

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 \pm 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 LRP5 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

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References

- [1] K.M. Cadigan, R. Nusse, Wnt signaling: a common theme in animal development, *Genes Dev.* 11 (1997) 3286–3305.
- [2] A. Wodarz, R. Nusse, Mechanisms of Wnt signaling in development, *Annu. Rev. Cell Dev. Biol.* 14 (1998) 59–88.
- [3] A. Bejsovec, Wnt signaling: an embarrassment of receptors, *Curr. Biol.* 10 (2000) R919–R922.
- [4] Y. Kawano, R. Kypta, Secreted antagonists of the Wnt signalling pathway, *J. Cell Sci.* 116 (2003) 2627–2634.
- [5] A. Bafico, G. Liu, A. Yaniv, A. Gazit, S.A. Aaronson, Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow, *Nat. Cell Biol.* 3 (2001) 683–686.
- [6] Y. Gong, R.B. Slee, N. Fukui, G. Rawadi, S. Roman-Roman, A.M. Reginato, H. Wang, T. Cundy, F.H. Glorieux, D. Lev, M.

- Zacharin, K. Oexle, J. Marcelino, W. Suwairi, S. Heeger, G. Sabatakos, S. Apte, W.N. Adkins, J. Allgrove, M. Arslan-Kirchner, J.A. Batch, P. Beighton, G.C. Black, R.G. Boles, L.M. Boon, C. Borrone, H.G. Brunner, G.F. Carle, B. Dallapiccola, A. De Paepe, B. Floege, M.L. Halfhide, B. Hall, R.C. Hennekam, T. Hirose, A. Jans, H. Juppner, C.A. Kim, K. Keppler-Noreuil, A. Kohlschuetter, D. LaCombe, M. Lambert, E. Lemyre, T. Letteboer, L. Peltonen, R.S. Ramesar, M. Romanengo, H. Somer, E. Steichen-Gersdorf, B. Steinmann, B. Sullivan, A. Superti-Furga, W. Swoboda, M.J. van den Boogaard, W. Van Hul, M. Vikkula, M. Votruba, B. Zabel, T. Garcia, R. Baron, B.R. Olsen, M.L. Warman, LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development, *Cell* 107 (2001) 513–523.
- [7] M. Kato, M.S. Patel, R. Levasseur, I. Lobov, B.H. Chang, D.A. Glass, C. Hartmann, L. Li, T.H. Hwang, C.F. Brayton, R.A. Lang, G. Karsenty, L. Chan, Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in *Lrp5*, a Wnt coreceptor, *J. Cell Biol.* 157 (2002) 303–314.
- [8] L.M. Boyden, J. Mao, J. Belsky, L. Mitzner, A. Farhi, M.A. Mitnick, D. Wu, K. Insogna, R.P. Lifton, High bone density due to a mutation in LDL-receptor-related protein 5, *N. Engl. J. Med.* 346 (2002) 1513–1521.
- [9] M.S. Patel, G. Karsenty, Regulation of bone formation and vision by LRP5, *N. Engl. J. Med.* 346 (2002) 1572–1574.
- [10] S. Harada, G.A. Rodan, Control of osteoblast function and regulation of bone mass, *Nature* 423 (2003) 349–355.
- [11] E. Canalis, Mechanisms of glucocorticoid action in bone: implications to glucocorticoid-induced osteoporosis, *J. Clin. Endocrinol. Metab.* 81 (1996) 3441–3447.
- [12] F. Manelli, A. Giustina, Glucocorticoid-induced osteoporosis, *Trends Endocrinol. Metab.* 11 (2000) 79–85.
- [13] R.S. Weinstein, R.L. Jilka, A.M. Parfitt, S.C. Manolagas, Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone, *J. Clin. Invest.* 102 (1998) 274–282.
- [14] K. Ohnaka, S. Shimoda, H. Nawata, H. Shimokawa, K. Kaibuchi, Y. Iwamoto, R. Takayanagi, Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts, *Biochem. Biophys. Res. Commun.* 287 (2001) 337–342.
- [15] K. Ohnaka, K. Numaguchi, T. Yamakawa, T. Inagami, Induction of cyclooxygenase-2 by angiotensin II in cultured rat vascular smooth muscle cells, *Hypertension* 35 (2000) 68–75.
- [16] J. Sambrook, D. Russell, *Molecular Cloning: A Laboratory Manual*, third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
- [17] B. Mao, W. Wu, G. Davidson, J. Marhold, M. Li, B.M. Mechler, H. Delius, D. Hoppe, P. Stanek, C. Walter, A. Glinka, C. Niehrs, Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling, *Nature* 417 (2002) 664–667.
- [18] A. Glinka, W. Wu, H. Delius, A.P. Monaghan, C. Blumenstock, C. Niehrs, Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction, *Nature* 391 (1998) 357–362.
- [19] M. Mukhopadhyay, S. Shtrom, C. Rodriguez-Esteban, L. Chen, T. Tsukui, L. Gomer, D.W. Dorward, A. Glinka, A. Grinberg, S.P. Huang, C. Niehrs, J.C. Belmonte, H. Westphal, Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse, *Dev. Cell* 1 (2001) 423–434.
- [20] V.E. Krupnik, J.D. Sharp, C. Jiang, K. Robison, T.W. Chickerling, L. Amaravadi, D.E. Brown, D. Guyot, G. Mays, K. Leiby, B. Chang, T. Duong, A.D. Goodearl, D.P. Gearing, S.Y. Sokol, S.A. McCarthy, Functional and structural diversity of the human Dickkopf gene family, *Gene* 238 (1999) 301–313.

- [21] W. Wu, A. Glinka, H. Delius, C. Niehrs, Mutual antagonism between dickkopf1 and dickkopf2 regulates Wnt/beta-catenin signalling, *Curr. Biol.* 10 (2000) 1611–1614.
- [22] A.P. Monaghan, P. Kioschis, W. Wu, A. Zuniga, D. Bock, A. Poustka, H. Delius, C. Niehrs, Dickkopf genes are co-ordinately expressed in mesodermal lineages, *Mech. Dev.* 87 (1999) 45–56.
- [23] P. Fedi, A. Bafico, A. Nieto Soria, W.H. Burgess, T. Miki, D.P. Bottaro, M.H. Kraus, S.A. Aaronson, Isolation and biochemical characterization of the human Dkk-1 homologue, a novel inhibitor of mammalian Wnt signaling, *J. Biol. Chem.* 274 (1999) 19465–19472.
- [24] L. Grotewold, U. Ruther, The Wnt antagonist Dickkopf-1 is regulated by Bmp signaling and c-Jun and modulates programmed cell death, *EMBO J.* 21 (2002) 966–975.
- [25] J. Wang, J. Shou, X. Chen, Dickkopf-1, an inhibitor of the Wnt signaling pathway, is induced by p53, *Oncogene* 19 (2000) 1843–1848.
- [26] J. Shou, F. Ali-Osman, A.S. Multani, S. Pathak, P. Fedi, K.S. Srivenugopal, Human Dkk-1, a gene encoding a Wnt antagonist, responds to DNA damage and its overexpression sensitizes brain tumor cells to apoptosis following alkylation damage of DNA, *Oncogene* 21 (2002) 878–889.
- [27] G. Rawadi, B. Vayssiere, F. Dunn, R. Baron, S. Roman-Roman, BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop, *J. Bone Miner. Res.* 18 (2003) 1842–1853.
- [28] E. Tian, F. Zhan, R. Walker, E. Rasmussen, Y. Ma, B. Barlogie, J.D. Shaughnessy, The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma, *N. Engl. J. Med.* 349 (2003) 2483–2494.

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-Taybi syndrome, 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 coactivator (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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Coregulator-Related Diseases

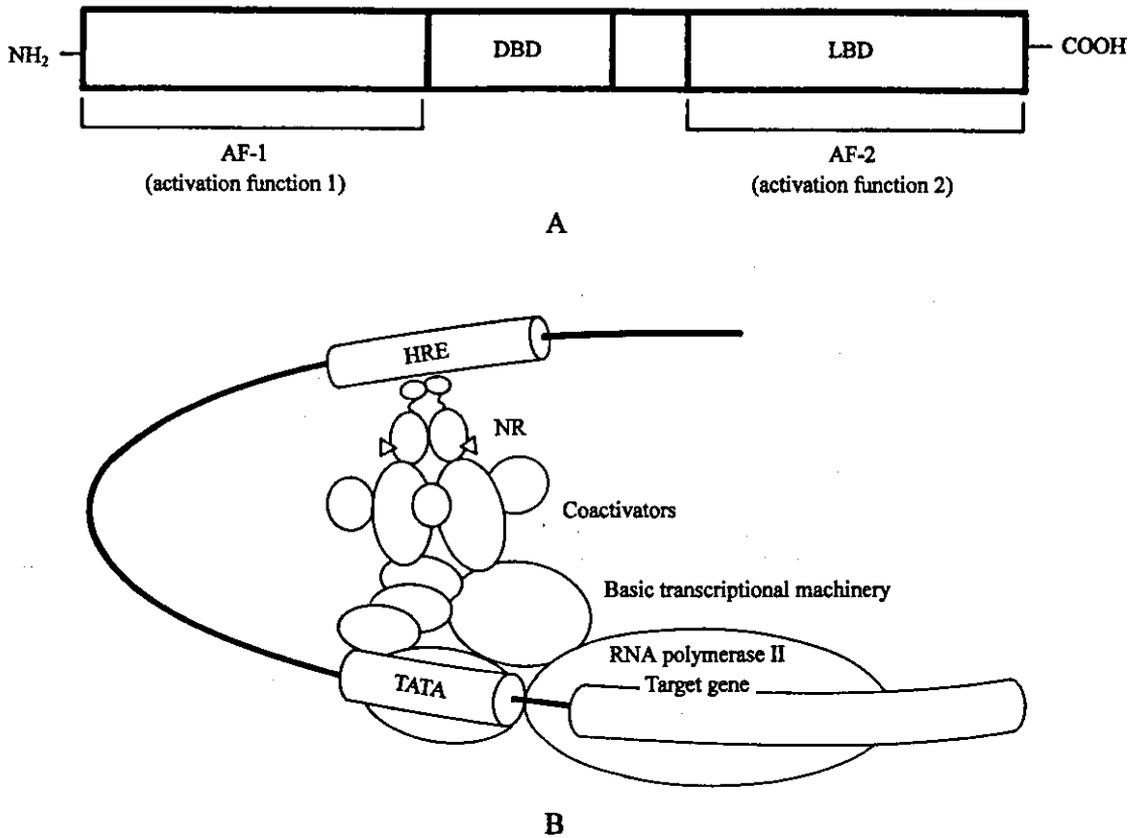


Figure 1. (A) Structure of nuclear receptors (NRs). DBD, DNA-binding domain; LBD, ligand-binding domain. (B) Interactions among the nuclear receptor (NR), coactivators and basic transcriptional machinery.

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, Reifenstein 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 phosphory-

lation-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 *TRβ*s (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

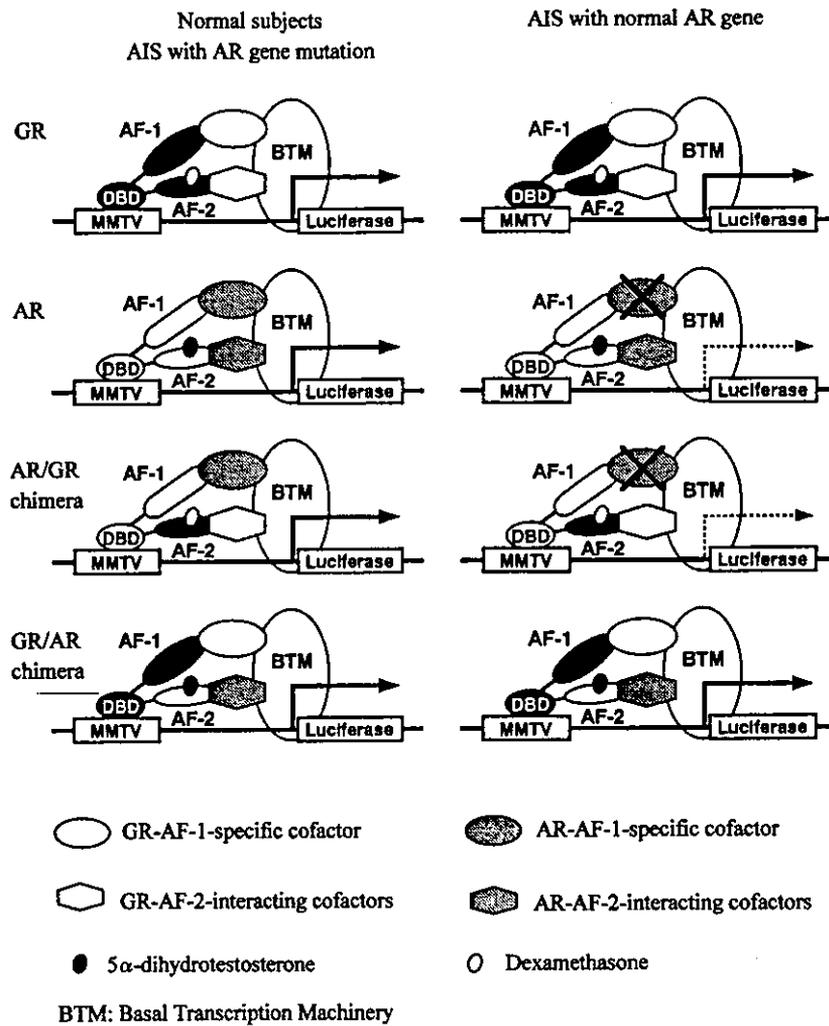


Figure 2. Schematic representation of the coactivator defects in AIS patients (ref. 5). In the fibroblasts of AIS patients with normal genes, transactivation by the transfected AR or AR-GR chimera was low because of defects in an AR AF-1 specific coregulator essential for transmission of the transactivating signal. In the fibroblasts of AIS patients with an AR gene mutation (Arg⁴⁸⁰→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

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 corepressors	
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.

References

- Mangelsdorf DJ, Thummel C, Beato M, et al. The nuclear receptor superfamily: the second decade. *Cell* **83**: 835–839, 1995.
- Horwitz KB, Jackson TA, Bain DL, Takimoto GS, Tung L. Nuclear receptor coactivators and corepressors. *Mol Endocrinol* **10**: 1167–1177, 1996.
- McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* **20**: 321–344, 1999.
- Chrousos GP. A new "new" syndrome in the new world: is multiple postreceptor steroid hormone resistance due to a coregulator defect? *J Clin Endocrinol Metab* **84**: 4450–4453, 1999.
- Adachi M, Takayanagi R, Tomura A, et al. Androgen-insensitivity syndrome as a possible coactivator disease. *N Engl J Med* **343**: 856–862, 2000 (Erratum in: *N Engl J Med* **344**: 696).
- Petrij F, Giles RH, Dauwerse HG, et al. Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature* **376**: 348–351, 1995.
- Ito M, Roeder RG. The TRAP/SMCC/Mediator complex and thyrid hormone receptor function. *Trends in Endocrinol & Metabolism* **12**: 127–134, 2001.
- Zhao Y, Goto K, Saitoh M, et al. Activation function-1 domain of androgen receptor contributes to the interaction between subnuclear splicing factor compartment and nuclear receptor compartment; Identification of the p102 U5 snRNP binding protein as a coactivator for the receptor. *J Biol Chem* **277**: 30031–30039, 2002.
- Saitoh M, Takayanagi R, Goto K, et al. The presence of the amino- and carboxy-terminal domains in androgen receptor is essential for the completion of a transcriptionally active form with coactivators and intranuclear compartmentalization common to the steroid hormone receptors: A three-dimensional imaging study. *Mol Endocrinol* **16**: 694–706, 2002.
- Quigley CA, De Bellis A, Marschke KB, el Awady MK, Wilson EM, French FS. Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev* **16**: 271–321, 1995.
- Ahmed SF, Cheng A, Dovey L, et al. Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. *J Clin Endocrinol Metab* **85**: 658–665, 2000.
- Rubinstein JH, Taybi H. Broad thumbs and toes and facial abnormalities. *Am J Dis Child* **105**: 588–608, 1963.
- Kamei Y, Xu L, Heinzel T, et al. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**: 403–414, 1996.
- Tanaka Y, Naruse I, Maekawa T, Masuya H, Shiroishi T, Ishii S. Abnormal skeletal patterning in embryos lacking a single Cbp allele: a partial similarity with Rubinstein-Taybi syndrome. *Proc Natl Acad Sci USA* **94**: 10215–10220, 1997.
- Yao TP, Oh SP, Fuchs M, et al. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional interator p300. *Cell* **93**: 361–372, 1998.
- Oike Y, Hata A, Mamiya T, et al. Truncated CBP protein leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. *Hum Mol Genet* **8**: 387–396, 1999.
- Murata T, Kurokawa R, Krones A, et al. Defect of histone acetyltransferase activity of the nuclear transcriptional coactivator CBP in Rubinstein Taybi syndrome. *Hum Mol Genet* **10**: 1071–1076, 2001.
- Petrij F, Dauwerse HG, Blough RI, et al. Diagnostic analysis of the Rubinstein-Taybi syndrome: five cosmid should be used for microdeletion detection and low number of protein truncating mutations. *J Med Genet* **37**: 168–176, 2000.
- New MI, Nimkarn S, Brandon DD, et al. Resistance to several steroids in two sisters. *J Clin Endocrinol Metab* **84**: 4454–4464, 1999.
- New MI, Nimkarn S, Brandon DD, et al. Resistance to multiple steroids in two sisters. *J Steroid Biochem Mol Biol* **76**: 161–166, 2001.
- Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* **279**: 1922–1925, 1998.
- Safer JD, Cohen RN, Hollenberg AN, Wondisford EF. Defective release of corepressor by hinge mutants of the thyroid hormone receptor found in patients with resistance to thyroid hormone. *J Biol Chem* **273**: 30175–30182, 1998.
- Yoh SM, Chatterjee VK, Privalsky ML. Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T3 receptors and transcriptional corepressors. *Mol Endocrinol* **11**: 470–480, 1997.
- Weiss RE, Xu J, Ning G, Pohlenz J, O'Malley BW, Refetoff S. Mice deficient in the steroid receptor coactivator 1 (SRC-1) are resistant to thyroid hormone. *EMBO J* **18**: 1900–1904, 1997.
- Ito M, Yuan C-X, Okano HJ, Darnell RB, Roder RG. Involvement of the TRAP220 component of the TRAP/SMCC coactivator complex in embryonic development and thyrid hormone action. *Molecular Cell* **5**: 683–693, 2000.
- Philibert RA, King BH, Winfield S, Cook EH, et al. Association of X-chromosome dedecamer insertional variant allele with mental retardation. *Mol Psychiatry* **3**: 303–309, 1998 (Erratum in: *Mol Psychiatry* **4**: 197, 1999).
- Bautista S, Valles H, Walker RL, et al. In breast cancer, amplification of the steroid receptor coactivator gene AIB1 is correlated with estrogen and progesterone receptor positivity. *Clin Cancer Res* **4**: 2925–2929, 1998.
- Kato S, Endoh H, Masuhiro Y, et al. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**: 1491–1494, 1995.
- Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol* **20**: 5041–5047, 2000.
- Zwijsen RM, Buckle RS, Hijmans EM, Loomans CJ, Bernards R. Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. *Genes Dev* **12**: 3488–3498, 1998.
- Planas-Silva MD, Shang Y, Donaher JL, Brown M, Weinberg RA. AIB1 enhances estrogen-dependent induction of cyclin D1 expression. *Cancer Res* **61**: 3858–3862, 2001.
- Lutterbach B, Westendorf JJ, Linggi B, et al. ETO, a target of t (8; 21) inn acute leukemia, interacts with the NCoR and mSin3 corepressors. *Mol Cell Biol* **18**: 7176–7184, 1998.

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 fac-

tor (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

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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.

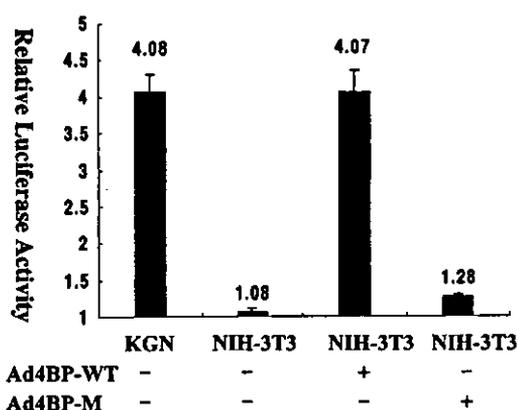


Fig. 1. CYP19 (Aromatase) Promoter II Activity in Response to Wild-Type or Mutant Ad4BP/SF-1 Stimulated by PKA

KGN cells and NIH-3T3 cells were transfected with the human ArPll firefly luciferase reporter plasmid, pGL3-ArPll, 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 \pm 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 adenylyl 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 ArPll activity. However, when wild-type Ad4BP/SF-1 was cotransfected into NIH-3T3 cells, overnight forskolin treatment elevated the ArPll 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 ArPll (Fig. 1). Therefore, it is evident that Ad4BP/SF-1 is actually a requirement for the augmentation of ovarian ArPll 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-ArPll 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

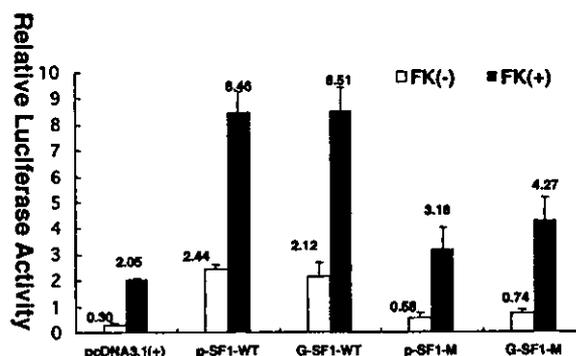


Fig. 2. Transactivation Activity of Wild-Type or Mutant Ad4BP/SF-1 Fused to GFP and Their Responsiveness to PKA

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-ArPll + 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.

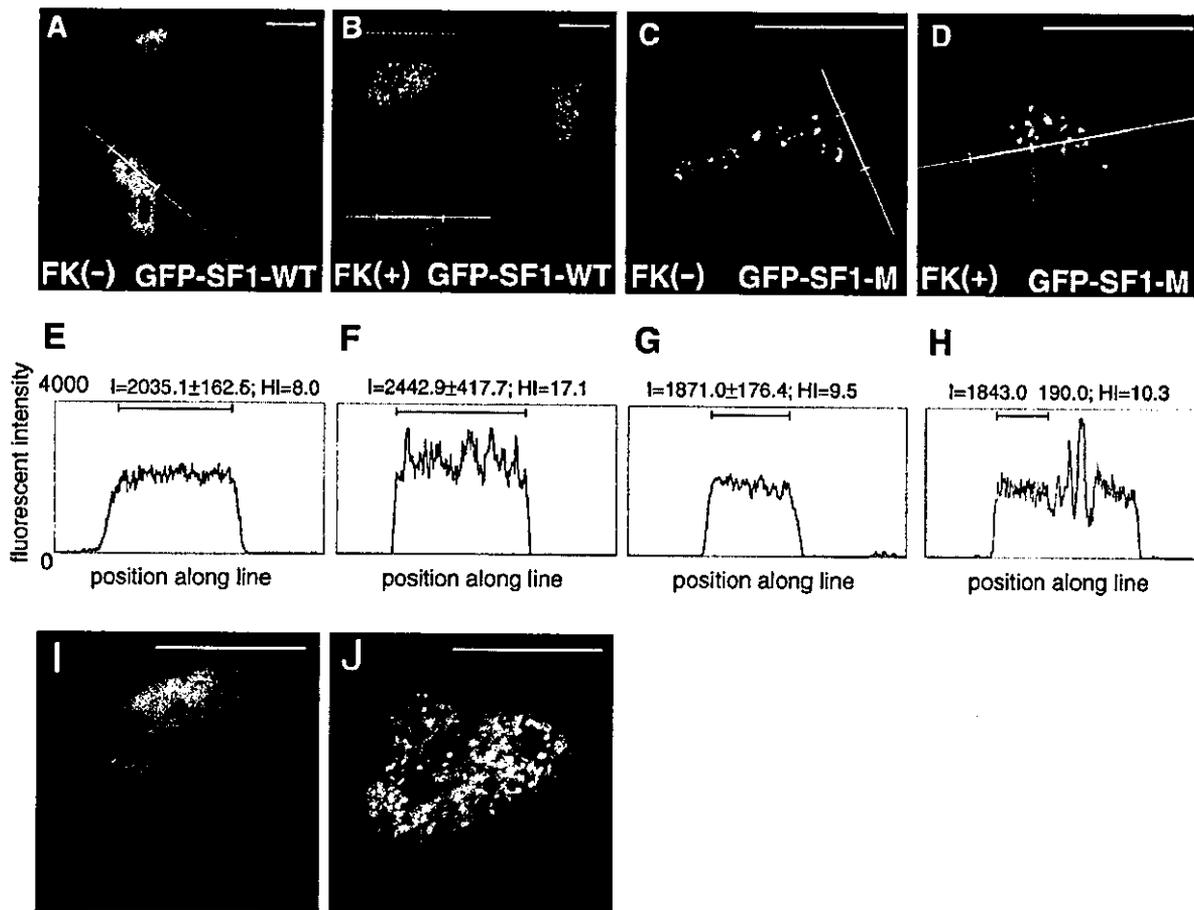


Fig. 3. Subnuclear Localization of GFP-Ad4BP/SF-1-WT and GFP-Ad4BP/SF-1-M in the Presence or Absence of 10^{-6} mol/liter Forskolin

KGN cells were transfected with $0.5\mu\text{g}/\text{dish}$ 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, $10\mu\text{m}$.

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 (nu-

cleus, 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 $\text{HI} = 100 \times \text{sd}/\text{mean}$. 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. ER α (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 pattern (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 GCN5 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

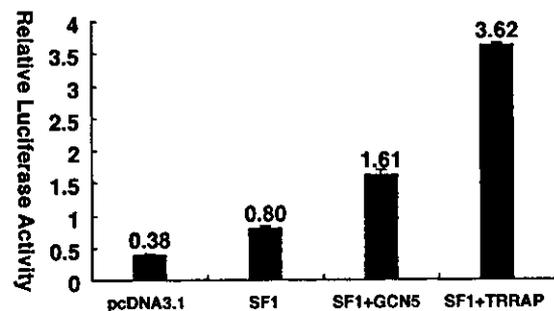


Fig. 4. Coactivators GCN5 and TRRAP Potentiate the Transactivation Activity of Ad4BP/SF-1 on the CYP19 Promoter

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.

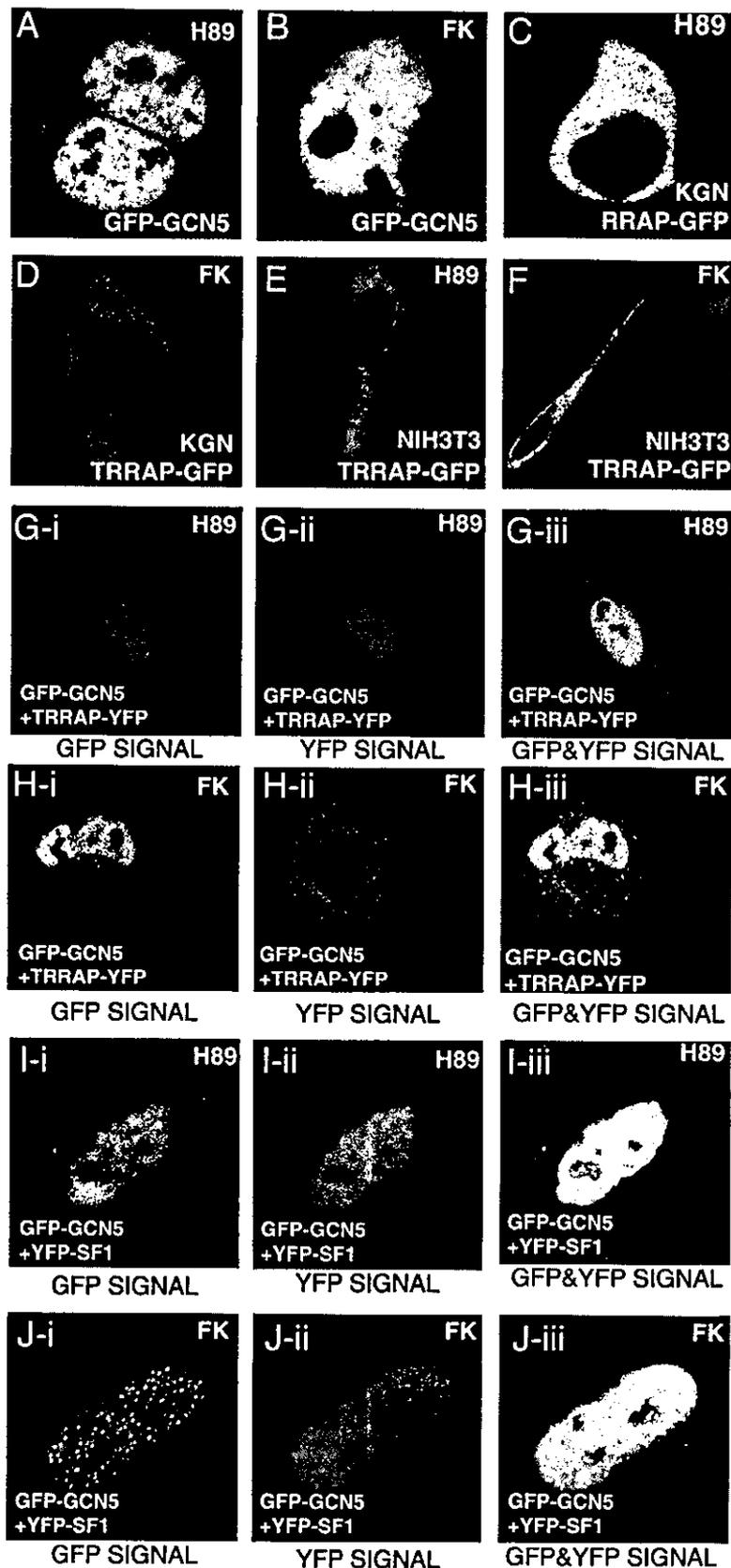


Fig. 5. Subcellular Localization of Fluorescent Protein-Fused GCN5 or Fluorescent Protein-Fused TRRAP, and Their Interrelationship with Fluorescent Ad4BP/SF-1, with the PKA Signal Pathway Either Blocked by H89 or Activated by Forskolin

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

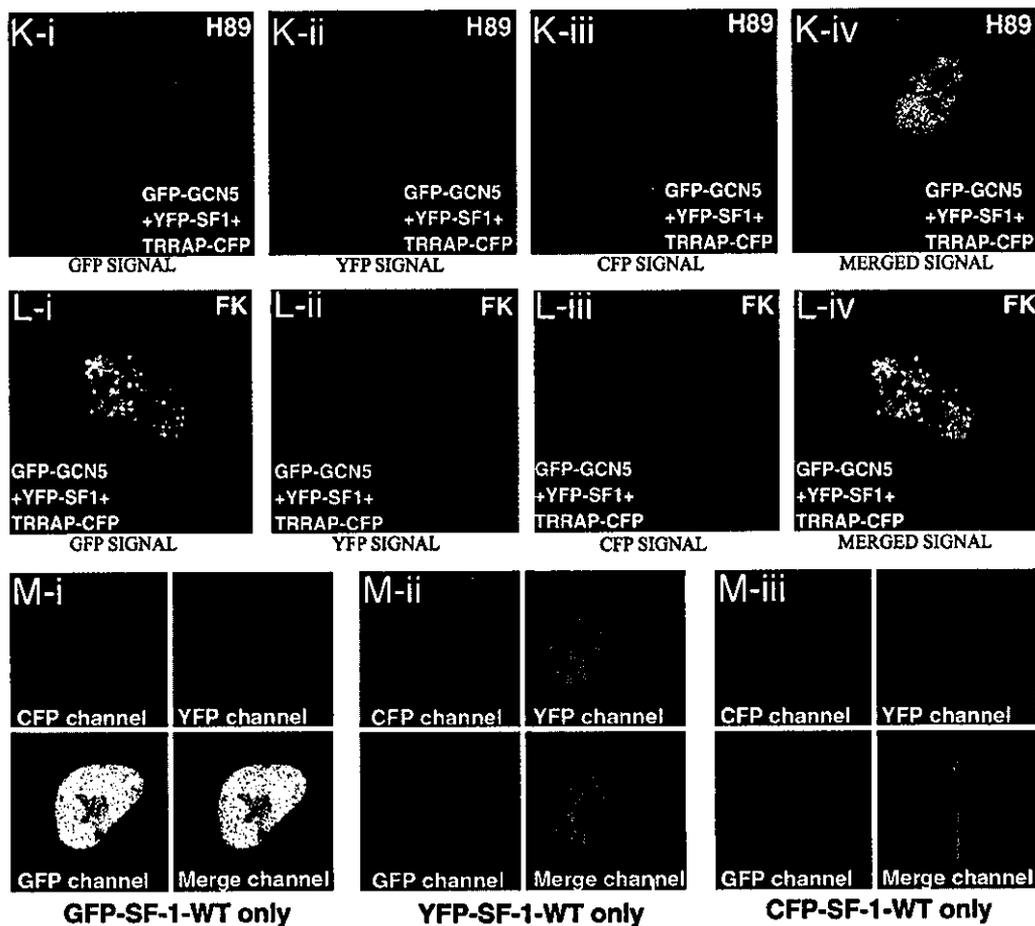


Fig. 5 Continued.

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 homogeneously 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 co-transfected 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.

CFP was diffusely distributed in a pan-cellular manner (Fig. 5K). However, foci formation took place inside the nucleus when forskolin was added. In this case, not only GCN5 and Ad4BP/SF-1 were colocalized in fluorescent dots, but TRRAP also made foci and finely colocalized with GCN5 and Ad4BP/SF-1 (Fig. 5L). These data suggest that PKA activation assembles Ad4BP/SF-1 into foci with an accompanying recruitment of coactivators like the GCN5/TRRAP complex.

DAX-1 Immobilized Ad4BP/SF-1 in the Nucleus, and this Process Was Rescued by PKA

DAX-1 is a suppressive protein for the transcriptional activation induced by Ad4BP/SF-1 and is thus considered to be an inhibitor of steroidogenesis. It has been proved that DAX-1 can bind directly to Ad4BP/SF-1 and antagonize the transcriptional activity of Ad4BP/SF-1, either via its silencing C-terminal domain (31) or by recruiting the corepressors N-CoR (11) or Alien (32) to Ad4BP/SF-1. We investigated the relationship between DAX-1 and Ad4BP/SF-1 during the process of activation of Ad4BP/SF-1 induced by PKA. The dual-luciferase assay revealed that the inhibition of the transcriptional activity of Ad4BP/SF-1 induced by DAX-1 could be recovered by PKA stimulation (Fig. 6). Next, the subcellular distributions of GFP-DAX-1 alone, and combined with YFP-Ad4BP/SF-1, were studied in living KGN cells the PKA signal pathway of which was either blocked or activated. When the cells were transfected by GFP-DAX-1 alone, the fluorescence signal was predominantly located in the nucleus in a homogenous manner, while a relatively weak diffuse fluorescence was observed in the cytosol (Fig. 7A). Forskolin (10^{-6} mol/liter) treatment caused no effect on this distribution pattern of DAX-1 (Fig. 7B). The same distribution pattern of endogenous DAX-1 in

KGN cells was observed by immunostaining (data not shown). However, cotransfection of GFP-DAX-1 and YFP-Ad4BP/SF-1 caused a dramatic change in the subcellular distribution patterns. Namely, both proteins assembled to form clear dots with no visible diffuse fluorescent background, and the GFP-DAX-1 and YFP-Ad4BP/SF-1 fluorescent dots completely overlapped (Fig. 7C, i, ii, and iii). This was further supported by the LSM semiquantitative colocalization analysis (Fig. 7C, iv). The weak GFP signal observed in the cytosol when GFP-DAX-1 was solely transfected also completely disappeared (Fig. 7C). More importantly, the completely overlapping GFP-DAX-1 and YFP-Ad4BP/SF-1 fluorescence signals were partially separated when the cells were stimulated by 10^{-6} mol/liter forskolin (Fig. 7D). The LSM colocalization analysis showed that the incomplete colocalization resulted from partial GFP-DAX-1 signal being dissociated from the overlapping dots (Fig. 7D, iv). This phenomenon was observed in most cells with proper expression of both GFP-DAX-1 and YFP-Ad4BP/SF-1, although it has been observed that different individual cells respond to forskolin stimulation to a variable extent (data not shown). Thus interaction between DAX-1 and Ad4BP/SF-1 might be interfered with or weakened by activation of PKA, and DAX-1 might be stripped from binding with Ad4BP/SF-1 when PKA is activated.

To further investigate this hypothesis, we applied the FRAP technique to examine the intranuclear dynamics of fluorescent Ad4BP/SF-1 and DAX-1 in living KGN cells. Proper mobility has recently been shown to be important for nuclear receptors to be transcriptionally functional (21). As shown in Fig. 8, coexpression of pRc/RSV-DAX-1 not only changed the subnuclear distribution pattern of YFP-Ad4BP/SF-1, but also clearly immobilized YFP-Ad4BP/SF-1. When PKA was blocked, the half-maximal recovery time ($t_{1/2}$) of YFP-Ad4BP/SF-1 with no cotransfection of DAX-1 was 0.84 ± 0.16 sec (mean \pm sd, $n = 20$, Fig. 8A), whereas the recovery $t_{1/2}$ of YFP-Ad4BP/SF-1 with cotransfection of DAX-1 was prolonged to 9.21 ± 2.36 sec (mean \pm sd, $n = 20$, Fig. 8B). These data strongly suggest that Ad4BP/SF-1 is quite mobile in the nucleus, and interaction of the receptor with DAX-1 may interfere with its mobility. Immobilization of Ad4BP/SF-1 might also be a mechanism through which DAX-1 exerts its inhibitory effect on Ad4BP/SF-1, in addition to the mechanism of direct interaction and recruitment of corepressors. We further studied the mobility of YFP-Ad4BP/SF-1 with the coexpression of DAX-1 with forskolin to activate the intracellular PKA pathway. Interestingly, the sharply reduced mobility of YFP-Ad4BP/SF-1 induced by DAX-1 partially recovered in the presence of forskolin (recovery $t_{1/2}$: 1.78 ± 0.34 sec, mean \pm sd, $n = 20$, Fig. 8C). From these data, activation of PKA might be able to disrupt or weaken the direct interaction of the inhibitory DAX-1 with Ad4BP/SF-1 and thus potentiate the transactivation

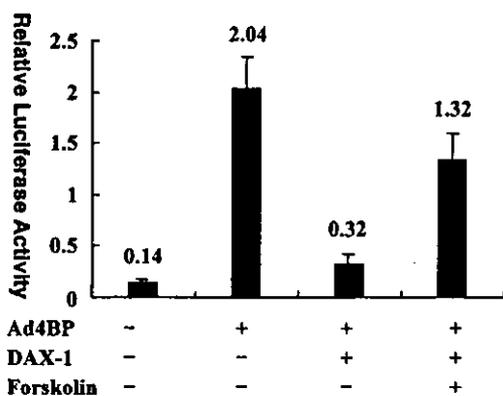


Fig. 6. Effect of Forskolin on DAX-1-Mediated Inhibition of Ad4BP/SF-1 Transactivation

KGN cells were cotransfected with pGL3-ArP11 and pRL-CMV. The strategy for cotransfection of Ad4BP/SF-1, DAX-1, or both, and the treatment with forskolin are indicated. DAX-1 could repress Ad4BP/SF-1-mediated transcription, whereas the DAX-1-inhibited Ad4BP/SF-1 transactivation could be rescued by forskolin.

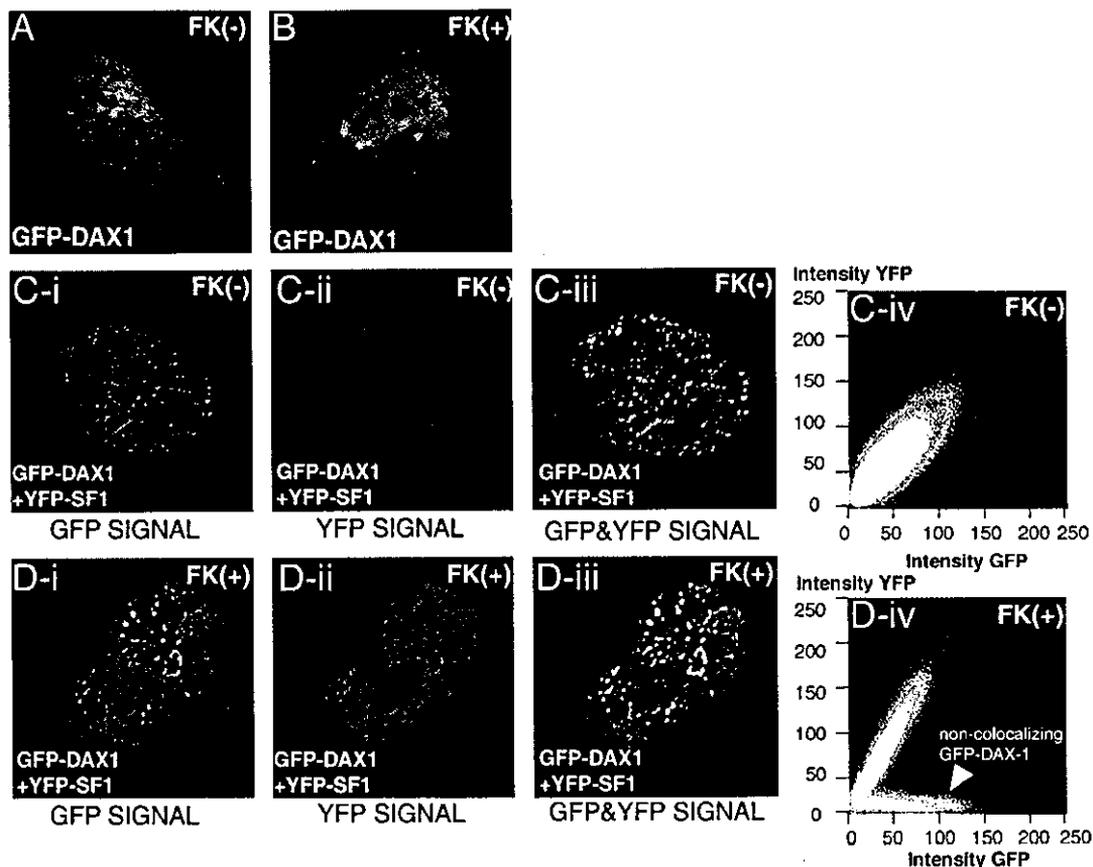


Fig. 7. Subcellular Distribution Interaction between GFP-DAX-1 and YFP-Ad4BP/SF-1

KGN cells were transfected with GFP-DAX-1 or GFP-DAX-1 + YFP-Ad4BP/SF-1 as indicated in each panel. A and B, DAX-1 is mainly diffuse in the nucleus with a weak signal detected in the cytoplasm, and this distribution is not altered by forskolin. C, Coexistence of GFP-DAX-1 and YFP-Ad4BP/SF-1 leads to the formation of clear dots with both fluorescent signals overlapping each other. The LSM colocalization analysis (C-iv) shows that GFP-DAX-1 and YFP-Ad4BP/SF-1 signals are colocalizing almost completely. No diffuse intranuclear fluorescence background or weak cytosol fluorescence was detected. D, Forskolin partially separated the two completely overlapping fluorescent signals. The colocalization analysis (D-iv) shows that a fraction of GFP-DAX-1 signal is not colocalizing with YFP-Ad4BP/SF-1, suggesting that the interaction between Ad4BP/SF-1 and DAX-1 is weakened, and partial DAX-1 is disassociated from the Ad4BP/SF-1-DAX-1 binding complex.

of Ad4BP/SF-1, as shown by the luciferase reporter assay in Fig. 6.

DISCUSSION

Various lipophilic ligands have been found to interact with ligand-binding domains, induce allosteric changes, and thus convert nuclear receptors into an active conformation that can actively regulate transcription. However, Ad4BP/SF-1 is an orphan nuclear receptor and has no known ligand. Cross-talk with the main intracellular signal transduction pathways and protein-protein interactions seem to play definitive roles in the transcriptional regulation by nuclear receptors, especially those orphan nuclear receptors like Ad4BP/SF-1.

It is well known that the transactivational activity of Ad4BP/SF-1 can be further activated by the cAMP-

PKA signal pathway. In this study, this phenomenon was also observed in a well known Ad4BP/SF-1 target gene's promoter, *human CYP19 ArPII*, in KGN cells. The precise mechanism by which cAMP potentiates Ad4BP/SF-1-dependent transactivation was investigated using this model. One hypothesis is that PKA may directly or indirectly activate Ad4BP/SF-1 transcriptional activity by phosphorylation of Ad4BP/SF-1 because Ad4BP/SF-1 has been demonstrated to be phosphorylated *in vitro* by the PKA or MAPK pathway (33, 34). However, mutations of the predicted potential phosphorylation sites for the PKA (Ser 430) and MAPK (Ser 203) pathways did not affect the ability of PKA to stimulate Ad4BP/SF-1-dependent transactivational activity (26). In fact, it was shown to be difficult to prove that elevated cAMP could stimulate the phosphorylation of Ad4BP/SF-1 *in vivo* (35). In this regard, the opposite theory has recently been proposed that during the process of PKA-mediated Ad4BP/SF-1

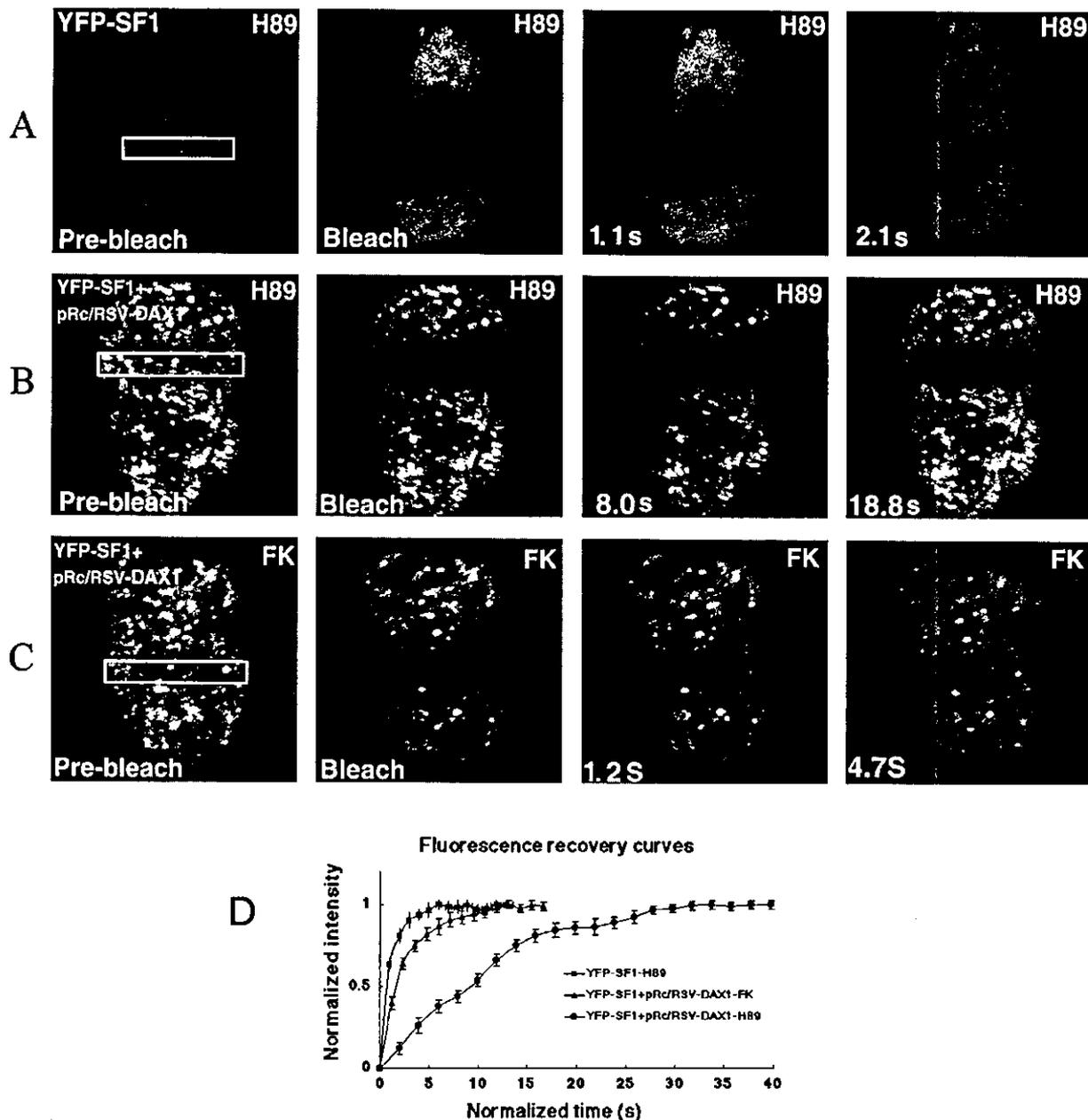


Fig. 8. FRAP Analysis of YFP-Ad4BP/SF-1 Cotransfected with pRc/RSV-DAX-1 after Forskolin or H89 Treatment

KGN cells were transfected with YFP-Ad4BP/SF-1 or YFP-Ad4BP/SF-1 + pRc/RSV-DAX-1, and treated with 10^{-6} mol/liter H89 or 10^{-6} mol/liter forskolin as indicated. Images show a single Z section and were obtained before and after bleaching at the time points indicated in each panel. The region of interest (ROI) of photobleaching is also indicated. A, When YFP-Ad4BP/SF-1 was solely transfected and cells were treated with H89, YFP-Ad4BP/SF-1 demonstrates a high intranuclear mobility. A definite bleach zone is detected after photobleaching. The total nuclear fluorescence reaches equilibrium within 1 sec. B, Cotransfection of pRc/RSV-DAX-1 and treatment with H89 prolonged the fluorescence recovery half-time to 9 sec, indicating that Ad4BP/SF-1 mobility is reduced. C, The prolonged fluorescence recovery time is rescued by forskolin. Ad4BP/SF-1 regains its mobility in the presence of forskolin, suggesting that PKA weakens the Ad4BP/SF-1-DAX-1 interaction. D, The recovery curves of the three groups of cells. The normalized intensity at each time point was averaged and plotted to the normalized time points. The $t_{1/2}$ value can be readily observed from the graph as the time at which the normalized intensity reaches 0.5 arbitrary units.

transactivation, phosphatase activity, but not kinase activity, may be critical (36). Another possible mechanism suggested was that PKA may increase the Ad4BP/SF-1 protein level by stabilizing it (26), but there is also conflicting evidence that the mRNA or

protein level of Ad4BP/SF-1 remains constant after elevation or decrease of the cAMP level (37, 38). Peroxisome proliferators activated receptor- γ coactivator 1 (PGC-1) is a unique coactivator that can be transcriptionally increased by cAMP-PKA signaling (39).

The possibility that PKA may augment Ad4BP/SF-1 transactivation by means of increasing the PGC-1 level, if PGC-1 could serve as a coactivator for Ad4BP/SF-1, seems to be unlikely because forced expression of PGC-1 could not further enhance Ad4BP/SF-1-mediated ArPII activity in either KGN or NIH-3T3 cells (data not shown).

Ligand-induced subnuclear compartmentalization (foci formation) is a common phenomenon of non-orphan nuclear receptors and usually is considered to be related to the transactivationally active form of receptors. Ligand-induced transactivationally active GR (40), vitamin D receptor (41), estrogen receptor (ER) (42), mineralocorticoid receptor (43), and androgen receptor (AR) (44) have been found to be distributed in the nuclei that produce the GFP fluorescent foci. Intracellular fluorescent foci formation depends closely on whether the receptor is transcriptionally active or inactive. Transcriptionally active AR treated with dihydrotestosterone produced 250–400 foci in the boundary region between euchromatin and heterochromatin (44). Although AR bound to antiandrogens like hydroxyflutamide also translocated to the nucleus, they spread homogeneously throughout the nucleus without producing any fluorescent foci (44). In addition, when ARs make foci, as induced by dihydrotestosterone, coactivators like SRC-1, TIF2, and CBP were found to be accumulated in identical locations, and CBP was found to be one of the factors essential for AR foci formation (25). It is thus speculated that transcriptionally activated nuclear receptors are transferred to common nuclear compartments (foci) in the nuclear matrix and form a complex with coactivators, and that this process is essential for full transactivation. However, it is unlikely that foci compartments directly represent the active transcription sites, because they were found usually not to overlap with activated RNA polymerase II or nascent mRNA. But currently, liganded GR has been observed to dynamically aggregate to an artificial promoter (a MMTV array that has been integrated to chromosome), where the binding sites are amplified many times (18, 19). Another corroborative study reported a strong correlation between aryl hydrocarbon receptor foci and the active transcription sites (45). Recent studies generally suggest that foci compartments of nuclear receptors may represent the sites for storage and/or assembly of activated nuclear receptor, and nuclear receptors can be dynamically recruited from foci compartments into the active transcription sites. It should be noted that foci are also possibly related to protein degradation compartment (46), because cognate ligand also induce proteolysis, a process that is tightly coupled with the ligand-induced activation and compartmentalization of nuclear receptors, and is believed to be a mechanism to precisely regulate the activity of liganded nuclear receptors.

In parallel with the elevated transactivation ability, GFP-Ad4BP/SF-1 underwent a compartmental shift and manifested foci formation in the nucleus when the

PKA signal pathway was activated. This phenomenon was observed only with wild-type Ad4BP/SF-1, and not in the functionally inactive mutant Ad4BP/SF-1 (G35E). The PKA-inducible foci formation appears to be a characteristic of functionally stimulated Ad4BP/SF-1 because it is consistent with the PKA-stimulated transactivation of Ad4BP/SF-1, suggesting that Ad4BP/SF-1 is assembled to a transactivationally active subnuclear compartment. In addition, in the process of PKA-triggered foci formation, recruitment of coactivators such as GCN5 and TRRAP also takes place. It should be noted that this PKA-induced foci formation occurs on a diffuse fluorescence signal background, suggesting that not all Ad4BP/SF-1 proteins, but rather a fraction of them, are assembled to the foci, and that the Ad4BP/SF-1 in the nuclear pool may be undergoing rapid exchange.

The GCN5/TRRAP complex is a newly identified third class of nuclear receptor coactivator complex, in addition to the previously described P160/CBP HAT complex and vitamin D receptor-interacting protein/thyroid hormone receptor-associated protein non-HAT complex. GCN5 contains HAT activity whereas the three LXXLL motifs of TRRAP are responsible for interaction with nuclear receptors, according to the reported case of ER α , in a ligand-dependent manner (30). Based on our data, TRRAP is a surprisingly dominant cytoplasmic protein but seems to be dragged into the nucleus by its partner, GCN5. Both of these proteins can be recruited to Ad4BP/SF-1 when the receptor is stimulated by PKA, suggesting that this nuclear receptor coactivator complex is involved in the Ad4BP/SF-1 activation process. Therefore, activation of the PKA signal pathway is able to alter the subnuclear distribution pattern of Ad4BP/SF-1 and assemble this orphan nuclear receptor to a functionally active state, with recruitment of coactivators. Forskolin, a stimulator of adenylyl cyclase, could induce Ad4BP/SF-1 to make foci, a phenomenon mimicking the role of cognate ligands to their respective nuclear receptors, raising a new concept that foci formation can also be a result of the activation of an intracellular signal pathway like PKA, which secondarily activates the nuclear receptors themselves. This result sheds new light on our understanding of the nature of foci, which has not yet been well defined.

The mutant Ad4BP/SF-1 (G35E) could not be transactivationally enhanced by PKA and could not make foci in the presence of forskolin. Surprisingly, however, mutant Ad4BP/SF-1 fluorescence aggregated in dots in the nucleoli. Although no significant nucleolar fluorescence was detectable in the case of the wild type, we cannot exclude the presence of a small fraction of Ad4BP/SF-1 molecules localizing to the nucleolus. The p-box of Ad4BP/SF-1, where the G35E mutation is located, or the region nearby, might be an important domain required for nuclear matrix binding of Ad4BP/SF-1 and thus prevent Ad4BP/SF-1 from entering the nucleoli, or this domain might be a nucleolus export