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The Nuclear Receptor Coactivators p300/CBP/Cointegrator-Associated Protein (p/CIP) and Transcription Intermediary Factor 2 (TIF2) Differentially Regulate PKA-Stimulated Transcriptional Activity of Steroidogenic Factor 1

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Steroidogenic factor-1 (SF-1) is a member of the nuclear receptor superfamily that plays essential roles in the development of endocrine organs. Steroid receptor coactivator 1 and transcription intermediary factor 2 (TIF2) belong to the p160 coactivator family that mediates transcriptional activation by several nuclear receptors, including SF-1. Here, it is reported that another of the p160 coactivators, p/CIP, interacts with SF-1 through the activation function-2 domain. Both p300/CBP/cointegrator-associated protein (p/CIP) and TIF2 potentiated SF-1-mediated transcription from two reporter gene constructs in transfected nonsteroidogenic COS-1 cells and in adrenocortical Y1 cells. PKA was shown to stimulate SF-1 transcriptional activity, and coexpression of p/CIP together with the PKA catalytic subunit stimulated SF-1-mediated transactivation even further. In contrast, PKA catalytic subunit overexpression impaired the ability of TIF2 to potentiate SF-1-dependent transcription. Activation of PKA also inhibited the TIF2-mediated coactivation of other nuclear receptors such as PPARα/-γ and liver X receptor-α. The TIF2 mRNA levels were not affected by PKA, but instead we found that PKA activation led to a decrease in the levels of TIF2 protein. Moreover, the C-terminal activation domain 2 of TIF2 was required for the inhibitory effect of PKA, suggesting that this region is the target for the PKA-mediated down-regulation. Thus, in contrast to the regulation of p/CIP and steroid receptor coactivator 1, we suggest that activation of PKA leads to selective down-regulation of TIF2 and subsequently repression of TIF2 coactivator function. (*Molecular Endocrinology* 16: 757–773, 2002)

STEROIDOGENIC FACTOR-1 (SF-1/AD4BP/NR5A1) is a member of the nuclear receptor superfamily of proteins that is essential for adrenal and gonadal development, steroidogenesis, and gonadal differentiation (1–4). Like other members of this family of transcription factors, SF-1 has a characteristic zinc finger DNA-binding domain, an intervening hinge region, and a carboxyl-terminal ligand-binding domain (5). However, unlike most nuclear receptors, SF-1 binds to DNA as a monomer (6, 7). SF-1 contains a short α-helix in the carboxy terminus, the activation function-2 domain (AF-2), that is conserved in essentially all nuclear receptors and that is important for transactivation of target genes (5, 8). It has been shown that increased PKA activity stimulates SF-1 transcriptional activity (9) and that mutations in the AF-2 core domain of SF-1 dominantly suppress PKA-dependent transactivation of the bovine CYP17 gene (8).

Nuclear receptors stimulate transcription upon activation by their cognate hormones or ligands and recruitment of coactivators through the AF-2 domain. These coactivators can form contacts between the nuclear receptors and the transcriptional initiation complex (10, 11). It has been reported that SF-1 interacts with several transcription factors as well as transcriptional cofactors such as steroid receptor coactivator-1 (SRC-1)/nuclear receptor coactivator-1 (12, 13) and transcription intermediary factor-2 (TIF2)/GR-interacting protein 1 (GRIP1)/nuclear receptor coactivator-2 (14). The AF-2 domain of SF-1 is essential for the interaction with these coactivators (12–14).

The coactivator p300/CBP/cointegrator-associated protein (p/CIP)/SRC-3/activator of TR and RA/ampli-

Abbreviations: aa, Amino acids; AD, activation domain; AF, activation function; CARM1, coactivator-associated arginine methyltransferase 1; CBP, cAMP response element binding protein-binding protein; 9-*cis*-RA, 9-*cis*-retinoic acid; ERR, estrogen-related receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRIP1, GR-interacting protein 1; LXR, liver X receptor; NTA, nitrilotriacetic acid; PKA-Cα, PKA catalytic subunit; PRMT1, protein arginine methyltransferase 1; RLU, relative light units; SF-1, steroidogenic factor-1; SRC, steroid receptor coactivator; TIF2, transcription intermediary factor 2.

fied in breast cancer 1 [p/300/CBP/cointegrator-associated protein] is a 152-kDa protein, which is related to SRC-1 and TIF2 (31% and 36% amino acid identity, respectively; Ref. 15). These coactivators interact with many nuclear receptors including RAR, TR, PPARα and PPARγ, RXR, liver X receptor (LXR), ER, as well as certain orphan receptors (16–18) (reviewed in Refs. 19 and 20). p/CIP, TIF2, and SRC-1 have similar and quite ubiquitous expression patterns (15). However, mice lacking SRC-1 and p/CIP show very distinct phenotypes, and it has been suggested that the wide range of reported expression patterns is due to expression in all vascular smooth muscle cells (21, 22). It is therefore possible that there are significant expression differences in nonendothelial cells. The SRC-coactivator family members are in turn associated with CBP in a complex required for transcriptional activity of nuclear receptors and other CBP/p300-dependent transcription factors (23). CBP and p300 are functionally conserved proteins that have intrinsic acetylase activity, and they are essential in transcriptional activation by a large number of regulated transcription factors, including nuclear receptors, at least in part by destabilizing chromatin (24).

In this report, we have examined the regulation of SF-1-dependent transcription by the cAMP pathway and the AF-2 coactivators p/CIP and TIF2. A direct interaction between SF-1 and p/CIP is demonstrated, and the AF-2 domain of SF-1 as well as the LXXLL motives of p/CIP are shown to be critical for this interaction. The association between SF-1 and TIF2 differed from that of SF-1 and p/CIP with respect to the role of specific residues within the AF-2 core domain. Moreover, whereas p/CIP (and SRC-1) increase SF-1-stimulated transcription in cells that overexpress the catalytic subunit of PKA (PKA-Cα), the TIF2-mediated potentiation of SF-1 activity is repressed by activation of PKA. We also report that activation of PKA inhibits the TIF2 coactivation through selective down-regulation of TIF2 protein. Our findings may suggest a new mechanism for selection of specific coactivators in response to extracellular signals that activate the cAMP-signaling pathway.

RESULTS

SF-1 Interacts Directly with the Coactivator p/CIP in an AF-2-Dependent Manner

The interaction between p/CIP and SF-1 was investigated using a yeast two-hybrid assay. We employed the yeast strain EGY191 and the reporter plasmid pJK103 with only two *Lex*A operons in the promoters of the reporter genes to lower the basal transcriptional activity. The cofactor p/CIP was fused to the B42 transcriptional activation domain (B42/p/CIP), and SF-1 was fused to the *Lex*A DNA binding domain (*LexA*/SF-1). The bait plasmid encoding *LexA*/SF-1 was transformed into the yeast strain L40 (MAT**a**), whereas the prey plasmid encoding B42/p/CIP was transformed into the EGY191 strain (MATα). Mating of the two strains with different MAT loci brought the bait and prey proteins together in the diploid. A liquid semi-quantitative β-galactosidase assay was performed to measure the two-hybrid interaction in mating colonies as relative β-galactosidase units related to the density of the yeast culture [relative light units (RLU)/OD_{600} (Fig. 1, A and B)]. Mating of the yeast strains expressing the B42/p/CIP- and the *Lex*A/SF-1-fusion proteins resulted in a significant increase (74-fold) of the reporter gene activity as compared with yeast expressing *Lex*A/SF-1-fusion protein and the B42 transcriptional activation domain alone (B42/$-$). No detectable activity was observed when B42/p/CIP was expressed together with the negative controls *Lex*A/Lamin or *LexA*/$-$. As SRC-1 has been reported to interact with SF-1 (12, 13), B42/SRC-1 was used as positive control in the two-hybrid system.

Nuclear receptors contain a transactivation domain, AF-2, within the carboxyl-terminal region that is required for ligand-induced transactivation (8, 25–27). This domain appears to be of fundamental importance for the interaction with certain coactivators, as demonstrated for the SF-1 interaction with SRC-1 (12, 13). To get further insight into the significance of the AF-2 domain for the interaction between SF-1 and p/CIP, we analyzed whether deletion of the AF-2 domain of SF-1 affected its ability to interact with p/CIP. No interaction was observed between *Lex*A/SF-1ΔAF-2 [amino acids (aa) 107–436] and B42/p/CIP (Fig. 1A), suggesting that a functional AF-2 domain is required for SF-1 to interact with p/CIP. To eliminate that the lack of interaction was due to lower expression levels of *Lex*A/SF-1ΔAF-2, we performed Western blot analysis of protein extracts prepared from transformed yeast using an anti-*Lex*A antibody. As shown in Fig. 1C, the *Lex*A/SF-1ΔAF-2 mutants were at least expressed at the same levels as the *Lex*A/SF-1 protein.

A number of coactivators, including p/CIP, have been shown to contain leucine-rich (LXXLL) helices termed NR boxes that are necessary for interaction with nuclear receptors (28–30). However, different nuclear receptors have specific LXXLL motif requirements for the interaction with coactivators (17, 29). To gain further insight into the SF-1-p/CIP interaction, we introduced mutations into each of the three LXXLL motifs (L1–3) in the central receptor interaction domain of p/CIP (Table 1). The mutated p/CIP variants were analyzed in the yeast two-hybrid assay (Fig. 1B). All three variants containing mutations in single LXXLL motifs (L1, L2, and L3) showed a significant decrease in the binding of SF-1 ($\sim$40–70% of wild type), suggesting that all motifs must be intact to obtain full interaction with SF-1. Mutation of NR box 1 (L1) affected the interaction most strongly, suggesting that this motif is the most important for interaction with SF-1 followed by NR box 2. It was noted that p/CIP mutated in NR box 1 and 2 (L1/L2) showed very slight interaction with SF-1, and mutations of all three NR

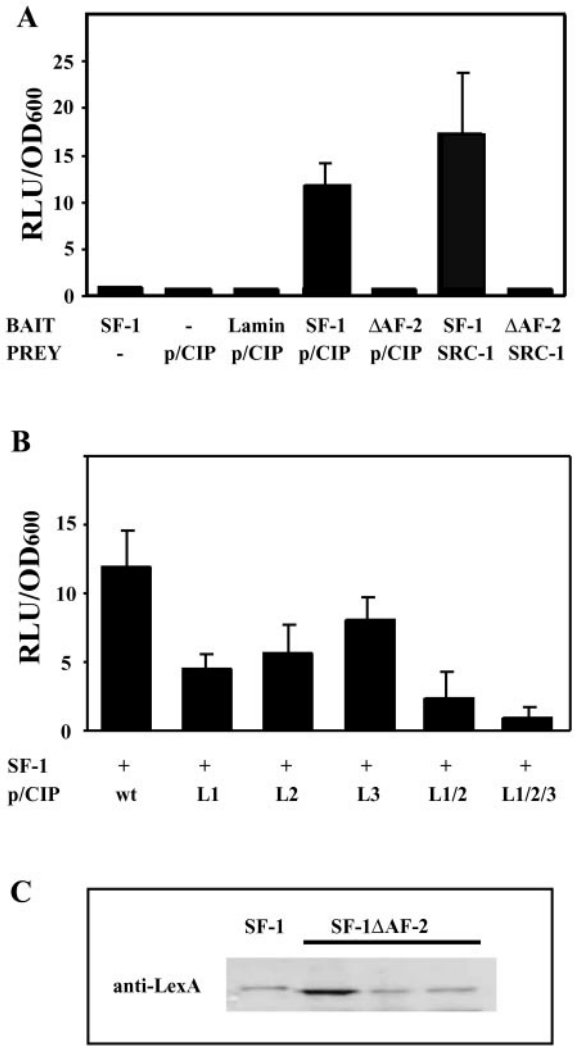

Fig. 1. Interactions Between SF-1 and p/CIP in the Yeast Two-Hybrid System

A, A liquid, semiquantitative β-galactosidase assay of the two-hybrid interactions between bait (*Lex*A/SF-1, *Lex*A/SF-1ΔAF-2, *Lex*A/Lamin, or *Lex*A/−) and prey proteins (B42/p/CIP, B42/SRC-1, or B42/−) in yeast. The different combinations of bait and prey are as indicated in the figure. Bait (*Lex*A) and prey (B42) vectors are included as negative controls and are marked (−). The bait plasmids were transformed into the yeast strain L40 (MAT**a**), whereas the prey plasmids were transformed into the EGY191 strain (MATα). Mating of the two strains with different MAT loci brought the bait and prey proteins together in the diploid. Measuring the β-galactosidase activity in the transformants identified the strength of the interaction between bait and prey. The relative β-galactosidase activity is given per unit of OD_{600} (RLU/OD_{600}) in the yeast cultures. The figure shows the mean value of at least four independent colonies. B, Yeast two-hybrid interactions between *Lex*A/SF-1 and B42/p/CIP with various mutations in the central receptor interaction (LXXLL) domains of p/CIP (L1, L2, L3, L1/2, and L1/2/3 as shown in Table 1). The relative β-galactosidase activities (RLU/OD_{600}) shown are the mean values of at least four independent mating colonies. C, Western blot with anti-*Lex*A antibody showing the expression of *Lex*A/SF-1 (one colony) and *Lex*A/SF-1ΔAF-2 (three independent colonies) in the L40 strain.

Table 1. Amino Acids of the p/CIP Mutants

	aa no.	Wild-Type	Mutations
L1	614-618	LLQLL	LAAAA
L2	677-682	ILHKLL	ILAAAA
L3	729-734	LLRYLL	LLAAAA

Amino acids of the different p/CIP mutants in the three putative NR box motives 1, 2, and 3 (L1, L2, L3, and the combinations L1/2 and L1/2/3) used in the transfection studies and in the yeast two-hybrid interaction assays.

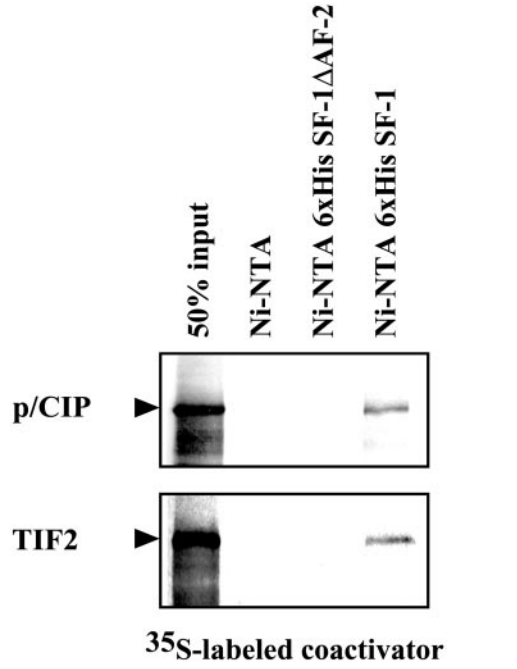

Fig. 2. Interaction Between SF-1 and the Coactivators *in Vitro*

[^{35}S]Methionine-labeled *in vitro* translated p/CIP and TIF2 (3 μl) were incubated with $(His)_6$-SF-1 (5 μg) or $(His)_6$-SF-1ΔAF-2 (5 μg). $(His)_6$-SF-1ΔAF-2 contains the AF-2 core (LLIEML) deletion mutation of SF-1. These protein mixtures or the [^{35}S]methionine-labeled coactivators (3 μl) alone were then incubated with Ni-NTA agarose. After extensive washing, the proteins were analyzed by SDS-PAGE (6% polyacrylamide). Fifty percent of the input radiolabeled p/CIP and TIF2 are shown on the *left*.

boxes (L1/L2/L3) nearly abolished the binding of p/CIP to SF-1.

The interaction between SF-1 and p/CIP was also demonstrated by a protein-protein interaction assay performed with a baculovirus-expressed $(His)_6$-SF-1-fusion protein. The cofactors p/CIP and TIF2 were radiolabeled with [^{35}S]methionine during *in vitro* translation. p/CIP bound to the $(His)_6$-SF-1-fusion protein linked to Ni-nitrilotriacetic acid (NTA) agarose, but not to the $(His)_6$-SF-1ΔAF-2-fusion (AF-2 core deletion) protein or to the Ni-NTA agarose beads alone (Fig. 2). The coactivator TIF2, which has been reported previously to interact with SF-1 (14), also interacted with the

$(His)_6$-SF-1 protein in a similar fashion, but not with the $(His)_6$-SF-ΔAF-2 protein. Taken together, these results confirm that SF-1 interacts directly with p/CIP (Figs. 1 and 2) and TIF2 (Fig. 2) (14), and the nondetectable bindings to the $(His)_6$-SF-ΔAF-2-fusion protein show that the AF-2 domain is required for the SF-1-p/CIP and SF-1-TIF2 interactions.

p/CIP Potentiates the Transcriptional Activity of SF-1

To assess the functional importance of the SF-1-p/CIP interaction, COS-1 cells were transfected with expression vectors encoding SF-1 and p/CIP together with the reporter construct pT81–4CRS2-luc, which contains a minimal thymidine kinase promoter and four copies of the SF-1 response element from the bovine CYP17 gene (9). As also previously reported (9), activation of the cAMP pathway stimulated SF-1-dependent transcription from the CRS2 element (Fig. 3). It should be noted that PKA-Cα did not stimulate this target gene in the absence of SF-1, indicating that the response is SF-1 dependent. p/CIP potentiated SF-1-mediated transactivation in a dose-dependent manner both in the presence and the absence of PKA-Cα overexpression (Fig. 3A and data not shown). A maximal increase (15-fold) in luciferase activity was observed after coexpression of p/CIP with PKA. In contrast, expression of p/CIP and PKA-Cα had no effect on CRS2-dependent transcription in the absence of SF-1 (Fig. 3A), indicating that the stimulatory effects of PKA and p/CIP are mediated through SF-1.

The functional significance of the SF-1 interaction with p/CIP in cells that naturally express SF-1 was investigated by transfection studies in the steroidogenic cell line Y1 (mouse adrenocortical cell line). Y1 cells endogenously express SF-1 in sufficient amounts to activate the reporter plasmid 4CRS2-luc (9). As shown in Fig. 3B, coexpression of p/CIP and PKA-Cα in Y1 cells increased the reporter gene activity significantly, whereas no such increase in CRS2-mediated transcription was observed in the absence of PKA. Next we tested the ability of p/CIP to modulate SF-1-stimulated transcription via an element from another SF-1 target gene. The SCC-luc reporter gene contains the region between −186 and +12 of the bovine CYP11A gene that includes a SF-1 binding site (28). As shown in Fig. 3C, p/CIP potentiated SF-1-induced stimulation of the SCC-luc activity in COS-1 cells, and similar to the data on 4CRS2-luc, coexpression of PKA-Cα further stimulated the SCC-luc activity. Thus, these findings strengthen the above results, suggesting that p/CIP acts as a coactivator of SF-1.

To examine the functional importance of the three central LXXLL motifs in p/CIP, COS-1 cells were transiently transfected with expression vectors encoding SF-1 and p/CIP with mutations in the LXXLL motifs (L1, L2, and L3; Table 1) along with the CRS2-reporter construct. A 10-fold increase in SF-1-induced transactivation was detected with the expression of SF-1 alone, as compared with the basal reporter gene activity, whereas an approximately 90-fold increase in SF-1-transcriptional activity was observed in cells coexpressing SF-1 and PKA-Cα. p/CIP-L1 exhibited an inhibitory effect when coexpressed with SF-1 alone, reducing the SF-1-induced transactivation to 5-fold, and a comparable reduction was observed in presence of PKA-Cα overexpression (Fig. 3D). Expression of p/CIP-L3 also decreased the SF-1 transcriptional activity (to ~4-fold) compared with the basal reporter gene activity, whereas expression of p/CIP-L2 gave a small but significant increase in the SF-1-induced transactivation. Thus, it appears the L1 and L3 mutants had a dominant negative effect on the SF-1-mediated transactivation. Moreover, it was noted that mutations in NR box 1 and 2 (L1/L2) or all three NR boxes (L1/L2/L3) nearly eliminated the transcriptional activity of SF-1. Taken together, these results suggest that all three NR boxes are required for the complete function of p/CIP as a coactivator of SF-1 transcriptional activity.

TIF2-Mediated Coactivation of SF-1 Is Inhibited by PKA

Data from Northern blot analysis suggest that SRC-1, p/CIP, and TIF2 are equally expressed in COS-1 and Y1 cells, and the expression levels were not affected by activation of the cAMP pathway (data not shown). Furthermore, we noted that p/CIP and SRC-1 both potentiated SF-1-stimulated CRS2-reporter gene activity in the presence of overexpressed PKA-Cα (Fig. 3) (29). An expression construct encoding TIF2 was transfected into both COS-1 cells and Y1 cells to study the effect of TIF2 on SF-1-induced stimulation of the CRS2 and SCC constructs. As shown in Fig. 4, TIF2 strongly potentiated SF-1-dependent transcriptional activation, indicating that TIF2 also acted as a coactivator of SF-1. In COS-1 cells overexpression of TIF2 stimulated the SF-1-induced transcription from the CRS2 construct 290-fold (Fig. 4A), whereas the effect on SCC-luc was 70-fold greater (Fig. 4C). Transcription from the CRS2-reporter gene was also significantly stimulated (54-fold) by TIF2 in Y1 cells (Fig. 4B). In contrast to the effects of p/CIP and SRC-1 on SF-1-dependent transcriptional activity, PKA-Cα overexpression markedly impaired the potentiation by TIF2 on both reporter gene constructs tested. These results suggest that PKA represses TIF2 coactivation, and that the different members of the SRC coactivator family act differently in the presence of increased PKA activity.

To assess further whether the effects of TIF2 and p/CIP on CRS2-mediated transcription are dependent on DNA-binding of SF-1, we used a reporter construct carrying a mutation in the SF-1 binding site (9). This mutation (M4) interferes with SF-1 binding as demonstrated by EMSA, and the M4 reporter has lost the ability to mediate SF-1-dependent transcription (9). As shown in Fig. 5, the reporter construct M4 did not

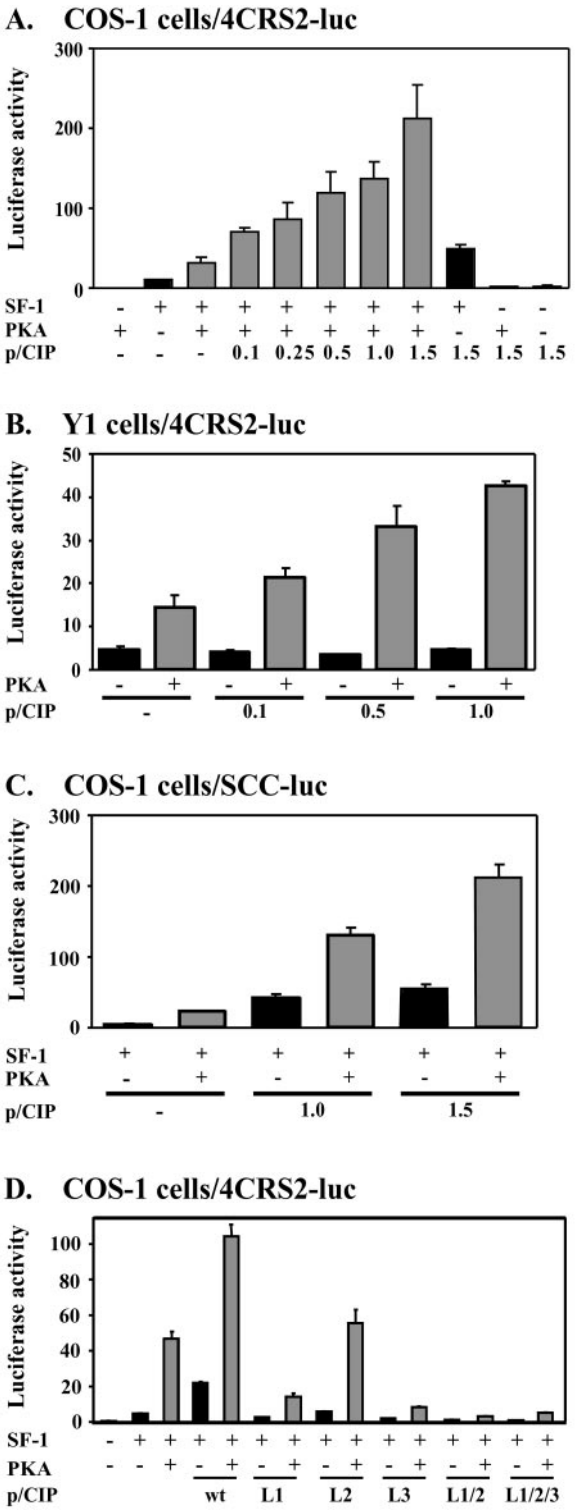

Fig. 3. p/CIP Enhances SF-1-Dependent Transcription in Mammalian Cells

A, COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg), p/CIP (0.1–1.5 μg), and PKA-Cα (0.1 μg) along with the 4CRS2-luc reporter construct (1.5 μg) as indicated in the figure. Forty-eight hours after transfection the cells were harvested and luciferase assays were performed. B, Y1 adrenocortical cells that express SF-1 endogenously were cotransfected with the reporter 4CRS2-luc (1.5 μg), increasing amounts of expression vectors encoding p/CIP (0.1–1.0 μg), and PKA-Cα (0.1 μg) as indicated. Luciferase assays were performed 24 h after transfection. C, Expression vectors encoding SF-1 (0.1 μg), p/CIP (1.0 and 1.5 μg), and PKA-Cα (0.1 μg) were cotransfected into COS-1 cells along with the SCC-luc reporter construct (1.5 μg) as indicated in the figure. Luciferase assays were performed 48 h after transfection. D, The 4CRS2-luc reporter construct (1.5 μg) and expression vectors encoding SF-1 (0.1 μg), PKA-Cα (0.1 μg), and p/CIP (1.0 μg) or different p/CIP LXXLL mutants (L1, L2, L3, L1/2, and L1/2/3; Table 1) were cotransfected into COS-1 cells as indicated in the figure. Luciferase assays were performed 48 h after transfection. For all experiments, the luciferase activities (mean ± SD) are based on triplicate transfections from representative experiments.

mediate any transcriptional activity in response to SF-1 alone or in combination with PKA-Cα. Furthermore, neither TIF2 nor p/CIP affected the luciferase activity from this construct. Thus, these results confirm that SF-1 is required for the coactivation by TIF2 and p/CIP.

Divergent Effects of AF-2 Mutations on the p/CIP- and TIF2-Mediated Potentiation of SF-1 Activity

The helix H12 at the carboxy terminus of the ligand-binding domains of the different receptors is required for AF-2 transcriptional activation function, and some mutations within this helix eliminate AF-2 activity completely, whereas others do not lead to reduced interactions with cofactors (30–32). Based on the findings that potentiation of SF-1 activity by p/CIP and TIF2 are differently modulated by PKA, we found it interesting to examine whether differential requirements of amino acids in the AF-2 domain of SF-1 could be detected. The ability of p/CIP and TIF2 to modulate the transcriptional activity of the different SF-1 mutants (Table 2) was analyzed in transfected COS-1 cells. Previously, it has been demonstrated that these mutants are expressed at approximately the same level as wild-type SF-1 in transfected COS-1 cells, and that they retain the ability to bind to DNA (8). The LL mutation represents a change of the AF-2 sequence LLIEML (wild type) to AAIEML at amino acids Leu451 and Leu452, whereas the ΔLL deletion mutation lacks these 2 aa. The E mutant carries a single amino acid substitution at Glu454 (LLIAML), and the ΔAF-2 core deletion mutant lacks the six carboxyl-terminal amino acids (451–456). A significant reduction in transcriptional activity of SF-1 was observed as a result of different mutations and deletions in the AF-2 domain (Fig. 6). Even though the mutants stimulated CRS2-mediated transcription after expression of PKA-Cα and p/CIP, their abilities to activate transcription were reduced significantly compared with wild-type SF-1. We observed a comparable relative stimulation of the CRS2-mediated transcription by the mutants after coexpression of PKA-Cα and p/CIP (Fig. 6A). These re-

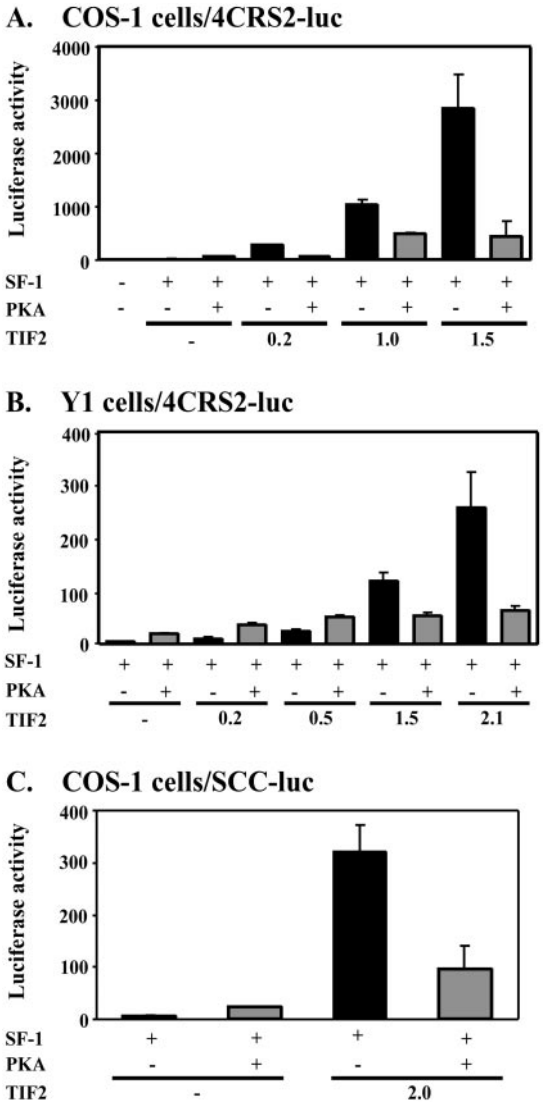

Fig. 4. PKA Affects the Ability of TIF2 to Potentiate SF-1-Dependent Transcription

A, COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg), TIF2 (0.2–1.5 μg), and PKA-Cα (0.1 μg) along with the luciferase reporter plasmid 4CRS2-luc (1.5 μg) as indicated in the figure. Luciferase assays were performed 48 h after transfection. B, Y1 adrenocortical cells were transfected with expression vectors encoding TIF2 (0.2–2.1 μg) and PKA-Cα (0.1 μg) together with the luciferase reporter plasmid 4CRS2-luc (1.5 μg), as indicated in the figure. Luciferase assays were performed 24 h after transfection. C, COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg), TIF2 (2.0 μg), and PKA-Cα (0.1 μg) along with the luciferase reporter plasmid SCC-luc (1.5 μg), as indicated in the figure. Luciferase assays were performed 48 h after transfection. Results are expressed as luciferase activities (mean ± SD) of triplicate transfections from representative experiments.

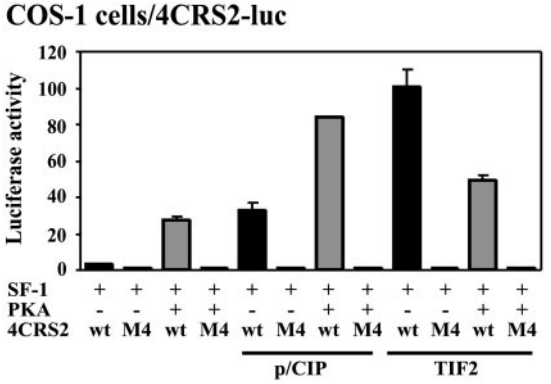

Fig. 5. Binding of SF-1 to the CRS2 Element Is Necessary for p/CIP and TIF2 Coactivation

COS-1 cells were transfected with expression vector encoding SF-1 (0.1 μg), PKA-Cα (0.1 μg), TIF2 (2.0 μg), or p/CIP (1.0 μg) together with wild-type 4CRS2-luc-reporter (wt) (1.5 μg) or 4CRS2-M4-luc (M4) (1.5 μg) containing a mutation in the SF-1 binding site. Luciferase assays were performed 48 h after transfection. The figure shows the mean value ± SD of triplicate transfections from a representative experiment.

Table 2. Amino Acids of the SF-1 Mutants

Wild-Type AF-2 activation domain (aa 451-456)	LLIEML
LLmut	AAIEML
ΔLL	—IEML
Emut	LLIAML
ΔAF-2 (6-aa core deletion)	———
ΔAF-2 (25-aa deletion)[a]	←

Amino acids of SF-1 mutants in the AF-2 domain used for transfection studies and in the yeast two-hybrid interaction assays ([a]). ——, Amino acid deletions. ←, Indicate that additional 19 amino acids prior to the 6-aa core deletion are deleted.

sults indicate that a functional AF-2 domain is required for optimal SF-1-activated transcription and for the further potentiation by p/CIP. The SF-1 mutants were also coexpressed with TIF2 in COS-1 cells. As shown in Fig. 6B, all mutants had a significantly compromised transcriptional activity when coexpressed with TIF2, except for the E mutant. Surprisingly, this mutant, with a substitution of amino acid Glu454 to Ala, had almost similar transcriptional activity as wild-type SF-1. These data suggest that the E mutation does not significantly affect the binding of overexpressed TIF2 to SF-1 in our system. Moreover, it suggests that the mechanisms of TIF2-SF-1 interactions involve different parts of the AF-2 domain than the p/CIP-SF-1 interactions. Whereas p/CIP requires an intact AF-2 core domain of SF-1 for maximal effect on transcription, TIF2 coactivation is not dependent on the glutamic acid residue in the AF-2 core domain.

Effects of the MAPK Signaling Pathway on TIF2-Mediated Coactivation of SF-1

It has been reported that SF-1 is phosphorylated at Serine 203 by the MAPK signaling pathway, and that the interaction with TIF2 is enhanced by MAPK-stim-

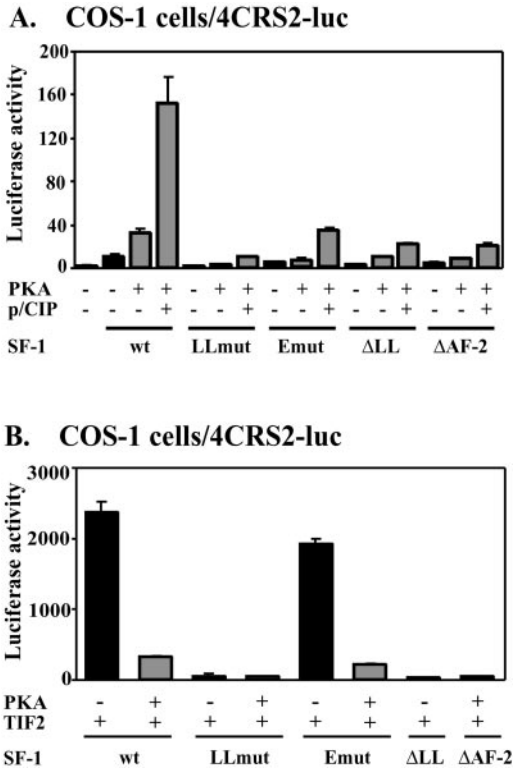

Fig. 6. Mutations in the AF-2 Core Domain of SF-1 Differently Affect Coactivation by p/CIP and TIF2

COS-1 cells were transfected with plasmids encoding different SF-1 AF-2 mutants (LL- and E-mutation or ΔLL- and ΔAF-2 deletions) (0.1 μg), PKA-Cα expression vector (0.1 μg), and the 4CRS2-luc-reporter construct (1.5 μg) and p/CIP (1.0 μg) (panel A) or TIF2 (1.5 μg) (panel B). Luciferase assays were performed 48 h after transfection. The figure shows the mean value ± SD of triplicate transfections from representative experiments.

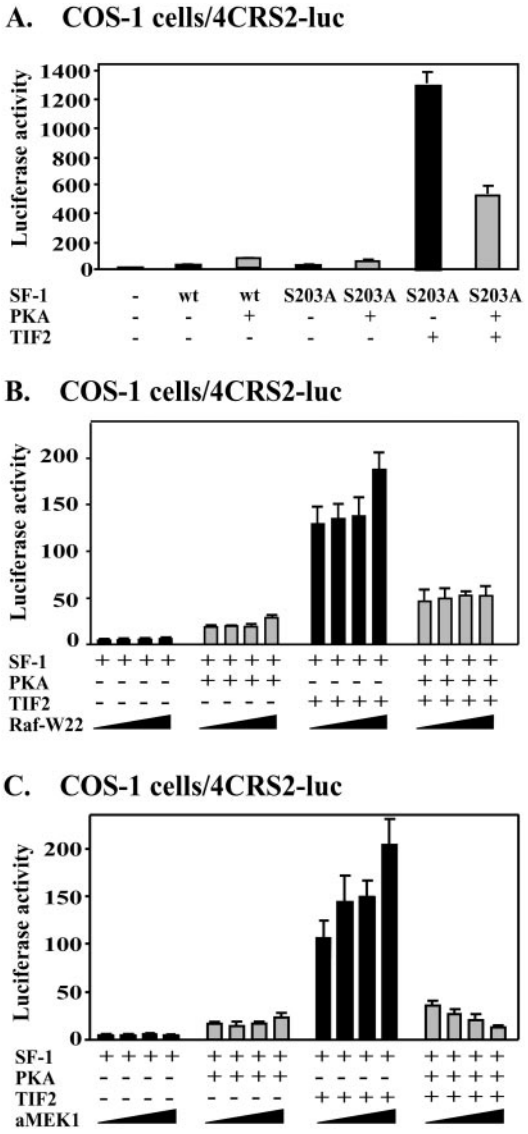

Fig. 7. The Raf-1/MAPK/ERK Signaling Pathway Does Not Affect the Ability of PKA to Inhibit TIF2-Mediated Coactivation of SF-1

A, COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg), SF-1^{S203A} (0.1 μg), TIF2 (1.0 μg), and PKA-Cα (0.1 μg) together with the 4CRS2-luc reporter construct (1.5 μg), as indicated in the figure. B and C, COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg), TIF2 (1.3 μg), PKA-Cα (0.1 μg), and the 4CRS2-luc reporter construct (1.3 μg) and increasing amounts of either Raf-22W [pZip-Neo-Raf-22W expression plasmid encoding the activated Raf-W22 protein (0–25-75–200 ng)] (panel B) or aMEK1 [R4F-MKK1ΔN3S218E/S222D, encoding constitutively active MEK1 (0–25-75–200 ng)] (panel C). Luciferase assays were performed 48 h after transfection. Results are expressed as luciferase activity (mean ± SD) of triplicate transfections from representative experiments.

ulated phosphorylation of SF-1 (14). In some cell types elevation of cAMP and activation of PKA inhibits the MAPK pathway through phosphorylation and subsequent inhibition of Raf-1 kinase (33–36), whereas in other cell types stimulation of PKA can result in an activation of the MAPK pathway (37–41). Therefore, we asked whether PKA acted to modulate the MAPK-stimulated phosphorylation of SF-1 and subsequently the association between SF-1 and TIF2. To examine this issue, an expression plasmid encoding SF-1 carrying a mutation in the MAPK phosphorylation site (Ser203Ala) (14) was coexpressed with TIF2 in COS-1 cells in the presence or absence of PKA-Cα overexpression. SF-1^{S203A} activated the CRS2 reporter activity by approximately 80% as compared with wild-type SF-1, and coexpression of TIF2 stimulated the SF-1^{S203A}-mediated transcription similarly to wild type (73-fold). Activation of PKA inhibited the TIF2-mediated coactivation of SF-1^{S203A}-induced transcription to the same extent as the wild-type SF-1-induced transcriptional activity (Fig. 7A), suggesting that the inhibitory effect of PKA was not attributed to dephosphorylation of SF-1 at Serine 203 and thereby reduced association with TIF2. To further examine the possibility that PKA acted through modulation of the MAPK signaling pathway to inhibit TIF2 coactivation of SF-1,

we performed transient transfection experiments with expression plasmids encoding the activated Raf-W22 protein and a constitutively active MEK1, both of which are upstream activators of the MAPK signaling pathway. Overexpression of activated Raf-W22 or the active MEK1 augmented the SF-1-mediated transcription in a dose-dependent manner, especially in the presence of coexpressed TIF2 (Fig. 7B). These results are consistent with previous observations by others (14). It was noted, however, that the PKA-mediated inhibition of the TIF2 coactivation was not reduced by overexpression of Raf-W22 or active MEK1 (Fig. 7, B and C). Taken together, these results suggest that the mechanisms underlying the ability of PKA to alter the interaction between SF-1 and TIF2 do not involve dephosphorylation of SF-1 at S203 through modulation of the Raf-1/MAPK/ERK signaling pathway.

TIF2-Mediated Coactivation of PPARα, PPARγ, LXRα, and RXRα Is Inhibited by PKA

The finding that stimulation of the PKA pathway inhibited the TIF2-mediated coactivation of SF-1 prompted us to question whether PKA could inhibit the ability of TIF2 to coactivate other members of the nuclear hormone receptor superfamily. To address this question, we determined whether PKA could inhibit the TIF2-mediated coactivation of the PPARα and PPARγ, the LXRα, and the RXRα. TIF2 has been reported to interact with and coactivate these nuclear receptors (16–18). First, we performed transient transfections of COS-1 cells with PPARα and PPARγ expression vectors along with the 3PPRE-luc reporter construct, which contains three copies of the peroxisome proliferator response element from the acyl-CoA promoter. Coexpression of p/CIP enhanced the activity of PPARα and PPARγ in the absence or presence of ligands (Wy 14,643 and BRL 49653, respectively), and PKA-Cα overexpression led to a further enhancement of the reporter gene activity (Fig. 8, A and B). As expected, TIF2 also enhanced the PPARα and PPARγ transcriptional activity. However, in contrast to the effects on p/CIP-mediated coactivation of PPARα/-γ activity, PKA-Cα overexpression markedly impaired the potentiation by TIF2, consistent with the above findings for SF-1. Comparable results were also obtained with the 3PPRE-luc-reporter construct and expression plasmids encoding PPARγ and the heterodimerization partner RXRα in the absence or presence of the RXR ligand 9-*cis*-retinoic acid [9-*cis*-RA (data not shown)]. Next, we examined whether the TIF2-mediated coactivation of LXRα was modulated by PKA using a reporter construct containing part of the 5′-flanking region of LXRα upstream of the luciferase gene (42) and expression vectors encoding LXRα and RXRα. Interestingly, we observed that PKA-Cα overexpression inhibited the LXRα-luc reporter gene activity in transfected COS-1 cells. However, TIF2 enhanced the LXRα transcriptional activity markedly in the absence or presence of the LXRα ligand (22)-OH-cholesterol and/or the RXRα ligand 9-*cis*-RA (Fig. 8C; data not shown). Moreover, PKA-Cα overexpression nearly abolished the potentiation of LXRα activity by TIF2. It should be noted that p/CIP overexpression did not stimulate the LXRα transcriptional activity in COS-1 cells transfected with the LXRα-luc reporter gene (data not shown). Taken together, the present results suggest that stimulation of the PKA pathway inhibits the TIF2-mediated coactivation of several other nuclear hormone receptors.

Activation of PKA Leads to a Selective Decrease of TIF2 Protein

To evaluate whether stimulation of the PKA pathway could modulate the association between SF-1 and TIF2, we performed transient transfections of COS-1 cells with expression vectors encoding SF-1 and HA-GRIP1/TIF2 in the presence or absence of the PKA-Cα expression plasmid. The CRS2 reporter construct was included to ensure that the transcriptional activities of the expression constructs in these experiments were comparable to the above results. SF-1 was immunoprecipitated from the COS-1 cells using a polyclonal anti-SF-1 antibody, and the amount of coimmunoprecipitated HA-GRIP1/TIF2 was then detected by Western blot analysis using an anti-HA antibody. Surprisingly, coexpression of PKA-Cα markedly decreased the level of TIF2 protein (Fig. 9; input), and we could not detect a significant decrease in the association between TIF2 and SF-1 in cells cotransfected with PKA-Cα expression plasmid (Fig. 9). These results were corroborated by Western blot analyses of protein extracts prepared from COS-1 cells transfected with expression vector encoding TIF2 in the presence and absence of PKA-Cα expression plasmid (Fig. 11B), which indicate that the protein level of TIF2 is downregulated after PKA-Cα coexpression. To determine whether PKA affected the expression of TIF2 we performed real-time quantitative RT-PCR using a standard curve generated with a pSG5-HA-GRIP1/TIF2 plasmid. The mRNA levels were normalized against the content of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same extracts. Agarose gel electrophoresis and melting point analysis demonstrated the presence of single PCR products (Fig. 10, A and B). As shown in Fig. 10C, no difference in the mRNA level of TIF2 was found in transfected COS-1 cells in the presence or absence of the PKA-Cα overexpression, indicating that activation of PKA did not decrease the level of TIF2 mRNA. These data indicate that the effect of PKA occurs at the posttranscriptional level, and it appears that activation of PKA leads to a selective decrease of TIF2 protein.

Deletion of the TIF2 Activation Domains (ADs) Impaired the Inhibitory Effect of PKA

The p160 coactivators generate their activation signals via two C-terminal ADs, AD1 and AD2, which act by

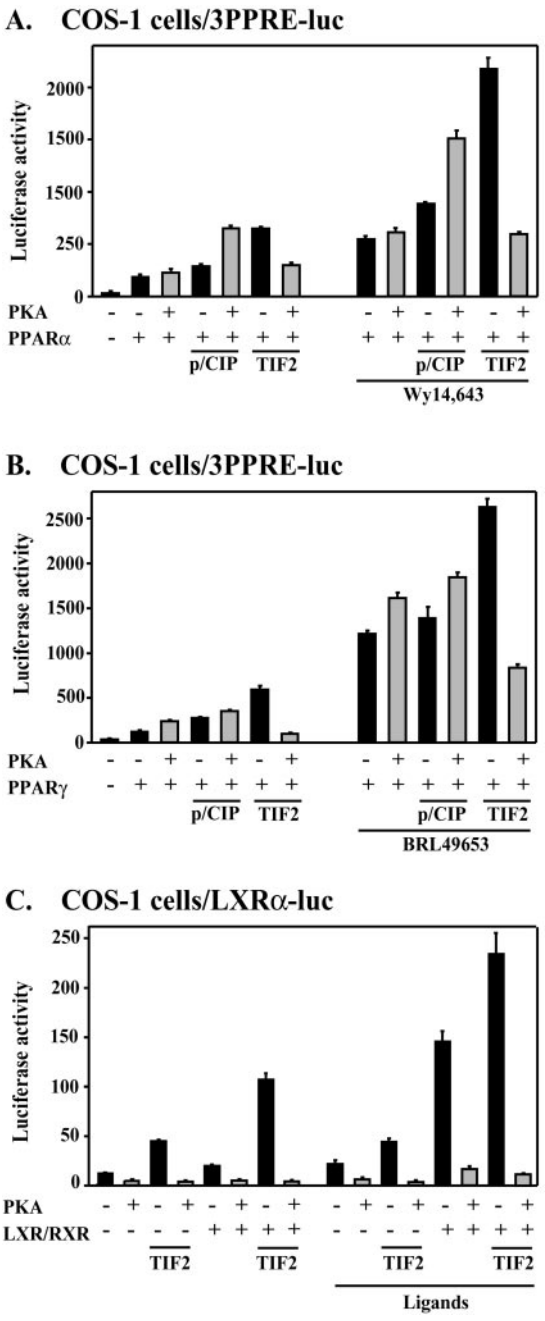

Fig. 8. PKA Inhibits the Ability of TIF2 to Potentiate Transcription Mediated by PPARα

A, Expression vectors encoding PPARα (150 ng), PKA-Cα (75 ng), p/CIP (1.5 μg), and TIF2 (1.5 μg) were transfected into COS-1 cells as indicated in the figure together with the reporter construct 3PPRE-luc (0.9 μg); 24 h post transfection the cells were treated with 30 μM Wy14,643. B, The expression vectors encoding PPARγ (150 ng), PKA-Cα (75 ng), p/CIP (1.5 μg), and TIF2 (1.5 μg) were transfected into COS-1 cells as indicated in the figure together with the reporter construct 3PPRE-luc (0.9 μg); 24 h post transfection the cells were treated with 1 μM BRL 49653. C, The expression vectors encoding LXRα (150 ng), RXRα (150 ng), PKA-Cα (75 ng), and TIF2 (1.5 μg) were transfected into COS-1 cells as indicated in the figure together with the reporter construct LXRα-luc (1.0 μg); 24 h post transfection the cells were treated with 20 μM 22(R)-OH-cholesterol and 20 μM 9-*cis*-RA. Luciferase assays were performed 48 h after transfection. The figure shows the mean value ± SD of triplicate transfections from representative experiments.

recruiting different secondary coactivator proteins. AD1 binds the related proteins p300 and CBP that serve as coactivators for several transcriptional factors, including nuclear receptors (24, 43, 44). The more C-terminal AD2 domain is independent from AD1 and can bind the coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1) that act as secondary coactivators for nuclear receptors (23, 45). To examine the roles of these activation domains with respect to the TIF2-mediated potentiation of SF-1 activity and regulation by the PKA pathway, COS-1 cells were transfected with expression plasmids encoding SF-1 and HA-GRIP1/TIF2 with deletions of the activation domains (AD1 and AD2) together with the 4CRS2-luc-reporter plasmid (Fig. 11A). The TIF2 mutants lacking AD1 and AD2 potentiated the SF-1-mediated transcription 6- to 7-fold, as compared with SF-1 alone, whereas wild-type TIF2 (1.0 μg) potentiated SF-1 transcriptional activity 133-fold. Interestingly, PKA-Cα overexpression inhibited the coactivator function of wild-type TIF2, but not the positive effects of the AD1 and AD2 deletion mutants. Moreover, coexpression of PKA-Cα with the AD2 deletion mutant even enhanced the transcriptional activity of SF-1 18-fold, whereas no changes in transcriptional activity were detected when PKA-Cα was coexpressed with the AD1 mutant. Deletion of both activation domains (AD1/AD2) abolished the coactivator function of TIF2. Because the AD1 domain binds CBP, it should be noted that coexpression of CBP together with TIF2 further stimulated SF-1 activity in transfected COS-1 cells. However, CBP overexpression did not affect the PKA-mediated inhibition of TIF2 (data not shown). These results suggest that the inhibitory effect of PKA on the TIF2 coactivator function is dependent on an intact C-terminal part of TIF2 and that the AD2 domain is essential.

Activation of PKA did not modulate the mRNA levels of wild-type TIF2 or the AD1 and AD2 deletion mutants in transfected COS-1 cells (Fig. 10). Although the amount of TIF2 ΔAD2 amplification product seemed to be lower than the amount of wild-type TIF2 and the TIF2 ΔAD1 mutant, no significant effects of PKA-Cα overexpression on the expression of TIF2 ΔAD2 were detected. It should also be noted that the protein expression levels of wild-type TIF2 and the TIF2 AD deletion mutants were comparably high (lanes 1–4, Fig. 11B). Interestingly, Western blot analysis clearly indicates that activation of PKA leads to a reduction in the protein levels of wild-type TIF2 and TIF2 AD1 deletion mutant, whereas the amounts of TIF2 AD2 and TIF2 AD1/AD2 deletion mutant remain unchanged after PKA activation (lanes 5–8, Fig. 11B). These results are consistent with the above effects on the SF-1

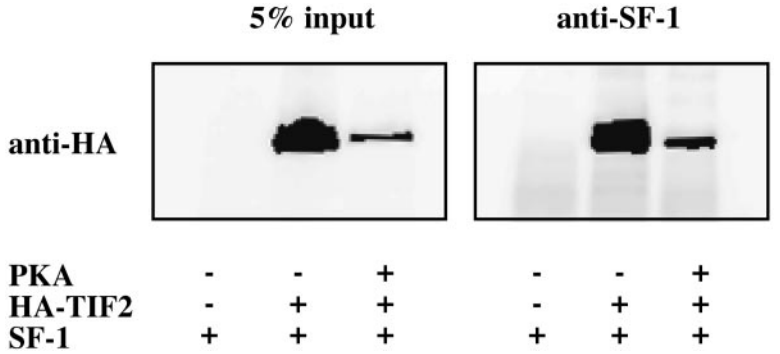

Fig. 9. SF-1-TIF2 Interaction in Transfected COS-1 Cells

COS-1 cells were transfected with the 4CRS2-luc-reporter construct (3.25 μg) and the expression vectors encoding SF-1 (0.22 μg), PKA-Cα (0.22 μg), and HA-GRIP1/TIF2 (4.3 μg) as indicated in the figure; 48 h after transfection the cells were harvested. Immunoprecipitation was performed on the cell lysates using an anti-SF-1 antibody. Five percent of the cell lysates (input) were saved before immunoprecipitation. Input and coimmunoprecipitated HA-GRIP1/TIF2 was detected by Western blotting using anti-HA antibodies. The results presented are representative of three independent experiments.

transcriptional activity and indicate that the PKA-mediated down-regulation of TIF2 protein is dependent on an intact AD2 domain.

DISCUSSION

Transcriptional activation by nuclear receptors involves interaction with the general transcription machinery and with proteins acting as coactivators or corepressors of transcription (10, 46). Most studies have focused on cofactor association with ligand-activated nuclear receptors. In these cases binding of ligands or hormones to the nuclear receptor induce conformational changes that allow the receptors to associate with cofactors, leading to increased or decreased transcription. Less is known about the role of cofactors in transcriptional activation of orphan nuclear receptors. Interestingly, the orphan receptors estrogen-related receptors (ERR1, ERR2, and ERR3) are shown to be activated by the coactivators GRIP1/TIF2, p/CIP, and SRC-1 in a ligand-independent manner, suggesting a mechanism differing from that previously shown for most ligand-dependent steroid hormone receptors (47, 48). SF-1 has been classified as a member of the orphan nuclear receptor family (1, 2), even though oxysterols can activate SF-1 under certain settings in some cell types (49, 50). In this paper we have examined how members of the SRC family affects transcriptional activation by SF-1. Previously it has been shown that SRC-1 (13) and TIF2 (14) interact with SF-1, and a key role for SRC-1 in regulation of SF-1 activity has been proposed (13). We now report that p/CIP also acts as a coactivator of SF-1. The expression of SF-1 was required for p/CIP to coactivate transcription from the CRS2 and SCC reporter constructs in COS-1 cells. Surprisingly, we found that endogenous SF-1 in Y1 cells must be stimulated by PKA to be potentiated by p/CIP. Thus, one interesting possibility might be that the cAMP pathway increases the synthesis or the availability of an unknown ligand in Y1 cells. The mechanisms behind the differential choice of cofactor recruitment by different nuclear receptors are not known, although different expression profiles of the cofactors in different cell types has been suggested as essential in cofactor recruitment preferences (21). p/CIP and SRC-1 were shown to have similar expression patterns in several tissues and cell lines (15), but a recent study suggest a more tissue-specific expression of p/CIP and, interestingly, this report shows that disruption of the SRC-3 (p/CIP) gene delays puberty and leads to an abnormal reproductive function (21). However, in our study Northern blot analyses of the Y1 and COS-1 cells revealed similar expression for p/CIP and SRC-1 in Y1 and COS-1 cells (data not shown), suggesting that different expression cannot explain our findings. Here we have shown that p/CIP interacts directly with SF-1. As expected, the LXXLL motifs of the central interaction domain of p/CIP are of major importance for this interaction, as well as for the coactivator function of p/CIP. It is clear from previous work that there are different requirements for LXXLL-containing helices used by different nuclear receptors (17, 25, 44, 51, 52). Our results suggest that, in the case of SF-1, the NR box 1 is the most important, followed by NR box 2, whereas the NR box 3 in p/CIP appears to have a low capacity for interaction. However, sequences surrounding the LXXLL core motifs may also be important for the SF-1-p/CIP interaction because residues on both sides of the LXXLL motifs have been suggested to contribute in the cofactor interaction with other nuclear receptors (17, 51). Interestingly, the yeast-two-hybrid interaction data and the results in transfected COS-1 cells revealed a different relative importance of NR box 3 in p/CIP. Whereas the mutation of NR box 3 in p/CIP only led to a 40% reduction in the interaction with SF-1, overexpression of p/CIP-L3 in COS-1 cells repressed

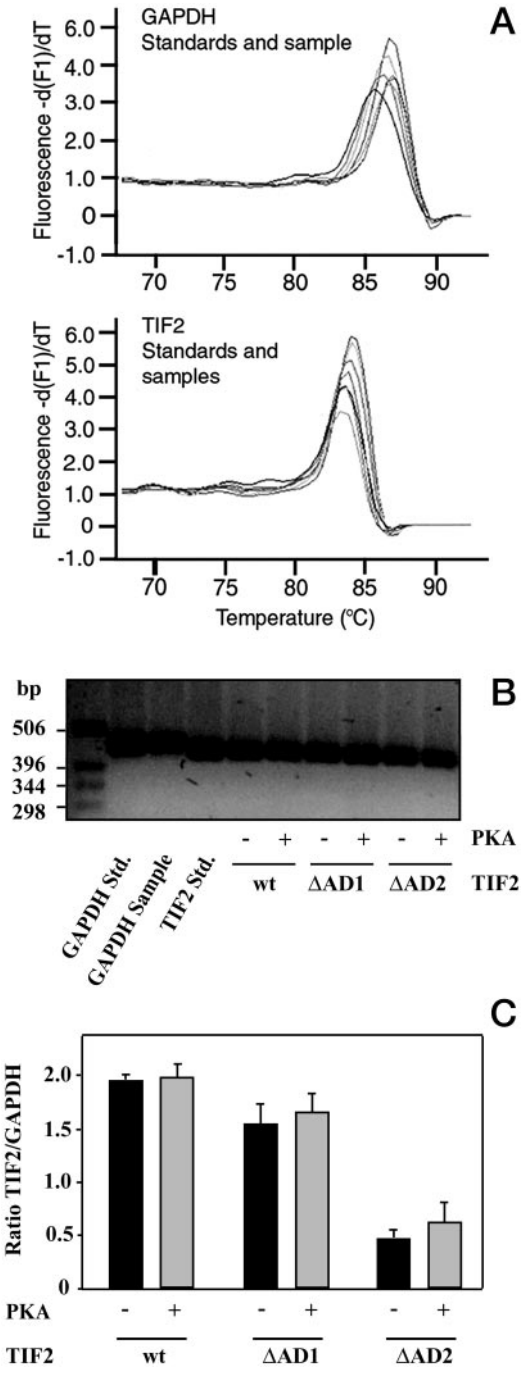

Fig. 10. PKA Activation Does Not Affect the mRNA Levels of TIF2 in Transfected Cells

Real-time RT-PCR using SYBR Green I fluorescence was used to measure product accumulation. A, Melting point analysis demonstrated single peaks and specific products for GAPDH (*upper panel*) and TIF2 (*lower panel*). COS-1 cDNA and the GAPDH cDNA standards were compared which show that there are no melting point differences (*upper panel*). cDNA from COS-1 cells transfected with expression vectors encoding HA-GRIP1/TIF2 wild-type or activation domain mutants (ΔAD1 and ΔAD2) were also compared with standard curves obtained from pSG5-HA-GRIP/TIF2 plasmid (*lower panel*). B, Product analysis. Each of the primer pairs amplified a single product of the appropriate predicted length as assessed by agarose gel electrophoresis and ethidium bromide staining. C, mRNA quantification. Expression of TIF2-, TIF2 ΔAD1-, and TIF2 ΔAD2 mRNA were not affected by PKA-Cα overexpression in transfected COS-1 cells. The figure shows the mean value ± SD of three separate experiments.

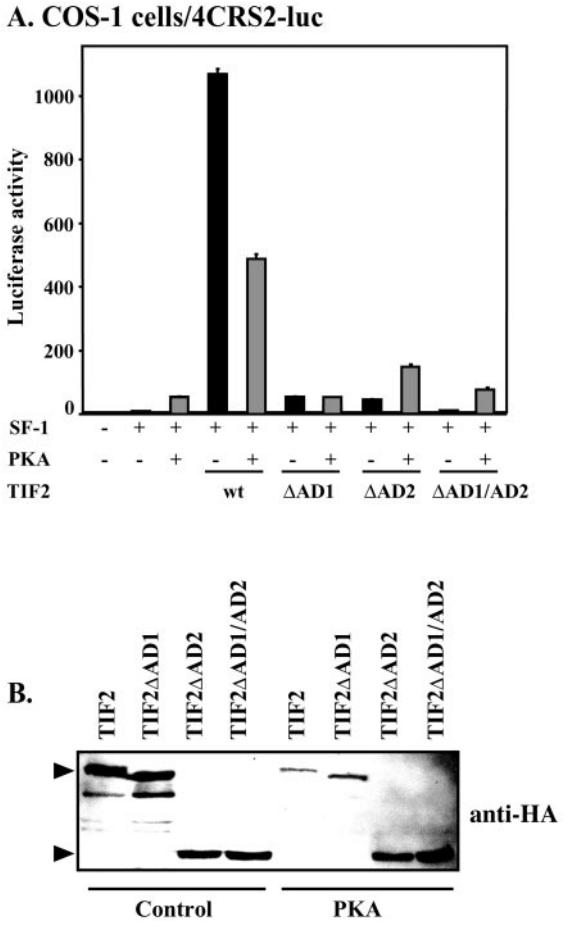

Fig. 11. Repression of TIF2 by PKA Is Dependent on the TIF2 Activation Domain 2

A, COS-1 cells were transfected with the 4CRS2-luc reporter construct (1.5 μg) and the expression vectors encoding SF-1 (0.1 μg), PKA-Cα (0.1 μg), and wild-type HA-GRIP1/TIF2 (1.0 μg) or HA-GRIP1/TIF2 activation domain mutants (ΔAD1, ΔAD2, or ΔAD1/2) (1.0 μg) as indicated in the figure; 48 h after transfection, luciferase assays were performed. The figure shows the mean value ± SD of triplicate transfections from a representative experiment. B, Western blot of cell lysates from COS-1 cells cotransfected with plasmids encoding wild-type HA-GRIP1/TIF2 or HA-GRIP1/TIF2 activation domain mutants (ΔAD1, ΔAD2, and ΔAD1/2) in the presence or absence of coexpressed PKA-Cα.

the transcriptional activity and appeared to have a dominant negative effect on the SF-1-mediated transactivation. Our results suggest that although NR box 3 is not essential for the physical interaction between p/CIP and SF-1, this p/CIP LXXLL motif seems to be of

functional importance in the stimulation of SF-1 activity. Thus, it would be interesting to examine in more detail whether there is a functional synergy among the different LXXLL motifs in p/CIP that is involved in the stimulation of SF-1 transcriptional activity.

The AF-2 domain within the carboxyl-terminal ligand-binding domain is essential for the interaction of nuclear receptors with certain cofactors (53), and the interaction between SF-1 and the coactivators SRC-1 and TIF2 is dependent on the AF-2 domain (12–14). Here we demonstrate that an intact AF-2 domain is also required for SF-1 interaction with p/CIP, and that different mutations in the AF-2 domain significantly impair PKA-stimulated SF-1 transactivation, as well as the further potentiation by p/CIP. In contrast, the substitution of Glu454 to Ala did not impair the TIF2-mediated potentiation of SF-1 activity significantly. This finding reveals differences in the SF-1-p/CIP and the SF-1-TIF2 interactions with regard to residue Glu454 that is a highly conserved amino acid in the AF-2 domain of nuclear receptors (54). Also in other nuclear receptors differential responses on transcriptional activity and cofactor interactions due to mutation of the glutamic acid residue in the AF-2 domain has been observed. Mutations at this residue have been reported to decrease the transcriptional activity of T3Rα and T3Rβ (55, 56), whereas no negative effects were observed for ERα and GR (54). Interestingly, a natural $T_3R\beta$ AF-2 domain mutant (E457D) bound T_3 with normal affinity and had normal interaction with the corepressor N-CoR, but did not bind SRC-1 (57). In a recent report, the substitution of Glu897 with Ala diminished androgen-dependent activation by AR (58). However, similar to our observations on the SF-1 E mutant, it was also shown that TIF2 overexpression significantly stimulated the AR mutant. TIF2 appeared to rescue the ligand-stimulated transactivation function of this AR mutant (58), as well as the loss of ligand-dependent transactivation of a similar $T_3R\alpha$ E mutant (59). Although this seems to be consistent with our data, it should be noted that in our system, TIF2 activates both the wild-type SF-1 and the E mutant in the apparent absence of ligand. Thus, it appears that E mutations in various nuclear receptors differentially affect the conformation and function of the receptors and thereby the interaction with cofactors.

Steroid hormone biosynthesis is under the control of ACTH, LH, and FSH from the pituitary (60). These hormones regulate steroidogenesis via increased intracellular cAMP levels and activation of PKA. Analysis of the promoter regions of the genes encoding the steroidogenic enzymes have led to identification of cAMP-responsive elements (60, 61), but so far little is known about the exact mechanisms behind the PKA-stimulated transcription of these genes. There are several possible explanations for the cAMP-dependent enhancement of SF-1-mediated transcriptional activation. SF-1 binding sites in the promoter regions of target genes are often within cAMP-responsive regions, *e.g*. in the promoters of CYP17 (62), CYP11A (63), CYP19 (64), CYP1B1 (65), the steroidogenic acute regulatory gene (66), and the human ACTH-receptor gene (67). One possibility, which has been considered (68–70), is that the cAMP pathway leads to phosphorylation of SF-1. It has, however, been difficult to show that elevation of cAMP stimulates the phosphorylation of SF-1 *in vivo* (71). Recently, we have observed that increased PKA activity leads to elevated levels of SF-1 protein without affecting the SF-1 mRNA level, indicating that PKA stimulates SF-1 activity through increased amounts of SF-1 protein (71a). Moreover, phosphorylation of SF-1 by the MAPK pathway, but not by the cAMP pathway, has been reported (14). Another possible mechanism for the cAMP-stimulated activation of SF-1 is that PKA phosphorylates, and thereby activates, another protein that interacts with SF-1. This protein may be a coactivator or another transcription factor. Interestingly, it was previously shown that SF-1 interacts with cAMP response element-binding protein, leading to recruitment of CBP and increased histone acetylation, thereby suggesting a mechanism for the synergism between the SF-1 and cAMP pathways (72). We did not, however, observe any stimulation by PKA on CRS2/reporter gene activity in the absence of SF-1, confirming the importance of SF-1 in the cAMP response. An alternative possibility is that the cAMP pathway leads to increased expression of transcription factors or cofactors interacting with SF-1. However, no changes in the expression levels of SRC-1, p/CIP, or TIF2 were detected in COS-1 or Y1 cells treated with forskolin or transfected with PKA-Cα (data not shown).

Unexpectedly, we found that PKA represses the stimulatory effect of TIF2 on SF-1-induced transcription, whereas no such effect was observed with p/CIP and SRC-1 (29). Our data indicate that this is not caused by modulation of SF-1 through inhibition of MAPK-induced phosphorylation at S203, but rather on a PKA-mediated regulation of TIF2. This is supported by the fact that PKA activation also inhibited the TIF2 coactivation of PPARα, PPARγ, and LXRα/RXRα. A previous report has shown that expression of GRIP1/TIF2 in HepG2 cells enhances ER-mediated transcriptional activity, but not in the presence of a cAMP analog (73). Thus, it seems that the transcriptional activity of several nuclear receptors can be repressed through PKA-mediated inhibition of TIF2. Interestingly, we observed that activation of PKA represses TIF2 function through selective down-regulation of TIF2 protein, and that this is dependent on an intact activation domain 2 (AD2) in TIF2. The mechanisms for coregulator down-regulation is not well understood, and there are several possible mechanisms behind this kind of regulation. PKA may act through direct phosphorylation of a residue in AD2 or by phosphorylation through indirect pathways inducing the activity of another kinase, and phosphorylation may serve as a signal for selective degradation (74). A second possible mechanism involves recruitment of another co-

regulator protein that interacts with TIF2. Recently, it was reported that SRC-1 is phosphorylated at two MAPK sites (T1179 and S1185) after elevation of cAMP in COS-1 cells (75). However, in contrast to the PKA-induced negative action on TIF2 that we observe, activation of PKA was found to increase the intrinsic transactivation function of SRC-1 (75). A detailed analysis of the phosphorylation sites in TIF2 will be necessary to determine whether phosphorylation regulates the interaction between TIF2 and nuclear hormone receptors. Recently, it has been reported that although TIF2 is essentially localized to the nucleus of proliferating myoblasts, weak expression can be observed in the cytoplasm (76). Thus, it would be interesting to perform localization studies of endogenous and overexpressed TIF2 in the presence and absence of PKA overexpression to determine whether changes in subcellular localization are coupled with the PKA-mediated down-regulation of TIF2. It is also of interest to examine whether PKA regulates the proteins that interact with TIF2. AD2 can bind CARM1 and PRMT1 (23, 45), and PKA might modulate the association between TIF2 and CARM1 or PRMT1.

In summary, we have demonstrated that SF-1 interacts with the coactivator p/CIP and that p/CIP and TIF2 interact with different amino acids in the AF-2 domain of SF-1. Furthermore, this is the first report demonstrating that the p160 coactivators are differentially regulated by the cAMP pathway. Our study indicates that PKA activation leads to selective down-regulation of TIF2, and this may reflect an important turn-off mechanism in response to extracellular signals.

MATERIALS AND METHODS

Plasmid Construct

The expression plasmid pCMV5-SF-1 and the luciferase reporter plasmid pT81–4CRS2-luc (4CRS2-luc), containing the SF-1 binding site from the proximal promoter region of the bovine CYP17 gene, are described in Ref. 9. The M4 reporter is mutated in the SF-1 binding site of pT81–4CRS2-luc (9). The scc-wt-luc (SCC-luc) reporter contains the region between −186 and +12 of the bovine CYP11A gene that includes a SF-1 binding site (28). The pCMV5-SF-1 mutations (LL-, E-mutations; and ΔLL-, ΔAF-2-deletions) have been described previously (8). The pCMV5-Cα plasmid expressing the catalytic subunit of PKA was a gift from Dr. G. S. McKnight (Seattle, WA). The pJG4–5-p/CIP prey vector and the expression plasmid pCMX-p/CIP were generously supplied by Dr. S. Westin (San Diego, CA) (15). Amino acid substitutions in the LXXLL motives of the pYESTrp-p/CIP prey vector and the pCMX-p/CIP expression plasmid were performed using the QuikChange Site-directed Mutagenesis kit from Stratagene (La Jolla, CA) (Table 1). The expression plasmids pSG5-HA-GRIP1, pSG5-HA-GRIP1ΔAD1, pSG5-HA-GRIP1ΔAD2, and pSG5-HA-GRIP1ΔAD1/AD2 were kindly provided by Dr. M. R. Stallcup (Los Angeles, CA) (45). The expression plasmid pSG5-GRIP1 is described in (26). pCMV5-TIF2 was kindly supplied by Dr. E. Treuter (Stockholm, Sweden). The PPREx3-tk-luc (3PPRE-luc) reporter construct containing three copies of the peroxisome proliferator response element from the acyl-CoA promoter and the expression plasmids pcDNA3.1-hPPARα and pcDNA3.1-hPPARγ1 were kindly provided by Dr. K. Kristiansen (Odense, Denmark) (77). The pGL3basic-LXRα (−1,515/+1,822)-luc (LXRα-luc) reporter construct and the expression plasmids pCMV-RXRα and pCMX-LXRα were kindly provided by Dr. H. I. Nebb (Oslo, Norway) (42). The pZip-Neo-Raf22W expression plasmid encoding the activated Raf-1 protein was kindly provided by Dr. C. J. Der (Chapel Hill, NC) (78), and the R4F-MKK1ΔN3S218E/S222D plasmid encoding constitutively active MEK1 was kindly supplied by Dr. N. G. Ahn (Boulder, CO) (79). pYESTrp-SRC-1 was constructed by insertion of a PCR-amplified DNA fragment encoding SRC-1 into *Eco*RI/*Xho*I-linearized pYESTrp (Invitrogen, San Diego, CA). pHybLex/Zeo-SF-1 contains the ligand-binding domain and hinge region of SF-1 and was used for interaction studies. pHybLex/Zeo-SF-1 was constructed by insertion of a PCR-amplified DNA fragment encoding aa 107–461 of SF-1 into *Xho*I/*Sac*I-linearized pHybLex/Zeo (Invitrogen). pHybLex/Zeo-SF-1ΔAF-2, a carboxyl-terminal 25 aa deletion mutation, was created by cloning of a PCR-amplified fragment of pCMV5-SF-1ΔAF-2 (aa 107–436) as described above for pHybLex/Zeo-SF-1. pHybLex/Zeo-Lamin (Invitrogen) was used as a negative control in the interaction studies.

Yeast Two-Hybrid Interaction and β-Galactosidase Assay

Yeast two-hybrid mating assay was used to examine the interaction between the *Lex*A-fusion bait proteins SF-1, SF-1ΔAF-2, and Lamin (negative control) and the B42-fusion prey proteins p/CIP and SRC-1. In addition to the wild-type p/CIP prey proteins, p/CIP mutants were used with amino acid substitutions in the LXXLL motives (L1, L2, L3) and combinations of these mutations (L1/2, L1/2/3). The *Lex*A-fusion proteins were introduced into the EGY191 yeast strain (MATα *ura3 trp1 his3::2lex*Aop-*LEU2)* and selected on plates containing zeocin (200 mg/ml), whereas the B42-fusion proteins were transformed into the L40 yeast strain (MAT**a** *his3 Δ200 trp1–901 leu2–3112 ade2 LYS2::*(4*lex*Aop-*HIS3) URA3::*(8*lex*Aop-*lacZ) GAL4)* and selected on plates without tryptophan. Selection for the presence of both bait and prey plasmids (mating colonies) were carried out on plates with zeocin, lacking tryptophan. The double transformants were transferred to plates with zeocin, lacking both tryptophan and leucine, to select clones with interaction between bait and prey. The strength of the interaction was measured in semi-quantitative liquid β-galactosidase assays. To examine the β-galactosidase activities, strain EGY191 was cotransformed with the 2*lex*Aop-*lacZ*-reporter plasmid pJK103. EGY191 and pJK103 were a gift from Dr. E. Golemis (Philadelphia, PA). The chemiluminescent reporter gene assay system from Galacto-*Star* (Tropix, Bedford, MA) was used to detect β-galactosidase activity in yeast cell extracts from at least four independent mating colonies. The yeast cells were grown in selective media with zeocin, but without tryptophan and uracil, for selection of bait, prey, and reporter plasmids, respectively. Galactose (2%) and raffinose (1%) were added to induce the GAL1 promoter of the β-galactosidase reporter gene. The activity was normalized to growth (OD_{600}), and both isolation of yeast cell extracts and luminometer assay time were according to the formula provided by CLONTECH Laboratories, Inc. (Palo Alto, CA).

In Vitro Protein Interaction Assay

Baculovirus was used to express the $(His)_6$-containing wild-type SF-1-fusion protein and the $(His)_6$-containing SF-1ΔAF-2 with an AF-2 core (LLIEML) deletion. The genes encoding wild-type SF-1 and the SF-1ΔAF-2 mutant were

cloned into pFastBac HTb donor plasmids (Life Technologies, Inc., St. Louis, MO) at the *Bam*HI and *Xho*I sites. The recombinant plasmids were transformed into DH10Bac-competent cells that carry a baculovirus shuttle vector (bacmid) and a helper plasmid, resulting in a site-specific transposition of the expression cassettes that contain the SF-1 genes from the donor plasmids into the bacmids. The recombinant baculoviruses, which were used to infect Sf21 insect cells for expression of the proteins, were generated by transfection of the insect cells with their corresponding recombinant bacmids. The expressed $(His)_6$-SF-1 and $(His)_6$-SF-1ΔAF-2 proteins were purified using affinity chromatography with Ni-NTA agarose (QIAGEN, Chatsworth, CA). [^{35}S]Methionine-labeled TIF2 and p/CIP were prepared by using the TNT reticulocyte lysate system (Promega Corp., Madison, WI) in the presence of [^{35}S]methionine.

For protein interaction assay, 3 μl of the [^{35}S]methionine-labeled *in vitro* translated coactivators were incubated with 5 μg of purified $(His)_6$-SF-1 or $(His)_6$-SF-1ΔAF-2 proteins at 30 C for 1 h with occasional gentle mixing. These protein mixtures or the [^{35}S]methionine-labeled coactivators (3 μl) alone were then incubated with 30 μl of Ni-NTA agarose in 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10% glycerol, 20 mM imidazole, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin to a total volume of 300 μl on a rotating wheel at room temperature for 1 h. After incubation, the Ni-NTA agarose beads were washed with 500 μl of the above buffer. The Ni-NTA agarose beads were subsequently boiled at 95 C for 5 min in the presence of 15 μl SDS-loading buffer (100 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and subjected to 6% SDS-PAGE followed by autoradiography.

Cell Culture and Transfections

Y1 mouse adrenocortical tumor cells and COS-1 cells were cultured in DMEM supplemented with 10% FCS, 100 U penicillin/ml, and 100 μg streptomycin/ml. For transfection, Y1 cells were seeded at a density of 2.5×10^5 cells per well of a six-well plate and transiently transfected the following day by using the calcium phosphate method (62). COS-1 cells were seeded as described for Y1 cells and transiently transfected the following day by the SuperFect transfection procedure according to the manufacturer's protocol (QIAGEN). Both Y1 and COS-1 cells were transfected with 0.9–1.5 μg reporter plasmid (4CRS2-luc, 4CRS2-M4-luc, SCC-luc, 3PPRE-luc, or RXRα-luc), 75–100 ng pCMV5-Cα, and 0.1–2.0 μg pCMX-p/CIP or alternatively 0.02–2.1 μg pSG5-GRIP1/TIF2 as indicated in the figures. The total amount of plasmid was kept constant by compensating with pCMV5. COS-1 cells were also cotransfected with pCMV5-SF-1 or pCMV5-SF-1 mutants as indicated in the figure legends. In the control experiments, plasmids encoding other nuclear receptors, such as pcDNA3.1-hPPARα (0.15 μg), pcDNA3.1-hPPARγ1 (0.15 μg), pCMV-RXRα (0.15 μg), and pCMX-LXRα (0.15 μg), were transfected into COS-1 cells in the presence of ligands as indicated. Wy14,643 (30 μM) was added to the transfections with PPARα, whereas 1 μM BRL 49653 was used in the experiments with PPARγ. 22(R)-OH-cholesterol (20 μM) and 20 μM 9-*cis*-RA acid were used as LXR and RXR ligands, respectively. The cells were washed once with PBS 24 h (Y1) or 48 h (COS-1) after transfection and assayed for luciferase activity. A portion (40 μl) of the cell extracts was used for luciferase determination on a LUCY-1 luminometer (Anthos, Austria). The luciferase assay was performed in accordance with the protocol of the Luciferase Assay Kit (BIO Thema AB, Sweden). All experiments were performed in triplicate and repeated three to five times, and measured luciferase activity is shown in the figures.

Coimmunoprecipitation

Plates (60 mm) were seeded at a density of 3.3×10^5 COS-1 cells per plate and transfected with SuperFect (CLONTECH Laboratories, Inc.) as recommended the following day. The 4CRS2-luc reporter construct (3.25 μg) and the expression vectors encoding SF-1 (0.22 μg), PKA-Cα (0.22 μg), and TIF2 (4.31 μg) were used in the same relative amounts as in the luciferase assays. Forty-eight hours after transfection, the cells were washed twice with PBS and selected in 1 ml PBS and thereby lysed by adding 200 μl ice-cold lysis buffer [50 mM Tris-HCl (pH7.5), 100 mM NaCl, 0.5% SDS, 1.0% Triton X-100] to the pelleted cells. Four volumes of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 50 μl protein A-Sepharose were added to the supernatants. The samples were then incubated for 2 h before the supernatant was added to 3 μl anti-SF-1 antibodies from Dr. K. Morohashi and incubated overnight, followed by a 2-h incubation with 50 μl protein A-Sepharose the next day, all at 4 C. The precipitated protein A-Sepharose was washed four times with ice-cold 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% SDS, 0.5% Triton X-100. Phenylmethylsulfonyl fluoride (0.2 mM) and 0.7 μg/ml of aprotinin, leupeptin, pepstatin, and soybean trypotinin were added to all the solutions before use. The precipitates were subsequently boiled with 30 μl 2× sample buffer and electrophoresed on an 8% SDS-polyacrylamide gel. Five percent of the cell lysate (input) was saved before coimmunoprecipitation with anti-SF-1, and both input and coimmunoprecipitated proteins were detected by Western blotting and with anti-HA antibodies.

Western Blot

Yeast extract, cell lysates, or coimmunoprecipitate was electrophoresed on a 6–10% SDS-polyacrylamide gel and transferred to nitrocellulose filters. The bait proteins *Lex*A/SF-1 and *Lex*A/SF-1ΔAF-2 were detected with anti-*Lex*A (Invitrogen) to compare expression levels, whereas HA-GRIP1/TIF2 proteins were detected with rabbit anti-HA (Zymed Laboratories, Inc., South San Francisco, CA). Goat antirabbit-IgG-horseradish peroxidase was used as a secondary antibody (Pierce Chemical Co., Rockford, IL).

Isolation of mRNA and Real-Time RT-PCR

COS-1 cells were cotransfected with expression plasmids encoding SF-1 and HA-GRIP1/TIF2 wild-type or activation domain deletion mutants (AD1 and AD2) in the presence or absence of PKA-Cα expression plasmid. At 48 h post-transfection, the cells were washed once with PBS before harvest. Isolation of mRNA was performed using the MagNA Pure LC mRNA Isolation Kit II and the MagNA Pure LC according to the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN). Quantitative real-time RT-PCR was carried out using a LightCycler rapid thermal cycler system (Roche Molecular Biochemicals). Forward (F) and reverse (R) PCR primers designed from the mouse TIF2/GRIP1 sequence were as follows: F, 5′-AAG-CCT-TTG-CCA-GAT-TCA-G-3′; R, 5′-CAA-CGA-GAG-TGC-CAT-CAG-AC-3′. The predicted size of the TIF2/GRIP1 PCR product was 430 bp. Please note that the TIF2/GRIP1 primers were not optimized for the African green monkey TIF2 sequence, and subsequently we did not amplify TIF2 cDNA from endogenously expressed TIF2 mRNA in COS-1 cells. Forward and reverse GAPDH primers were:, F, 5′-ACC-ACA-GTC-CAT-GCC-ATC-AC-3′; R, 5′-TCC-ACC-ACC-CTG-TTG-CTG-TA-3′. The predicted size of the GAPDH PCR products was 480 bp. Reactions were performed using the LightCycler-RNA Master SYBR Green I Kit (Roche Molecular Biochemicals), and the PCR products were detected via intercalation of the fluorescent dye SYBR-Green. TIF2 and GAPDH standards were prepared by 10-fold serial dilutions of linearized pSG5-HA-GRIP1/TIF2 plasmid

and DNA fragments containing the full-length GAPDH cDNA sequence, respectively. Standards were used over the range of 100 pg/μl to 0.01 pg/μl. The negative controls were prepared by replacing the mRNA template with PCR-grade H_2O. The protocols included a 20-min reverse transcription step at 61 C, a 5-sec denaturation step, and then 45 cycles consisting of denaturation at 95 C for 5 sec, annealing at 55 C (TIF2) or 60 C (GAPDH) for 5 sec, and an extension phase at 72 C for 18 sec (TIF2) or 20 sec (GAPDH). Fluorescence was measured at the end of the 72 C extension phase. The quality of the RT-PCR products was controlled by melting point curve analysis. Additionally, the amplification products were subjected to agarose gel electrophoresis (1.6%) and stained with ethidium bromide (0.5 μg/ml) to ensure specificity of amplification.

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