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Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis

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Received 2 April 2004

Abstract

To clarify the underlying mechanism of glucocorticoid-induced osteoporosis, we investigated the effect of glucocorticoid on the expression of dickkopf-1 (Dkk-1), an antagonist of Wnt signaling, in primary cultured human osteoblasts. Dexamethasone markedly induced the expression of mRNA for Dkk-1 in a dose- and time-dependent manner. The expression of Kremen1, a receptor for Dkk, did not change by the treatment with dexamethasone, while that of low-density lipoprotein receptor-related protein 5 (LRP5), a Wnt coreceptor, slightly decreased by the treatment with dexamethasone. Dexamethasone increased the transcriptional activity of the Dkk-1 gene promoter in human osteoblasts. Serial deletion and mutation analyses of the Dkk-1 promoter showed that one putative glucocorticoid responsive element-like sequence located from −788 to −774 bp is essential for the enhancement of the Dkk-1 promoter activity by dexamethasone in human osteoblasts. Since the Wnt signal is now recognized as a crucial regulator for bone formation, the Dkk-1 enhanced by glucocorticoid may inhibit the Wnt signal in osteoblasts, which may be involved in the pathogenesis of glucocorticoid-induced osteoporosis.
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Keywords: Glucocorticoid; Wnt; Dickkopf-1; Osteoblast; Osteoporosis

The Wnt family is a secreted glycoprotein that participates in morphogenesis, determination of cell polarity, and regulation of cell proliferation and differentiation during embryogenesis [1,2]. The Wnt proteins bind to frizzled family of seven transmembrane domain receptor and its coreceptor low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6 [3]. Canonical Wnts inactivate glycogen synthase kinase-3β (GSK-3β) and inhibit phosphorylation and consequential degradation of intracellular β-catenin [1–3]. Accumulated β-catenin translocates into the nucleus and activates target genes by complex with transcription factors of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family [1–3]. The Wnt signal is regulated by two classes of extracellular antagonists [4]. Secreted frizzled-related protein (sFRP), Cerberus, and Wnt inhibitory factor-1 (WIF-1) are inhibitors that bind Wnts and restrict the Wnt function. Dickkopf (Dkk) family is another class of secreted Wnt antagonist. Dkk interacts with the Wnt coreceptor LRP5 and LRP6, and inhibits Wnt signaling by disturbing the binding of LRP5/6 to the Wnt/frizzled ligand–receptor complex [5].

Recent analyses of patients with the LRP5 gene mutation and LRP5 knockout mice revealed that LRP5 plays pivotal roles in bone metabolism [6–8]. It is reported that the missense mutations of LRP5 gene cause osteoporosis-pseudoglioma syndrome in which bone and eyes are abnormally developed [6]. LRP5 knockout mice also showed similar phenotype in which low bone density, decreased osteoblast proliferation that is independent of *Runx2/Cbfa1*, and abnormal eye development were observed [7]. On the other hand, it is demonstrated that patients with other mutation of LRP5 gene have high bone mass [8]. In this mutation,

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the mutated LRP5 receptor has a low binding affinity to Dkk-1 and causes decrease in inhibitory function of Dkk-1 against Wnt signaling. Thus, the Wnt signal is now recognized as a novel regulator of bone formation and an important molecular target for the treatment of osteoporosis [9,10].

Glucocorticoid-induced osteoporosis is one of the serious problems during glucocorticoid therapy [11,12]. The major cause of glucocorticoid-induced osteoporosis is considered to be impairment of bone formation [11,12]. Glucocorticoid in an excess dose has inhibitory actions on osteoblastic replication, maturation, and differentiation. Glucocorticoid suppresses the expression of *Runx2/Cbfa1*, a critical factor for osteoblastogenesis, and reduces the synthesis of osteocalcin, type I collagen, and insulin-like growth factor-I (IGF-I) in osteoblasts [11,12]. It also promotes the apoptosis of osteoblasts and osteocytes [13]. However, detailed mechanism underlying glucocorticoid-induced osteoporosis remains to be fully elucidated.

In the present study, we hypothesized that glucocorticoid would affect the Wnt signal of bone formation in osteoblasts, and examined the effect of glucocorticoid on the expression of Wnt signal-related molecules in primary cultured human osteoblasts. We found that dexamethasone induces the expression of Dkk-1, an antagonist of Wnt, through the activation of trascription via glucocorticoid responsive element (GRE) of the Dkk-1 gene promoter.

Materials and methods

Materials. Eagle's α MEM, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) was purchased from Sanko Junyaku (Tokyo, Japan). Dexamethasone, 17β-estradiol, dihydrotestosterone, and 1,25-dihydroxyvitamin D3 were purchased from Sigma (St. Louis, MI). All other reagents were of analytical grade.

Cell culture. Human osteoblasts were prepared from the bone fragments of femur neck as described previously [14]. The cells were grown in Eagle's α MEM with 10% FCS, 100 mU/ml penicillin, and 100 mU/ml streptomycin. Cells at ~80% confluence in 100-mm culture dishes (Falcon, Lincoln Park, NJ) were made quiescent through incubation with serum-free medium for 1 day before experiments.

Quantification of RNA. Total RNA was isolated from cultured cells using a RNeasy RNA Extraction kit (Qiagen, Germany). Reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis were performed as described previously [14,15]. The primer sets for DKK-1 (396 bp) were 5′-TGATGAGTACTGCGCTAGTC-3′ (sense) and 5′-CTCCTATGCTTGGTACACAC-3′ (antisense), the primer sets for LRP5 (377 bp) were 5′-CCGTCATTGGCATCA TCCTC-3′ (sense) and 5′-GTCCATGTTGTACAGGGAGG-3′ (antisense), the primer sets for Kremen1 (354 bp) were 5′-GTTTGCTGGG ATGGAGTCAG-3′ (sense) and 5′-GTGTAGCCATCCAGAAGC TC-3′ (antisense), and the primer sets for GAPDH (321 bp) were 5′-GGGCTCTCCAGAACATCATC-3′ (sense) and 5′-CAAAGTGGTC GTTGAGGGCA-3′ (antisense). The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI), sequenced, and then used as cDNA probes for Northern blot analysis.

Determination of the initiation site of transcription for the Dkk-1 gene was performed by a RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) kit (Ambion, Austin, TX) according to the manufacturer's instruction. The outer and inner antisense primers used for 5′ RLM-RACE for Dkk-1 were 5′-CTGCAGGCGAGACAGA TTTG-3′ and 5′-GGCTGGTAGTTGTCAATGGT-3′, respectively.

Construction of human Dkk-1 promoter/luciferase chimeric plasmids. Human gemonic DNA was purified from a Japanese man with a QIAamp DNA Blood Kit (Qiagen). The approximately 0.8 kb upstream region of the human Dkk-1 promoter was amplified by PCR using KOD-plus DNA polymerase (Toyobo, Tokyo, Japan), subcloned into the pCR-Blunt II-TOPO vector (Invitrogen), and sequenced to confirm the validity of the PCR product. The primer sets were 5′-CTCACGCGTCTGCCTAATCA-3′ (sense) and 5′- AAGCTTTCAG AAGGACTCAAGAGGGA-3′ (antisense, *Hin*dIII-linker added). After digestion with *Mlu*I and *Hin*dIII, the fragment was subcloned into the *Mlu*I/*Hin*dIII site of a promoterless luciferase expression vector, pGL3-Basic vector (Promega) and designated as pGL3-Dkk-1(−837/+151). Serial 5′-deletion constructs of the Dkk-1 promoter were generated by PCR, using pGL3-Dkk-1 (−837/+151) as template. To generate the mutant, PCR-mediated site-directed mutagenesis was performed by a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All the deletion and mutation constructs were confirmed by DNA sequencing.

Transient transfection and reporter assay. Human osteoblasts were transiently transfected by means of calcium phosphate precipitation [16]. Briefly, cells (5×10^4 cells/well) were seeded in a 12-well plate (Falcon) prior to transfection: each well received 5 μg of the Dkk-1 promoter-reporter firefly luciferase plasmid (pGL3) and 100 ng pRL-CMV (a *Renilla* luciferase vector, Promega) as an internal control. Two hours after transfection, the cells were incubated in Eagle's α MEM with 10% dextran/charcoal-treated FCS in the presence or absence of 10^{-7} M dexamethasone. Firefly and *Renilla* luciferase activities were measured at 36 h after transfection with a dual luciferase assay kit (Promega), and the values were adjusted for the activity of the internal control (*Renilla* luciferase activity).

Statistical analysis. Data are expressed as means ± SD. Statistical analyses were performed with ANOVA followed by Fisher's protected least significant difference test. Significance was accepted at $P < 0.05$.

Results

We first screened the mRNA expression for the Dkk family (Dkk-1, -2, -3, and -4) by RT-PCR, and found that only Dkk-1 mRNA is expressed in primary cultured human osteoblasts (data not shown). We then examined the expression level of Dkk-1 mRNA by Northern blot analysis (Fig. 1). Although quiescent human osteoblasts expressed a low level of Dkk-1 mRNA transcript (1.8 kb), dexamethasone (10^{-7} M) significantly induced the expression of Dkk-1 mRNA (more than 10-fold) compared to that in unstimulated condition (Fig. 1A). This effect was observed at 6 h and reached maximum at 24 h (Fig. 1B). This increase was in a dose-dependent manner (10^{-9}–10^{-7} M of dexamethasone) (Fig. 1A). We also examined the effect of other steroid hormones on the expression of Dkk-1 mRNA. Addition of 10^{-7} M of 17β-estradiol (E2), dihydrotestosterone (DHT) or 1,25-dihydroxyvitamin D3 (VD3) did not affect the expression level of Dkk-1 mRNA (Fig. 2). These results

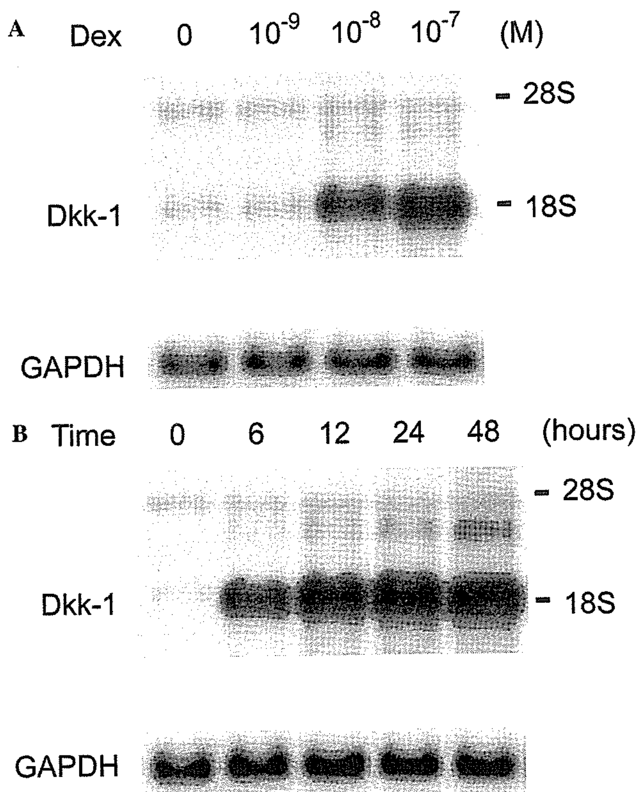

Fig. 1. Effect of dexamethasone on the expression level of Dkk-1 mRNA in primary cultured human osteoblasts. (A) Human osteoblasts were incubated for 24 h with vehicle (ethanol) or dexamethasone (Dex, 10^{-9}–10^{-7} M). (B) Human osteoblasts were incubated for 6–48 h with dexamethasone (10^{-7} M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments are shown.

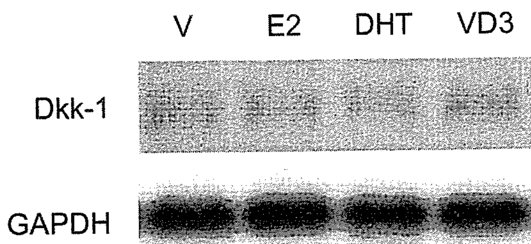

Fig. 2. Effect of various steroid hormones on the expression level of Dkk-1 mRNA in primary cultured human osteoblasts. Human osteoblasts were incubated for 24 h with vehicle (V, ethanol), 17β-estradiol (E2, 10^{-7} M), dihydrotestosterone (DHT, 10^{-7} M), or 1,25-dihydroxyvitamin D3 (VD3, 10^{-7} M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments is shown.

suggest that glucocorticoid specifically induces the expression of Dkk-1 mRNA in primary cultured human osteoblasts.

We then examined whether dexamethasone affects the expression of other Wnt signal-related molecules in cultured human osteoblasts. Cultured human osteoblasts expressed the mRNA transcript for Kremen1, a receptor for Dkk [17], and that for LRP5. As shown in Fig. 3, dexamethasone (10^{-7} M) did not alter the expression level of the mRNA for Kremen1. On the other hand, the expression of LRP5 mRNA slightly decreased by the treatment with dexamethasone (10^{-7} M).

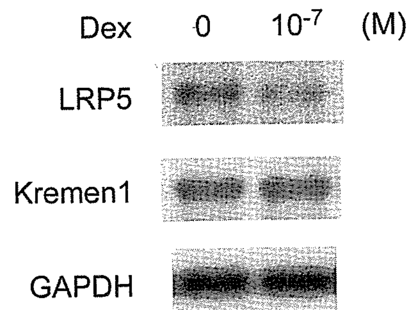

Fig. 3. Effect of dexamethasone on the expression levels of LRP5 and Kremen1 mRNA in primary cultured human osteoblasts. Human osteoblasts were incubated for 24 h with vehicle (ethanol) or dexamethasone (Dex, 10^{-7} M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments is shown.

To clarify the mechanism by which dexamethasone up-regulates the expression of the Dkk-1 mRNA, we investigated the effect of dexamethasone on the promoter activity of the human Dkk-1 gene in cultured human osteoblasts. At first, we determined the transcription initiation site for the Dkk-1 gene in human osteoblasts by RLM-RACE. One major product was observed by PCR, and the sequence analysis of this product revealed that the transcription initiation site is 'A' at −152 bp relative to the translation start site. We then examined the 5′-promoter region on the transcriptional activity. When the reporter plasmid containing of the 0.8 kb upstream region was transfected into human osteoblasts, the basic promoter activity was 8–10 times higher than that of promoterless control plasmid. The addition of dexamethasone (10^{-7} M) increased 6–8 times the luciferase activity of the 0.8 kb construct (Fig. 4). The magnitude of the enhancement of the dexamethasone-induced transcription was comparable to that of the Dkk-1 mRNA induction by dexamethasone (Fig. 1A). Deletion of the 5′-promoter region from −837 to −540 bp, which contains one of three putative GRE-like sequences located within the 0.8 kb upstream region, abolished the effect of dexamethasone. No significant change of the dexamethasone effect on the promoter activity was observed by the deletion from −540 to −409 bp and that from −409 to −314 bp, each of which contains one putative GRE (Fig. 4). Mutation of the GRE-like sequence (−788 to −774 bp) in the promoter region from −837 to −540 bp also abrogated the induction effect of dexamethasone. These results suggest that the GRE located from −788 to −774 bp functions in the induction of Dkk-1 in response to glucocorticoid in human osteoblasts.

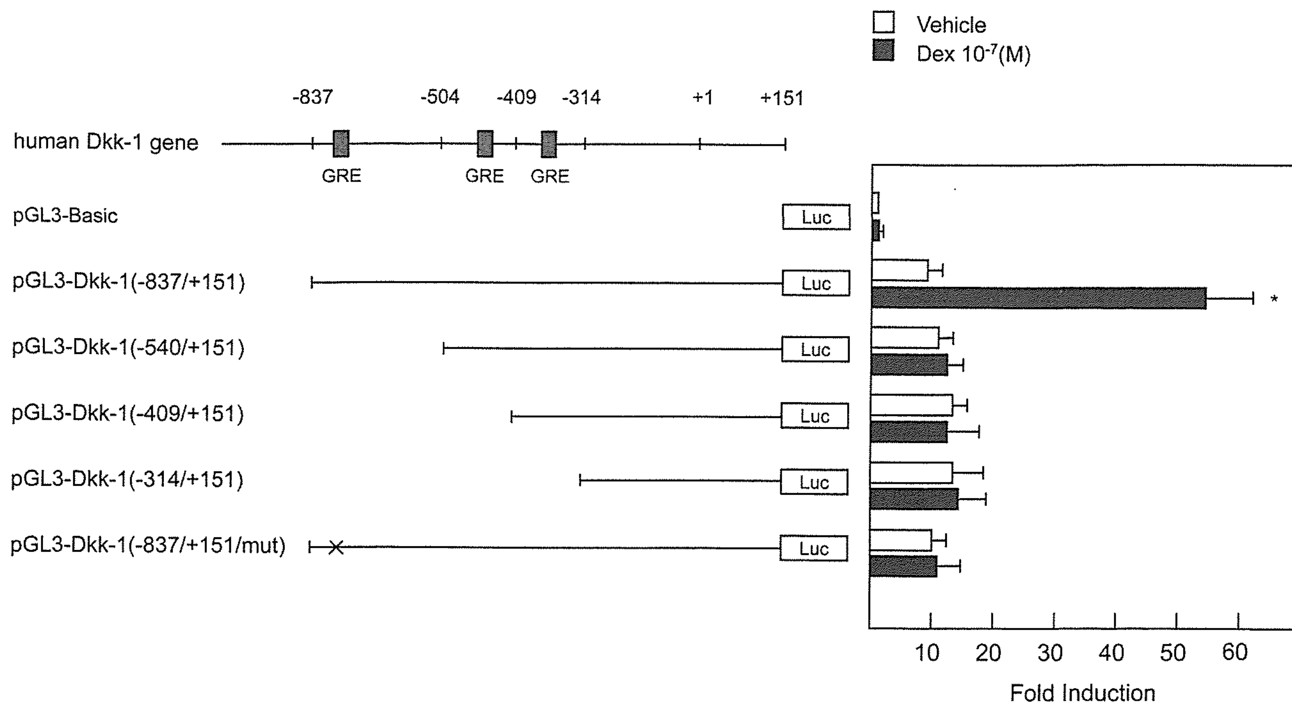

Fig. 4. Effect of dexamethasone on the promoter activity of the human Dkk-1 gene in primary cultured osteoblasts. Human osteoblasts were transiently transfected with various lengths of Dkk-1 promoter region/luciferase (Luc) chimeric plasmids or GRE-mutated Dkk-1 promoter region/luciferase chimeric plasmid as described in Materials and methods. Three putative GRE-like sequences are located in the human Dkk-1 gene promoter regions from −788 to −774 bp, from −448 to −434 bp, and from −380 to −366 bp. The sequence of GRE from −788 to −774 bp (5′-AGAACAACATTAAAT-3′) of pGL3-Dkk-1(−837/+151) was mutated to 5′-AGAGTTACATTAAAT-3′, and designated as pGL3-Dkk-1(−837/+151/mut). The reporter luciferase activity was expressed as fold over the activity of pGL3-Basic in the absence of dexamethasone. Data are shown as means ± SD ($n = 4$). *$P < 0.01$ vs. vehicle. One representative data of three independent experiments is shown. Vehicle, ethanol (open column) and Dex, dexamethasone (closed column).

Discussion

In the present study, we clearly demonstrated that dexamethasone markedly induces the expression of Dkk-1 mRNA in primary cultured human osteoblasts. We also showed that this induction is mainly mediated through the activation of transcription via GRE of the Dkk-1 gene promoter.

In this study, we found that only the mRNA for Dkk-1 was expressed in cultured human osteoblasts by RT-PCR and Northern blot analysis. Dkk-1 was originally identified as a head inducer in *Xenopus* [18] and was shown to be involved in skeletal development [19]. The Dkk family consists of Dkk-1, Dkk-2, Dkk-3, Dkk-4, and Dkk-3-related protein Soggy, and each Dkk possesses different properties on Wnt signaling [20,21]. It is important to address which Dkk plays a role in bone physiologically. Developmental analysis of mouse embryo showed that Dkk-1, Dkk-2, and Dkk-3 are expressed in undifferentiated mesenchymal cells on day E12.5 post coitum (p.c.), but only Dkk-1 is expressed in mesenchymal cells of mineralizing bone on day E15.5 p.c. [22]. These results may suggest a possible regulatory role of Dkk-1 in osteoblasts.

The expression of Dkk-1 is regulated both developmentally and tissue-specifically [18,22,23], and also induced by several factors such as bone morphogenetic protein-4 (BMP-4), *c-Jun*, p53 tumor suppressor, UV ray, and DNA damaging agents [24–26]. However, to our knowledge, this is the first report about the regulation of Dkk-1 expression by glucocorticoid in human osteoblasts.

Since Dkk-1 is a potent inhibitor of Wnt signaling, it is considered that the Dkk-1 induced markedly by dexamethasone suppresses the Wnt signal of bone formation in osteoblasts, which may be involved in the impairment of bone formation by glucocorticoid. Indeed, over-expression of Dkk-1 repressed the Wnt3a-induced alkaline phosphatase activity and reduced the extracellular matrix mineralization in mouse preosteoblastic MC3T3-E1 cells [27]. The recent study also demonstrated that Dkk-1 produced by multiple myeloma cells is associated with development of osteolytic lesions in bone [28]. The results that dexamethasone did not change the expression level of Kremen1, which participates in endocytosis of the LRP/Dkk complex from the cell surface [17], and slightly suppressed the expression of LRP5 may support a possibility that glucocorticoid suppresses bone formation by the induction

of Dkk-1, although we did not examine whether dexamethasone affects the expression of the Wnt receptor, frizzled. It remains to be fully elucidated which frizzled receptor functions for the Wnt signal in osteoblasts and further studies will be required.

Analysis of the Dkk-1 promoter region revealed that the induction of Dkk-1 by dexamethasone mainly resulted from activation of transcription through GRE in the Dkk-1 gene promoter. There are several putative GRE-like sequences within approximately 1 kb upstream from the transcription initiation site in the human Dkk-1 gene. Deletion and mutation analyses showed that the GRE located from −788 to −774 bp is responsible for the activation of the promoter activity in response to dexamethasone in human osteoblasts.

The recent report about LPR5 null mice demonstrated that loss of function in LRP5 causes low bone mass by decrease in osteoblastic proliferation and function [7]. It is noteworthy that the Wnt/LRP5 signal is independent of *Runx2/Cbfa1*, a master transcriptional factor for osteoblastic differentiation [7]. On the other hand, patients with the mutation of LRP5 (G171V), a gain-of-function mutation of LRP5, show high bone mass with no fragility, probably because of normal osteoclastic function [8]. Therefore, the enhancement of the Wnt signal in osteoblast by a specific inhibitor for Dkk-1 interacting with LRP5 is expected to promote bone formation by stimulating osteoblastic proliferation and function in the *Runx2/Cbfa1*-independent pathway. Our present findings and further study about the regulation of LRP5 function by Dkk-1 in osteoblasts may lead to the development of new drugs to promote bone formation for the treatment of glucocorticoid-induced osteoporosis.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (B) and the grant for the 21st Century COE Program from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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Coregulator-Related Diseases

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Abstract

Coregulators are a group of proteins, which modulate the nuclear receptor transactivation function. In this study, a new "coregulator disease" concept was proposed from observations of a case of androgen insensitivity syndrome (AIS) and cases involving Rubinstein-Taybi syndrome and X-linked dementia and hypothyroidism syndrome. In addition, coregulators are thought to be closely associated with the pathogenesis of several diseases such as hormone-dependent cancers and leukemia. Based on these observations, the clinical disorders associated with some coregulator abnormalities were reviewed. (Internal Medicine 43: 368–373, 2004)

Key words: coregulator, coactivator, corepressor, androgen insensitivity syndrome (AIS), Rubinstein-Tabi sundrome, Refetoff syndrome, hormone-dependent cancer, leukemia

Introduction

Steroid hormone receptors such as the androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR) are ligand-dependent transcription factors that belong to the nuclear receptor superfamily. Nuclear receptors bind to their cognate response elements in the promoter region of target genes, and regulate their expressions (1, 2). Some abnormalities in the structures of transcription factor cause the disturbed transactivation of target genes, leading to various physiological abnormalities. Such a disease state is well established as a "transcription factor disease." However, there are several cases that show no mutations in the corresponding nuclear receptors, although clinical and biochemical profiles completely match the disease concept. Coregulators are most likely associated with such a mechanism since coregulators interact with various nuclear receptor proteins and modulate the transcriptional activity (2–4). We have proposed a new disease concept of coregulator disease from a patient with androgen insensitivity syndrome (AIS) who showed no *AR* gene mutation (5). Rubinstein-Taybi syndrome and X-linked dementia and hypothyroidism syndrome, which are known to be caused by abnormalities of CBP and TR associated protein (TRAP) 230, respectively, have already been established as a coregulator disease (6, 7). This review focuses on several clinical disorders, which have been proven or are thought to be related to coregulator abnormalities.

Mechanism of Steroid Receptors

Nuclear receptors share a common structure, which consists of a transcription active domain, a DNA-binding domain and a ligand-binding domain. There are two transcription activation domains; the activation function-1 (AF-1) domain in the N-terminal region and the activation function-2 (AF-2) domain in the C-terminal region. While the AF-2 domain is relatively conserved among nuclear receptors, the AF-1 domain differs widely (Fig. 1) (1, 2). When a ligand is bound to a receptor, the receptor changes in structure, translocates from the cytoplasm to the nucleus and then binds to the promoter region of the target gene. Coregulator proteins bind to the nuclear receptors and modulate the transcriptional activity of the nuclear receptors in a promoter- and cell-specific manner (Fig. 2). There are two types of coregulators, coactivator proteins, which activate transcription, and corepressor proteins, which repress transcription. CBP/p300, p160 family (steroid receptor coativator (SRC) -1/NcoA1, transcriptional intermediary factor (TIF) 2/GRIP etc), VDR interacting protein (DRIP)/TRAP and others have been reported as typical coactivators, and nuclear receptor corepressor (NcoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT), as typical corepressors (2, 3). Although most coregulators are AF-2 binding proteins, p300/CBP and SRC-1 interact with both AF-1 and AF-2. In addition, several AF-1 binding coregulators such as breast cancer susceptibility gene 1 (BRCA1), SRA cyclin E and AR N-terminal domain transactivating protein-1 (ANT-1) (8) have also been identified. Interaction of the AF-1 and AF-2 domains is important

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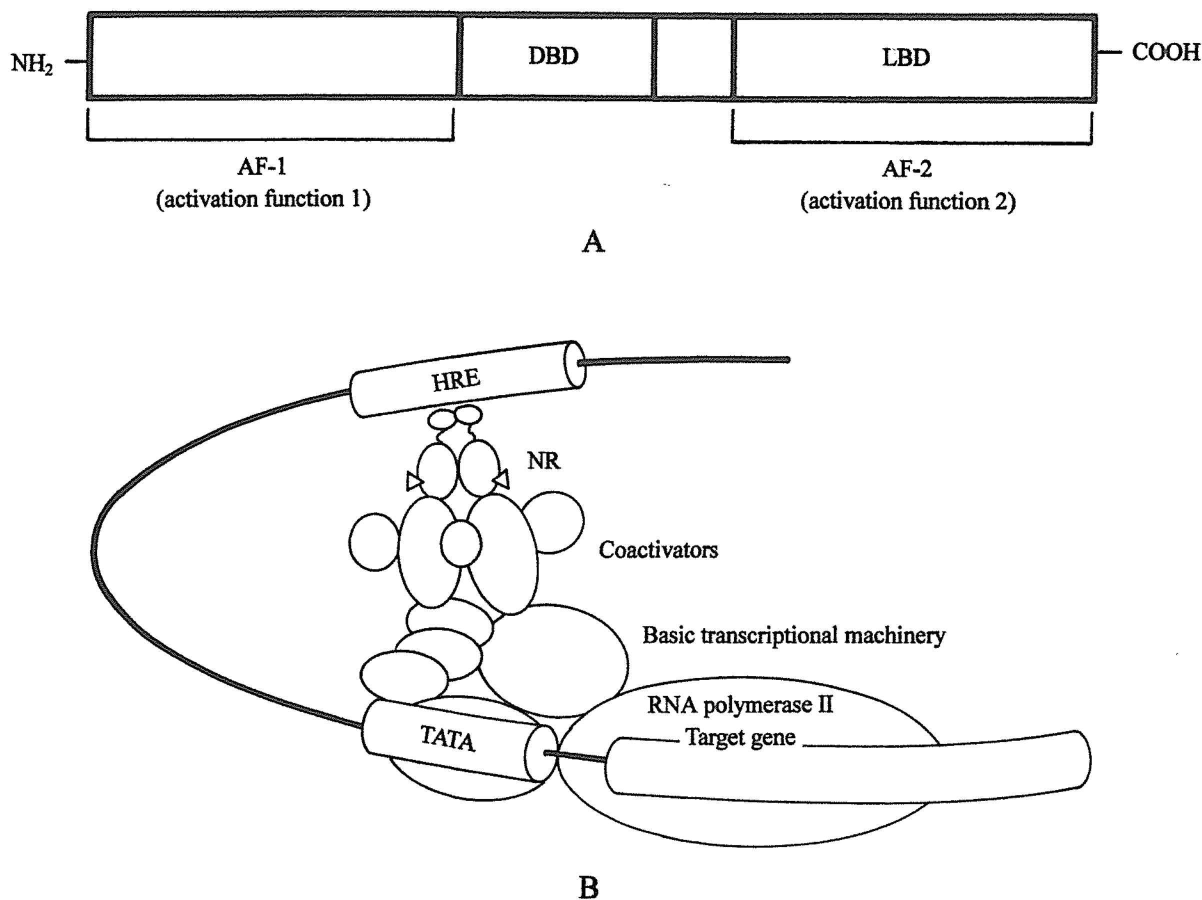

Figure 1. (A) Structure of nuclear receptors (NRs). DBD, DNA-binding domain; LBD, ligand-binding domain. (B) Interactions among the nuclear receptor (NR), coactivators and basic transcriptional machinery.

for exerting the full nuclear receptor transactivation capacity (9). Recently, it was found that rather than operating individually these coregulators operate together by forming an enormous protein complex. For example, a complex composed mainly of CBP/p300 and the p160 system, has histone acetyltransferase (HAT) activity that acetylates a basic amino acid of the histone protein and alters the chromatin structure. This makes it easier for transcription factors to be recruited on the DNA and thus promotes transcription. However, complexes such as the DRIP/TRAP complex do not have HAT activity. On the contrary, the corepressor SMRT/NCoR complex represses transcription activity by coupling with the nuclear receptor that is unbound to the ligand and recruits the histone deacetylation enzyme (HDAC), which has an opposing effect to HAT on the promoter. It is thus speculated that once ligand binds to the nuclear receptor, the corepressor complex dissociates from the receptor and the coactivators are recruited onto the promoter (2, 3).

AIS and Coactivator Disease

Of several steroid hormone resistance syndromes such as AIS, primary glucocorticoid resistance due to GR abnormalities, and type II vitamin D resistance due to vitamin D receptor (VDR) abnormalities, AIS is thought to be the most common. AIS occurs in genetically 46XY males who have testis but suffer from various degrees of virilization failure because of androgen insensitivity (10). This disease can be classified into four types depending on the degree of virilization failure as follows: complete AIS, partial AIS, Reifeinstein syndrome and male infertility. Patients with complete AIS show male pseudohermaphroditism, that is, they have female external genitalia and female type breast development. Patients with Reifenstein syndrome have hypospadias, a micropenis and gynecomastia. Most cases of AIS are the result of *AR* gene mutations located on chromosome Xq11-12. More than 300 AIS mutations have been identified. The degree of AR dysfunction due to *AR* gene abnormality generally correlates with the clinical severity (feminization) (10, 11).

We previously examined a complete AIS patient without an *AR* gene mutation (5). The patient also expressed a normal androgen binding capacity in the pubic skin fibroblast. Therefore, it was postulated that the pathogenesis of this patient was due to an unknown factor that might have modulated AR function. The degree of transcriptional activation by the normal AR in the genital fibroblast of the patient was less than 9% of that in the fibroblasts of normal subjects. However, the fibroblasts of another complete AIS patient with an *AR* gene mutation (Arg 840→His) were similar to those of the normal subjects. The degree of transcriptional activation by the AR-GR chimera that consisted of the N-terminal and DNA-binding domains of the AR and the C-terminal domain of the GR, in the genital fibroblast of the patient was 12 to 17% of that in the fibroblasts of the normal and complete AIS patient. In contrast, the degree of transcriptional activation by the GR and GR-AR chimera that consisted of the N-terminal and DNA-binding domains of the GR and the C-terminal domain of the AR was similar among the three types of fibroblasts. Furthermore, the degree of ligand-independent transcriptional activation by the N-terminal domain fragment including the AF-1 region of the AR in the genital fibroblasts of this patient was approximately 20% of that in the fibroblasts of the normal and complete AIS patients. However, the degree of transcriptional activation by the N-terminal domain fragment of the GR was similar among the three types of fibroblasts. The degree of ligand-dependent transcriptional activation by the C-terminal domain fragments of the AR and GR was similar among the three types of fibroblasts.

Together, these findings suggest that transmission of the transactivating signal from the AF-1 region of the AR to the basal transcriptional machinery might be impaired in this patient. Finally, as a result of the AR AF-1 binding protein investigation of the pubic skin fibroblasts using glutathione-S-transferase (GST) pull down analysis, it was revealed that the absence of 90 kDa protein, which is present in normal fibroblasts, is most likely the cause of AIS in this case. From these findings, we proposed a new clinical concept of steroid hormone resistance, a coactivator disease, in which a physiologically indispensable AF-1-specific coactivator crucial to the transactivation activity of AR is responsible for complete AIS (Fig. 2) (5).

Other Kinds of Coregulator Disease

Rubinstein-Taybi (RTS) syndrome

Rubinstein-Taybi (RTS) syndrome is another coregulator disease. In 1963, RTS syndrome was reported as a disease that presents autosomal dominant inheritance and clinically characterized by, for example, short stature, craniofacial malformation, heart malformation and mental retardation (12). In 1995, CREB (cAMP response element binding protein)-binding protein *CBP* gene defects was reported as the cause of this disorder (6). CBP is a protein originally identified as a coactivator that stimulates the phosphorylation-dependent transcriptional activation of CREB. CBP also works as a nuclear integrator when various classes of transcription factors crosstalk with each other (13). In a CBP heterozygous (CBP +/–) mouse created by gene targeting, several bone malformations and cardiac anomalies similar to those of RTS were observed, supporting a correlation between RTS and CBP (14). However, how CBP abnormality causes RTS has not been well established. Unlike CBP +/– mice, CBP –/– embryos did not exhibit cardiac anomalies (15), suggesting that at least the cardiac anomaly in CBP +/– mice might be caused by the dominant-negative action of the truncated CBP mutant (16). The impaired HAT activity observed in the CBP mutant might also be a cause of RTS in humans (17). However, since it has been reported that only 20% of 194 patients with RST were found to have *CBP* gene mutations there is the possibility that other gene mutations are also involved in the diverse RST symptoms (18).

Multiple hormone resistance

A case involving sisters with resistance to multiple steroid hormones such as glucocorticoid, androgen and mineralocorticoid, revealed the possibility that some abnormalities in the coactivators that interact with GR, MR and AR might be present (4, 19, 20). Investigations showed that SRC-1 null mice exhibited partial resistance to sex hormones, estrogen, progesterone and androgen with impaired development of testis, and uterine and mammary glands (21). However, since no abnormalities of the known coregulators such as SRC-1, TIF-2, AIB1 and SRA were found, the etiology of these cases remains unknown (19, 20).

Refetoff syndrome

Resistance to thyroid hormone (RTH) is an inherited disorder called Refetoff syndrome that involves reduced tissue sensitivity to the thyroid hormone. Most cases associated with RTH are caused by *TRβ* gene mutations. The resulting mutant receptors suppress the functioning of normal TR as a result of a dominant negative effect. It has been reported that dissociation of corepressors such as NcoR and SMART or recruitment of the coactivator SRC-1 are impaired in mutant TRβs (22, 23). However, some patients with RTH do not have *TRβ* gene mutations. Since SRC-1 null mice exhibited a phenotype similar to RTH, it can, however, be speculated that some coregulator abnormalities are present in these patients (24). Nevertheless, no known coregulator defects such as SRC-1 mutations have been found in such cases.

TRAP complex-related phenotypes

Another coactivator complex, the human TR-associated protein (TRAP) complex was originally identified through its intracellular ligand-dependent association with TRα. TRAP-related complexes have also been isolated and identified as being completely or partly SRB/MED-containing cofactor complex (SMCC) or DRIP/TRAP complexes and so on (25). The TRAP complex lacks HAT activity but does interact with RNA polymerase II and is thought to be a component of an

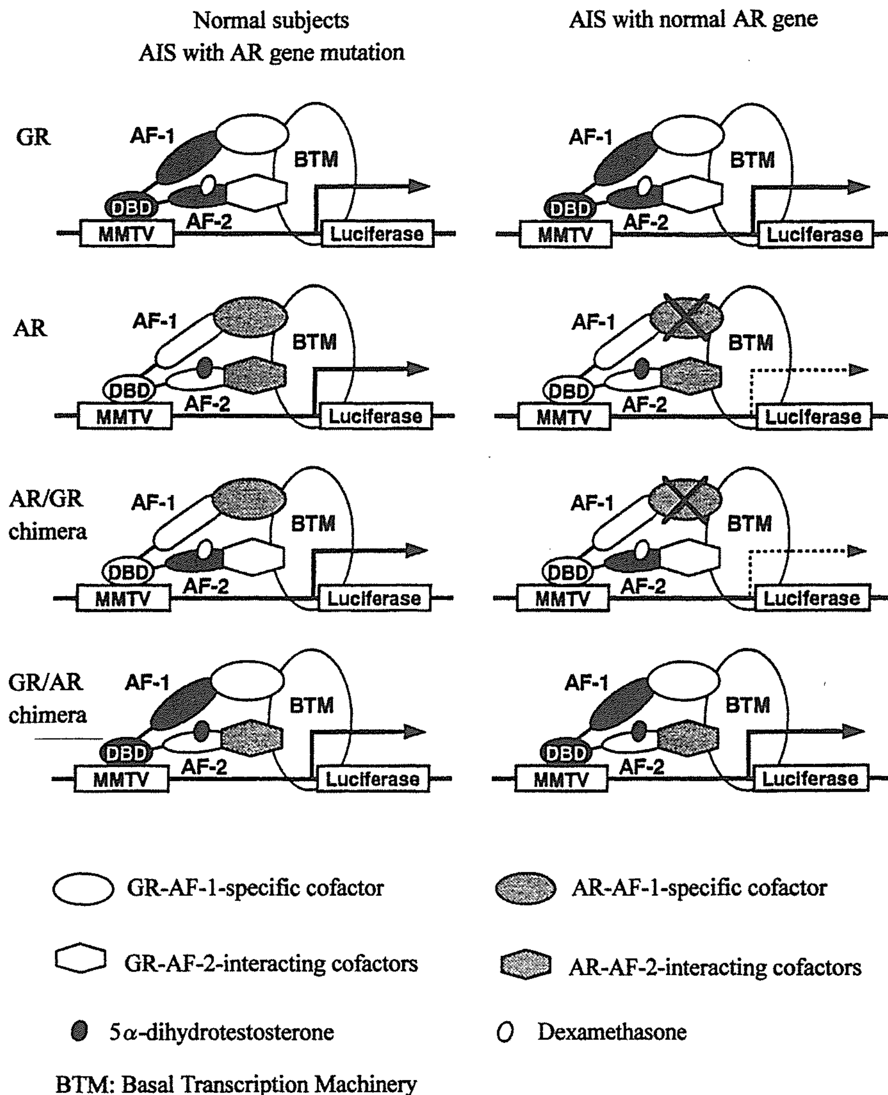

Figure 2. Schematic representation of the coactivator defects in AIS patients (ref. 5). In the fibroblasts of AIS patients with normal genes, transactivation by the transfected AR or AR-GR chimera was low because of defects in an AR AF-1 specific coregulator essential to transmission of the transactivating signal. In the fibroblasts of AIS patients with an AR gene mutation ($Arg^{840} \rightarrow His$), the endogenous AR is inactive, but the transfected AR and AR-GR chimera are active, because the AR-AF-1-specific cofactor is intact. The transfected GR and AR-AR chimera are active among the three types of fibroblasts, since the GR AF-1 specific coregulator is intact. LBD: ligand-binding domain, BTM: basal transcriptional machinery.

RNA polymerase II holoenzyme. The TR complex interacts with a variety of activators that include not only nuclear receptors such as TR and VDR but also others such as p53, VP16, Sp1 and so on. As suggested by yeast mediator functions, this mammalian complex appears to function through direct interactions with both DNA-bound activators and RNA polymerase II, thus mediating the activator-enhanced recruitment of RNA polymerase II and other general transcription factors to the promoter. TRAP 220 is a component of the TRAP complex, and a mammalian homologue of the yeast mediator that shows diverse coactivation functions, interacting directly with nuclear receptors and RNA polymerase II. Complete disruption of the murine *Trap 220* gene produced null mutants that died during early gestation as a result of heart failure while exhibiting impaired neuronal development. Primary embryonic fibroblasts derived from these null mutants showed a prominent decrease in thyroid hormone receptor function that was restorable by ectopic TRAP 220. In

Table 1. Coregulator-related Diseases

Disease	Coregulator abnormality
A. Coregulator disease	
Rubinstein-Taybi syndrome	CBP gene deletions or mutations
Androgen insensitivity syndrome	AR AF-1 specific coactivator abnormality
Multiple hormone resistance	Absence of any defects in the coregulators examined
Refetoff syndrome	Absence of any defects in the coregulators examined
X-linked dementia and hypothyroidism	TRAP230
B. Diseases with coregulator abnormalities or altered interactions with the coregulators	
a. Altered expression or phosphorylation of the coregulators	
Breast cancer	increased expression of AIB1
	phosphorylation of AIB1
	increased expression of cyclin D1
	increased expression of SRA isoform
Prostate cancer	increased expression of TIF2 or SRC-1
	synergistic action of BRCA1 with the AR-TIF2 complex
	activation of cyclin E for AR transcription
Adrenal tumor	correlation between COUP-TF-1 and N-CoR mRNA expression
Huntington's disease	increased expression of CA150
Meningioma	increased expression of TIF2
AIDS	activation of Vpr for GR transactivation
b. Altered binding affinity with the coactivators	
Prostate cancer	increased binding affinity between the mutant ARs and TIF2
Kennedy's disease	decreased binding affinity between the AR polyglutamine repeats and TIF2 or SRC-1
Refetoff syndrome	increased binding affinity between the mutant TRβs and SRC-1
c. Altered binding affinity with the corepessors	
Refetoff syndrome	impaired dissociation of corepressors from the mutant TRβs

addition, haploinsufficient animals showed growth retardation and pituitary hypothyroidism (7, 25). Furthermore, it was reported that a mutation within the CAG repeat of the TRAP 230 subunit gene of the TRAP complex is correlated with X-linked dementia and hypothyroidism syndrome (7, 26). These results suggest a close functional association between the TRAP complex and pituitary-thyroid axis.

Various Clinical Situations That are Thought to be Related to Coregulators

It is well recognized that proliferations of mammary and uterine cancers are estrogen-dependent whereas those of prostate cancer are androgen-dependent. In such hormone-dependent cancers, it has been suggested that the degree of expression or phosphorylation of steroid hormone receptors and coregulators in cancerous tissues is closely associated with the extent of cancer cell proliferation or the prognosis. Although some changes in the expression of steroid hormone receptors such as ER, progesterone receptor (PR) or AR occur in accordance with tumorigenesis, it is reported that these changes are regulated by the expression of the coactivators or corepressors. In addition, it has been shown that signal crosstalk between the steroid hormone receptor and growth factor provokes phosphorylation of both the steroid hormone receptor and coregulators, consequently leading to more powerful activation of the steroid hormone receptors. One such example is AIB1, which is a coactivator and is especially overexpressed in mammary cancer (27). The mechanism by which growth factors such as insulin-like growth factor (IGF), which is an epidermal growth factor (EGF), promote mammary cancer proliferation involves MAPK activation by these growth factors leading to phosphorylation of ER (28) and AIB1 (27). The phosphorylated AIB1 couples tightly with p300/CBP leading to the enhancement of ER-mediated transactivation as a result of increased HAT activity (29). While ER also stimulates the expression of a cell cycle regulator, cyclin D1, AIB1 enhances the ER-mediated transcriptional activity of Cyclin D1 gene promoter. It is reported that Cyclin D1 is overexpressed from the early stages of breast cancer, suggesting that AIB1 also plays an important role in the regulation of Cyclin D1-associated proliferation mechanisms during the development of breast cancer (30, 31). Similar effects have also been reported with other coregulators, namely SRC-1 and TIF-2 on the estrogen-dependent transcription of Cyclin D1.

The mechanisms associated with the onset of leukemia, involve chromosomal translocation resulting in the formation of a chimera-type fusion protein composed of hematopoietic transcription factor, which leads to the suppression of hematopoietic differentiation as a result of a dominant negative effect. A co-repressor complex is involved in this mechanism. For example, the hematopoietic transcription activation factor, AML1, is associated with the onset of leukemia because it forms fusion proteins as a result of chromosomal translocations such as t (8, 21), t (12, 21), t (3,

21). Furthermore, the fusion protein, AML-ETO, that results from t (10, 23) translocation recruits corepressors such as NCoR and SMRT and the corepressor complex, thus suppressing differentiation of the hematopoietic cell and resulting in the production of leukemia (AML-M2) (32).

Since it is difficult to refer to each disease that is thought to be associated with coregulators, a summary of possible coregulator-related diseases is presented in Table 1.

Conclusion

The discovery of AR coregulator disease in association with AIS has verified the importance of the AF-1 region during AR transcription activities. Identification of the exact molecule that underlies this disease will be an important issue in future studies. Coregulators are essential in determining the tissue-specific actions and the specific biological functions of nuclear receptors. Therefore, extensive research of coregulators is expected to clarify the mechanisms associated with nuclear receptors as well as the pathogenesis of steroid resistance syndrome and hormone-dependent cancers.

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Protein Kinase A Potentiates Adrenal 4 Binding Protein/Steroidogenic Factor 1 Transactivation by Reintegrating the Subcellular Dynamic Interactions of the Nuclear Receptor with Its Cofactors, General Control Nonderepressed-5/Transformation/Transcription Domain-Associated Protein, and Suppressor, Dosage-Sensitive Sex Reversal-1: a Laser Confocal Imaging Study in Living KGN Cells

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The mechanism through which protein kinase A (PKA) potentiates the transactivation ability of adrenal 4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) is currently unclear. In the present study, we investigated the mechanism by applying laser confocal microscopy and fluorescence recovery after photobleaching technique. In KGN cells, forskolin (a PKA stimulator) could reorganize wild-type Ad4BP/SF-1, but not mutant Ad4BP/SF-1 (G35E), from a diffuse distribution pattern to foci formation in the nucleus. The subcellular distributions of GCN5 (general control nonderepressed) and TRRAP (transformation/transcription domain-associated protein), both of which were recently proved to be working in the same complex as the third class of nuclear receptor coactivators, were unexpectedly diffuse inside and outside the nucleus, respectively, when they were separately transfected. However TRRAP was translocated into the nucleus in the presence of GCN5, and together with GCN5 colocalized with Ad4BP/SF-1 in the same foci when PKA was activated. A luciferase assay also indicated that these two cofactors enhanced Ad4BP/SF-1 transactivation.

Dosage-sensitive sex reversal (DAX-1) interacts with and thus inhibits Ad4BP/SF-1 transactivation. The coexistence of the two proteins dramatically altered their respective subnuclear distributions. They colocalized extensively, suggestive of binding, and Ad4BP/SF-1 was sharply immobilized when DAX-1 was coexpressed, whereas PKA could maintain mobility, as evidenced by Fluorescence Recovery After Photobleaching showing that Ad4BP/SF-1 mobility recovered after forskolin treatment.

Therefore, the PKA signal pathway may modify the interaction between Ad4BP/SF-1 and its activators and repressor (GCN5 and TRRAP are integrated, whereas DAX-1 is disassociated), and thus stimulate the Ad4BP/SF-1 transactivation. (*Molecular Endocrinology* 18: 127–141, 2004)

AD4BP, ALSO KNOWN as SF-1 and formally designated NR5A1 (nuclear receptor subfamily 5, group A, member 1) is a mammalian homolog of *Drosophila* fushi-tarazu factor 1 (1). Ad4BP/SF-1 was originally identified as a steroidogenic tissue-specific transcription factor (2) and belongs structurally to a member of the nuclear receptor superfamily that includes receptors for steroid, thyroid, and retinoid hormones. Ad4BP/SF-1 contains a characteristic zinc finger DNA-binding domain, an intervening hinge region, and a putative carboxyl-terminal ligand-binding domain. Ad4BP/SF-1 is designated as an orphan nuclear receptor because no definite ligand has been identified to date. Ad4BP/SF-1 is essential for the development of steroidogenic tissue (3–5) because disruption of mouse Ad4BP/SF-1 caused a lack

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

of adrenal and gonadal development, XY sex reversal, persistence of Müllerian structure in males, and abnormalities of the hypothalamus and pituitary gonadotropes (6, 7). In humans, there have been three patients reported thus far with Ad4BP/SF-1 mutations. The first Ad4BP/SF-1 mutation in humans was a heterozygous mutation (G35E) in a karyotypically male patient who showed complete XY sex reversal and primary adrenal failure (8).

Dosage-sensitive sex reversal (DAX-1) is an unusual orphan receptor with an expression profile that overlaps that of Ad4BP/SF-1, namely, in the hypothalamus-pituitary-adrenal and gonadal axis (9, 10). Naturally occurring loss-of-function mutations of the DAX-1 gene cause the human disorder adrenal hypoplasia congenital (AHC) and hypogonadotropic hypogonadism. DAX-1 is an inhibitor of steroidogenesis because it suppresses the transcriptional activation induced by Ad4BP/SF-1. One mechanism for suppression of the Ad4BP/SF-1 transactivation by DAX-1 is that DAX-1 can recruit the nuclear receptor corepressor N-CoR to Ad4BP/SF-1, and this corepressor recruitment capability was found to be markedly diminished in some of the naturally occurring DAX-1 mutations in patients with AHC and hypogonadotropic hypogonadism (11).

It is well known that activation of the cAMP-protein kinase A (PKA) signal pathway can strongly potentiate Ad4BP/SF-1 transactivation activity. Ad4BP/SF-1 binds as a monomer to its responsive element located in the promoter of steroidogenic genes. Ad4BP/SF-1 has been shown to be able to greatly increase both the basal and cAMP-dependent promoter activity of steroidogenic genes, including *CYP17, CYP11A,* and *CYP19* genes (12–14) and *inhibin-α* promoter (15). However, the mechanism by which cAMP augments Ad4BP/SF-1-dependent transactivation activity has not been well elucidated.

In the presence of ligand, steroid receptors have been thought to remain statically bound to regulatory sites in the target genes. In contrast, the vast majority of nuclear proteins, including steroid/nuclear receptors, are now believed to be highly dynamic with a wide range of mobility (16, 17). Recent intensive studies of glucocorticoid receptor (GR) (18–20) and estrogen receptor (ER) (21, 22) revealed that receptors undergo continuous exchange between chromatin regulatory elements and the nucleoplasm compartment when ligand is constantly available. The ligand-induced steroid receptor-coactivator complex, and even the individual components of those complexes, also undergo rapid exchange (18, 22). GR cycles continuously on and off the chromatin target as demonstrated as a hit and run model, in which GR first binds to chromatin after ligand activation, recruits a remodeling activity, facilitates transcription factor binding, and is simultaneously lost from the template (20). Rapid exchange of a nuclear receptor with regulatory sites may have important consequences, because the dynamic receptor would be continuously available for modification by some second pathway, such as multicellular signal pathways, which may quickly modulate nuclear receptor activity. Nuclear receptors such as retinoid acid receptor and thyroid hormone receptor (TR) have also been proven to be moving rapidly in the nucleus (23), hinting the dynamic exchange process might be a general feature of many nuclear receptors. Ligand-binding and protein-protein interaction seem to affect the intracellular mobility of some nuclear receptors and thereby may contribute to their biological activity (23). High mobility is thought to be critical for nuclear receptors to exert their effects on transcription (21).

By taking advantage of the technique of laser confocal microscopy and fluorescence recovery after photobleaching (FRAP) study, we found that, in living granulosa-like KGN cells, activation of the PKA signal pathway altered the Ad4BP/SF-1 subnuclear distribution pattern, leading to the formation of fluorescent foci. This process was accompanied by the recruitment of a newly identified third class of nuclear receptor coactivator complex, the GCN5/TRRAP complex, and also the disassembly of DAX-1, which interacted with Ad4BP/SF-1 and immobilized Ad4BP/SF-1. Our data thus suggest that the reintegration of the protein-protein interaction between Ad4BP/SF-1 and its coactivators or its repressor protein, DAX-1, might be a possible mechanism explaining how PKA potentiates Ad4BP/SF-1 transactivation.

RESULTS

Ad4BP/SF-1 Is Critical for the Augmentation of Aromatase Promoter II (ArPII) by PKA

The expression of the *human cytochrome P45019 aromatase* gene in the ovary is specifically driven by ArPII, a well known target promoter of Ad4BP/SF-1 (24). FSH, via membrane G protein, can stimulate aromatase expression in ovarian granulosa cells by increasing the intracellular cAMP level and thus activating the PKA signal pathway. Activation of the PKA pathway inside the cells can further increase the Ad4BP/SF-1-stimulated ArPII activity. As shown in Fig. 1, the steroidogenic human granulosa-like cell line KGN, which expresses aromatase, and the nonsteroidogenic NIH-3T3 fibroblast cell line were transfected with the human ArPII firefly luciferase reporter plasmid, pGL3-ArPII, together with the renilla luciferase plasmid phRL-CMV, which constitutively expresses the renilla luciferase to serve as an internal control. The expression vectors for wild-type or mutant

Abbreviations: Ad4BP/SF-1, Adrenal 4 binding protein/steroidogenic factor 1; AF-2 domain, activation function-2 domain; AHC, adrenal hypoplasia congenital, X-linked; AR, androgen receptor; ArPII, aromatase promoter II; CFP, cyan fluorescence protein; DAX-1, dosage-sensitive sex reversal; DMSO, dimethylsulfoxide; ER, estrogen receptor; FRAP, fluorescence recovery after photobleaching; GCN5, general control nonderepressed; GFP, green fluorescence protein; GR, glucocorticoid receptor; HI, heterogeneity index; PGC-1, peroxisome proliferators activated receptor-γ coactivator 1; PKA, protein kinase A; TRRAP, transformation/transcription domain-associated protein; YFP, yellow fluorescence protein.

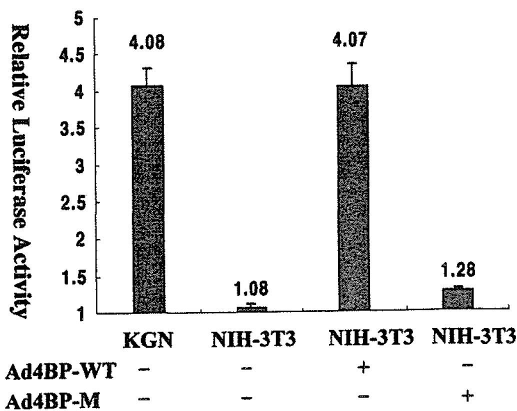

Fig. 1. CYP19 (Aromatase) Promoter II Activity in Response to Wild-Type or Mutant Ad4BP/SF-1 Stimulated by PKA

KGN cells and NIH-3T3 cells were transfected with the human ArPII firefly luciferase reporter plasmid, pGL3-ArPII, together with the renilla luciferase plasmid, phRL-CMV, as an internal control. pcDNA3.1-Ad4BP/SF-1-WT (wild type) or pcDNA3.1-Ad4BP/SF-1-M (mutant, G35E) was cotransfected into two groups of NIH-3T3 cells as indicated. All cells were treated overnight with either 10^{-6} mol/liter forskolin or the solvent DMSO. The multiple of relative luciferase activities induced by forskolin to that of control (induced by DMSO) are expressed as the mean ± SD.

(G35E) human Ad4BP/SF-1, or the control empty vector pcDNA3.1(+), were also cotransfected into NIH-3T3 cells. One night after the transfection, cells were treated overnight with 10^{-6} mol/liter forskolin (an adenyl cyclase stimulator that activates the PKA signal pathway by increasing cAMP) or the solvent dimethylsulfoxide (DMSO), and then a dual-luciferase assay was performed. In KGN cells, which endogenously express Ad4BP/SF-1, treatment with 10^{-6} mol/liter forskolin overnight increased the promoter activity 4-fold under the current experimental condition. On the other hand, in the nonsteroidogenic NIH-3T3 cells, which do not endogenously express Ad4BP/SF-1, the same treatment exhibited almost no effect on the ArPII activity. However, when wild-type Ad4BP/SF-1 was cotransfected into NIH-3T3 cells, overnight forskolin treatment elevated the ArPII activity 4-fold, as observed in KGN cells, whereas the transactivationally inactive mutant Ad4BP/SF-1 (G35E) could not convey the stimulatory effect of forskolin to ArPII (Fig. 1). Therefore, it is evident that Ad4BP/SF-1 is actually a requirement for the augmentation of ovarian ArPII activity induced by PKA. A similar effect of PKA on Ad4BP/SF-1-dependent transcription of the CYP 11A promoter was also observed in another pair of steroidogenic and nonsteroidogenic cells, Y1 and CV1 (data not shown).

Activation of PKA Altered the Subnuclear Distribution Pattern of Human Ad4BP/SF-1 from Homogeneity to Foci Formation

Research using nuclear receptors fused to green fluorescent protein (GFP), yellow fluorescent protein (YFP), or cyan fluorescent protein (CFP) under various stimuli in living cells has proved to be a powerful tool for deepening our understanding of the transcriptional activation of nuclear receptors (25). In this study, the intracellular distribution pattern of wild-type, or mutant Ad4BP/SF-1 containing G35E, in response to PKA, was examined dynamically by observing the fusion proteins GFP-Ad4BP/SF-1 and YFP-Ad4BP/SF-1 under a confocal laser microscope. The functional validity of these chimeric proteins was tested by a dual luciferase assay using the pGL3-ArPII reporter described above in KGN cells. As shown in Fig. 2, the transactivation activity of GFP-Ad4BP/SF-1-WT (wild type) was up to 87% conserved compared with pcDNA3.1-SF1-WT, and the responsiveness of GFP-SF1-WT to PKA was almost completely conserved. Both GFP-Ad4BP/SF-1-M (mutant) and pcDNA3.1-Ad4BP/SF-1-M were transactivationally inactive compared with Ad4BP/SF-1-WT.

After transient transfection, and treatment with or without 10^{-6} mol/liter forskolin, the intracellular distribution of each variant of Ad4BP/SF-1 fused to GFP was visualized in both steroidogenic KGN cells and nonsteroidogenic monkey kidney CV1 cells using a LSM-510-META laser confocal scanning microscope (Carl Zeiss, Oberkochen, Germany). GFP-Ad4BP/SF-1-WT (Fig. 3A) was predominantly located inside the nuclei, as previously reported in Y1 cells (26). The nucleoli demonstrated almost no fluorescence, suggesting that nucleoli are almost devoid of Ad4BP/SF-1. The cytoplasm was also devoid of fluorescence. In the absence of forskolin, the GFP-Ad4BP/SF-1-WT

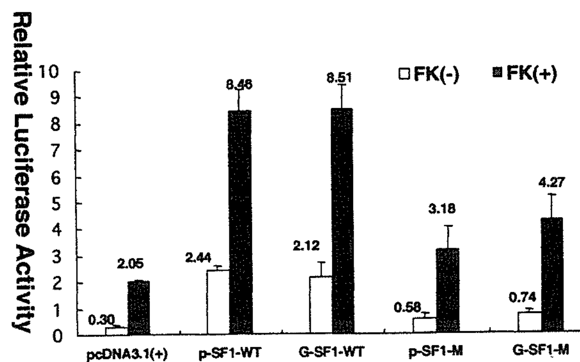

Fig. 2. Transactivation Activity of Wild-Type or Mutant Ad4BP/SF-1 Fused to GFP and Their Responsiveness to PKA

KGN cells were transfected with pcDNA3.1-Ad4BP/SF-1-WT or pcDNA3.1-Ad4BP/SF-1-M (G35E) or their respective GFP-fusion plasmids together with pGL3-ArPII + phRL-CMV. The cells were then treated overnight with 10^{-6} mol/liter forskolin or the solvent DMSO. *Solid and hollow bars* represent the luciferase activities with treatment of forskolin or DMSO, respectively. The transactivation activity of GFP-Ad4BP/SF-1-WT was preserved up to 87% compared with pcDNA3.1-Ad4BP/SF-1-WT, and its responsiveness to forskolin was almost completely conserved. Both pcDNA3.1-Ad4BP/SF-1-M and GFP-Ad4BP/SF-1-M were transactivationally inactive.

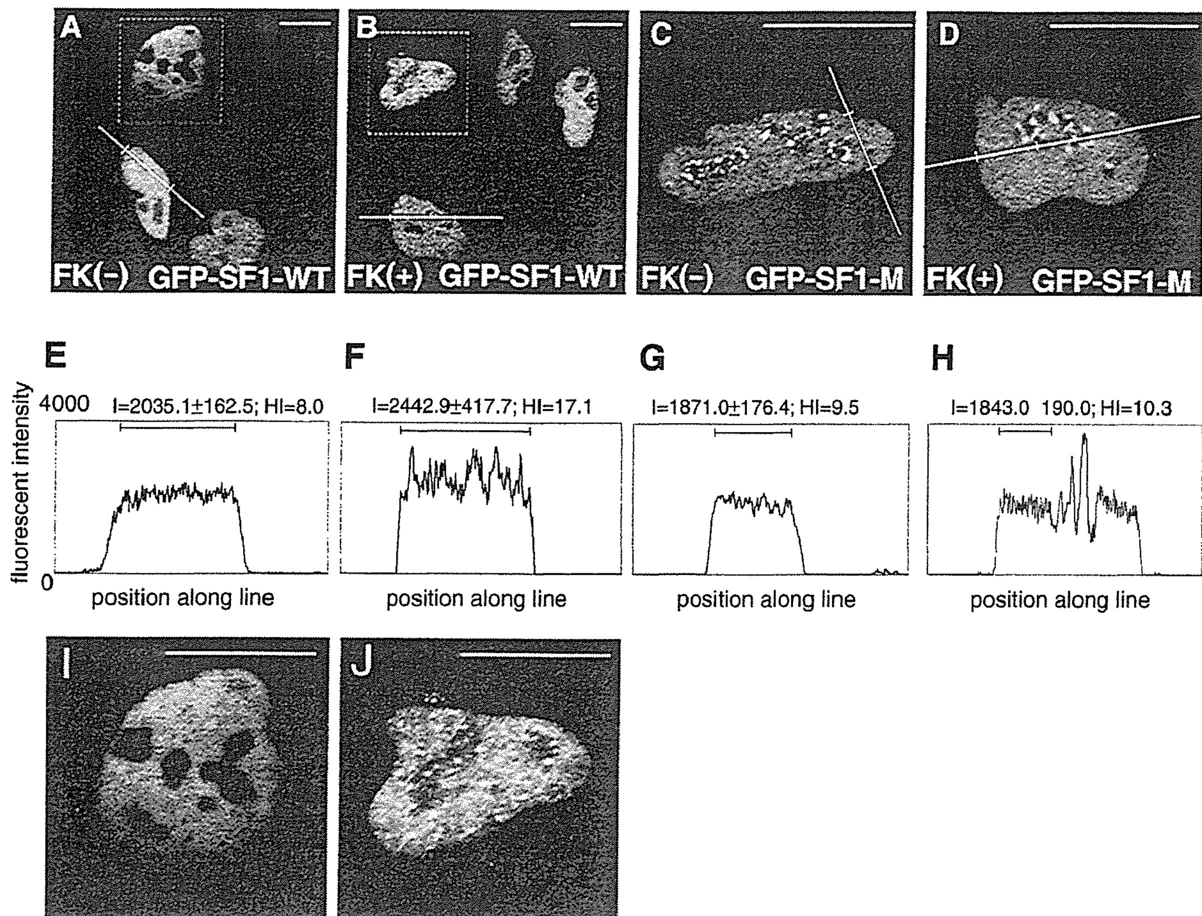

Fig. 3. Subnuclear Localization of GFP-Ad4BP/SF-1-WT and GFP-Ad4BP/SF-1-M in the Presence or Absence of 10^{-6} mol/liter Forskolin

KGN cells were transfected with 0.5μg/dish of GFP-Ad4BP/SF-1-WT (A and B) or GFP-Ad4BP/SF-1-M (C and D). The chimeric fluorescent proteins expressed were observed in living cells using a Zeiss LSM 510 META laser confocal microscope as described in *Materials and Methods*. Wild-type Ad4BP/SF-1 is diffuse in the nucleus (A and I) and is assembled into foci on a diffuse fluorescence background when PKA is activated (B and J). Mutant Ad4BP/SF-1 is diffuse in the nucleus whereas dots are manifested in the nucleoli (C), and forskolin has no effect on its distribution pattern (D). The lines for line scan analysis were shown on each representative cell, with the segment for HI (heterogeneity index) analysis also indicated. The fluorescent intensity fluctuation graph of each representative cell is shown as panels E–H, in relation to panels A–D, respectively. X axis is the position along lines and Y axis is fluorescent intensity. A *bar within each graph* marks the segment of the line for which the HI analysis is performed; the corresponding I (intensity) and HI values are indicated on the *top* of each *graph*. Panels I and J are magnified views of the cells outlined by the *hatched line* in panel A and B, respectively. *Magnification scale bar*, 10 μm.

fluorescence signal was quite diffuse in the nuclei of most cells (Fig. 3, A and I).

The treatment of 10^{-6} mol/liter forskolin overnight caused more than 60% of studied cells to manifest clear foci formation, which happened inside the nucleoplasm but not the nucleoli (Fig. 3, B and J), suggesting an intranuclear rearrangement of the Ad4BP/SF-1 distribution pattern induced by PKA. The intranuclear distribution pattern of GFP-Ad4BP/SF-1-WT was further quantitatively analyzed. Digital figures obtained by a LSM-510-META microscope were subjected for Linescan analysis by the LSM software (version 3.0). A straight line was made through a target cell, and the fluorescent intensity along the line was recorded by the software. The mean and SD values of the fluorescent intensity signals for the segment of interest (nucleus, avoid nucleoli) were calculated. The heterogeneity of fluorescent intensity along the segment of interest was evaluated by the parameter of HI (heterogeneity index), which was calculated by the formula of HI = 100 × SD/mean. A fluorescent intensity fluctuation graph, which apparently demonstrates the heterogeneity, was made by plotting intensity against distance of the line. Panels E–H of Fig. 3 represent the Linescan of panels A–D, respectively. As shown in Fig. 3E, the fluorescent intensity of the representative cell from forskolin(−) group remains quite constant (HI = 8.0), whereas forskolin treatment (Fig. 3F) causes a 2-fold fluorescent intensity increase (HI = 17.1), indicating the reorganization of GFP-Ad4BP/SF-1-WT in the nuclear. Fifty cells with proper expression of GFP-Ad4BP/SF-1-WT from each group were analyzed by

Linescan, and the HI value of the forskolin (+) group is more than two times higher than that of the forskolin (−) group (17.9 ± 3.07 *vs.* 7.9 ± 1.7, $P < 0.01$).

However, on transfection with GFP-Ad4BP/SF-1-M (mutant), the fluorescence signal was still found to localize inside the nucleus in the absence of forskolin (Fig. 3C), but it was also diffusely distributed even in the presence of 10^{-6} mol/liter forskolin (Fig. 3D). Linescan shows that the intranuclear distribution pattern is almost not altered by forskolin (Fig. 3, G and H). Interestingly, clear fluorescent dots were observed inside the nucleoli in the case of the mutant, and the pattern was unchanged in the presence or absence of forskolin.

The similar phenomenon could be observed in CV1 cells (data not shown). Precisely, a time course study found that, in KGN cells, wild-type Ad4BP/SF-1 made foci within 3 h after addition of forskolin, while in CV1 cells, only a small amount of cells began manifesting foci 5–6 h after forskolin treatment, indicating a delayed reaction of Ad4BP/SF-1 to PKA signal pathway in this cell line as compared with the KGN cell line.

Coactivators GCN5 and TRRAP Are Recruited to Ad4BP/SF-1 Foci When the PKA Signal Pathway Is Activated

Foci formation in the nuclear localization of nuclear receptors usually correlated with a functionally active state of the nuclear receptors as a result of a compartmental shift upon activation of the nuclear receptors (25). Activation of PKA seems to provoke the assembly of wild-type Ad4BP/SF-1 protein into this active foci state but had no effect on the mutant Ad4BP/SF-1, which could not respond transactivationally to PKA (Fig. 1). The question to be addressed next is the nature of this foci formation of Ad4BP/SF-1 induced by PKA. Coactivators such as p300/CBP (CREB-binding protein), SRC-1 (steroid receptor coactivator-1), and GCN5 exhibit HAT (histone acetyltransferase) activity. These HAT proteins acetylate nucleosomal histone, which further increases the accessibility of transcription factors to their DNA targets. Recently, it has been pointed out that not only histone, but also several transcription factors such as p53, E2F transcription factor 1, and AR *etc.*, can also be acetylated by the HAT coactivators. GCN5 was found to be able to acetylate Ad4BP/SF-1 *in vitro* and thus stimulate the transactivation of Ad4BP/SF-1 (27). GCN5 can be recruited to Ad4BP/SF-1 as a newly identified Ad4BP/SF-1 coactivator. The c-Myc-interacting protein TRRAP (28) was recently proved to be working together with the coactivator GCN5 and other partners such as TAFII30 (29) as the third class of coactivator complex for nuclear receptors in addition to the first class of the p160/CBP-HAT coactivator complex and the second vitamin D receptor interacting protein/thyroid hormone receptor-associated protein non-HAT coactivator complex (30). The three LXXLL motifs of TRRAP serve as a direct and ligand-dependent interaction surface for nuclear receptors, *e.g.* ERα (30). As shown in Fig. 4, both GCN5 and TRRAP were found to further potentiate the Ad4BP/SF-1-stimulated ArPII activity with a more powerful effect seen in the case of TRRAP. Both of these factors may work as coactivators for Ad4BP/SF-1 and enhance the transactivation ability of Ad4BP/SF-1.

We next studied the intracellular distribution dynamics of both GCN5 and TRRAP in living KGN cells and their relationship with Ad4BP/SF-1 in the presence or absence of activation of the PKA signal pathway. The transcriptional coactivation ability of these fusion proteins measured by the dual-luciferase reporter assay was similar to that observed in Fig. 2 (data not shown). Considering that the PKA pathway is a critical signal pathway for cells, autonomous activation of this pathway might interfere with the results and thus possibly make the difference less obvious. Therefore, for the control groups of cells, we blocked the PKA pathway with 10^{-6} mol/liter H89, seeking a clearer difference between the control and forskolin groups. When GFP-GCN5 was transfected into KGN cells, fluorescence was mainly located inside the nucleus in a uniform pattern with the nucleoli being devoid of fluorescence. This distribution pattern was not altered even after PKA was stimulated by 10^{-6} mol/liter forskolin (Fig. 5, A and B). When TRRAP-GFP was transfected into KGN cells, TRRAP-GFP predominantly resided in the cytosol in a diffuse manner, and again 10^{-6} mol/liter forskolin had no effect on this distribution manner (Fig. 5, C and D). To verify this result, TRRAP-GFP was also transfected into the nonsteroidogenic NIH-3T3 cells, and the same result was observed (Fig. 5, E and F). This observation of dissociated localization between GCN-5 and TRRAP in the basal state was unexpected because TRRAP/GCN5 has been shown to work as cofactor in the same complex. When we next cotransfected both GFP-GCN5 and TRRAP-YFP into KGN cells, the distribution pattern of GCN5 was almost identical to the one observed when GFP-GCN5 was

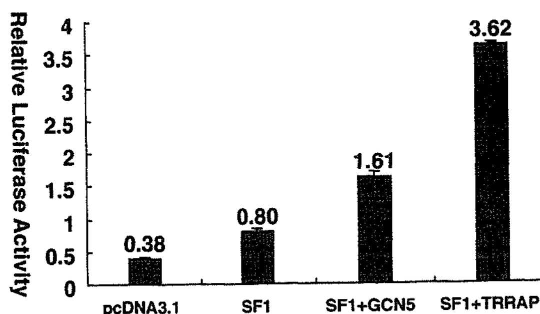

Fig. 4. Coactivators GCN5 and TRRAP Potentiate the Transactivation Activity of Ad4BP/SF-1 on the CYP19 Promoter

NIH-3T3 cells were transfected with pGL3-ArPII + phRL-CMV. pcDNA3.1-Ad4BP/SF-1-WT, or in combination with the same amount on a molar basis of either pcDNA3-GCN5 or pcDNA3-TRRAP were also cotransfected. Both cofactors could potentiate Ad4BP/SF-1-mediated transcription.

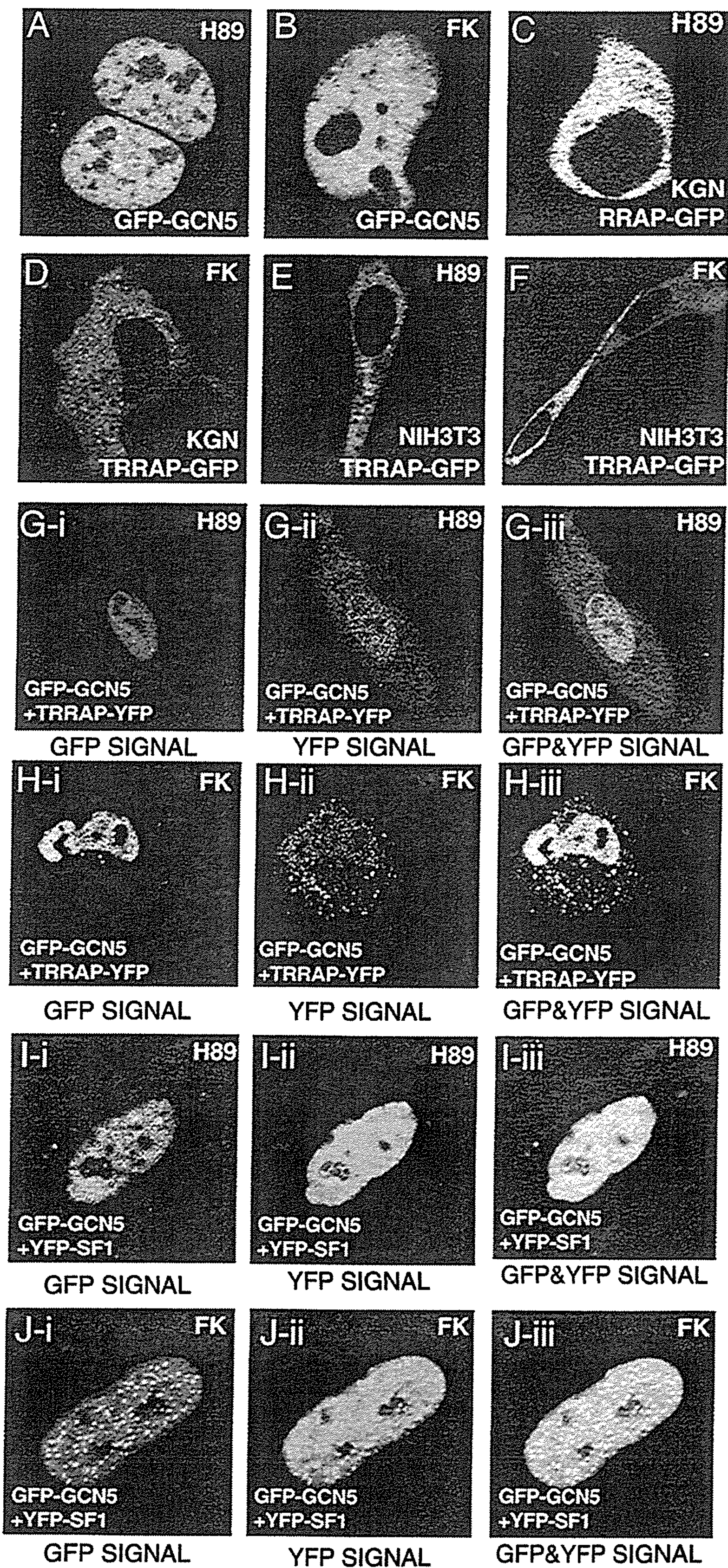

Fig. 5. Subcellular Localization of Fluorescent Protein-Fused GCN5 or Fluorescent Protein-Fused TRRAP, and Their Interrelationship with Fluorescent Ad4BP/SF-1, with the PKA Signal Pathway Either Blocked by H89 or Activated by Forskolin

KGN cells or NIH-3T3 cells (panels E and F) were transfected with the expression plasmids indicated in each panel. The

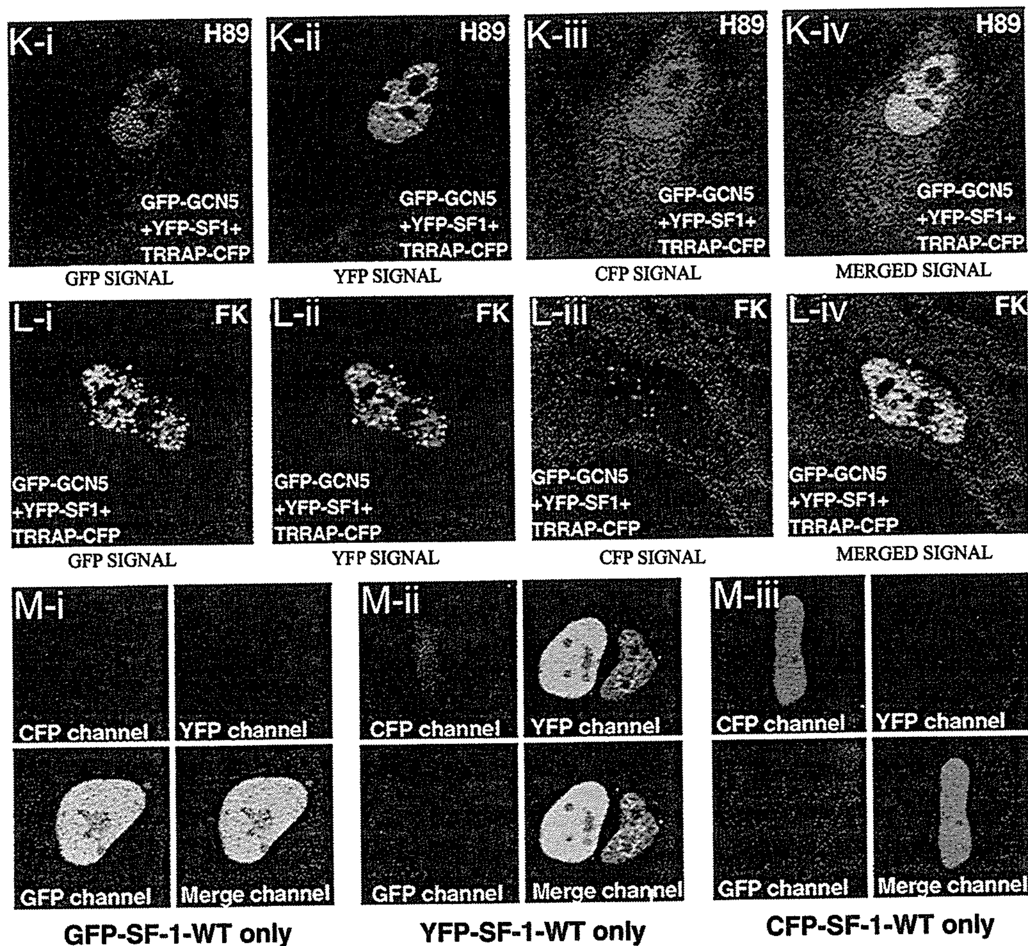

Fig. 5 Continued.

solely transfected. However, in contrast to the restricted localization of TRRAP in the cytosol when TRRAP-GFP was solely transfected, TRRAP-YFP with the coexistence of GFP-GCN5 resided homogenously both inside and outside the nucleus, with a little more intensity inside the nucleus (Fig. 5G). This pan-cellular distribution of TRRAP with the coexistence of GCN5 was unchanged by 10^{-6} mol/liter forskolin treatment (Fig. 5H). TRRAP may originate as a cytoplasmic protein, but GCN5 might be able to bind TRRAP and drag it into the nucleus, and then both work together as coactivators in the same complex.

The intracellular distribution relationships between GCN5, TRRAP, and Ad4BP/SF-1 in the presence and absence of PKA activation were subsequently studied. When GFP-GCN5 and YFP-Ad4BP/SF-1 were cotransfected into KGN cells, both fluorescence signals were predominantly located in the nucleus in a uniform pattern (Fig. 5I). Forskolin (10^{-6} mol/liter) changed YFP-Ad4BP/SF-1 to a speckled distribution pattern with the formation of foci. Furthermore, GFP-GCN5 was also induced to make foci by forskolin treatment and precisely colocalized with YFP-Ad4BP/SF-1 in the same fluorescent foci (Fig. 5J). When GFP-GCN5, YFP-Ad4BP/SF-1, and TRRAP-CFP were cotransfected together in the presence of a PKA blocker, H89, both GFP-GCN5 and YFP-Ad4BP/SF-1 showed a diffuse distribution pattern in the nucleus while TRRAP-

amounts of each plasmid transfected in each panel were equivalent on a molar basis. Treatment with forskolin (FK) or H89 is indicated *in or just below each panel*. In the cases of multifluorescent protein chimeras cotransfection, each fluorescent signal and the merged signals are also indicated. GCN5 is diffuse in the nucleus (A and B) of KGN cells, whereas TRRAP is predominantly located in the cytoplasm in both KGN cells (C and D) and NIH-3T3 cells (E and F). TRRAP is dragged into the nucleus when GCN5 is also present (G and H). Forskolin has no effect on their distribution pattern. When cotransfected with Ad4BP/SF-1, GCN5 (I and J) and GCN5 and TRRAP together (K and L) were recruited to Ad4BP/SF-1 foci when Ad4BP/SF-1 was activated by forskolin. M-i, M-ii, and M-iii are controls demonstrating the unmixing algorithms reliability of simultaneous imaging of GFP, YFP, and CFP. Cells containing only GFP are clearly visible in only the GFP channel with no bleed through in the YFP and CFP channels (M-i). Similarly, cells containing only YFP (M-ii) or CFP (M-iii) are only visible in the YFP or CFP channel, with no bleed through in the other two channels.