Direct interaction of C/EBP δ and Sp1 at the GC-enriched promoter region synergizes the IL-10 gene transcription in mouse macrophage

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Summary

We previously reported that LPS activates the transcription of the IL-10 gene through the Sp1 and C/EBP binding sites and indicated that Sp1, C/EBP β and C/EBP δ can coactivate the IL-10 gene expression in mouse macrophage cells [Liu Y.-W., Tseng H.-P., Chen L.-C., Chen B.-K., Chang W.-C. *J. Immunol.* 171: 821–828, 2003]. In the present report, we demonstrated the direct physical interaction between C/EBP δ and Sp1, and also mapped the interaction domains of these two proteins. C/EBP δ binds to Sp1 via its basic region leucine zipper domain. The C-terminus of Sp1 was also the major region interacting with C/EBP δ . However, both glutamine- and serine/threonine-rich homologous regions of Sp1 also interacted with C/EBP δ . The binding of Sp1 and C/EBP δ as a complex to the Sp1 binding site on the promoter of IL-10 was further confirmed by using the DNA affinity precipitation assay. By using Sp1-deficient SL2 cells, we also found that the overexpressions of C/EBP δ and Sp1 synergistically activate the transcriptional activity of IL-10 gene. Taken together, our present results revealed a novel mechanism of a superactivation of Sp1 by C/EBP δ via a direct interaction between these two transcription factors leading to the activation of the IL-10 gene in mouse macrophage cells.

Introduction

GC (GGGGCGGGG) boxes are important cis-acting DNA regulatory elements required for the transcriptional regulation of many housekeeping, tissue-specific, viral and inducible genes [1–3]. The transcription factor Sp1 binds and acts through GC boxes to regulate gene expression. The Sp1 protein contains several important domains including the N terminus-inhibitory domain (ID), two serine/threonine-rich domains, two glu-

tamine-rich domains, three conserved Cys2His2 zinc finger DNA binding domains and a C terminus D domain [4]. Sp1 has been shown to associate directly with members of the basal transcription machinery such as TBP components [5]. In addition, Sp1 could physically interact and cooperate functionally with other transcriptional activators including ER α [4], c-Jun [6, 7], p53 [8], SMRT, NcoR, BcoR [9], and C/EBP β [10]. However, little is known about the interaction between Sp1 and C/EBP δ .

C/EBP δ is a member of the C/EBP family. Six of the members of the C/EBP family had been identified, and were designated as C/EBP α , $-\beta$,

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$-\delta$, $-\gamma$, $-\epsilon$ and $-\zeta$ [11, 12]. All of these members contain a conserved basic leucine zipper (bZIP) domain at the C-terminus. All C/EBP isoforms share a $> 90\%$ homology in the 55–65 amino acid residues at the C-terminus, which contains the bZIP domain, whereas the N-termini of C/EBP proteins are quite divergent ($< 20\%$ homology). C/EBP family members may form homodimers or heterodimerize with other members of the family [13–17]. They may also heterodimerize with other bZIP members and form thus dimers including C/EBP α –NF- κ B [18], C/EBP β –NF- κ B [19–22], C/EBP δ –NF- κ B [22], C/EBP β –C/ATF (C/EBP-related activating transcription factor) [23], and C/EBP β –CREB (cAMP response element (CRE)-binding protein) [24]. Of these, the leucine zipper of C/EBP β and the Rel homology domain of NF- κ B were reported to be involved in their binding [19–22].

Interleukin-10 is a pleiotropic cytokine that regulates a variety of functions in hemopoietic cells. The principal function of IL-10 is to mediate the down-regulation of Th1 responses by inhibiting the production of IL-12 in macrophages, IFN- γ in NK cells, and most proinflammatory cytokines [25–33]. The immunosuppressive functions of IL-10 are important in protecting the host from harmful effects caused by prolonged inflammatory responses after microbial infections [26, 29]. In addition to possessing anti-inflammatory effects, IL-10 can also impair the immune function inasmuch as T cell responses are restored by the treatment of anti-IL-10 antibodies in leprosy and in HIV⁺ patients [34, 35]. Besides these anti-inflammatory functions, many studies have demonstrated the association of abnormal expression of IL-10 with certain diseases. For example, a high expression of IL-10 has been suggested to play an exacerbating role in systemic lupus erythematosus patients. Polymorphisms in the human IL-10 promoter have also been shown to associate with the severity of multiple autoimmune disorders such as lupus [36–38], rheumatoid arthritis [39] as well as various cancers [40–43]. Two areas of multiple CA dinucleotide repeat polymorphism in the 5'-flanking region of human IL-10 are involved in genetic predisposition in certain diseases [44].

In a previous study [45], we reported that lipopolysaccharide (LPS) activates the transcription of the IL-10 gene promoter through the Sp1 and C/EBP binding sites, and that Sp1, C/EBP β or

C/EBP δ could coactivate IL-10 gene expression in mouse macrophage cells. The protein interaction between Sp1 and C/EBP β or between Sp1 and C/EBP δ upon the LPS treatment was also confirmed by coimmunoprecipitation [45]. In the present study, we studied further the interaction between C/EBP δ and Sp1, mapped the protein–protein interaction domains, and found that the major interaction domains of C/EBP δ and Sp1 were located in their C-termini. We also demonstrated that the interaction between C/EBP δ and Sp1 causes Sp1 to bind to the GC-rich binding site on the promoter, thus initiating the transcriptional activation of mouse IL-10.

Materials and Methods

Materials

LPS (*Salmonella typhosa*) was from Sigma-Aldrich (St. Louis, MO). The luciferase assay system and TNT coupled reticulocyte lysate system were from Promega (Madison, WI). The endotoxin-free plasmid purification kit was from QIAGEN (Valencia, CA). [³⁵S]Methionine (1000 Ci/mmol) and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Biosciences (Buckinghamshire, UK). DMEM, Schneider's *Drosophila* medium, and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY). Antibodies against Sp1, C/EBP δ and GST were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents used were of the highest purity obtainable.

Construction of expression vectors

pET28a-C/EBP δ ₁₂ was obtained from the 1.0 kb fragment of the murine C/EBP δ (generously provided by Dr. S. Mcknight, University of Texas Southwestern Medical Center) that the N-terminal murine C/EBP δ was fused in-frame with HIS polypeptide into the *Eco*RI/*Hind*III sites of pET-28a vector (Novagen) into the *Eco*RI/*Hind*III sites. To create pGEM4T-1 C/EBP^N(1–124), pGEM4T-1 C/EBP^M(121–191), pGEM4T-1 C/EBP(189–268)^C and pGEM4T-1 C/EBP(1–268), PCR-generated fragments encoding amino acids 1–124, 121–191, 189–268 and 1–268 of the murine C/EBP δ were inserted into the *Bam*HI/*Eco*R I

sites of pGEM4T-1 (Amerham Biotech), respectively. pPacC/EBP δ was generated by inserting the murine C/EBP δ into *SpeI/KpnI* sites of pPac5c-PL vector (generously provided by Dr. C. Thummel, University of Utah School of Medicine).

Expression of GST-fusion proteins

Escherichia coli containing GST-Sp1, GST-C/EBP δ (1–268), GST-C/EBP δ^N (1–124), GST-C/EBP δ^M (124–192), and GST-C/EBP δ^C (189–268) expression vectors were cultured in LB medium, and GST-fusion proteins were induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 6 h at 37°C. Expression of GST-C/EBP δ (1–268), GST-C/EBP δ^N (1–124), GST-C/EBP δ^M (124–192), GST-C/EBP δ^C (189–268) was induced with 250 μ M IPTG for overnight at 25°C. Cells were harvested, and incubated in PBS buffer containing 0.2–1% Triton X-100, 2 μ g/ml lysozyme, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 2 μ g/ml leupeptin and 0.5 μ g/ml pepstatin at 37°C for 1 h. After sonication, samples were centrifuged at 5000 $\times$ g for 30 min, and the supernatant was used for GST-pull down assay.

Expression of HIS-fusion proteins

HIS-C/EBP δ (1–268) was expressed in BL21 (DE3) cells in LB medium, followed by the induction with 1 mM IPTG at 37°C for 4 h. Cells were harvested and incubated in 20 mM phosphate buffer (pH 7.4) containing 5.4 mM KCl, 1.276 M NaCl, 2% glycerol, 1% Triton X-100, 2 mM PMSF, 4 μ g/ml leupeptatin, 1 μ g/ml pepstatin, 8 μ g/ml lysozyme and 0.4 U/ml DNase I at 37°C for 1 h and then sonicated. Samples were centrifuged at 5000 $\times$ g for 30 min. The supernatant containing His-fusion protein was denatured in 8 M urea and then kept on ice for 1 h. Denatured proteins were loaded and rotated on Talon Metal Affinity Resin (BV Biosciences) overnight at 4°C and then washed with 10 volumes of 10 mM phosphate buffer (pH 7.4) containing 2.7 mM KCl, 638 mM NaCl, 0.5% Triton X-100, 8 M urea for once. Samples were further washed with 10 mM phosphate buffer (pH 7.4) containing 2.7 mM KCl, 638 mM NaCl for twice. Proteins were eluted with elution buffer containing 50 mM sodium phosphate (pH 6.0) with further addition of 500 mM NaCl and

150 mM imidazole, and dialyzed sequentially in buffer containing 25 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA and 10% glycerol. After dialysis, the purified HIS-C/EBP δ was aliquoted and stored at –80°C.

Synthesis of the [³⁵S]methionine-labeled proteins

pKS Sp1 was constructed by inserting the 2.4 kb fragment of the human Sp1 (generously provided by Dr. G. Suske, University of Marburg, Germany) into *XbaI/HindIII* sites of pBluescript KS vector by *XbaI/HindIII* sites. To create pTOPO-T7 Sp1 Δ A(Δ 1–253), pTOPO-T7 Sp1 Δ B(Δ 254–533), pTOPO-T7 Sp1 Δ C(Δ 533–607), pTOPO-KS-T7 Sp1 Δ D(Δ 605–778), pTOPO-Sp1D(612–778), PCR-generated fragments were ligated into pCRII-TOPO by TA cloning (Invitrogen), respectively. Expression of proteins *in vitro* was performed by using the coupled *in vitro* transcription/translation (TNT) system according to the manufacturer's instructions. Labeling of the *in vitro* expressed proteins was performed by the inclusion of 20 μ Ci of [³⁵S]methionine in the TNT reaction mixture.

GST pull-down assay

Glutathione-Sepharose 4B beads were equilibrated in PBS and mixed with bacterially expressed GST fusion proteins on a rotary shaker overnight at 4°C. The beads were washed three times with 10 volumes of PBS containing 1% Triton X-100, and then washed one time with interaction buffer containing 20 mM HEPES, 100 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mg/ml BSA, 0.5 mM DTT, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 1 mM orthovanadate, 0.5 mM PMSF and 1 mM NaF. Fifty microliters of a 1:1 bead slurry were combined with 300–500 ng of purified protein (HIS-C/EBP δ) or 25 μ l of ³⁵S-labeled reticulocyte lysates in a final volume of 300 μ l of interaction buffer on a rotary shaker for 2 h at 4°C. The beads were then washed three times with interaction buffer, and the bound proteins were eluted by boiling in Laemmli SDS-PAGE loading buffer and subjected to SDS-PAGE. Bound proteins were visualized by autoradiography or Western blotting.

Cell culture

Murine macrophage RAW264.7 cells were grown and subcultured at 37 °C under 5% CO₂ in 75-cm² plastic flasks containing 12 ml of DMEM supplemented with 10% FBS. SL2 cells were grown at 25 °C in 75-cm² plastic flasks containing 10 ml of Schneider's *Drosophila* medium supplemented with 10% FBS.

Nuclear extract preparation

Confluent cells were collected in 1 ml of PBS, centrifuged at 400 × *g* for 10 s and then resuspended in 0.4 ml of buffer A containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM PMSF, 1 mM orthovanadate, 2 µg/ml pepstatin A and 2 µg/ml leupeptin at 4 °C for 10 min. Nuclei were pelleted by centrifugation at 400 × *g* for 10 s. Pellets were resuspended in 0.1 ml of buffer B containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol (v/v), 0.5 mM PMSF, 1 mM orthovanadate, 2 µg/ml pepstatin A and 2 µg/ml leupeptin at 4 °C for 20 min. The suspension was pelleted by centrifugation at 400 × *g* for 2 min, and supernatants were collected and stored at −80 °C.

Transfection of SL2 cells by calcium phosphate method

SL2 cells were plated onto 12-well plastic dishes at a density of 4 × 10⁵ cells and were transfected by the calcium phosphate method [46]. Cells were transfected with 0.2 µg of luciferase reporter plasmid, 0.8 µg of pPac-Sp1, and 0.4 µg of pPac-C/EBPδ for 48 h, and then luciferase activity assay was performed as previous report [45]. Variable amounts of expression plasmids were compensated with the empty vector pPac.

DNA affinity precipitation assay (DAPA)

The assay was performed according to the method of Zhu et al. [47] with a slight modification. The binding assay was performed by mixing 100 µg of nuclear extract proteins or 300 ng of purified HIS-C/EBPδ or 25 µl of *in vitro* transcribed-translated Sp1 with 2 µg of biotinylated IL-10-specific Sp1 or C/EBP oligo-

nucleotides, and 20 µl of streptavidin-agarose beads in 380 µl of binding buffer containing 20 mM Tris-HCl (pH 7.9), 10% glycerol, 1 mM EDTA, 5 mM MgCl₂, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 M NaCl and 1 mM DTT. Samples were incubated at room temperature for 1 h with rotating. Beads were pelleted and washed three times with cold PBS containing 1% Triton X-100. The binding proteins were eluted by loading buffer and separated by SDS-PAGE, followed by Western blot analysis probed with specific antibodies. 5'-Biotinylated wild-type and mutated Sp1 sequences were 5'-biotin TTAGAAGAGGGAG-GAGGAGCCTGA-3' (Sp1-WT) and 5'-biotin-TTAGAAGAGGGATTATTAGCCTGA-3' (Sp1-Mut), respectively. 5'-Biotinylated wild-type and mutated C/EBP sequences were 5'-biotin-AACAATTATTTCTCAATCCTAATA-3' (C/EBP-WT) and 5'-biotin-AACAATTAGTACTCAATCC TAATA-3' (C/EBP-Mut), respectively.

Western blotting

Analytical 10% SDS-polyacrylamide slab gel electrophoresis was performed. Samples including 30 µg of nuclear protein, 30–50 ng of purified protein, glutathione-Sepharose beads of GST pull-down assay or Streptavidin-agarose beads of DAPA experiment were mixed with SDS loading buffer, followed by boiling for 5 min. For immunoblotting, proteins on the SDS gels were transferred to a polyvinylidene difluoride membrane by an Electrobolt apparatus (Bio-Rad, Hercules, CA). After blocking with 5% non-fat dried milk in PBS buffer, the blots were incubated with antibodies against specific proteins as the primary antibodies, individually, and the horseradish peroxidase-conjugated secondary antibodies were added. Bands were detected by an enhanced chemiluminescence kit from Pierce Biotechnology (SuperSignal, Rockford, IL).

Results

Direct interaction between Sp1 and C/EBPδ

Previously we found the coimmunoprecipitation of Sp1 and C/EBPδ in the nuclear extracts of RAW264.7 cells when cells were treated with

Figure 1. Interaction between Sp1 and C/EBP δ *in vitro*. GST-Sp1 and HIS-C/EBP δ fusion proteins were expressed in *E. coli* and purified as described in Materials and methods. GST pull-down assay was performed. Purified GST (250 ng) or GST-Sp1 crude extract (500 μ l) was allowed to react and conjugate with glutathione-Sepharose beads. The GST- and GST-Sp1 coupled Sepharose beads were then incubated with 300 ng of purified HIS-C/EBP δ in the interaction buffer, and the His-C/EBP δ that bound to GST-Sp1 was then analyzed by Western blotting using anti-C/EBP δ antibodies (lane 3). The GST and GST-Sp1 conjugated on Sepharose beads were also confirmed by Western blotting using antibodies against GST and Sp1 antibodies, respectively (lanes 3 and 4). Purified HIS-C/EBP δ and GST-Sp1 conjugated with Sepharose beads were loaded as input control (lanes 1 and 2), respectively.

LPS, and that an overexpression of C/EBP δ could activate the IL-10 gene expression in a synergistic manner [45]. In order to study whether Sp1 interacts directly with C/EBP δ , we first performed an *in vitro* GST pull-down assay, in which bacterially expressed HIS-C/EBP δ was purified and analyzed for its retention by a GST-Sp1 affinity resin. The interaction between purified HIS-C/EBP δ and GST-Sp1 was elucidated by using the GST pull-down assay (Figure 1; lanes 1 and 2). In the assay, HIS-C/EBP δ was retained by the GST-Sp1 but not by the GST resin (Figure 1; lane 3 versus lane 4, respectively). In a reciprocal experiment, the *in vitro* synthesized 35 S-labeled Sp1 bound specifically to a resin carrying GST-C/EBP δ but not to a resin carrying only GST (upper panel of Figure 2B, compare lane 2 with lane 3). These results indicated that Sp1 and C/EBP δ directly interact with each other.

The C terminus of C/EBP δ is required for physical interaction with Sp1

In order to study the C/EBP δ domain involved in the interaction with Sp1, GST pull-down assay was performed by using GST-linked mutant proteins of C/EBP δ and a wild-type Sp1 labeled with 35 S. A diagram of the truncated mutants of C/EBP δ is shown in Figure 2A. C/EBP δ ^N(1–124) lacks the inhibition domain and the C-terminal tail; C/EBP δ ^M(124–192) lacks the activation domains of an N-terminus and a C-terminal tail; C/EBP δ ^C(189–268) contains the bZIP at C-terminus but lacks the N-terminus activation and inhibition domains. Direct protein–protein interaction assay indicated that C/EBP δ ^C(189–268), in contrast to C/EBP δ ^M and C/EBP δ ^N, contains a major domain able to interact with Sp1 (upper panel of Figure 2C, lane 1 versus lanes 2, 3 and 4) under the same experimental conditions with equal amounts of mutant proteins in the assay (Figure 2C, bottom panel). The truncated mutant of C/EBP δ with a C-terminal region (189–268) containing a bZIP domain bound specifically to 35 S-labeled Sp1, whereas C/EBP δ ^M and C/EBP δ ^N, which lack the C-terminal region, were unable to bind to Sp1. These results indicated that the C-terminal region (189–268) of C/EBP δ is required for its interaction with Sp1.

C-terminus region containing the DNA binding and the activation D domain of Sp1 essential for interaction with the C-terminus of C/EBP δ

To study the region of Sp1 involved in its interaction with C/EBP δ , the same GST pull-down assay was performed by using GST-C/EBP δ and 35 S-labeled Sp1 mutants synthesized *in vitro*. The truncated mutants include Sp1 Δ A (Δ 1–253), Sp1 Δ B (Δ 254–553) and Sp1 Δ C (Δ 553–607), lacking respectively the activation A domain, the activation B domain and the activation C domain (Figure 3A). The fourth mutant Sp1 Δ D (Δ 605–778) lacks the C-terminus DNA binding domain (DBD) and the activation D domain (Figure 3A). In the GST pull-down assay, same amount of GST-C/EBP δ was used in the reaction mixtures as confirmed by the SDS-PAGE stained with Coomassie blue (lower panel of Figure 3B). The binding of 35 S-labeled Sp1 and its truncated mutants to GST-C/EBP δ was detected by autora-

Figure 2. The C-terminal C/EBP δ is required for physical interaction with Sp1. (A) Schematic representation of the GST-fused full-length and truncated C/EBP δ forms used in GST pull-down assays is shown. The transactivation domains (AD) are shown as gray boxes, inhibition domain (ID) as striped boxes and basic leucine zipper (bZIP) domain as black boxes. Interaction of wild-type C/EBP δ (B) and various truncated C/EBP δ forms (C) with Sp1 was analyzed by the GST pull-down assay. GST and GST-C/EBP δ fusion proteins were expressed in *E. coli*, and purified as described in Materials and methods. The extracts of GST, GST-C/EBP δ^N (1-124), GST-C/EBP δ^M (124-192), GST-C/EBP δ^C (189-268) and GST-C/EBP δ (1-286) were conjugated with glutathione-Sepharose beads, respectively, and then reacted with 25 μl of ^{35}S -labeled Sp1 synthesized *in vitro*. After reaction, the bound GST-fused C/EBP δ proteins and ^{35}S -labeled Sp1 were separated by SDS-PAGE. ^{35}S -labeled Sp1 reacted with GST-C/EBP δ proteins was analyzed by autoradiography (upper panels of B and C). The GST and GST-C/EBP δ proteins reacted with Sp1 were detected by using Coomassie blue staining (lower panels of B and C). The relative position of molecular mass standards is shown in kD (lane 5). Input controls using 10% of the *in vitro* transcription-translation reaction mixture used in the GST pull-down assay, and the bovine serum albumin (BSA) were also performed. Asterisks indicate the mobility position of GST, and various fragments of GST-C/EBP δ on SDS-PAGE.

diography (upper panel of Figure 3B). The interaction between GST-C/EBP δ and wild-type Sp1 was confirmed (compare lane 3 with lane 2). In experiments using the truncated mutants of Sp1, the interaction between Sp1 ΔC and C/EBP δ was most apparent (lane 12 versus lane 11), indicating that the activation C domain was not required for the interaction. Contrast to that seen with Sp1 ΔC , the interaction of C/EBP δ with Sp1 ΔA (compare lane 6 with lane 5) or Sp1 ΔB (compare lane 9 with lane 8) was weaker, indicating the activation domains of A and B on Sp1 might play a role in Sp1 interaction with C/EBP δ . Among the four structural domains on Sp1, the C-terminus including the DNA binding domain and the activation D domain is the major Sp1 region interacting with

C/EBP δ since binding between Sp1 ΔD and C/EBP δ was observed (compare lane 15 with lane 14). This notion was further supported in the GST pull-down assay by using ^{35}S -labeled Sp1D (612-778) synthesized *in vitro* (Figure 4A). The interaction between Sp1D (612-778) and C/EBP δ was used as a positive control (Figure 4B; lane 3 versus lane 2). We studied the interaction between Sp1D (612-778) and the truncated C/EBP δ mutants including C/EBP δ^N , C/EBP δ^M and C/EBP δ^C . We found that Sp1D only interacted with C/EBP δ^C (Figure 4C; lane 4 versus lanes 2 and 3). These results clearly indicated that the C-terminus (612-778) containing the DNA binding domain and the activation D domain of Sp1 is the major site Sp1 used to interact with C/EBP δ . Moreover,

Figure 3. Analysis of Sp1 domains interacting with C/EBP δ . (A) Schematic representation of the full-length and truncated Sp1 forms used in the GST pull-down assay is shown. The serine/threonine-rich subdomains of the transactivation domains are shown as gray boxes, the glutamine-rich subdomains as striped boxes and the zinc fingers on C-terminus DNA binding domains (DBD) are shown as three small black squares. (B) The domains of Sp1 interacted with C/EBP δ were then analyzed by GST pull-down assay. An aliquot of GST-C/EBP δ was incubated with 25 μ l of the *in vitro* synthesized ³⁵S-labeled Sp1, Sp1 Δ A, Sp1 Δ B, Sp1 Δ C or Sp1 Δ D proteins. The bound ³⁵S-labeled Sp1 with GST-C/EBP δ was analyzed by SDS-PAGE. The quantity of GST-fusion proteins was confirmed by PAGE stained with Coomassie blue (lower panel), and the bound ³⁵S-labeled Sp1 and its truncated mutants proteins were detected by autoradiography (upper panel). One-tenth of the *in vitro* transcription-translation reaction mixture used in the GST pull-down assay was used as input control. Asterisks indicate the mobility positions of Sp1 and its truncated mutant mutants on gel of SDS-PAGE.

the two homologous glutamine- and serine/threonine-rich regions A and B of Sp1 might also play, albeit to a lesser extent, some functional role in the Sp1 interaction with C/EBP δ .

Sp1-C/EBP δ complex binds to the Sp1-responsive motif on the IL-10 promoter

Since both Sp1 and C/EBP δ directly interacted with each other mainly through their C-termini,

we studied the binding site of the Sp1-C/EBP δ complex on the gene promoter of IL-10 by DAPA experiments. Several potential response elements are presented in the 553-bp promoter of the mouse IL-10 gene (Figure 5A) including one Sp1 and three C/EBP binding sites. The C/EBP binding sequence (−403/−380 bp) and the Sp1 binding site (−98/−75 bp) selected as DAPA probes are the major LPS response elements [45]. The C/EBP and Sp1 binding sites were mutated as shown in

Figure 4. Direct physical interaction between C-terminal domains of Sp1 and C/EBPδ. (A) Schematic representation of the C-terminus fragment (612–778) of Sp1 (Sp1D) used in the GST pull-down assay is shown. Interaction of Sp1D with C/EBPδ (B) and the truncated mutant proteins of C/EBPδ (C) was studied. An aliquot of purified GST-fused C/EBPδ and its truncated mutant proteins was incubated with 25 μ l of the *in vitro* synthesized ³⁵S-labeled Sp1 D. After reaction, the bound GST-fused proteins and ³⁵S-Sp1D were analyzed by SDS-PAGE. The amount of GST-fused proteins including C/EBPδ and its mutants were confirmed by SDS-PAGE stained with Coomassie blue (lower panels of B and C), and the ³⁵S-labeled Sp1D interacted with GST-fused proteins was detected by autoradiography (upper panels of B and C). One-tenth of the *in vitro* transcription–translation reaction mixture used in the GST pull-down assay was used as the input control.

Figure 5A. From our previous report [45], we know that Sp1 was constitutively expressed whereas the expression of C/EBPδ was induced upon the LPS treatment in mouse macrophage cells (see Figure 5B and 5C; input lanes). We studied by DAPA the binding of Sp1 and C/EBPδ complex to the Sp1 and C/EBPδ binding sequences in the nuclear extracts of the cells treated with or without LPS. As shown in Figure 5B, C/EBPδ in the nuclear extracts prepared from LPS-treated cells, but not that prepared from control cells, significantly binds the wild-type C/EBP probe (Figure 5B, lane 5 versus lane 3). The binding of C/EBPδ to C/EBP binding sequence was abolished if a mutated probe was used (Figure 5B, lane 6), indicating that the binding of C/EBPδ to the C/EBP binding sequence on the IL-10 gene promoter was specific. There was no Sp1 binding to

either wild-type or mutated probe derived from the C/EBP binding sequence. On the other hand, the constitutively expressed Sp1 in the nuclear extracts prepared from LPS-treated and control cells bound equally well to the wild-type Sp1 probe (Figure 5C, lanes 4 and 6). Interestingly, C/EBPδ, prepared from the nuclear extract of LPS-treated cells, bound to wild-type Sp1 probe (Figure 5C, lane 6). However, the binding was abolished when the Sp1 mutant probe was used (Figure 5C, lane 5). These results suggest that the constitutively expressed Sp1 and LPS-induced C/EBPδ in the cells forms a C/EBPδ–Sp1 complex that binds to the gene promoter of IL-10 through the Sp1 binding site. This notion was further confirmed by similar experiments using purified C/EBPδ and ³⁵S-labeled Sp1 synthesized *in vitro*. As shown in Figure 5D, C/EBPδ did not bind to the Sp1

Figure 5. Complex of Sp1 and C/EBP δ binds to GC-rich but not to C/EBP binding sequences on the gene promoter of IL-10. (A) The sequences of the wild-type (WT), Sp1 and C/EBP binding sites on the IL-10 gene promoter and those of mutated (mt) Sp1 and C/EBP binding sites are indicated. (B) and (C) Nuclear extracts were prepared from RAW264.7 cells treated with or without 5 ng/ml LPS for 5 h. The binding of Sp1 and C/EBP δ to Sp1 and C/EBP δ binding sites on gene promoter of mouse IL-10 was analyzed by DAPA as described in Materials and methods. (D) Binding of the *in vitro* synthesized ³⁵S-labeled Sp1 and purified C/EBP δ to the Sp1 binding site on mouse IL-10 gene promoter was analyzed by DAPA. Binding of HIS-C/EBP δ , and ³⁵S-Sp1 to Sp1 binding DNA probe was detected by Western blot analysis for C/EBP δ and by autoradiography, respectively.

binding probe (lane 3), although Sp1 bound directly to the Sp1 probe (lane 4). However, if C/EBP δ was through the pre-existed Sp1 bound to the Sp1 binding site of the IL-10 promoter, an association of C/EBP δ and Sp1 on the probe was observed (lane 5). These results indicated that C/EBP δ binds to the gene promoter of IL-10 through Sp1 which targets the Sp1 binding site in the nucleus when cells were stimulated with LPS.

Functional synergism between Sp1 and C/EBP δ is mediated through the Sp1 element

In order to investigate the functional contribution of the C/EBP and Sp1 binding elements and their

cognate factors to the activation of IL-10 gene, the promoter constructs bearing the C/EBP binding site either with a mutated Sp1 binding site (IG553 Δ Sp1), a Sp1 binding site only (IG122), or with both C/EBP and Sp1 binding sites (IG553), respectively as shown in Figure 6A, were transfected into the Sp1-deficient *Drosophila* Schneider SL2 cells. Effects of the overexpression of C/EBP δ and Sp1 on the transcriptional activity of various promoter constructs are summarized in Figure 6B. Using the promoter (IG553) encompassing C/EBP and Sp1 binding sites, overexpression of cells with C/EBP δ activated the promoter activity only marginally (lane 2 versus lane 1), while overexpression with Sp1 significantly induced the

Figure 6. Synergistic effect of C/EBP δ and Sp1 on the promoter activity of the mouse IL-10 gene through the Sp1 binding site. (A) Appellation of wild-type or Sp1 site-directed mutant and 5'-flanking deletion of the IL-10 promoter fragment bearing luciferase expression plasmid is indicated. (B) Sp1-deficient SL2 cells were transfected with C/EBP δ , Sp1 and luciferase reporter plasmids by calcium phosphate method for 48 h. The luciferase activity was determined and normalized by protein concentrations. In each group, luciferase activity in cells transfected with empty vector pPac was defined as the basal control, and the induction fold was obtained by comparing the luciferase activity induced by expression vectors with the basal activity of vector pPac. Values are means $\pm$ SEM from five independent experiments. Statistical analysis was performed by Student's *t* test.

promoter activity (lane 3 versus lane 1). As expected, the overexpression of the cells with both C/EBP δ and Sp1 synergistically activated the promoter activity of vector IG553 (lane 4 versus lane 3). By using the vector with promoter bearing only the Sp1 binding site (IG122), C/EBP δ overexpression had no effect on the gene promoter activity (lane 6 versus lane 5), whereas an overexpression of cells with Sp1 caused a small induction of the gene promoter activity (lane 7 versus lane 5). Overexpression of cells with both C/EBP δ and Sp1 synergistically activated the promoter activity of vector IG122 (compare lane 8 with lane 7). However, Sp1 and C/EBP δ overexpressions had no effect on the promoter activity of a vector

IG553 Δ Sp1 which contains a mutated Sp1 binding site (lanes 9, 10, 11 and 12). Take together, these results indicated that Sp1 binding site on the gene promoter played a functional role in the regulation of the promoter activity of IL-10 where C/EBP δ interacts with Sp1, leading to the binding of Sp1 to its binding site on the promoter, thus causing the promoter activation.

Discussion

We previously reported that in mouse RAW264.7 macrophage cells the Sp1 binding site residing on the -89/-78 bp of the gene promoter is required

for a basal promoter activity, whereas a C/EBP binding site residing on the $-398/-385$ bp plus the Sp1 binding site are required for the IL-10 gene activation upon the LPS stimulation [45]. We also found in these cells that the LPS treatment induces a C/EBP β or C/EBP δ expression and enhances the interaction between Sp1 and C/EBP β , or C/EBP δ , as demonstrated by coimmunoprecipitation experiments. Since C/EBP δ is more potent in inducing the IL-10 gene activation than C/EBP β in LPS-treated mouse macrophage cells [45], we focused in this report on C/EBP δ , especially on its direct interaction with Sp1, and examined thus protein interaction domains. The direct interaction between C/EBP δ and Sp1 was demonstrated either by using purified GST-Sp1 and HIS-C/EBP δ proteins (Figure 1A), or by purified GST-C/EBP δ and 35 S-labelled Sp1 (Figure 2B). The major interaction domains of Sp1 were mapped and found to reside mainly at the C-terminus (612–778) and, to a lesser extent, in the glutamine- and serine/threonine-rich region A and B. The interacting domain of C/EBP δ with Sp1 was on its

C-terminus (189–268). The major interaction domain of Sp1 comprises the DNA binding domain and a D domain, while that of C/EBP δ comprises a bZIP domain. It was reported that C/EBP δ forms both the homodimers, and heterodimers with bZIP containing proteins through an interaction with the bZIP domains of these proteins similar to that seen in the interaction between C/EBP δ and NF- κ B [22]. However, the C-terminus DNA binding domain of Sp1 contains three conserved Cys2His2 zinc fingers without a leucine zipper. Therefore, the binding of C/EBP δ with the C-terminus of Sp1 may differ from that seen with the bZIP protein family. Interaction domains between Sp1 and c-Jun have been well documented [7]. However, only a subtle difference exists between the two Sp1 binding domains interacting respectively with c-Jun and C/EBP δ . The c-Jun region interacting with Sp1 was mapped within the basic region leucine zipper domain that is very similar to that seen with the C/EBP δ domain interacting with Sp1 as demonstrated in the present study. However, the interaction between

Figure 7. A scheme for the transcriptional regulation of IL-10 gene promoter in mouse RAW264.7 cells. A diagram of response elements and the transcription factors recruited by LPS stimulation to the IL-10 gene promoter described in this text is shown. Binding of Sp1 to Sp1 responsive element is important for the basal level expression of IL-10 gene in control cells. Upon LPS treatment, biosynthesis of C/EBP δ is induced. The induced-C/EBP δ not only binds to C/EBP binding site but also is recruited to Sp1 binding site through the direct interaction with Sp1, followed by the binding of Sp1 in C/EBP δ -Sp1 complex to Sp1 binding site on gene promoter, to synergically activate the gene promoter activity of IL-10 in cooperation with Sp1 in LPS-treated mouse macrophages.

c-Jun and Sp1 requires at least one of the two homologous glutamine- and serine/threonine-rich region A and B and/or the DNA binding zinc finger domain of Sp1 [7]. In this report, we found that the C-terminus DNA binding region of Sp1 is the major domain interacting with C/EBP δ , and that both homologous glutamine- and serine/threonine-rich regions A and B of Sp1 also play a minor role in its interaction with C/EBP δ .

One of the most interesting findings in this report is that the complex of C/EBP δ and Sp1 act synergistically to activate the IL-10 gene promoter through the Sp1 binding to the Sp1 binding site on the IL-10 promoter. C/EBP δ induced by the LPS treatment in the nuclear extract not only bound to the C/EBP binding site but also to the Sp1 binding site through an interaction with Sp1 which acts as an anchor protein recruiting C/EBP δ to the gene promoter after Sp1 binds to the IL-10 promoter (Figure 5). The analysis of protein-protein interaction indicates that the interaction domain between C/EBP δ and Sp1 overlapped with the C/EBP δ DNA binding domain. The result may explain why the complex of Sp1-C/EBP δ was unable to bind to the C/EBP binding site on the gene promoter. On the other hand, the Sp1 interacts with C/EBP δ not only via the Sp1 DNA binding domain, but also partly via the A, B and D domains. Therefore the complex of Sp1 and C/EBP δ was able to bind to the Sp1 binding site on the gene promoter. Two possible models on the binding of the C/EBP δ -Sp1 complex to the Sp1 binding site on the IL-10 promoter are shown in Figure 7. The first model depicts the C/EBP δ -Sp1 complex to be formed in the nucleus, followed by a binding of this complex to the IL-10 gene promoter. The interaction between C/EBP δ and Sp1 was first detected when cells were treated with LPS followed by coimmunoprecipitation [45]. Results from the DAPA experiment (Figure 5C) indicating a specific binding of both Sp1 and C/EBP δ to Sp1 binding site on the IL-10 gene promoter further support this model. An alternative model of formation involves the direct binding of Sp1 to its binding site on the IL-10 gene promoter, followed by the C/EBP δ binding to Sp1 already bound on the Sp1 binding site. The result shown in Figure 5D supports this alternative model.

The interaction between C/EBP δ and Sp1 leading to Sp1 binding to the gene promoter was functional because the interaction activates the

transcriptional activity of IL-10 gene (Figure 6). Since both the C/EBP and Sp1 binding sites on the gene promoter are required for the LPS-induced gene expression of IL-10 in mouse macrophage cells [45], our present results indicate that C/EBP δ acts on both the C/EBP and Sp1 binding sites on the gene promoter of IL-10 to activate the gene transcription. A transcription factor acting through its own response element and also through its interaction with Sp1, followed by the binding of the latter formed complex to the Sp1 response element on a gene promoter was also observed in our previous study examining the functional role of c-Jun and Sp1 in epidermal growth factor (EGF)-induced gene activation of disease-associated keratin 16 in human keratinocytes [48]. In the study of keratin 16 gene expression, we found that both AP1 and Sp1 binding elements on the gene promoter are required for the EGF-induced gene expression. While EGF induces c-Jun to bind directly to AP1 binding element, c-Jun also indirectly binds to the Sp1 binding element through Sp1 to activate the transcriptional activation of keratin 16 gene promoter. Sp1 belongs to a zinc finger family of transcription factors that recognizes the GC-rich DNA sequence. Sp1 also interacts with several other transcription factors that bind to their respective response elements in the regulation of responsive genes including, e.g., YY1 [49, 50], the p65 subunit of NF- κ B [51], GATA1 [52], helicase-like transcription factor [53], OTF-1 [54], and BPV enhancer E2 [55]. Among these transcription factors interacting with Sp1, YY1, GATA-1 and BPV enhancer E2 were also found to indirectly bind to the Sp1 binding site on the gene promoter to activate the gene transcription through their direct interactions with Sp1. However their interactions with Sp1 do not involve the leucine zipper domain whereas C/EBP δ or c-Jun does.

It has been reported that the transcription factor C/EBP family and Sp1 cooperatively activate the gene promoters of rat CYP2D [56], human CD11c [57], lactoferrin [58], and mouse aldose reductase-like gene [59] as studied by the transfection and gel shift assays. However, the direct physical interaction between C/EBP family protein and Sp1 was only speculated but was never proven. Lee et al. [56] also found that glutamine- and serine/threonine-rich domains are required for the Sp1 interaction with C/EBP β in the presence of

DNA. In this study, we provide strong evidence to support the notion that Sp1 directly interacts with C/EBP δ , and that the complex of Sp1 and C/EBP δ transactivates the gene promoter of IL-10 through the complex binding to the Sp1 binding site of the gene promoter, thus contributing to the activation of IL-10 gene transcription in mouse macrophage cells when treated with LPS. Recently, we also found that Sp1 and C/EBP δ cooperatively regulate the promoter activity of rat Mrp 3 gene using the reporter gene assay and the gel shift assay [60]. The results of the present study shed light on the mechanism of gene regulation by showing a cooperation between Sp1 and C/EBP δ which may thus help elucidate the mechanism of action of other transcriptional regulation on the gene expression.

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