CHAPTER II

Molecular mechanism of transcriptional regulation via physical interaction between RAR and Sp1: Analysis on a role of RAR/RXR-derived transactivation

II-1. Introduction

In the chapter I, I described that RA up-regulates the expression of uPA mRNA via a novel mechanism. RARs/RXRs directly interact with a general transcription factor Sp1 and potentiate its binding to the GC box motif within the uPA promoter, culminating in an enhanced transcription of uPA gene. Among several questions remain to be elucidated, one such unresolved argument is concerning the role of RAR/RXR. Do RAR/RXR only modulate Sp1-derived transcription activity via potentiating its DNA binding or do they also exert their transcription activity by binding to the GC box via Sp1? In this chapter, I tested the latter possibility by analyzing the physical interaction between RAR/RXR and a fragment of Sp1, which lacks the transactivation activity but can work as a tether between RAR/RXR and GC box. The data suggested that RAR/RXR did not exhibit their transactivation activity by binding to the GC box via the Sp1 fragment.

II-2. Materials and Methods

Plasmids construction

The expression vectors encoding deletion mutants of Sp1 were constructed by PCR amplification, which utilized the Sp1 cloning vector, pBluescript-Sp1, as a template. Each deletion mutant cDNA was prepared as follows and subcloned into the *Xba* I/*Acc* I site of the pCIneo mammalian expression vector (Promega). For constructing Sp1 ΔN cDNA, which lacks the N-terminus encoding sequence (amino acids 2-257), PCR product was amplified with the primer pairs 5'-ACCTTGCTACCTGTCAACAGC-3' (based on the sense sequence corresponding to nucleic acids 772 to 792 of Sp1 cDNA) and 5'-CATGGGGGGATCCACTAGTT-3' (based on the antisense sequence of the vector located upstream of Sp1 cDNA plus initial methionine), religated at blunt-end, and excised with *Xba* I/*Acc* I. Similarly, ΔM cDNA was constructed by deleting the M region (amino acids 265-548) using the primer pairs 5'-AATGCCCCAGGTGATCATGG-3' (based on the sense sequence corresponding to 1645 to 1664 of Sp1 cDNA) and 5'-GCTGTTGACAGGTAGCAA GG-3' (based on the antisense sequence corresponding to 773 to 792 of Sp1 cDNA). ΔC cDNA was constructed by deleting the C-terminus (amino acids 552-778) of Sp1 using the primer pairs 5'-GCTTCTGAGATCAGGCAC-3' (based on the sense sequence corresponding to 2330 to 2347 of Sp1 cDNA) and 5'-CACCTGGGGCATTTGCTATAGC-3' (based on the antisense sequence corresponding to 1636 to 1657 of Sp1 cDNA). Sp1 C-terminus cDNA fragment was constructed by PCR amplification of cDNA fragment encoding amino acids 549-778 of Sp1 with primer pairs 5'-ATGAATGCCCCAGGTGATCAT-3' (based on the sense sequence corresponding to 1645 to 1662 of Sp1 cDNA plus initial methionine) and 5'-TGCCTGATCTCAGAAGCCATT-3' (based on the antisense sequence corresponding to 2326 to 2346 of Sp1 cDNA), subcloned first into the pGEM-T

Easy vector (Promega), and then finally inserted into the *Eco* RI site of either the pCIneo vector or pGEX6P-3 vector (Pharmacia).

GST fusion protein interaction assay

The *in vitro* interaction assays with [^{35}S]methionine-labeled, *in vitro*-translated mutant proteins were performed as described in the chapter I.

Transient transfection and luciferase assay

Transient transfection using the Lipofectamine Plus liposome reagent (Gibco BRL) and luciferase assay were performed as described in the chapter I, except that pCH110 (β-galactosidase expression vector, Pharmacia, 25 ng/dish) was used as the standard vector co-transfected with the pUK GC-Luc reporter construct. Luciferase activities were normalized to the numbers of the cells stained by X-gal.

Gel shift assay

Gel shift assay was carried out as described in the chapter I. Oligonucleotide corresponding to the uPA GC box, located in –63 to –32 of the uPA promoter (49), was synthesized, double-stranded, and end-labeled with [γ-^{32}P]ATP by T_4 polynucleotide kinase. For binding reactions, 10-20 ng of either Sp1 (Promega) or GST-Sp1 C-terminus fragment were pre-incubated with or without 20 ng each of RARα-GST and RXRα-GST plus/minus 9cRA for 15 min on ice, and then 40 fmol of labeled oligonucleotide (10,000 μCi/mol) was added in the presence of 1 μg of dI-dC (Pharmacia) in 40 μl of binding buffer (20 mM Hepes, pH 7.4, containing 1 mM $MgCl_2$, 10 mM $ZnSO_4$, 20 mM

KCl, and 8% glycerol). GST-Sp1 C-terminus fragment was purified from bacteria BL21 using the glutathione-Sepharose. The reaction mixture was incubated for 15 min on ice and separated on a 4% polyacrylamide gel at 4°C. The gel was dried and exposed on films for a Fujix BAS 2,500 Bio-imaging analyzer. Relative amounts of the Sp1-DNA complexes were determined by densitomeric analyses of the autoradiogram and plotted against Sp1 dosages.

II-3. Results

RAR interacts with the C-terminal region of Sp1

In order to appreciate a contribution of RAR/RXR-derived transactivation activity in the transcriptional regulation through RAR/RXR-Sp1 interaction, I planned to use a Sp1 fragment that can physically interact with RAR/RXR, but does not have a transcriptional activity. Hence, first, to map which domain(s) in Sp1 is necessary for physical interaction with RARα, deletion mutants of Sp1 were constructed. As depicted in Fig. II-1, Sp1 contains two activation domains abundant in glutamine and serine/threonine and one DNA-binding domain composed of three zinc fingers (55, 56). I made three deletion mutants deficient in each of the N-terminus (N), Mid (M), and C-terminus (C) regions (Fig. II-1), and examined their ability to physically associate with RARα by GST-pulldown assays. ^{35}S-labeled deletion mutants were synthesized by *in vitro* translation reaction in the reticulocyte lysates and incubated with RARα-GST proteins immobilized on the glutathione-Sepharose beads. The bound proteins were eluted and analyzed by SDS-PAGE followed by autoradiography. The results are shown in Fig. II-2. Lanes 1-4 show autoradiogram of the input samples guaranteeing the presence of labeled each mutant in each sample. Lanes 6-9 show autoradiogram of a protein bound to RARα-GST. Full-length Sp1 bound to RARα-GST but not to GST alone (compare lanes 5 and 6). Sp1 ΔN mutant and Sp1 ΔM mutant also interacted with RARα-GST (lanes 7 and 8, respectively). In contrast, Sp1 ΔC mutant failed to associate with RARα-GST (lane 9), suggesting that the C-terminus region of Sp1 might physically interact with RARα. This hypothesis was examined using ^{35}S-labeled C-terminus prepared by *in vitro* translation reaction (Fig. II-3). As seen in lane 5, the C-terminus of Sp1 bound to RARα-GST. The C-terminus region contains the zinc finger motifs responsible for

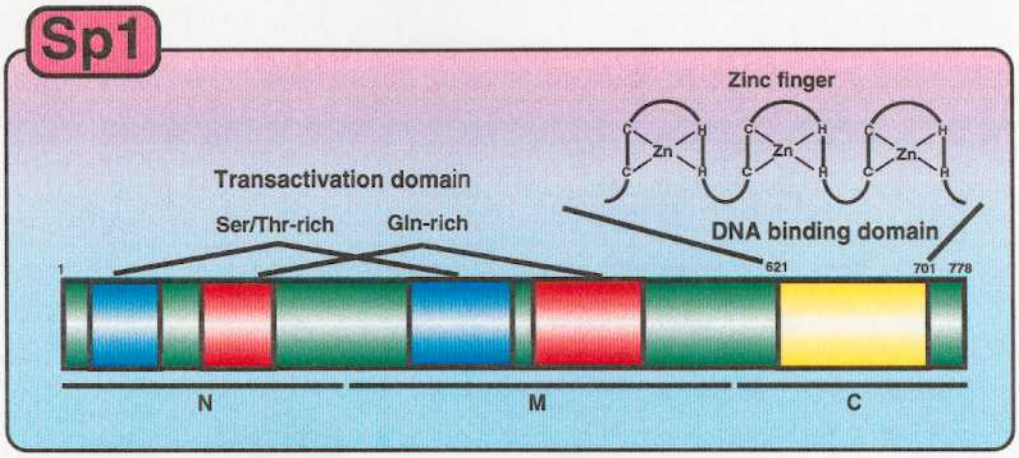

Fig. II-1. Schematic structure of the Sp1 protein. Sp1 contains two activation domains abundant in glutamine and serine/threonine, and one DNA-binding domain composed of three zinc finger motifs.

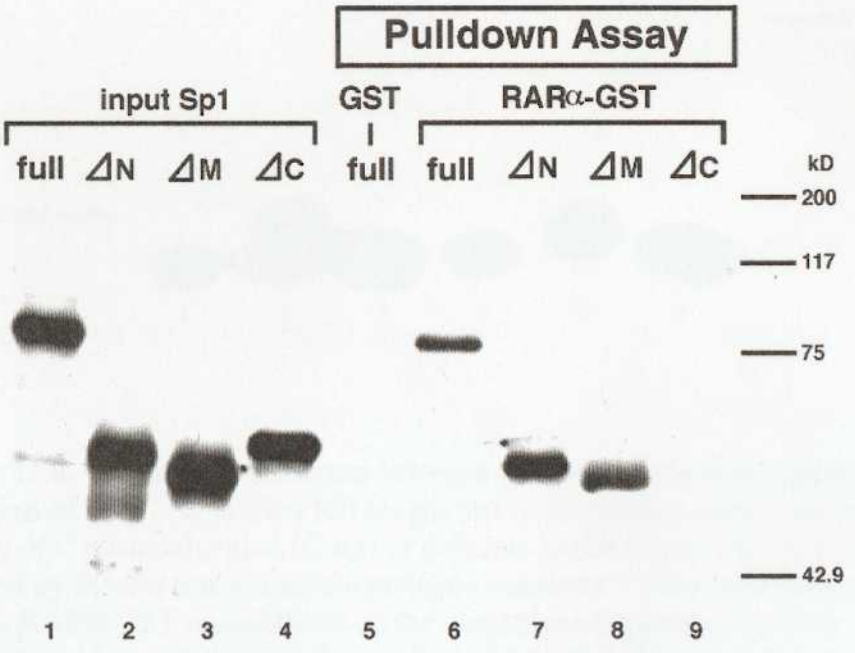

Fig. II-2. The C-terminus region of Sp1 is important for physical interaction with RARα in vitro. Plasmids expressing three deletion mutants of Sp1 were constructed. Each deletion mutant was ^{35}S-labeled by *in vitro* transcription/translation reaction and incubated with GST or RARα-GST immobilized on the glutathione-Sepharose beads as described in the Materials and Methods. After extensive washings, the bound proteins were eluted and subjected to SDS-PAGE followed by autoradiography. Lanes 1-4, input proteins; lane 5, proteins bound to GST; lanes 6-9, proteins bound to RARα-GST. Lanes 1 and 6, full-length Sp1; lanes 2 and 7, ΔN mutant; lanes 3 and 8, ΔM mutant; lanes 4 and 9, ΔC mutant. A representative result from three similar experiments is shown.

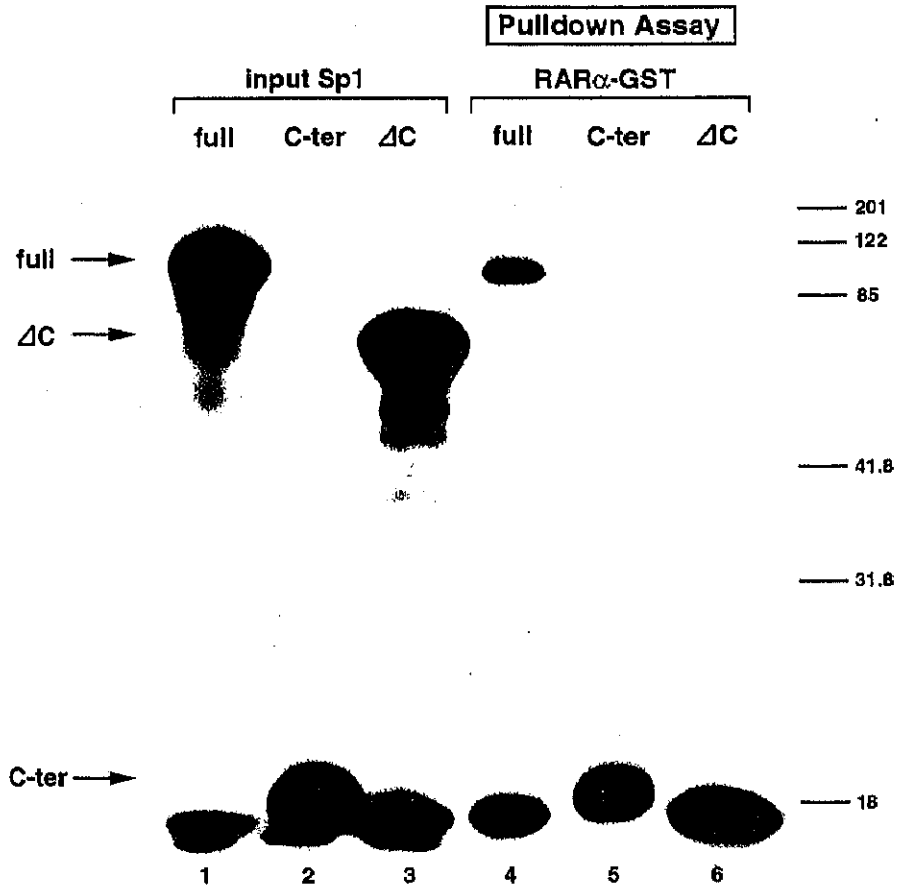

Fig. II-3. Physical interaction between RARα and the C-terminus region of Sp1. ^{35}S-labeled full-length Sp1 or its mutants either consisting only of C-terminal region (C-ter) or deficient in this region (ΔC) were prepared by *in vitro* transcription/translation reactions. They were incubated with RARα-GST immobilized on the glutathione-Sepharose beads as before. After extensive washings, the bound proteins were eluted and subjected to SDS-PAGE followed by autoradiography. Lanes 1-3, input proteins; lanes 4-6, proteins bound to RARα-GST. Lanes 1 and 4, full-length Sp1; lanes 2 and 5, C-terminus; lane 3 and 6, ΔC mutant. A representative result from three similar experiments is shown.

DNA binding. Therefore, I next examined whether the C-terminus alone could bind to GC box and whether its binding was potentiated by RAR as what had been observed for full-length Sp1. Fig. II-4 shows the result of gel shift experiment using the uPA GC box as a probe. The C-terminus of Sp1 alone bound to GC box (lane 4), and this binding was potentiated by addition of RARα/RXRα (lane 5), suggesting that RAR/RXR enhance the binding of the C-terminus of Sp1 as well as full-length Sp1 through the physical interaction. These results suggested that the C-terminus region of Sp1 is necessary and sufficient to mediate the direct protein-protein interaction between Sp1 and RARα.

Examination of a role of RAR/RXR as transactivator

Because the C-terminus region does not contain transactivation domains, it did not show any transactivation activities (Fig. II-5, lane 7). Therefore, it was expected that the C-terminal region may tether RARα/RXRα and GC box as an adapter molecule. In another word, RARα/RXRα may bind to GC box via the C-terminus fragment of Sp1. If RARα/RXRα-derived transactivation activity contributes to the transcription potentiated by RARα/RXRα-Sp1 interaction, RARα/RXRα should promote transactivation activity of GC box-containing reporter construct upon binding to its GC box via the C-terminal region, which alone is unable to promote transactivation. This was examined using pUK GC-Luc construct transfected into BAECs. Changes in the luciferase activity were monitored after BAECs were co-transfected with the reporter construct plus either each of full-length Sp1, C-terminal region of Sp1, or RARα/RXRα alone, or combination of full-length Sp1 plus RARα/RXRα or C-terminal region of Sp1 plus RARα/RXRα (Fig. II-5). As shown by column 4, transfection with full-length Sp1 enhanced the reporter activity about 8.5- fold.

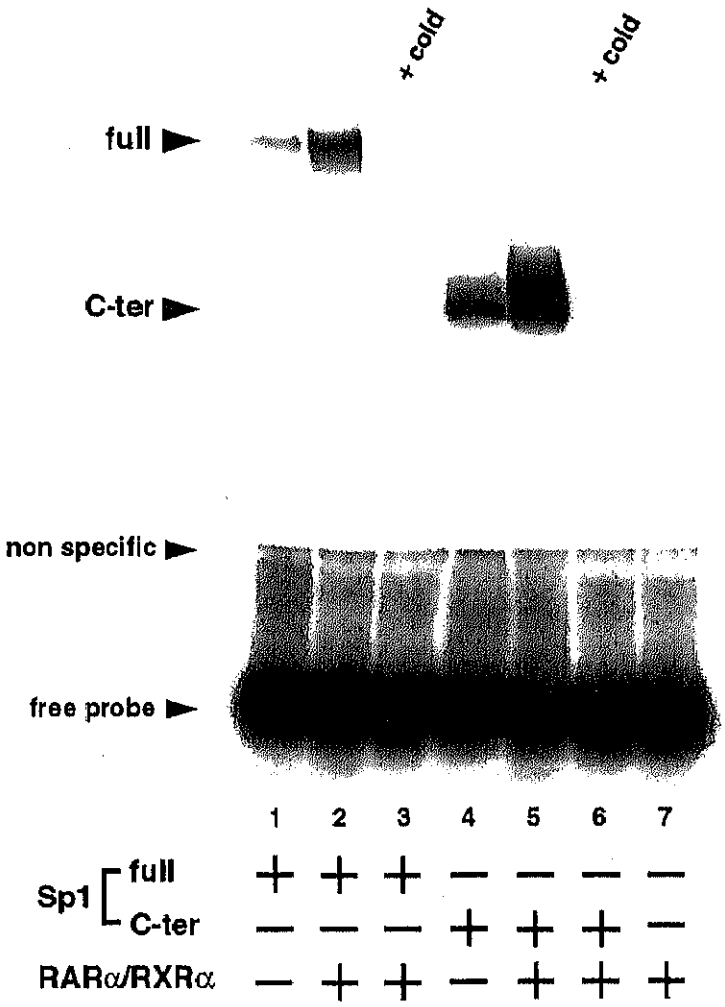

Fig. II-4. Binding of the C-terminus region of Sp1 to the uPA GC box. After 15 ng of either full-length Sp1 (full) or its C-terminus fragment (C-ter) were preincubated with or without 20 ng each of RARα-GST and RXRα-GST, the reaction mixture was indicated with ^{32}P-labeled uPA GC box, and thereafter protein-DNA complexes were separated by a 4% polyacrylamide gel electrophoresis and visualized by autoradiography. Lane 1, full-length Sp1; lane 2, full-length Sp1 and RARα/RXRα; lane 3, full-length Sp1 and RARα/RXRα + 50-fold excess of unlabeled oligonucleotide (cold); lane 4, C-terminus; lane 5, C-terminus and RARα/RXRα; lane 6, C-terminus and RARα/RXRα + 50-fold excess of unlabeled oligonucleotide (cold); lane 7, RARα/RXRα. A representative result from two similar experiments is shown.

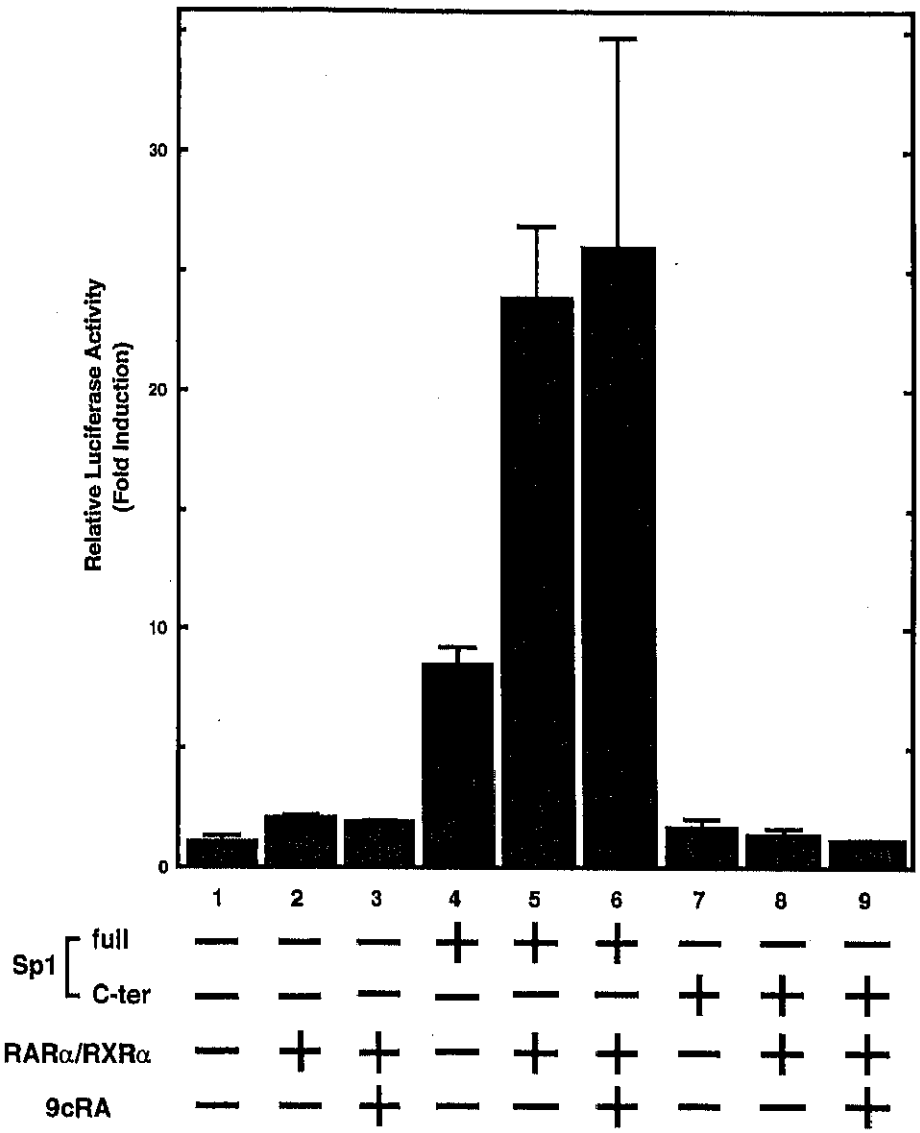

Fig. II-5. Disappearance of the RAR/RXR-Sp1 effect by eliminating Sp1 transactivation domain. Transfections into BAEC cultures grown on 35 mm-dishes were performed using the pUK GC-Luc construct as a reporter. One day after co-transfection with 750 ng of either full-length Sp1 or its C-terminus fragment expressing vector plus/minus 125 ng each of RARα and RXRα expressing vectors, cells were incubated for 16 h either with vehicle or with 1 μM 9cRA. Cell lysates were prepared from each dish, and luciferase activity in each lysate was measured. Sample 1, reporter only; sample 2, RARα/RXRα; sample 3, RARα/RXRα + 9cRA; sample 4, full-length Sp1; sample 5, full-length Sp1 and RARα/RXRα; sample 6, full-length Sp1 and RARα/RXRα + 9cRA; sample 7, C-terminus; sample 8, C-terminus and RARα/RXRα; sample 9, C-terminus and RARα/RXRα + 9cRA. Data are represented as the mean ± SD (n = 3).

Although RARα/RXRα at this dosage alone did not promote the transactivation regardless of the presence of ligand (columns 2 and 3), it strongly potentiated the transactivation activity when co-transfected together with full-length Sp1 (column 5; about 24-fold) without an obvious ligand-dependency (column 6). In contrast, transfection with Sp1 C-terminus fragment scarcely enhanced luciferase activity (column 7) and this activity was not potentiated by co-transfection with RARα/RXRα (column 8). Addition of 9cRA did not affect it (column 9). These results suggested that the ligand-inducible transcriptional activity of RAR/RXR might not contribute to the enhanced transactivation of the uPA promoter through RAR/RXR-Sp1 interaction. However, there remains a possibility that RARα/RXRα drive transcription when making a complex with full-length Sp1. This possibility was explored in the next.

Under the absence of ligands it is hard to discriminate RARα/RXRα-driven transactivation activity from Sp1-derived transactivation activity. However, if RARα/RXRα-driven transactivation activity has a role, by adding the ligand I should see a further enhancement in the reporter activity which had been already potentiated by RARα/RXRα -Sp1 interaction, because generally RAR/RXR-derived transactivation activity is raised upon binding of the ligand (1, 2). I examined this point using the BAEC cultures transfected with the pUK GC-Luc (Fig. II-6A). As shown by *curve* a, reporter activity was dose-dependently enhanced following transfection of full-length Sp1 and increased to about 8.5–fold at 750 ng of Sp1 expressing vector/35 mm dish. Co-transfection of 125 ng each of RARα and RXRα with Sp1 dramatically potentiated Sp1's effect, and resulted in about 24–fold induction at 750 ng of Sp1 expressing vector/35 mm dish (*curve* b). Treatment of the cells with 1 μM 9cRA, a ligand for both RARα and RXRα, minimally enhanced the reporter activity, but its effect was statistically not significant (*curve* c). Finally, I examined

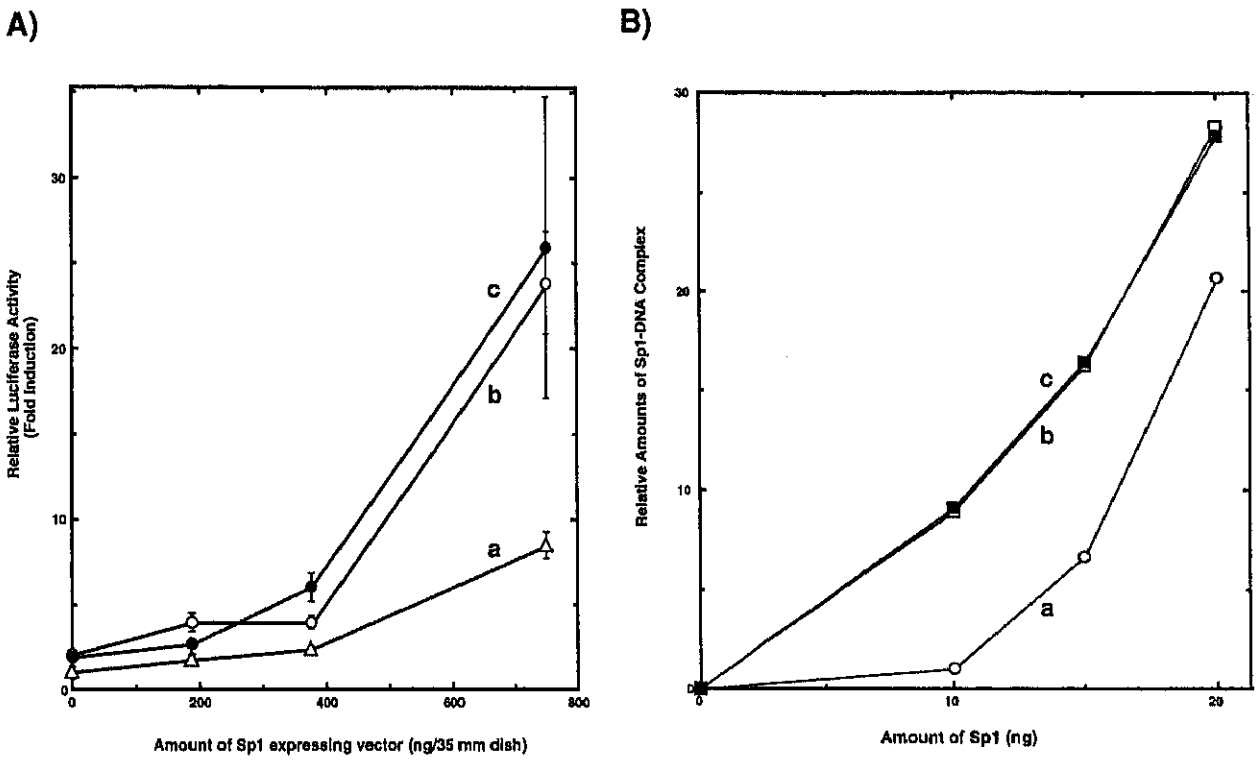

Fig. II-6. Ligand-independent potentiation of Sp1 activity by RAR/RXR. A) Transfection studies were performed as described before using the pUK GC-Luc construct as a reporter. One day after co-transfection with indicated amounts of full-length Sp1 expressing vector plus 125 ng each of RARα and RXRα expressing vectors, cells were incubated for 16 h either with vehicle or with 1 μM 9cRA. Luciferase activity in each cell lysate was measured and plotted against Sp1 dosages. Curves a and b, untreated cells; curve c, 9cRA-treated cells. Curve a, Sp1 alone; curves b and c; Sp1 plus RARα/RXRα. Each point represents the mean ± SD (n = 3). B) Relative amounts of Sp1 bound to the uPA GC box. Gel shift experiments were performed with the indicated amounts of Sp1 plus/minus 20 ng each of RARα-GST and RXRα-GST. Relative amounts of the Sp1-DNA complex were determined by densitomeric analyses of the autoradiogram and plotted against Sp1 dosages. Curve a, Sp1 alone; curve b, Sp1 and RARα/RXRα; curve c, Sp1 and RARα/RXRα + 9cRA.

whether this small enhancement could be caused at the step of potentiation of Sp1 binding to the GC box. Gel shift assays were performed and the amounts of Sp1-GC box complex estimated by densitometric analyses of the autoradiogram were plotted against the amounts of Sp1 (Fig. II-6B). As can be indicated by a comparison between *curves* b and c, addition of the ligand did not show any effects.

II-4. Discussion

In the present study, I first demonstrated that RARα directly interacted with the C-terminus region of Sp1 (Fig. II-3), which contains DNA-binding domain composed of three zinc finger motifs. Next, I presented that whereas RAR/RXR potentiated the transactivation activity of the reporter gene by full-length Sp1, they failed to enhance the transactivation activity by C-terminus of Sp1, which was expected to serve as an adapter between RAR/RXR and the GC box within the reporter construct (Fig. II-5). Furthermore, I could not see any obvious ligand dependency (Figs. II-5 and II-6), which have been reported to be necessary for RAR/RXR to exert their full transactivation activities (6, 7). From these results, I speculated that an enhancement of the transcriptional activity through the physical interaction between RAR/RXR and Sp1 may mainly be due to an enhancement of Sp1-derived transactivation activity at least in part through potentiation of DNA-binding.

It might be possible that RAR/RXR not only potentiate DNA-binding of Sp1, but also potentiate the transcriptional activity of Sp1. The result of Fig. I-5 in the chapter I shows that RARα and RXRα enhanced transactivation of the Gal4-UAS-luciferase reporter construct via interaction with a chimeric transcription factor, Sp1-Gal4 (51-54), suggesting that RAR/RXR would modulate the transcriptional activity of Sp1. I predict two possible mechanisms. 1) RAR/RXR directly modulate Sp1-derived transactivation activity as a coactivator. 2) RAR/RXR indirectly enhance the transcriptional activity of Sp1 by recruiting coactivator(s).

What is a role of ligand in this novel transcriptional mechanism? One likely answer is that ligand is needed to increase cellular RAR levels at the very first as shown in Fig. I-1 of chapter I. Because RAR genes contain canonical RAREs in their promoters, the expressions of RARs increase

upon stimulation with RA (6-8). Once the concentration of RARs increases the system appears to proceed without a requirement for ligand. However, I can not exclude the possibility that endogenous RARs/RXRs require ligand to keep corepressors away and to associate with Sp1, whereas large numbers of exogenously transfected RARs/RXRs might be free from limited numbers of corepressors and therefore might not require a ligand.

Several further questions still remain unresolved. For example, it will be important to clarify how the physical interaction between RAR/RXR and Sp1 strengthens DNA-binding activity of Sp1. It might be possible that RARα interacts with the suburbs of the zinc fingers, changes their conformations, and stabilizes them, leading to an enhanced DNA-binding of Sp1 to GC box. The involvement of corepressors/coactivators should also be examined. I am now trying to map which domain(s) in RAR molecule is necessary for physical interaction with Sp1 as the first step towards answering these questions.

Together with the results in chapter I, I demonstrated that RAR/RXR stimulated the transcription of the uPA gene via enhancing transactivation activity of Sp1, at least by potentiating its binding to the GC box through physical interaction between RAR/RXR and Sp1. Most recently, it is found that promoters of several other GC box containing genes known to be induced by RA in the endothelial cells can be transactivated through a similar mechanism (J. Shimada et al., unpublished observation). These genes include transglutaminase, TGF-β, and its signaling receptors. Namely, RA is revealed to enhance these gene expressions via RAR-Sp1 interaction. In the next two chapters, I will discuss about potential biological consequences of this passway.

II-5. Summary

I have described a novel mechanism for transcriptional regulation through physical interaction between RARs/RXRs and Sp1. Here, I investigated whether RAR/RXR might act as transactivator(s) in this process. GST-pulldown assays revealed that RARα directly interacted with the C-terminus region of Sp1, which contains DNA-binding domain. Co-transfection studies showed that RARα/RXRα did not exhibit transactivation activity via physical interaction with this Sp1 fragment that served as a scaffold. Moreover, the effects of RARα/RXRα to potentiate both DNA-binding of full-length Sp1 and its transcription activity were ligand-independent. These results suggest that the gene expression augmented by the RAR/RXR-Sp1 interaction may be mainly due to an enhancement of Sp1-derived transcription activity at least in part through a potentiation of DNA-binding of Sp1 by RAR/RXR.