp1 ZFDBD (lane 6, T; lane 7, S) is 37.78 kDa, GST-Sp3 ZFDBD (lane 8, T; lane 9, S) is 40.97 kDa, GST-Sp4 ZFDBD (lane 10, T; lane 11, S) is 40.09 kDa (T, total protein. S, soluble protein). **(B)** Purification of recombinant proteins A, GST only; B, GST-Sp1 ID; C, GST-Sp3 ID; D, GST-Sp1 ZFDBD; E, GST-Sp3 ZFDBD; F, GST-Sp4 ZFDBD

2. The ZFDBDs and IDs of Sp-family proteins can repress transcription once targeted to proximal promoter by GAL4 DBD system

Our preliminary experiments showed that the Sp1 ZFDBD can interact with mSin3A, HDAC and POZ-domain. Accordingly, we suspected that transcription factor modules such as ZFDBDs and IDs of Krüppel-like zinc finger superfamily may interact with other polypeptides and this interaction may be important in the regulation of transcription. We constructed several GAL4 fusion expression plasmids by subcloning genes encoding Sp-family ZFDBDs or IDs into pBIND (Promega). CV-1 cells were transiently transfected with the GAL4 fusion expression plasmids. Our data showed that ZFDBDs and IDs were able to repress potently (Fig. 3). The repression extent of Krüppel-like ZFDBD was 35% to 90%. The IDs of Sp1 and Sp3 repress transcription by more than 90%. Most of the GAL4 fused ZFDBDs and IDs reduced transcription of the reporter gene compared to GAL4 DBD control. However, GAL4 Sp1 ZFDBD was not able to repress transcription below the control level. These data suggest that most of the ZFDBDs or IDs can repress transcription. And transcriptional repressions were more prominent with the IDs than with the ZFDBDs. The transcription repression varies significantly depending on the member of the Sp-family.

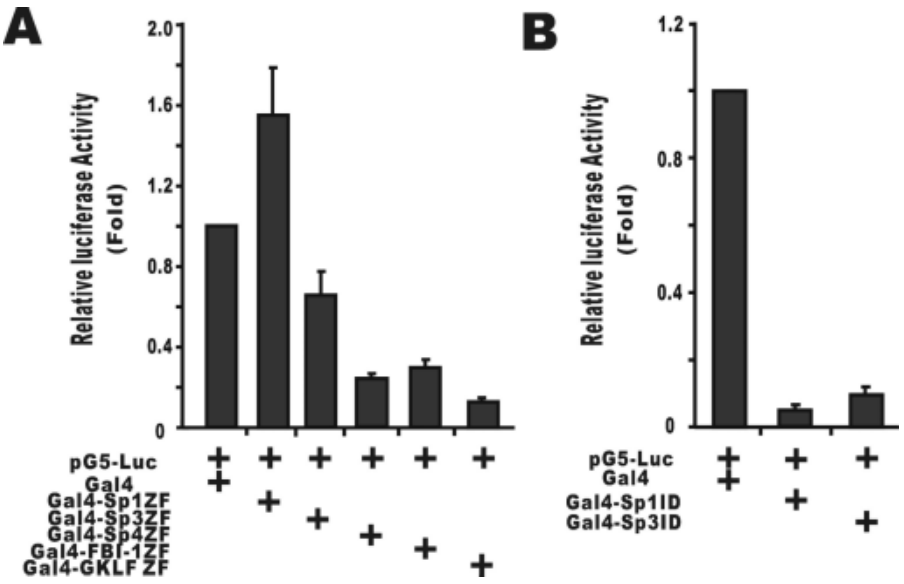

Figure 3. Transcription repression by Sp-family ZFDBDs and IDs. CV-1 cells were transiently transfected with luciferase reporter plasmid pG5-Luc and GAL4-ZFDBDs, GAL-IDs fusion protein expression plasmids of Krüppel-like family proteins. Cell extracts were analyzed for reporter activities and normalized with β-galactosidase activity. **(A)** Promoter targeted ZFDBDs of Sp3, Sp4, FBI-1, GKLF but not Sp1 ZFDBD repress transcription. **(B)** The IDs of Sp1 and Sp3 potently repress transcription.

3. Dose-dependent transcription repression by ZFDBD and ID of Sp1

The ZFDBDs and IDs were targeted to proximal promoter repressed transcription. To examine the dosage effect, CV-1 cells were transfected with increasing amount of expression vectors (0.1 μg to 0.7 μg) of GAL4-ZFDBD and ID of Sp1. Sp1 ID significantly repressed transcription by more than 80% at 0.7 μg. GAL4-Sp1 ZFDBD did not repress transcription compared to GAL4-DBD control as we observed in Fig. 4. Although GAL4-Sp1 ZFDBD activated transcription by 2 fold at 0.1 μg, additional transcription GAL4-Sp1 ZFDBD did not repress transcription below the control. This data showed that both Sp1 ZFDBD and ID repressed transcription and Sp1 ID repressed transcription much more potently than Sp1 ZFDBD.

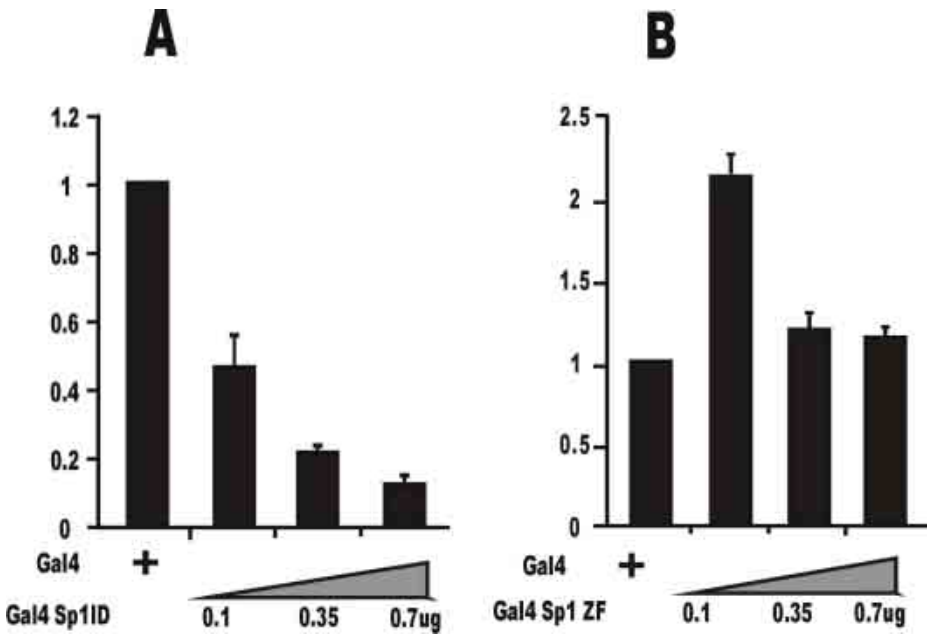

Figure 4. Dose dependent transcription repression by ZFDBD and ID of Sp1. CV-1 cells were transiently transfected with increasing amount of expression of GAL4-Sp1 ZFDBD, Sp1 ID and luciferase reporter plasmid pG5-Luc. Cell extracts were analyzed for reporter activities and normalized with β-galactosidase activity. **(A)** Promoter activity is significantly decreased by increasing amount of Sp1 ID expression vector. **(B)** Sp1 ZFDBD do not decrease transcription below the control level.

4. The ZFDBDs and IDs of Sp-family interact with corepressors *in vivo*

Our preliminary experiments showed that Sp1 ZFDBD interacts with mSin3A, HDAC and the POZ domain of FBI. These lines of data suggest that ZFDBD is an important protein-protein interaction motif between Sp1 and other proteins including corepressor. Here, we investigated using mammlian two hybrid system whether the Sp-family ZFDBDs interact with other corepressors such as SMRT, NCoR, and BCoR or not. We constructed GAL4-Sp-family ZFDBDs or IDs and VP16-corepressor expression plasmids. CV-1 cells were transiently transfected with pG5-Luc, GAL4 fusion plasmid, and VP16 fusion plasmid. Then luciferase activity was measured.

Interestingly, the molecular interaction between ZFDBDs or IDs and corepressors varies significantly (Fig. 5A-C). The Sp1 ZFDBD interacted more strongly with SMRT, BCoR but slight weakly with NCoR (Fig. 5A). The interaction between Sp3 ZFDBD and corepressors was very different from other ZFDBDs (Fig. 5B). The interaction of Sp3 ZFDBD and corepressor was clear with NCoR but not so certain with SMRT and BCoR. The interaction of Sp4 ZFDBD and corepressors was strongest among the ZFDBDs tested. The interaction between Sp4 ZFDBD and SMRT was more than 27 fold stronger than the control. And the interaction of Sp4 ZFDBD and NCoR or BCoR was about 10 fold, 17.5 fold respectively. The interaction between IDs of Sp-family and corepressor was about 3.5 fold to 9 fold of the control, and SMRT and NCoR interacted stronger than BCoR. Our data suggest that Sp-family ZFDBDs interact with corepressors. The intensity of molecular interaction vary significantly depending on the Sp-family ZFDBDs. The data suggest that the Sp-family ZFDBDs are not in naked state and form complexes with corepressors. And

the difference in the molecular interaction with corepressors may be the key factor in the transcriptional regulation of Sp-family transcription by modulating the naked state of ZFDBDs.

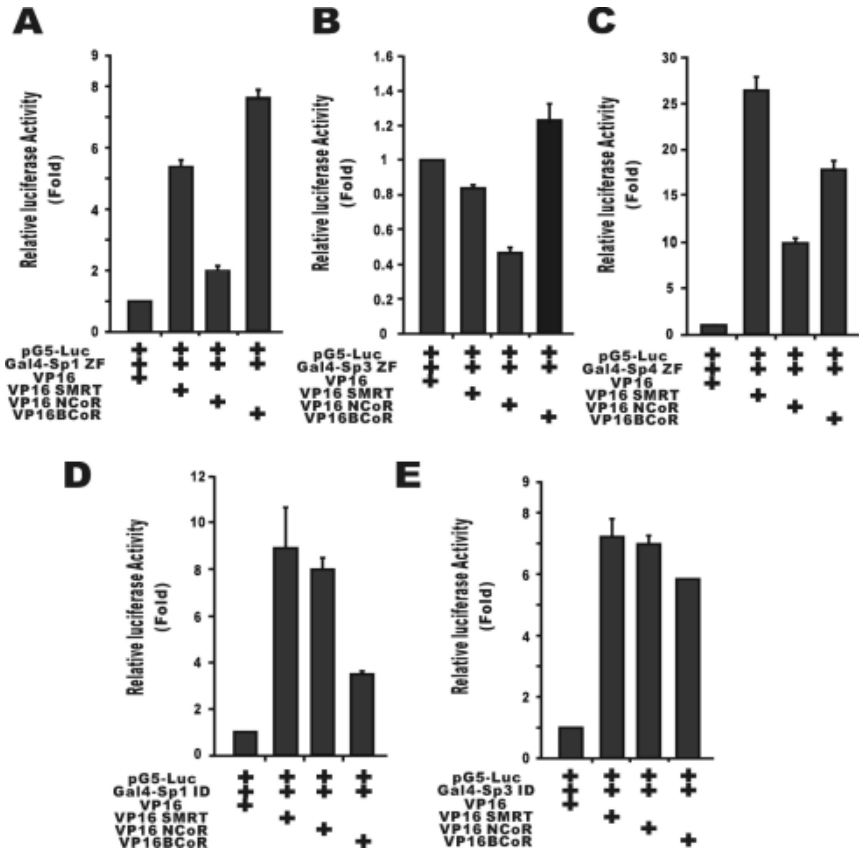

Figure 5. Sp-family ZFDBDs and IDs interact with corepressors *in vivo*. CV-1 cells were transfected with pG5-Luc, GAL4 bait expression plasmid, VP16 expression plasmid, and control pCMV β-gal plasmid. Cells were harvested and analyzed for reporter luciferase activities. The Sp-family ZFDBDs interact with corepressors (**A-C**). Also the IDs of Sp1 and Sp3 interact with corepressors (**D-E**). The interaction considerably differs depending on the domains tested. Sp4 ZFDBD interacts with corepressors most strongly. The interaction of Sp3 ZFDBD with NCoR is clear but not so certain with SMRT and BCoR.

5. The ZFDBDs and IDs of Sp-family interact with corepressors *in vitro*

To investigate whether the interaction between Sp-family ZFDBDs and corepressors is direct or not, we performed *in vitro* GST-pull down assay. The GST and GST-Sp-family ZFDBDs or GST-IDs bound to agarose was incubated with *in vitro* translated [^{35}S]-Methionine labelled corepressors. After precipitation and washing of the complex, the precipitants were analyzed by SDS-PAGE and autoradiography. The Sp-family ZFDBDs or IDs directly interacted with corepressors (Fig. 6). The size of SMRT, NCoR, and BCoR polypeptides used was 47.6 kDa, 55 kDa, 69.6 kDa, respectively. Our data show that Sp-family ZFDBDs and IDs are key module mediating the direct molecular interaction between Sp-family and corepressor proteins.

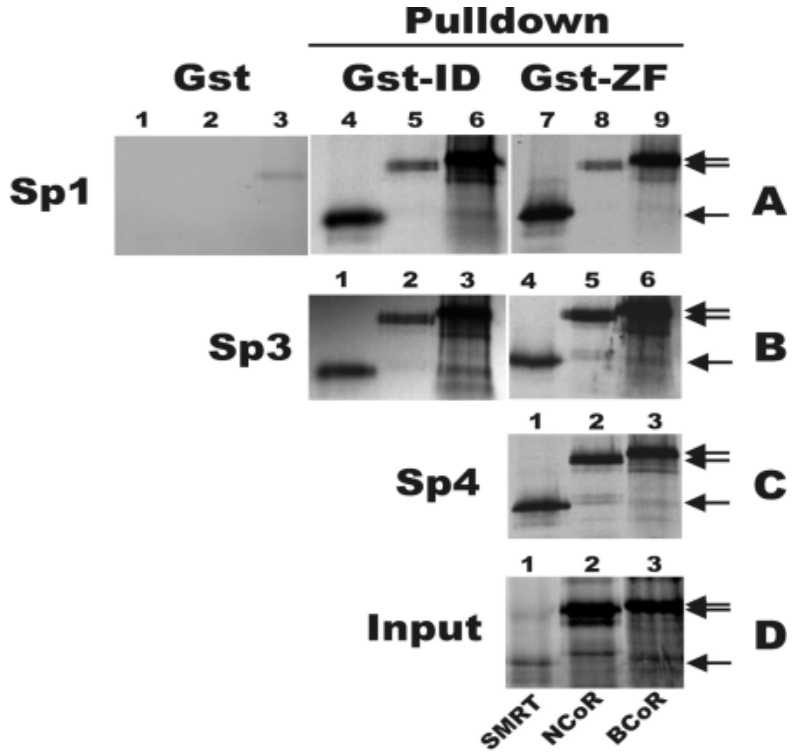

Figure 6. The ZFDBDs and IDs of Sp-family protein directly interact with the corepressors *in vitro*. GST-ZFDBD or GST-Sp ID fusion proteins were incubated with *in vitro* synthesized [^{35}S]-Methionine labelled corepressor polypeptides, and pulled down. Precipitated samples were resolved by 10% SDS-PAGE and autoradiographyed. **(A)** GST-agarose pull down of ID (lanes 4-6) and ZFDBD of Sp1 (lanes 7-9). Lanes 1-3, pull down with GST-agarose only. **(B)** GST-agarose pull down of ID (lane 1-3) and ZFDBD of Sp3 (lane 4-6). **(C)** GST-agarose pull down of ZFDBD of Sp4. **(D)** Input, corepressors added in binding reactions. Arrows indicated the positions of the corepressors pulled down.

6. The interaction of Sp1 ZFDBD with corepressors may be regulated by MAP kinase signaling pathway

It was reported that Sp1 is phosphorylated by Erk2, PKC-ζ, casein kinase, or PKA and dephosphorylated. Also, Sp1 was shown to be glycosylated, and deglycosylated by lipopolysaccharide. Interestingly, Sp3 is silenced by SUMOylation.[37-41] The modifications are important in the regulation of Sp-family transcription functions.

We suspected that the molecular interaction of Sp-family ZFDBDs and corepressors may be controlled by various signaling pathways. First, we suspected that Sp1 binding to GC-box is modified with MAP kinase pathway. We transiently transfected CV-1 cells with reporter plasmid pG5-GC-Luc and the transfected cells were treated with MAP kinase control reagents such as EGF (activator), and U0126 (MEK inhibitor). Our result showed that Sp1 binding to GC-box is activated by EGF and repressed by U0126 (Fig. 7A). Second, we tested whether the interaction between Sp1 ZFDBD and corepressors is influenced by MAP kinases signaling pathway. It was reported that different corepressor pathways regulated differentially. We transiently transfected CV-1 cells with pG5-Luc and GAL4-Sp1 ZFDBD and VP16-SMRT, VP16-NCoR, VP16-BCoR and the transfected cells were treated with MAP kinase control reagents. The interaction of Sp1 ZFDBD between SMRT is decreased by MAP kinase reagents regardless of activator and repressor (Fig. 7B). It was reported that interaction surface of protein is shieled by phosphorylation.[44] We guess that this phenomenon is due to the phosphorylation of SMRT by MAP kinase signaling pathway. Also, our data showed that the interaction of Sp1 ZFDBD between NCoR is activated by EGF and repressed by U0126. The interaction of Sp1 ZFDBD

between BCoR is activated by EGF and repressed by U0126 but not to control. Our data suggest that the interaction of Sp1 ZFDBD between corepressors is regulated MAP kinase signaling pathway variously although the details of regulation is obscure at this time.

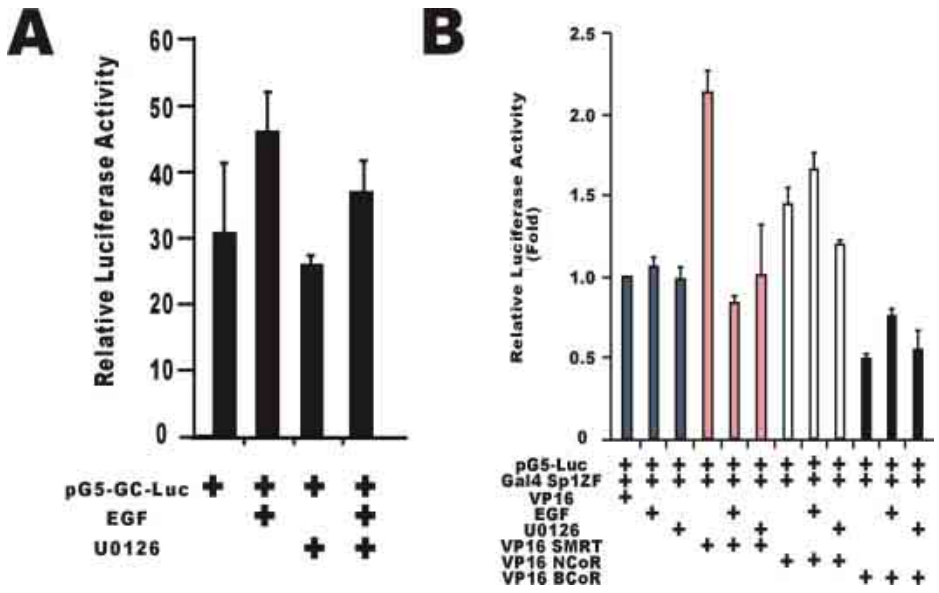

Figure 7. Interaction of Sp1 ZFDBD with corepressors may be regulated by MAP kinase signaling pathway. (A) CV-1 cells were transiently transfected with reporter plasmid pG5-GC-Luc. After 24 hrs, cells were treated with MAP kinase regulatory reagents such as EGF (50 ng/ml), U0126 (10 μM). **(B)** The interaction between the bait (ZFDBD) and fishes (corepressor) was analyzed by mammalian two hybrid assays in the presence or absence of activator (EGF) or inhibitor (U0126) of MAP kinase signaling pathway. Interaction of Sp1 ZFDBD with VP16 is not affected either by EGF or U0126, but interaction of Sp1 ZFDBD with corepressors (SMRT, NCoR, BCoR) is regulated by MAP kinase regulators.

IV. Discussion

The molecular mechanism of key biological processes can be understood by investigating the network of protein-protein interactions involved. Sp1 transcription factor regulates many cellular and viral gene expression by binding to the GC-boxes located in the proximal promoter. Sp1 was firstly purified and isolated as a transcription factor recognizing the adenovirus major late promoter (AdML) and form a family with the factors like Sp2, Sp3, and Sp4.[45] So far, Sp1 was shown to interact with various proteins (e.g., TBP, dTAF110, TAFII130, TAFII55, Rb, YY1, E2F, CRSP) and the interaction proved to be very important in the regulation of many cellular functions.[6-10]

We and other investigators recently found that the most highly conserved part of Sp1, i.e. the ZFDBD, is involved in the protein-protein interaction with other proteins such as the POZ-domains of FBI-1, mSin3A, and histone deacetylase 1 (HDAC1). In this study, we investigated and found that the Sp1 ZFDBD, previously known to be important only in the recognition of its target sequence, is involved in molecular interaction with other regulatory proteins and the interaction is important in the regulation of Sp1 activity. In addition, we also investigated the novel protein-protein interactions involving Sp-family IDs located at N-terminus with corepressors (SMRT, NCoR, BCoR). The molecular interaction provides a novel and intriguing way of regulating Sp-family transcription factors. Although Sp1 and Sp4 are known as transcription factors in general, the interaction with corepressors can explain how they can act as a transcription repressors in certain instances.[6,12-16] Also, it is expected that the repressor members of the Krüppel like

zinc finger transcription factors family including Sp-family member interact with corepressors and repress transcription by corepressor associated HDAC dependent chromatin compaction.

Our data clearly suggest that the ZFDBDs of Sp family are not in 'naked' state and accordingly cannot freely access its target regulatory sequence. Instead, the ZFDBDs are associated with other molecules, e.g. corepressors in our case. Sp family may be one component of large multiprotein complex having corepressor molecule in it. The intensity of molecular protein-protein interaction varies significantly depending on the ZFDBDs. The ZFDBD of Sp4 interacts with 3 times more strongly than that of Sp1 or Sp3. The difference in the intensity of molecular interaction may be significant in the regulation of various gene expression because the zinc fingers of Sp1, Sp3, and Sp4 recognize their target GC-boxes with almost the same affinity and specificity. Without the difference in the interaction, the transcription of a specific gene may be simply regulated by the context or composition of Sp-family members. We can suspect that transcription may be largely contributed by Sp1 because the molecular interaction involving Sp1 ZFDBD and corepressor is relatively weak compared with Sp3 or Sp4, and ZFDBD of Sp1 are relatively in more 'naked' form. However, in certain instances where the molecular interaction between Sp3 or Sp4 ZFDBDs are weaken by cellular needs, the ZFDBDs of Sp3 or Sp4 become naked, bind to its target GC-box, and control gene transcription.

Our data suggest that the molecular interaction between the ZFDBDs and corepressor may an important regulatory aspect of numerous cellular and viral gene expressions. The molecular interaction may be the target of regulation by various cellular processes. Previously, it was reported that members of Sp-family could be drastically regulated by

various means of modification such as phosphorylation, glycosylation, acetylation and SUMOlylation.[37-41] Also, the Sp1 binding site of $p21^{Waf/Cip1}$ promoter is the target of MAP kinase signaling pathway.[46] We investigated whether MAP kinase signaling pathway regulated the molecular interaction involving ZFDBD. We transfected the cells with mammalian two hybrid vectors and subsequently treated the cells with EGF and U0126. Sp1 reporter pG5-GC-Luc itself responds well to EGF and U0126, suggesting that MAP kinase pathway really control Sp1 activity although its mechanism is obscure at this moment. Interestingly, we also observed some effect of these drugs on the molecular interaction between ZFDBDs and corepressors. Although premature, our data suggest that the interaction can be regulated by cellular signaling process and the type of regulation differs depending on the types of corepressors interacted.

In short, our investigations suggest that the activities of Sp-family transcription factors can be regulated by novel protein-protein interaction involving the ZFDBDs or IDs. The molecular interaction may be important in the regulation of many cellular and viral gene expressions.

V. Conclusion

1. The ZFDBDs of Sp-family transcription factors directly interact with corepressors (SMRT, NCoR, BCoR).
2. The IDs of Sp1 and Sp3 directly interact with corepressors (SMRT, NCoR, BCoR).
3. The molecular interaction of Sp-family proteins with corepressors is highly conserved and their interactions may be important in the regulation of many viral and cellular gene expressions.
4. The interaction of Sp-family protein with corepressors is highly variables and may be regulated by MAP kinase signaling pathway.
5. The ZFDBD, a module previously known to function only in binding to the promoter regulatory *cis*-element, may harness diverse cellular regulatory information via protein-protein interactions and regulates biological processes by controlling transcription.

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국문요약

Sp-family 전사인자의 새로운 단백질-단백질 상호작용에 의한 전사조절

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이정안

Sp1은 수많은 세포 및 바이러스 유전자의 발현에 중요한 전사인자이다. Sp1은 주로 proximal promoter 상의 GC-box에 결합하여 전사를 활성화한다. 우리는 현재까지 promoter의 발현 조절 부위의 인식에만 중요한 것으로 알려진 전사인자의 DNA binding domain (DBD)인 zinc finger가 단백질-단백질 상호작용을 통하여 전사 수준에서의 유전자 발현조절에 중요한 역할을 한다는 것을 발견하였다. Sp1 zinc finger DBD는 POZ-domain, histone deacetylase 등과 결합할 수 있으며, 이러한 상호작용에 의하여, 전사활성에 필요한 Sp1의 조절부위 인식과정이 결정적으로 영향을 받는다. 또한 놀랍게도, 전사활성자 Sp1, Sp4나 억제부류인 Sp3의 경우, 이들의 zinc finger부위가 SMRT, NCoR, BCoR, mSin3A와 같은 종류의 corepressor 단백질과 결합함을 발견하였다. 또한 이들이 갖는 inhibitory domain (ID)들도 이들 단백질들과 단백질-단백질 상호작용함을 보여주었다. 따라서 Sp1 계열의 단백질들은 세포 내에서 노출된 zinc finger로

존재하는 것이 아니라, 이들 단백질들과 결합하여 일차적으로 target 인식이 봉쇄된 형태로 존재하는 것으로 생각된다. 흥미로운 것은 각 단백질간의 이러한 상호작용의 정도가 상당한 차이를 보이며, 이러한 차이가 비록 이들 zinc finger가 노출된 상황에서 GC-Box에 동일한 친화성을 갖고 결합할 수 있지만, 생체 내에서 어떤 Sp-family 전사인자가 실제로 target을 인식하는가를 결정하는 중요한 요인이 될 수 있다. 아울러 이러한 zinc finger-corepressor 단백질간의 상호작용은 MAP kinase 신호전달 경로의 조절자들에 의하여 그 상호작용이 조절되어, 노출된 zinc finger의 양을 조절함으로서 유전자 발현조절을 달성하는 것으로 생각되고 있다.

핵심되는 말: Sp1, zinc finger DNA binding domain (ZFDBD), inhibitory domain (ID), corepressor, MAP kinase