Paper I

ELSEVIER

Molecular and Cellular Endocrinology 203 (2003) 91–103

MCE
Molecular and Cellular Endocrinology

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Characterization of receptor-interacting protein RIP140 in the regulation of SF-1 responsive target genes

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Received 15 July 2002; accepted 6 February 2003

Abstract

Receptor-interacting protein (RIP) 140 interacts with several nuclear receptors, but its function in regulation of nuclear receptor action has been debated. Here we have examined the role of RIP140 in regulation of Steroidogenic factor-1 (SF-1)-dependent transcription. SF-1 interacts with RIP140 through its activation function-2 (AF-2) domain. Several domains of RIP140 interact directly with SF-1, but the carboxyl-terminal region containing 4 of its 9 LXXLL motifs showed the strongest SF-1 interaction. Coexpression of RIP140 and SF-1 in different cell types demonstrated that RIP140 acts as a potent corepressor of transcription from the SF-1 responsive cAMP regulatory sequence 2 (CRS2) element of the CYP17 gene and a variety of SF-1 responsive promoter genes. RIP140 also counteracted the stimulatory action of p160/SRC coactivators. The inhibitory effect of RIP140 was partially reversed by Trichostatin A, suggesting a role of histone deacetylase (HDAC) activity in RIP140-mediated repression of SF-1. Quantitation of endogenous coregulator mRNA levels revealed cell type specific differences that could affect the repressor action by overexpressed RIP140.

Keywords: SF-1; RIP140; Nuclear receptor; Coregulator

1. Introduction

Nuclear hormone receptors are transcription factors that modulate the promoter activities of target genes by binding to specific response elements. The nuclear orphan receptor steroidogenic factor-1 (SF-1) is found to be pivotal in adrenal development and sexual differentiation (Luo et al., 1994), and is expressed in the adrenal cortex, the gonads, pituitary gonadotrope cells, hypothalamus, and spleen (Morohashi et al., 1999; Ikeda et al., 1993). SF-1 regulates the cell specific expression of a variety of different proteins involved in steroidogenesis, reproduction and male gonadal differentiation (Parker and Schimmer, 1997). In the adrenal cortex, SF-1 regulates genes encoding the cytochrome P450 steroid hydroxylases (Morohashi and Omura, 1996), 3β-hydroxysteroid dehydrogenase, the ACTH receptor (Naville et al., 1998), StAR (Sugawara et al., 1996) and the high density lipoprotein (HDL) receptor SR-B1 (Lopez et al., 1999) by binding to SF-1 responsive elements of which several are also modulated by cAMP. In contrast to the classic steroid hormone receptors that bind to DNA as dimers, SF-1 binds the AGGTCA motif of the target genes as a monomer. The carboxyl terminus of nuclear receptors contains an amphipathic α-helix, the activation function-2 domain (AF-2), which is essential for transcriptional activation. Although oxysterols have been reported to activate SF-1 in certain cell types (Lala et al., 1997), no obligatory SF-1 ligand has been identified so far (Mellon and Bair, 1998).

Several nuclear receptor accessory proteins, referred to as coactivators and corepressors, act as bridging factors between the nuclear receptors and the transcription machinery. A number of coactivators such as the p160/SRC family and p300/CBP have been shown to

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0303-7207/03/$ - see front matter
doi:10.1016/S0303-7207(03)00097-2

encode intrinsic histone acetyltransferase activities (Chen et al., 1997; Liu et al., 1999). The p160/SRC members SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2 and p/CIP/ACTR/RAC3/AIB1/NcoA-3 interact with the AF-2 domain of several nuclear receptors including SF-1 (Børud et al., 2002; Crawford et al., 1997; Ito et al., 1998; Hammer et al., 1999). On the contrary, corepressors such as N-CoR/SMRT are found to interact with histone deacetylases (HDACs).

The human 140-kDa protein Receptor-interacting protein (RIP)140 was first identified as a coactivator of estrogen receptor in breast cancer cells. RIP140 interacted with the AF-2 domain of ER, and the interaction as well as the transcriptional activity, were increased by estrogen (Cavailles et al., 1995). RIP140 is ubiquitously expressed and has been demonstrated to interact with several other nuclear receptors including the retinoic acid, retinoid X, androgen, glucocorticoid, vitamin D and thyroid receptors in a ligand-dependent manner through the AF-2 domain (Ikonen et al., 1997; L'Horset et al., 1996; Cavailles et al., 1995; Masuyama et al., 1997; Subramaniam et al., 1999). Mouse RIP140 was cloned by screening libraries with the ligand binding domain of the orphan receptor TR2, and RIP140 was found to act as a corepressor of TR2 activity (Lee et al., 1998). RIP140 was recently also found to modulate the transcription of StAR through interactions with SF-1 and DAX-1 (Sugawara et al., 2001). Moreover, examination of female RIP140 knock out mice has demonstrated a failure of oocyte release, and the level of RIP140 seems to be important for female fertility (White et al., 2000). Although no phenotypic changes have been demonstrated in other endocrine tissues of the RIP140 null mice, a detailed examination of adrenal function has not been reported. RIP140 was initially proposed to compete with the p160/SRC family of coactivators for binding to the ligand-activated nuclear receptors (Treuter et al., 1998), and, more recently, the direct recruitment of HDACs such as HDAC1 and HDAC3 to the transcriptional machinery has also been proposed as a mechanism for RIP140-mediated repression of nuclear receptor transcriptional activity (Wei et al., 2000). In contrast to other nuclear receptor corepressors that recruit HDACs through Sin3 complexes, RIP140 interacts directly with HDACs through its amino-terminal domain (Wei et al., 2000, 2001). In addition, RIP140 has been shown to interact with the carboxyl-terminal binding protein (CtBP) that is associated with an array of transcriptional repressors (Vo et al., 2001).

Short α-helical sequences, LXXLL motifs, have been shown to mediate the interaction of several coregulators with different nuclear receptors (Heery et al., 1997). However, the number and the flanking sequences of these LXXLL motifs varies between the different coregulators, and this may account for the differences in binding to different nuclear receptors (Heery et al., 2001). RIP140 contains 9 LXXLL motifs (Lee et al., 1998), but it appears that other domains of the protein may also be involved in interaction with certain nuclear receptors and to date different nuclear receptors have been reported to interact with RIP140 through different motifs (Wei et al., 2001).

In this paper, we examine the role of RIP140 in regulation of SF-1-dependent transcription using different cell lines and a number of SF-1 responsive promoters including steroidogenic enzyme genes. The SF-1-interacting domains of RIP140 are detailed, and co-transfection experiments indicate that RIP140 counteracts the positive action of the p160/SRC coactivators p/CIP and TIF2 on SF-1 transcriptional activity. We also demonstrate a partial contribution of HDCA activity to RIP140 inhibition of SF-1. In addition, differences in the mRNA expression levels of RIP140 and TIF2 in COS-1 cells and steroidogenic Y1 cells are shown. These findings may explain cell type specific differences in RIP140-mediated repression of SF-1 dependent transcription.

2. Materials and methods

2.1. Plasmid constructs

The expression plasmid pCMV5-SF-1 and the luciferase reporter plasmid pT81-4CRS2-luc (4CRS2-luc) are described in (Bakke and Lund, 1995). 4CRS2-luc contains four copies of the SF-1 binding site from the proximal promoter region of the bovine CYP17 gene. The luciferase reporter plasmids pGL3-MIS-380 and p7β1.5H-Luc (P45011β) contain the promoter region between −380 and +6 of the mouse Müllerian inhibiting substance (MIS) and a 1.5-kb long up-stream promoter region of the bovine CYP11B gene, respectively, and were generously supplied by Dr K. Morohashi (Okazaki, Japan). The scc-wt-luc (CYP11A-luc) reporter contains the region between −186 and +12 of the bovine CYP11A gene (Ahlgren et al., 1999). The luciferase reporter plasmids pGL_2 1.3-kb StAR containing a 1.3 kb *Hin*d III fragment of the StAR promoter region (nucleotides −1293–+93) and the pGL_2 StAR which contains the region between −235 and +39 of the StAR promoter were generously supplied by Dr T. Sugawara (Sapporo, Japan). pGL3-290CYP17 carries the −290 CYP17 promoter region of the bovine CYP17 gene ahead of a luciferase reporter gene and was provided by Dr M. Waterman (Nashville, TN). The different mutations were introduced into the pGL3-290CYP17 reporter by using the QuickChange Site-directed Mutagenesis kit from Stratagene (La Jolla, CA): M1; GAG ACG TTG ATG GAC AGT GAG CAA G ⇒GAG ACG TTG CGG CTC AGT GAG CAA G, M2; AAG TCA AGG AGA AGG TCA ⇒

AAG TCA AGG AGA ATT TCA, and M3; ⇒AAG TCA TTT CTC AGG TCA. The pCMV5-Cα plasmid expressing the catalytic subunit of PKA, was a gift from Dr G.S. McKnight (Seattle, WA). The pSG5-HA-RIP140 and pCMV5-TIF2 plasmids were provided by Dr E. Treuter (Stockholm, Sweden) (Treuter et al., 1998), and the pSG5-HA-GRIP1 was provided by Dr M.R. Stallcup (Los Angeles, CA). The expression plasmid pCMX-p/CIP was supplied by Dr S. Westin (San Diego, CA). The β-galactosidase expression vector (pCMV-β-gal) is obtained from Stratagene. pHybLex/Zeo-SF-1 contains the LBD and hinge region of SF-1 and was used for interaction studies. pHybLex/Zeo-SF-1 (amino acid (aa) 107–461), pHybLex/Zeo-SF-1ΔAF-2 (aa 107–436), pHybLex/Zeo-Lamin (negative control plasmid) and pYESTrp-SRC-1 are described previously (Børud et al., 2002). The pYESTrp-RIP140 and the RIP140 deletion mutants were constructed by insertion of PCR-amplified DNA fragments encoding full length or fragments of RIP140 into the *Eco*R I/*Xho* I-linearized pYESTrp. The following fragments of RIP140 were inserted into linearized pYESTrp: aa1–1129, aa1–491, aa494–948, and aa431–1158.

2.2. Yeast two-hybrid interaction and β-galactosidase assay

A 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 RIP140 and SRC-1. The *Lex*A-fusion proteins were introduced into the EGY191 yeast strain (MATα *ura3 trp1 his3::*2*lex*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. The yeast strain EGY191 was cotransformed with the 2*lex*Aop-*lacZ* reporter plasmid pJK103. EGY191 and pJK103 was a gift from Dr E. Golemis (Philadelphia, PA). The chemiluminescent reporter gene assay system (Galacto-*Star*™ from Tropix, Bedford, MA) was used to measure the β-galactosidase activity in yeast cell extracts from at least four independent mating colonies. The yeast cultures 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 the growth (OD_{600}), and both these yeast methods were according to the formula provided in the Clonetech Yeast Protocol Handbook.

2.3. 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 (Lifetech), and the recombinant plasmids were transformed into DH10Bac competent cells that carry a baculovirus shuttle vector (bacmid) and a helper plasmid (Børud et al., 2002). Site-specific transposition of the expression cassettes moves the sequences that express SF-1 from the donor plasmids into the bacmids. The recombinant baculoviruses that 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-nitrilotriacetic (NTA) agarose (Qiagen). [^{35}S]methionine-labeled RIP140 fragments were prepared by using the TNT reticulocyte lysate system (Promega) in the presence [^{35}S]methionine.

In the protein interaction assay, 3 μl of the [^{35}S]methionine-labeled in vitro translated RIP140 fragments 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. The protein mixtures 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 PMSF and 5 μg/ml aprotinin to a total volume of 300 μl for 1 h. After the incubation period, the Ni-NTA agarose beads were washed. The Ni-NTA agarose beads containing the proteins of interest 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 the 6% SDS-polyacrylamide gel electrophoresis and autoradiography. Quantitation of the RIP140 fragments bound to SF-1 were performed on the autoradiographic films after having been digitized by scanning using the NIH Image 1.62 program.

2.4. Cell culture and transient transfection experiments

Y1 mouse adrenocortical tumor cells, COS-1 monkey kidney cells and MCF-7 human breast cancer cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units penicillin

per ml and 100 μg streptomycin per ml. The day before transfection, the Y1 cells were seeded at a density of 2.5×10^5 cells per well of a 6-well plate and transiently transfected the following day by using the calcium phosphate method (Lund et al., 1990). COS-1 and MCF-7 cells were seeded at a density of 85 000 cells per well of a 12-well plate, and transiently transfected with different reporter- and expression plasmids the following day by the SuperFect transfection procedure according to the manufacturer protocol (QIAGEN). The total amount of plasmid was kept constant by compensating with the respective empty vectors. The cells were washed once with PBS 24 h (Y1) or 48 h (COS-1 and MCF-7) after transfection, and assayed for luciferase activity. Thirty-five μl of the cell extracts were used for luciferase determination on a LUCY-3 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 triplicates and repeated three to five times. To ensure that the transfection experiments carried out at different times were reproducible and not subjected to nonspecific toxicity, selected cell cultures were cotransfected with pCMV-β-GAL and assayed for β-galactosidase activity.

2.5. Isolation of mRNA and real time RT-PCR

COS-1 and Y1 cells were cultured as described above. 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). 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 RIP140 sequence were as follows: F, 5′-CAT-GCT-GCA-CCT-CAA-AAA-3′; R, 5′-TGC-TAT-CCT-GTT-TGC-CTT-3′. The predicted size of the RIP140 fragment was 144 base pairs. The primers amplified both the human and the mouse RIP140 cDNA segments. Primers designed from the GRIP1 (the mouse TIF2 homologue) sequence were as follows: F, 5′-TAT-GGG-CAG-GTT-TGG-TGG-TT-3′; R, 5′-GCT-GCT-TTG-CGA-GGG-ACT-GT-5′. The predicted size of the GRIP1 PCR product was 93 base pairs. Forward and reverse TIF2 (the human GRIP1 homologue) primers were: F, 5-GCC-GAT-TTC-TCT-TGG-ATT-TG-3′; R; 5′-TGG-AGG-GGT-CAG-AGG-TAT-TT-3′. The predicted size of the TIF2 PCR product was 98 base pairs. 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 base pairs. 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. RIP140, TIF2, GRIP1 and GAPDH standards were prepared by 10-fold serial dilutions of linearized pSG5-HA-RIP140, pCMV5-TIF2, pSG5-HA-GRIP1 plasmids and DNA fragments containing the full length GAPDH cDNA sequence, respectively. Standards were used over the range of 100–0.01 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 s denaturation step, and then 40 cycles consisting of denaturation at 95 °C for 5 s, annealing at 58 °C (RIP140, TIF2, GRIP1) or 60 °C (GAPDH) for 5 s, and an extension phase at 72 °C for 15 s (RIP140, TIF2, GRIP1) or 20 s (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.

3. Results

3.1. SF-1 interacts with several NR box-containing RIP140 domains

To examine the interaction between SF-1 and RIP140, a yeast two-hybrid system with the EGY191 yeast strain and the reporter plasmid pJK103 was employed. The B42 transcriptional activation domain was fused to RIP140, whereas the *Lex*A DNA binding domain was fused to the LBD and hinge region of SF-1 (aa 107–461) (*LexA*/SF-1) and to SF-1 containing an activation function-2 domain (AF-2) deletion (aa 107–436) (*Lex*A/SF-1ΔAF-2). Liquid semi-quantitative β-galactosidase assays were performed to measure the two-hybrid interaction in mating colonies. Mating of the yeast strains expressing B42/RIP140 (aa 1–1129) and the *Lex*A/SF-1-fusion proteins resulted in a significant increase of the reporter gene activity (Fig. 2). SRC-1 was used as a positive control in the two-hybrid assay since it is known to interact with SF-1 (Crawford et al., 1997). The SF-1 AF-2 domain in the carboxyl-terminal region is essential for the interactions with several coactivators such as SRC-1 (Crawford et al., 1997; Ito et al., 1998), TIF2 (Hammer et al., 1999), and p/CIP (Børud et al., 2002). RIP140 has also been shown to depend on the AF-2 domain for interaction with other nuclear receptors (Lee et al., 1998; Schaufele, 1999; Treuter et al., 1998). As expected, no interaction was observed between *Lex*A/SF-1ΔAF-2 (aa 107–436) and B42/RIP140, indicating that a functional AF-2 domain is required for SF-1 to interact with RIP140.

Similar to other coregulators, RIP140 contains several short leucine rich (LXXLL) helices referred to as NR-boxes (Heery et al., 1997). The LXXLL motifs are responsible for binding of several other coregulators to nuclear receptors (Torchia et al., 1997), and RIP140 contains as many as nine such motifs (Heery et al., 1997). However, the role of these LXXLL motifs in the interaction between RIP140 and different nuclear receptors has been debated (Treuter et al., 1998; Lee and Wei, 1999; Kumar et al., 1999), and different nuclear receptors seem to have different requirements for specific LXXLL motifs (Kodera et al., 2000). In order to map the RIP140 domains of importance for the SF-1 interaction, deletion mutants of RIP140 were made (Fig. 1) and assessed for binding to SF-1. The RIP140 DNA fragments were inserted into linearized pYESTrp2 and subjected to yeast two-hybrid interaction β-galactosidase assays. RIP140 (aa 1–1129), which contains all of the nine LXXLL motifs, showed a 13-fold increase in reporter gene activity as compared with the *Lex*A/SF-1 fusion protein alone (Fig. 2). The amino-terminal RIP140 fragment (aa 1–491) containing five LXXLL motifs showed a weak, but detectable, interaction with SF-1 (2.5-fold), whereas the carboxyl-terminal fragment of RIP140 (aa 431–1158) that contains four LXXLL motifs interacted strongly with SF-1 (10-fold). The middle fragment of RIP140 (aa 494–948) also possesses the four carboxyl-terminal LXXLL motifs, and showed a strong SF-1 interaction (7-fold). Of note, all RIP140 fragments that were examined had the capability to interact with SF-1.

To confirm the direct interaction between SF-1 and RIP140 observed with the yeast two-hybrid system, we employed a protein–protein interaction assay based on baculovirus-expressed $(His)_6$-SF-1 fusion proteins linked to Ni-NTA agarose. RIP140 and its fragments were radiolabeled with [^{35}S]methionine during in vitro translation. As shown in Fig. 3, the fragments of RIP140 (aa 1–1129, aa 1–491, aa 494–948) all bound to the $(His)_6$-SF-1 fusion protein linked to Ni-NTA agarose, whereas

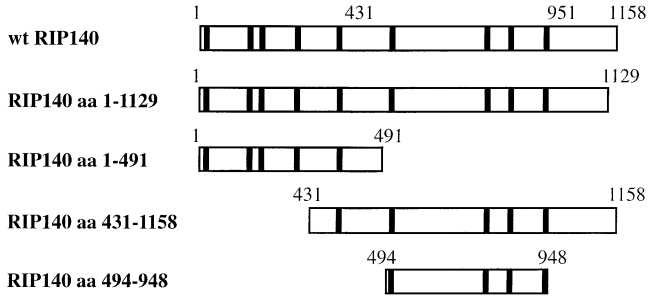

Fig. 1. A schematic representation of the protein fragments of RIP140 used in the present study. Numbers refer to amino acid positions. Black bars indicate the short leucine rich (LXXLL) helices referred to as NR-boxes. The depicted constructs were used for interaction assays.

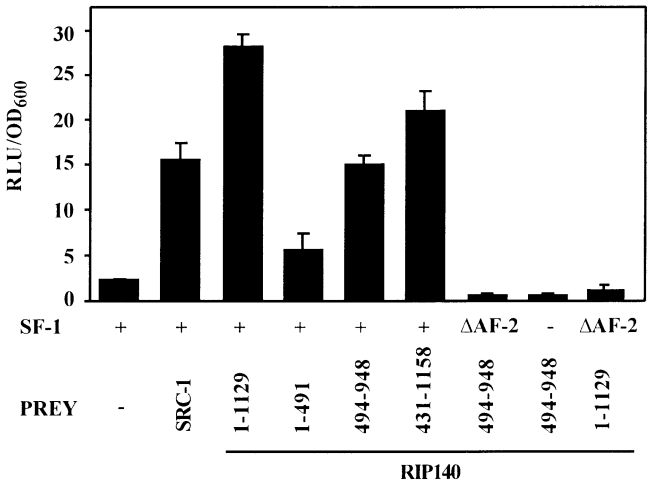

Fig. 2. Interactions between SF-1 and RIP140 fragments in the yeast two-hybrid system. A liquid, semi-quantitative β-galactosidase assay of the two-hybrid interaction between bait (*Lex*A/SF-1, *Lex*A/SF-1ΔAF-2 or *Lex*A/−) and prey proteins (B42/RIP140 fragments as indicated in the figure, B42/SRC-1 or B42/−) in yeast. The different combinations of bait and prey are 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). 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}). Results are expressed as the mean value ± S.D. of at least four independent colonies.

no binding (of RIP140 aa 1–1129) to the $(His)_6$-SF-1ΔAF-2 (AF-2 core deletion) fusion protein or to the Ni-NTA agarose alone was detected. The binding of RIP140 was specific to SF-1 because Ni-NTA alone did

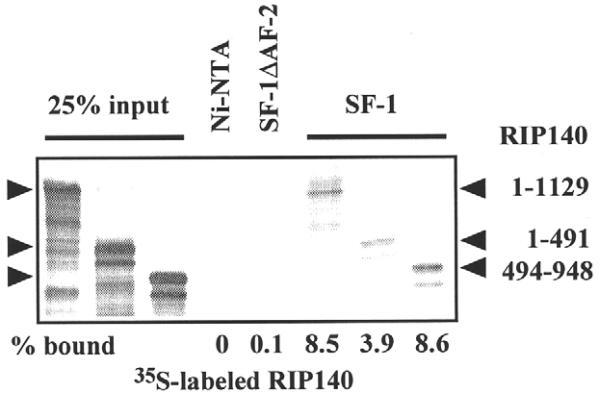

Fig. 3. In vitro interaction between SF-1 and RIP140 fragments. [^{35}S]methionine-labeled in vitro translated RIP140 fragments (aa 1–1129, aa 1–491 and aa 494–948) (3 μl) were incubated with $(His)_6$-SF-1 (5 μg). The RIP140 aa 1–1129 fragment was also incubated with $(His)_6$-SF-1ΔAF-2 (5 μg), which contains the AF-2 core (LLIEML) deletion mutation of SF-1. The protein mixtures or the [^{35}S]methionine-labeled RIP140 1–1129 fragment (3 μl) alone were then incubated with Ni-NTA agarose. After extensive washing, the proteins were analyzed by SDS-PAGE (6% polyacrylamide). 25% of the input radiolabeled RIP140 fragments are shown on the left. The level of interaction is subjected to quantitation (% bound) as indicated.

not retain any labeled protein under our assay conditions. SF-1 also interacted with full length RIP140 and with the RIP140 aa 431–1158 fragment in the protein–protein binding assay (data not shown). Consistent with the yeast two-hybrid data presented above, we detected a significantly reduced SF-1 binding to the amino-terminal fragment of RIP140 (aa 1–491) as compared with the interaction between SF-1 and the other RIP140 fragments examined. Taken together, these results clearly indicate that SF-1 interacts directly with RIP140. The different LXXLL containing domains of RIP140 showed variable affinity for SF-1, and the results of the yeast two-hybrid assay and the in vitro interaction assay suggest that the carboxyl-terminal region of RIP140 interacts more strongly with SF-1 than the RIP140 amino-terminal region.

3.2. RIP140 inhibits SF-1-induced transcriptional activation in transfected COS-1 and MCF-7 cells

The transcriptional activity of SF-1 was examined using a reporter plasmid, pT81-4CRS2-luc, which contains a minimal TK promoter and four SF-1 response elements (CRS2) from the bovine CYP17 gene placed upstream of the luciferase gene (Bakke and Lund, 1995). COS-1 cells lacking endogenous SF-1 were transiently transfected with expression plasmids encoding SF-1 and RIP140. Since SF-1 is reported to be a strong cAMP inducible activator of cAMP regulatory sequence 2 (CRS2)-dependent transcription (Bakke and Lund, 1995), cells were also cotransfected with a plasmid encoding the catalytic subunit of cAMP-dependent protein kinase or protein kinase A (PKA-Cα). The introduction of various amounts of RIP140 plasmid markedly inhibited the CRS2-dependent transcription in COS-1 cells transfected with an expression vector encoding SF-1 both in the presence and absence of PKA-Cα overexpression (Fig. 4A). The repression by RIP140 was dependent on the concentration of transfected RIP140 expression plasmid. RIP140 inhibited the CRS2-reporter gene activity by approximately 90%, and we observed no effects of RIP140 on the reporter gene activity in the absence of coexpressed SF-1 (data not shown). Previous reports by others have suggested that RIP140 competes with nuclear receptor coactivators for

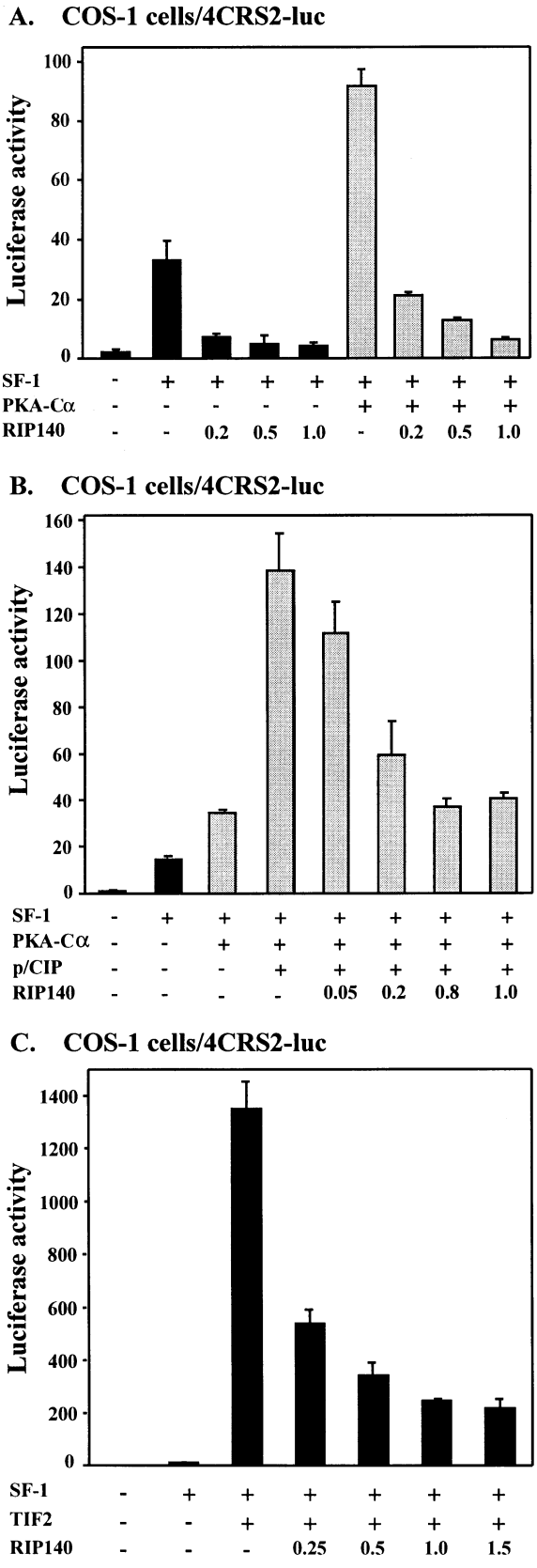

Fig. 4

Fig. 4. RIP140 inhibits SF-1-dependent transcription and antagonizes the coactivators p/CIP and TIF2 in transfected COS-1 cells. A, COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg), RIP140 (0.05-1.5 μg) and PKA-Cα (0.1 μg) along with the 4CRS2-luc reporter construct (1.5 μg) as indicated in the figures. The effects of RIP140 and two p160/SRC members were examined after coexpression of B, p/CIP (1.0 μg) and C, TIF2 (1.0 μg). For all experiments luciferase assays were performed 48 h after transfection. The figure shows the mean value ± S.D. of triplicate transfections from representative experiments.

the interaction with nuclear receptors (Treuter et al., 1998). Since SF-1 interacts directly with the p160/SRC coactivators p/CIP (Børud et al., 2002) and TIF2 (Hammer et al., 1999; Børud et al., 2002), we asked whether RIP140 could antagonize the p/CIP and TIF2 coactivation of a SF-1 responsive reporter gene. COS-1 cells were cotransfected with plasmids encoding the coactivators p/CIP or TIF2 together with increasing amounts of the expression plasmid encoding RIP140. As can be seen from Fig. 4B and C, RIP140 partially suppressed the stimulation of SF-1 reporter activity by the coactivators p/CIP and TIF2, possibly through competition for binding to the AF-2 domain of SF-1. To confirm the above results in another cell environment that lack the endogenous expression of SF-1, breast cancer MCF-7 cells were transfected with expression plasmids encoding SF-1 and RIP140. Consistent with the findings described above, introduction of RIP140 into MCF-7 cells resulted in a dose-dependent decrease in SF-1 stimulated CRS2-reporter gene activity and RIP140 also antagonized the positive effect of coexpressed TIF2 (Fig. 5).

3.3. The inhibitory effect of RIP140 was partially reversed by trichostatin A (TSA)

The negative effect of RIP140 on nuclear receptor actions has been linked to a direct interaction with HDACs (Wei et al., 2000). To examine the role of HDACs in RIP140 mediated repression of SF-1-dependent transcription, COS-1 cells were cotransfected with the CRS2-reporter gene and expression vectors encoding SF-1 and RIP140 followed by treatment with the HDAC inhibitor Trichostatin A (TSA). The presence of TSA stimulated the SF-1-dependent transcription alone (Fig. 6). However, we also detected a partial release of the RIP140 corepressor activity. In the presence of TSA, RIP140-mediated repression of CRS2-reporter gene activity was reduced by approximately 50%, suggesting a role of HDAC activity in RIP140-mediated corepression of SF-1 dependent transcription.

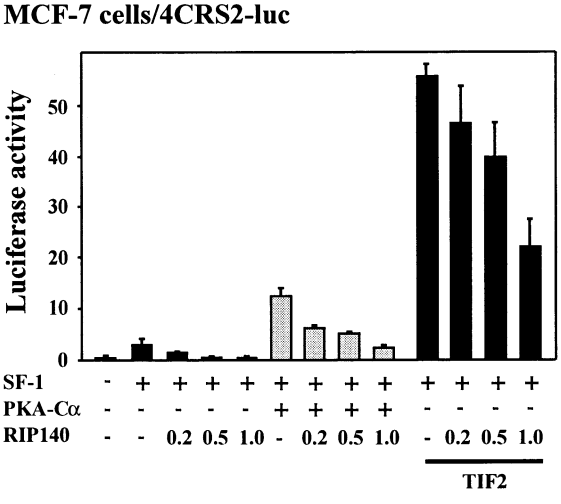

Fig. 5. RIP140 inhibits SF-1-dependent transcription in transfected MCF-7 cells. MCF-7 cells were transfected with expression vectors encoding SF-1 (0.1 μg), RIP140 (0.2–1.0 μg), PKA-Cα (0.1 μg) and TIF2 (1.0 μg) along with the 4CRS2-luc reporter construct (1.5 μg) as indicated in the figure. Luciferase assays were performed 48 h after transfection. The figure shows the mean value±S.D. of triplicate transfections from a representative experiment.

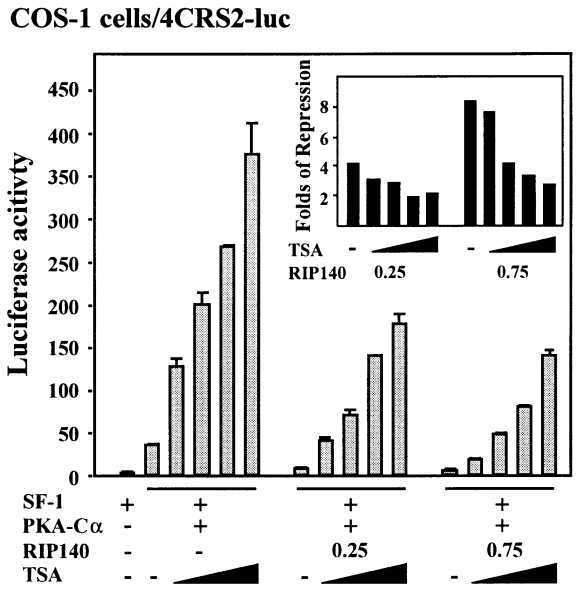

Fig. 6. RIP140-mediated repression of SF-1 was partially reversed by trichostatin A. COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg), RIP140 (0.25 or 0.75 μg), PKA-Cα (0.1 μg) and the 4CRS2-luc reporter construct (1.5 μg), followed by treatment with different concentrations of trichostatin A (TSA) (25, 50, 100, and 250 nM) 24 h after transfection. Results are expressed as the mean value±S.D. of triplicate transfections from a representative experiment. The inset shows the effect of different concentrations of TSA on the transrepressive activity of RIP140.

3.4. Modulation of SF-1-dependent transcription by RIP140 in adrenocortical Y1 cells

In order to characterize the role of RIP140 in regulation of SF-1 transcriptional activation in steroidogenic cells, an adrenocortical mouse cell line (Y1) was employed. These cells express SF-1 in sufficient amounts to activate the reporter plasmid 4CRS2-luc, and activation of PKA further stimulates the reporter gene activity (Bakke and Lund, 1995; Æsøy et al., 2002). Surprisingly, coexpression of RIP140 in Y1 cells did not significantly repress the CRS2 reporter gene activity (Fig. 7A). It is possible that this was caused by a lower availability of endogenous SF-1 as compared with the COS-1 and MCF-7 cells after introduction of exogenous SF-1. Therefore, we examined the function of RIP140 in Y1 cells after cotransfection with the SF-1 expression plasmid. However, the inability of RIP140 to act as a

repressor of CRS2-reporter gene activity in Y1 cells was not changed by the introduction of exogenous SF-1 (data not shown). Thus, it appeared that the Y1 cells differed from the other two cell lines examined with respect to the effect of RIP140 on SF-1 activity. To examine this further, RIP140 was coexpressed with the p160/SRC members p/CIP and TIF2. Similar to the COS- and MCF-7 cells, we observed that RIP140 antagonized coactivator stimulated SF-1 transactivation in Y1 cells. p/CIP-mediated coactivation of SF-1 activity was reduced by approximately 25% after the introduction of RIP140 (Fig. 7B), whereas the TIF2-enhanced SF-1 transactivation was reduced to 50% in Y1 cells transfected with RIP140 expression plasmid (Fig. 7C). Recently, we have reported that PKA inhibits TIF2 coactivator function through selective downregulation of TIF2 protein (Børud et al., 2002). As expected, PKA repressed TIF2-mediated coactivation of SF-1, but we did not observe any effects of the cAMP-signaling pathway on the RIP140-mediated modulation of CRS2-reporter gene activity (Fig. 7C). Taken together, it appears that RIP140 has the capability to inhibit p160/SRC-induced stimulation of the CRS2-reporter gene activity in Y1 cells. To assess further the role of RIP140 in regulation of SF-1-dependent transcription in Y1 cells, a SF-1 responsive reporter gene from the bovine CYP17 promoter was employed. The CYP17-luc reporter plasmid has one SF-1 binding site and contains the −290 CYP17 promoter region of the bovine CYP17 gene that encodes the cytochrome P450c17 (Bakke and Lund, 1995). Since RIP140 has previously been reported to inhibit the human StAR promoter activity in presence of SF-1 (Sugawara et al., 2001), we also examined the effects of RIP140 on a 1.3 kb StAR-luc reporter gene. As shown in Fig. 7D, overexpression of RIP140 in Y1 cells significantly repressed both the CYP17- and the StAR-reporter gene activities. An inhibitory action of RIP140 was also observed in presence of PKA Cα overexpression (data not shown). These results indicate that RIP140 inhibits the activity of two different natural SF-1 responsive promoters expressed in Y1 cells.

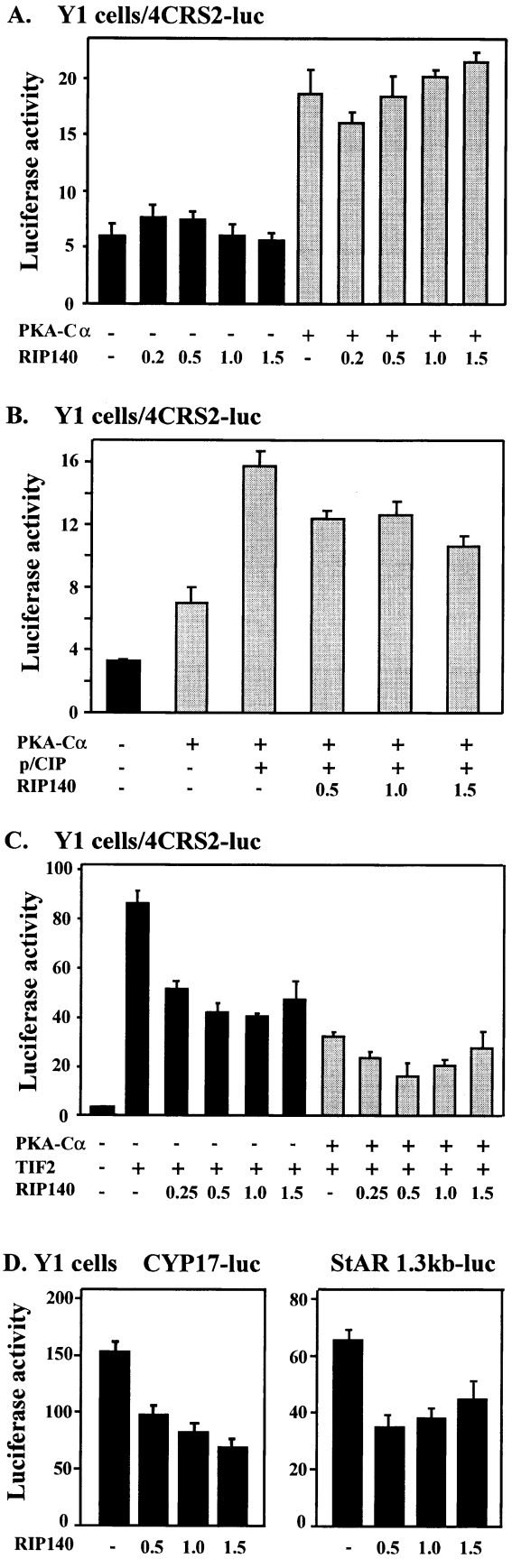

Fig. 7. Effects of RIP140 on SF-1-dependent transcription in Y1 cells. A, Y1 adrenocortical cells were transfected with expression vectors encoding RIP140 (0.2–1.5 μg) and PKA-Cα (0.1 μg) along with the 4CRS2-luc reporter construct (1.5 μg) as indicated in the figures. The effects of RIP140 and two p160/SRC members were examined after coexpression of B, p/CIP (1.0 μg) and C, TIF2 (1.0 μg) as indicated in the figure. D, Y1 cells were transfected with vectors encoding RIP140 (0.5–1.5 μg) and along with the CYP-17-(1.5 μg) or the pGL_2 1.3-kb StAR (1.5 μg) reporter constructs. For all experiments luciferase assays were performed 24 h after transfection. The luciferase activities (mean ± S.D.) are based on triplicate transfections from representative experiments.

Fig. 7

3.5. Role of RIP140 in regulation of SF-1-responsive promoters

The observation that RIP140 acted somewhat differently as a coregulator of SF-1 dependent transcriptional activation in various cell types prompted us to examine the function of RIP140 in regulation of other SF-1 responsive target genes. SF-1 regulates the transcription of steroidogenic P450 genes such as the CYP11A gene encoding cytochrome P450scc (Clemens et al., 1994), the CYP11B gene that encodes the cytochrome P450c11β (Morohashi et al., 1993), and the CYP17 gene (Bakke and Lund, 1995). Transiently transfected COS-1 cells were used to test whether RIP140 modulated SF-1 stimulated transcription from the CYP17-luc reporter plasmid (Bakke and Lund, 1995), a reporter plasmid containing the region between −186 and +12 of the bovine CYP11A gene containing one SF-1 binding site (Ahlgren et al., 1999), and a CYP11B-luc reporter plasmid carrying the 1.5-kb long up-stream promoter region of the bovine CYP11B gene placed ahead of a luciferase reporter gene (Morohashi et al., 1993). In addition, a SF-1 responsive reporter construct that contains the region between −380 and +6 of the murine MIS promoter was tested. MIS is known as a key factor in male endocrine development and the promoter region of the MIS gene contains two SF-1 binding sites (Watanabe et al., 2000). As shown in Fig. 8, SF-1 enhanced luciferase expression from the four reporter plasmids mentioned above. With the exception of the CYP11A-luc reporter construct, coexpression of RIP140 markedly reduced the SF-1 stimulated reporter gene activities. This indicates that RIP140 does not only modulate the activity of an artificial reporter gene construct such as pT81-4CRS2-luc that contains four repetitive SF-1 binding sites, but also SF-1 responsive reporter genes containing endogenous promoter sequences. No inhibitory effects of RIP140 were detected in the transfected COS-1 cells in absence of coexpressed SF-1, suggesting that the RIP140-mediated repression of reporter gene activity is dependent on the presence of SF-1 (Fig. 9A).

Transcription of the bovine CYP17 gene is regulated by ACTH via two cAMP regulatory sequences (CRS1 and CRS2) that bind to the homeodomain proteins Pbx1, Meis1 and Pknox (Bischof et al., 1998), and the orphan nuclear receptors chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1) and SF-1 (Bakke and Lund, 1995), respectively. To study the potential role of the CRS elements in RIP140 mediated regulation of the CYP17 promoter, we examined the action of RIP140 on SF-1 transcriptional activation of CYP17-luc reporter constructs containing mutations in the Pbx 1/Meis 1 binding element CRS1 and SF-1/COUP-TF1 binding element CRS2 (Bischof et al., 1998). Mutagenesis of the CRS1 element eliminating

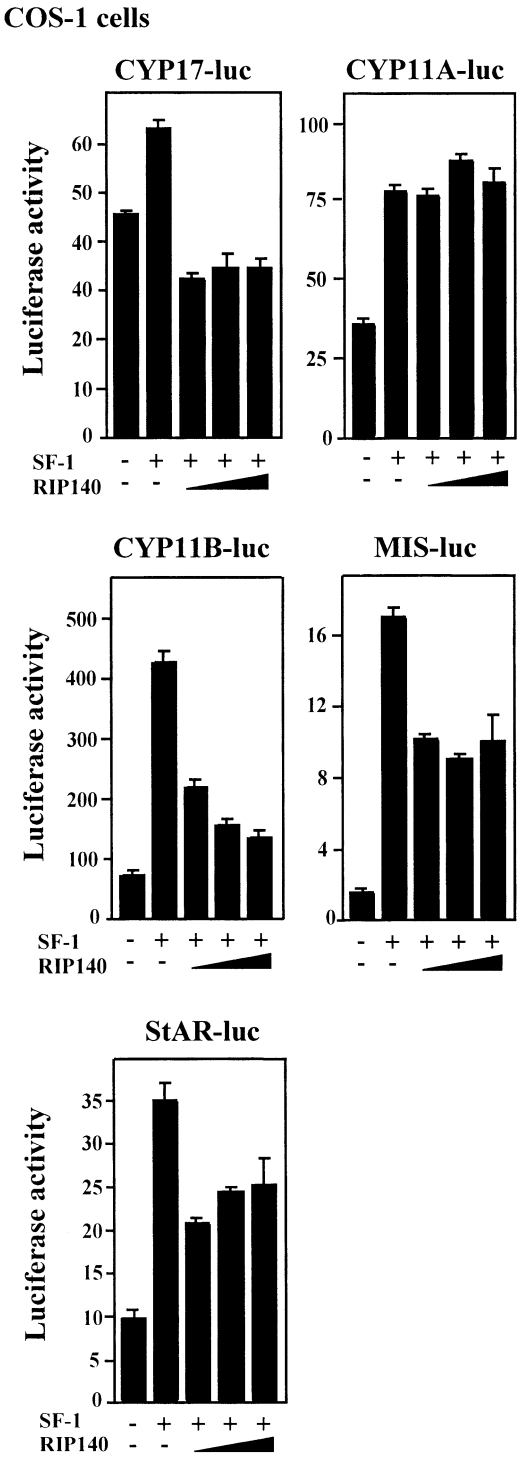

Fig. 8. RIP140 repression of different SF-1 regulated promoters. COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg) and RIP140 (0.2–1.0 μg) along with 1.5 μg of the CYP17-luc reporter construct, CYP11A-luc reporter construct, CYP11B-luc reporter construct, MIS-luc reporter construct (1.5 μg) or 1.3-kb StAR reporter construct (1.5 μg) as indicated in the figure. For all experiments luciferase assays were performed 48 h after transfection. The figure shows the mean value ± S.D. of triplicate transfections from representative experiments.

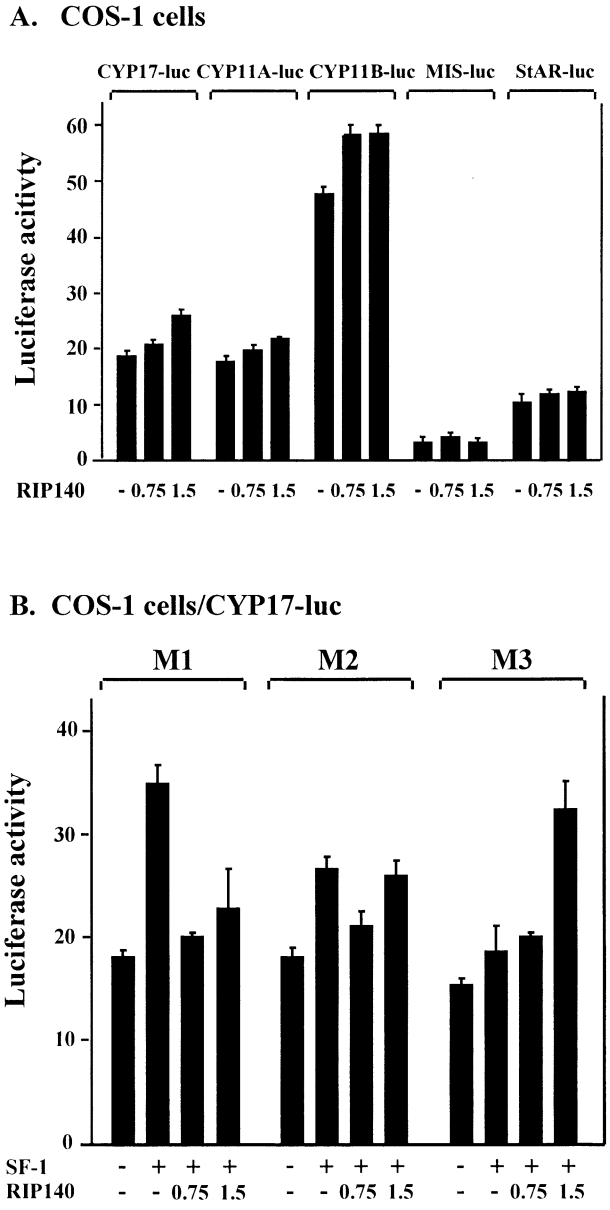

Fig. 9. RIP140 repression of SF-1 responsive target genes is SF-1 dependent. A, COS-1 cells were transfected with expression vectors encoding RIP140 (0.75–1.5 μg) and the CYP17-luc-, CYP11A-luc-, CYP11B-luc-, and MIS-luc reporter constructs (1.5 μg) as indicated in the figure, and, B, expression vectors encoding SF-1 (0.1 μg) and RIP140 (0.5–1.0 μg) along with the CYP17-luc reporter construct (1.5 μg) containing mutations in the cAMP regulatory sequence 1 (CRS1): GAGACGTTGATGGACAGTGAGCAAG⇒M1; GAGACGTTG **CG**G**CT**CAGTGAGCAAG, and in the cAMP regulatory sequence 2 (CRS2): AAGTCAAGGAGAAGGTCA⇒M2; AAGTCAAGGAGAA**TT**TCA, and ⇒M3; AAGTCA**TTTCTC**AGGTCA. Luciferase assays were performed 48 h after transfection. The figure shows the mean value±S.D. of triplicate transfections from representative experiments.

the binding of Pbx1/Meis1 (M1) (Bischof et al., 1998) did not affect the inhibitory action of RIP140 on SF-1 transcriptional activation (Fig. 9B). However, introduction of mutations in the CRS2 element that decreased the binding of SF-1 and disrupted the binding of COUP-TF (M2) reduced the inhibitory effect of RIP140, and mutations disrupting the binding of SF-1 but not COUP-TF (M3) (Bakke and Lund, 1995) abolished the RIP140-mediated repression of the CYP17 reporter gene activity. These data confirm the above results, and indicates that RIP140 repression of the CYP17 reporter gene was dependent on an intact CRS2 element that is able to bind to SF-1.

3.6. Expression levels of RIP140 and TIF2/GRIP1 in Y1 and COS-1 cells

Based on the observed differences in repression of CRS2-reporter gene activity after introduction of exogenous RIP140 in various cell types, we hypothesized a difference in the expression levels of a coactivator or of RIP140 itself in Y1- and COS-1 cells. Therefore, mRNA was isolated from cultured cells and quantitative RT-PCR was carried out using a Lightcycler rapid thermal cycler system. The mRNA levels of RIP140, TIF2 (COS-1 cells) and GRIP1 (Y1 cells) were measured using a standard curve generated from linearized plasmids (pSG5-HA-RIP140, pCMV5-TIF2 and pSG5-HA-GRIP1,) containing the full-length RIP140 or TIF2/GRIP1 cDNA, respectively. The values were normalized against the content of GAPDH mRNA in the same extracts. Melting point analyses of the PCR products demonstrated single peaks and specific products for GAPDH, RIP140 (Fig. 10A) and TIF2/GRIP1 (data not shown). As shown in Fig. 10B, our results suggest that Y1 cells express higher levels of both RIP140 and TIF2/GRIP1 than COS-1 cells. These differences in endogenous expression of coregulators may explain the distinctions in repression of the CRS2-reporter gene activity in Y1- and COS-1 cells after introduction of exogenous RIP140.

4. Discussion

In this study, we have examined the role of RIP140 in regulation of the SF-1-dependent transactivation. RIP140 is one of the most enigmatic nuclear receptor coregulators. Since its discovery as a coactivator of estrogen receptor in breast cancer cells (Cavailles et al., 1995), it has been reported that RIP140 represses the action of several nuclear receptors including the thyroid, estrogen, glucocorticoid, retinoic acid and retinoid X receptors (L'Horset et al., 1996; Cavailles et al., 1995; Subramaniam et al., 1999; Lee and Wei, 1999; Treuter et al., 1998), as well as the orphan nuclear receptor TR2 (Lee and Wei, 1999). Recently, it has also been demonstrated that RIP140 interacts with SF-1 and DAX-1, and an inhibitory effect of RIP140 in the regulation of the human steroidogenic acute regulatory protein pro-

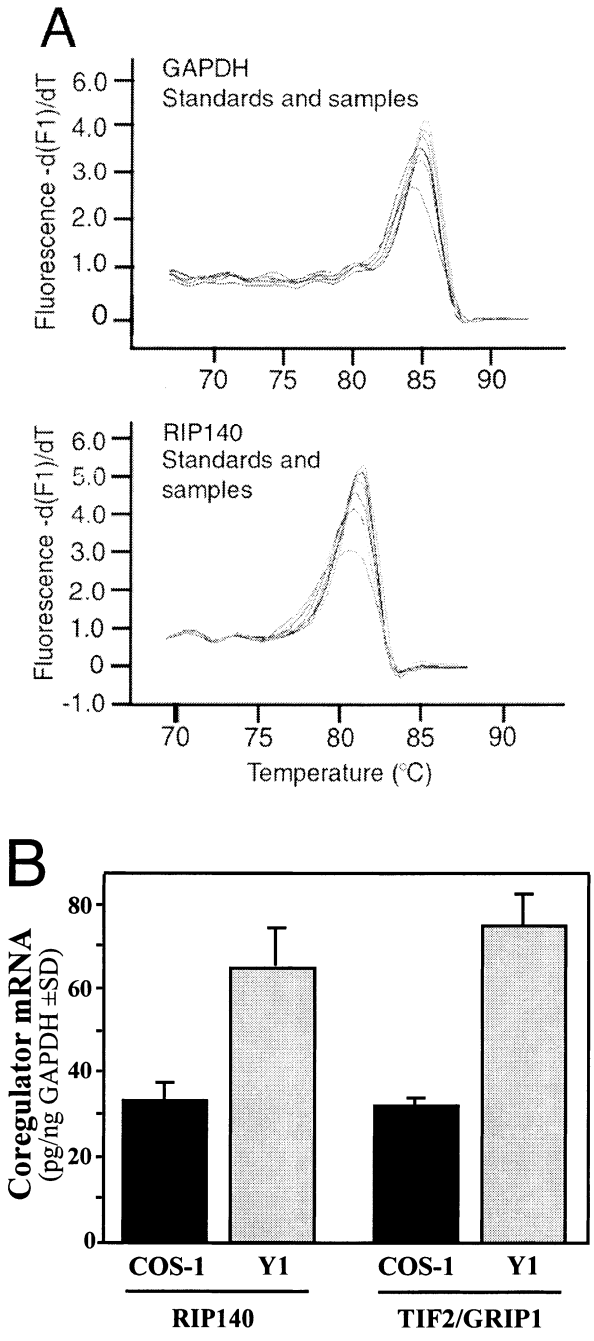

Fig. 10. RIP140 and TIF2/GRIP1 mRNA levels in COS-1 and Y1. Real time RT-PCR using SYBR Green I fluorescence was used to measure product accumulation. A, Melting point analyses demonstrated single peaks and specific products for GAPDH (upper panel) and RIP140 (lower panel). Each of the primer pairs amplified a single product of the appropriate predicted length. B, Coregulator mRNA expression levels. All samples were subjected to PCR amplification with primers specific for the constitutively expressed gene glyceraledehyde-3-phosphate dehydrogenase (GAPDH) and normalized. Coregulator mRNA levels are expressed as picograms of mRNA per nanogram of GAPDH mRNA±S.D. The figure shows the mean value±S.D. of at least three separate experiments.

moter was shown in adrenocortical Y1 cells (Sugawara et al., 2001). However, the mechanisms by which RIP140 modulate nuclear receptor action have been debated, and different hypotheses have been presented to explain the molecular action of RIP140 as a negative modulator of nuclear receptor regulated gene expression (Treuter et al., 1998; Wei et al., 2001; Vo et al., 2001).

In the present study, we have analyzed the role of RIP140 in regulation of SF-1-dependent transcription in more detail. We demonstrate that RIP140 can repress SF-1 action by antagonizing the stimulatory effects of true coactivators such as p/CIP and TIF2. This is consistent with previous reports showing competitive binding of RIP140 and SRC-1 to PPARγ (Treuter et al., 1998) and of RIP140 and TIF2 to GR (Subramaniam et al., 1999). However, we also demonstrate that it is possible to partially inhibit RIP140-mediated repression of SF-1 through inhibition of HDACs with TSA. RIP140 has been reported to interact directly with the class I HDACs (Wei et al., 2000, 2001). Thus, the repressive function of RIP140 in SF-1 transcriptional activation may involve several mechanisms. The CtBP is known as a corepressor associated with HDACs and other proteins involved in transcriptional silencing (Sewalt et al., 1999; Zhang et al., 2001). Recently, RIP140 has been shown to interact with CtBP through its amino-terminal portion, and acetylation of RIP140 by p300/CBP has been proposed as a regulatory mechanism for disruption of the RIP140-CtBP complex and subsequently derepression of nuclear receptor regulated genes (Vo et al., 2001). Whether CtBP is involved in RIP140 repression of SF-1 action remains, however, to be determined.

As expected, the AF-2 domain of SF-1 is required for the RIP140 interaction, as is the case with the p160/SRC family of coactivators (Børud et al., 2002; Crawford et al., 1997; Ito et al., 1998). Our findings indicate further that the carboxyl-terminal domain of RIP140 that contains 4 LXXLL motifs showed the strongest SF-1 interaction. This has also been reported to be the case for PPARγ (Treuter et al., 1998), whereas GR seemed to interact strongly with both the amino-terminal and the carboxyl-terminal fragments of RIP140 (Zilliacus et al., 2001). Both the number and the sequence of LXXLL core motifs as well as the flanking sequences have been proposed to influence on the affinity and selectivity of RIP140 for different nuclear receptors (Heery et al., 2001). Of note, all LXXLL containing RIP140 domains that we examined were found to interact with SF-1. In contrast, RAR/RXR utilizes the extreme carboxyl-terminal part of RIP140 (aa 1063–1076) that does not contain any of the nine LXXLL core motifs (Lee et al., 1998; Wei et al., 2001). Thus, it appears that the receptor interacting domains of RIP140 varies among the different receptor systems.

The ability of a transcription factor such as RIP140 to function as a repressor or activator depends on several factors, including the cell and promoter contexts, as well as the interacting nuclear receptor. In transfected COS-1 and MCF-7 cells RIP140 did not only inhibit SF-1-dependent transcription from the artificial reporter gene construct pT81-4CRS2-luc. We also report a significant RIP140 repression of SF-1-stimulated CYP17-, CYP11B- and MIS-reporter reporter gene activities. In adrenocortical Y1 cells RIP140 inhibited the CYP17- and StAR- reporter gene activities, but we did not detect a significant inhibitory effect on the 4xCRS2 reporter gene activity in absence of overexpressed coactivator. The higher levels of endogenous RIP140 in Y1 cells than in COS-1 cells may lead to a reduced effect of exogenously added RIP140. However, Y1 cells also contain higher levels of the SF-1 interacting coactivator TIF2/GRIP1 which is shown to counteract the RIP140 repression. To date, no obligatory SF-1 ligand has been identified (Mellon and Bair, 1998), and RIP140 appears to inhibit SF-1 in transfected COS-1 cells in the absence of a putative ligand. It is also possible, therefore, that the presence of a hitherto unidentified ligand in Y1 cells could modulate the RIP140 repressor function. Since it has been reported that the acetylation of RIP140 by p300/CBP leads to gene activation rather than repression, it would also be interesting to determine the acetylation state of RIP140 in different cell types including Y1 cells (Vo et al., 2001).

In conclusion, the current study demonstrates a suppressive role of RIP140 in the regulation of a number of SF-1 responsive target genes. RIP140 interacts directly with SF-1 preferentially through its carboxyl-terminal domain. However, our results also suggest that RIP140 coregulator function depend on other factors such as cell type, promoter context and the level of transcriptional coregulators.

Acknowledgements

This work was supported by grants from the Norwegian Research Council, the Norwegian Cancer Society and the Novo Nordisk Foundation. We thank K-I. Morohashi, E. Treuter, E. Golemis, S. Westin, M.G. Rosenfeld, M. Waterman, M.R. Stallcup and T. Sugawara for providing plasmids. Technical assistance from C. Cook, A.M. Sellevold, A-E. Larsen and Ingvild Fenne is highly appreciated.

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