Acetylation of Steroidogenic Factor 1 Protein Regulates Its Transcriptional Activity and Recruits the Coactivator GCN5*

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Steroidogenic factor-1 (SF-1), or Ad4BP, is a transcription factor belonging to the nuclear receptor superfamily, which is necessary for the expression of essentially all components of the steroidogenic pathway as well as for the development of the steroidogenic tissues (1–3). SF-1 is expressed in steroidogenic tissues such as adrenal glands, gonads, and placenta. It is also found in the pituitary and in the ventromedial nucleus of the hypothalamus (4). SF-1 is involved in the transcriptional regulation of a variety of genes such as the steroid hydroxylase genes, the luteinizing hormone receptor, the adrenocorticotropic hormone receptor, the prolactin receptor, and the steroidogenic acute regulatory protein (5–9). The SF-1 binding site in the genes, the luteinizing hormone receptor, the adrenocorticotropin receptor, the prolactin receptor, and the steroidogenic acute regulatory protein (5–9). The SF-1 binding site in the genes, the luteinizing hormone receptor, the adrenocorticotropin receptor, the prolactin receptor, and the steroidogenic acute regulatory protein (5–9).

SF-1 can be acetylated in vitro. Histone acetyltransferase GCN5 acetylates SF-1 in vitro. Moreover, we found that SF-1 recruited a novel coactivator GCN5, which can be a newly identified coactivator for SF-1. Acetylation of SF-1 stimulates its transcriptional activity. Inhibition of deacetylation by trichostatin A, a histone deacetylase inhibitor, increased SF-1-mediated transactivation and stabilized the nuclear export of the SF-1 protein.

Modulation of SF-1 activity is also mediated by protein-protein interactions. SF-1 interacts directly with the coactivators CBP (CREB binding protein) and steroid receptor coactivator 1, and transactivation was enhanced synergistically in the presence of both coactivators (23, 24). The precise mechanisms responsible for the stimulatory effect on the transcriptional activity of SF-1 by these coactivators are not yet known. Both structural and mutational analyses have indicated that the presence of LXXLL domains in the coactivators is important for the interactions with nuclear receptors and may be involved in the recruitment of still other proteins into the transcriptional complex (25, 26).

Nuclear receptor coactivators such as p300/CBP, GCN5 (general control nonderepressed), steroid receptor coactivator 1, p300/CBP-associated factor, and activator of thyroid and retinoic acid receptor exhibit HAT (histone acetyltransferase) activity. These HAT proteins acetylate nucleosomal histones, which further increase the accessibility of transcription factors to their DNA targets (27, 28). Acetylation of several transcription factors such as p53, GATA-binding protein 1, E2F1, erythropoietin Kruppel-like factor, and the androgen receptor have been demonstrated recently (29–33).

In this study we demonstrate that SF-1 can be acetylated in vivo. SF-1 interacts with a HAT coactivator GCN5, and GCN5 acetylates SF-1 in vitro. Acetylation by GCN5 enhanced SF-1-mediated transactivation. Lysines 34, 38, and 72 in the C-terminal domain of zinc finger motifs of SF-1 might be among the target lysines being acetylated. Inhibition of deacetylation by trichostatin A (TSA) regulates the SF-1 activity at multiple levels.

MATERIALS AND METHODS

Cell Culture and Transient Transfection—COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (low glucose), and Y-1 cells were maintained in DMEM (high glucose). COS-1 cells were transfected by the Superfectin (Qiagen) method according to standard procedures. For gene reporter assays, COS-1 cells (1.25 × 10⁵ cells/well in 105 cells/well in 35-mm dishes) were transfected with 10 ng of reporter plasmids and 100 ng of expression plasmids using Superfectin (Qiagen). After 24 h, cells were harvested and luciferase activity was measured using a Promega kit.

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12-well plates) and Y-1 cells (2.5 × 10⁶ cells/well in 12-well plates) were transfected. The calcium phosphate coprecipitation method was used to transfected Y-1 cells (34). Luciferase assays were performed as described previously (22).

Plasmid Constructions and Recombinant Proteins—Constructions of the reporter plasmid pT81-4CRS2LUC and the expression plasmid pCMV5-SF-1 are as described (35). A Myc-His-tagged SF-1 plasmid was generated by cloning a polymerase chain reaction-amplified fragment of SF-1 into pcDNA3.1/Myc-His A vector (Invitrogen). For mutagenesis the QuickChange™ site-directed mutagenesis kit (Stratagene) was used (22). Mutations were confirmed by sequencing. The SF-1 full-length cDNA was cloned into the pGEX-2TK vector (Amersham Pharmacia Biotech). The different GST constructs were expressed in the Escherichia coli strain BL21-codonplus-RIL. Bacterial cells were grown in 37 °C and lysed with 50 mm Tris, 2 mM EDTA buffer containing 2% Triton X-100, 0.5% Nonidet P-40, and 0.1% phenylmethylsulfonyl fluoride. The GST-SF-1 fusion protein was purified with glutathione beads (Amersham Pharmacia Biotech) from bacterial lysates according to the manufacturer’s instructions.

Immunoprecipitations and Western Blot Analysis—Transfected COS-1 cells were washed with PBS and lysed with EBC (20 mM Hepes, 120 mM NaCl, 0.5% Nonidet P-40) buffer. The lysate was centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was incubated with polyclonal antibodies against SF-1, and antibodies were captured by [1-14C]acetyl-CoA (59 mCi/mmol, Amersham Pharmacia Biotech) for 2 h at 37 °C. Escherichia coli used (22). Mutations were confirmed by sequencing. The SF-1 full-length cDNA was cloned into the pGEX-2TK vector (Amersham Pharmacia Biotech). The different GST constructs were expressed in the Escherichia coli strain BL21-codonplus-RIL. Bacterial cells were grown in 37 °C and lysed with 50 mm Tris, 2% EDTA buffer containing 2% Triton X-100, 0.5% Nonidet P-40, and 0.1% phenylmethylsulfonyl fluoride. The GST-SF-1 fusion protein was purified with glutathione beads (Amersham Pharmacia Biotech) from bacterial lysates according to the manufacturer’s instructions.

In Vivo Labeling of SF-1—15 μg of SF-1 expression plasmid or mutant plasmids was transfected into 1 × 10⁶ COS-1 cells/10-cm dish. After 24 h of transfection, the medium was replaced with 2 ml of DMEM containing 0.05 mCi/ml sodium [1-14C]acetate (39 mCi/mmol) (Sigma) and 2 μg/ml TSA/ml for 2 h. Extracts were immunoprecipitated with an anti-SF-1 polyclonal antibody. In control experiments, nontransfected cells were pulse labeled and immunoprecipitated using the same conditions. Immunoprecipitated samples were resolved on a 10% SDS-PAGE and exposed to autoradiography. Extracts from wild-type SF-1 (lane 1), RFQK mutant (lane 2), RGFFK mutant (lane 3), and the control cells (lane 4) are shown. Panel D, the expression of wild-type SF-1 protein (lane 1), mutant RFQK (lane 2), and mutant KGFFK (lane 3) in the radiolabeled extracts is shown. Whole cell extracts (20 μg) were used in each lane for 10% SDS-PAGE analyses. SF-1 protein expression was detected with an antibody against SF-1. The nontransfected cell lysates (lane 4) was used as a control.

RESULTS

SF-1 Is Acetylated in Vivo—The acetylation of lysine groups in transcription factors modulates their activity, both in vivo and in vitro (29–32). The presence of GK amino acids in a motif seems to be required for acetylation, as shown by mutational analyses done on the importin-α protein (36). Structural analyses of histone peptide acetylation by the HAT GCN5 also identified GXXXP as the recognition motif (37). In SF-1, the potential lysine motifs are identified C-terminal to the zinc finger domains, based on the previously reported consensus acetylation sequences (Fig. 1B). In this study the lysines of the RFQK and KGFFK motifs were mutated to alanine.

To determine SF-1 acetylation in vivo, COS-1 cells were transfected with an SF-1 expression plasmid or with mutant forms of SF-1. After 24 h of transfection, the cells were pulse labeled with 0.05 mCi/ml sodium [1-14C]acetate (39 mCi/mmol) for 2 h in the presence of histone deacetylase inhibitor TSA (2 μM). The cells were immediately lysed with EBC buffer and immunoprecipitated with an SF-1 antibody. As a control, nontransfected cells were pulse labeled and immunoprecipitated using the same conditions as above. The autoradiograph shown in Fig. 1C demonstrates the acetylation of the SF-1 and mutant proteins in COS-1 cells. No band is detected in the mock control. The presence of SF-1 and mutant proteins in the lysates is shown by Western blot (Fig. 1D).

Mutations of Lysine Residues Affect Transactivation of SF—RFQK and KGFFK mutants were characterized in COS-1 and mouse adrenocortical Y-1 cells. COS-1 cells were cotransfected with the reporter plasmid pT81-4CRS2LUC, and either wild- type SF-1 or mutant plasmids. The reporter plasmid contains the SF-1 binding site of the cytochrome P-450 17α-hydroxylase
Acetylation of SF-1. Panel A, COS-1 cells were transfected with SF-1 constructs. Wild-type or mutant SF-1 plasmid (0.5 μg) and pT81-4CRS2LUC reporter plasmid (0.5 μg) were used for transfection. The figure shows the luciferase activity measured. The mean values are from three independent experiments. Panel B, Y-1 cells were transfected with 1.5 μg of pT81-4CRS2LUC reporter plasmid alone and with 1.5 μg of SF-1 mutant plasmids. The figure shows the luciferase activity of the mean values of two independent experiments. Panel C, expression of wild-type SF-1 and mutant SF-1 protein in COS-1 cells was analyzed by 10% SDS-PAGE. 20 μg of whole cell extracts was used in each lane. SF-1 protein expression was detected with an antibody against SF-1. The expression of wild-type SF-1 protein (lane 1), mutant RFQK protein (lane 2), the mutant KGFFK protein (lane 3), and nontransfected cells (lane 4) is shown.

Gene (CYP17). The transactivation effect of wild-type SF-1 and its mutants in COS-1 cells is shown in Fig. 2A. Transactivation activity by the mutants in Y-1 cells, which express endogenous SF-1, is shown in Fig. 2B. Transfection of the plasmid with mutation in the RFQK domain, located C-terminal to the second zinc finger domain, positively increased transactivation in both cell types. Transfection of the plasmid with mutations in the KGFFK domain, which is C-terminal to the first zinc finger domain, showed reduced activity in both Y-1 and COS-1 cells. COS-1 cells transfected with either wild-type or mutant plasmids expressed equal amounts of the SF-1 protein (Fig. 2C).

In Vitro Acetylation of SF-1—The HAT GCN5, the first identified acetyltransferase (38–40), was used to study the in vitro acetylation of SF-1. Acetylation of transcription factors p53, E2F1, erythroid Kruppel-like factor by GCN5 family members has been reported previously (29, 32, 41). We used immunoprecipitated GCN5 protein from COS-1 cells to analyze the in vitro acetylation of a GST-SF-1 fusion protein. Fig. 2A shows that GCN5 acetylates the GST-SF-1 protein. A GST protein control was not acetylated by GCN5. Mutations in the RFQK or KGFFK motifs in the SF-1 protein show clear hypoacetylation (Fig. 3B).

SF-1 Interaction with GCN5 HAT Protein—To study the in vivo interaction of GCN5 with SF-1, 1 × 10^6 COS-1 cells/10-cm dish were transfected with a myc-His-tagged SF-1 expression vector and incubated in presence of 1 μM TSA for 12 h. After 40 h of transfection the cells were lysed with EBC buffer and immunoprecipitated with antibodies against hGCN5. Detection of SF-1 (Fig. 3D) by Western blot analyses demonstrated that GCN5 was able to pull down detectable amounts of SF-1. Nontransfected COS-1 cells were immunoprecipitated as above, in which no SF-1 band is detected. Immunoprecipitation of endogenous GCN5 with hGCN5 antibodies is shown in Fig. 3E.

GCN5 as a Coactivator of SF-1—The effect of the HAT proteins GCN5 and CBP on SF-1 transactivation was analyzed. COS-1 cells were cotransfected with the reporter plasmid pT81-4CRS2LUC and either the SF-1 plasmid alone or with the GCN5 expression plasmid. The results in Fig. 4A indicate that GCN5 stimulates SF-1 transactivation in a dose-dependent
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In the presence of GCN5 (Fig. 4B), transcription of SF-1 with CBP expression plasmid is consistent with previous results (Fig. 4C). In the presence of CBP, TSA enhanced transactivation of SF-1.

Inhibition of Deacetylation Stimulates SF-1 Transactivation—To determine the functional consequences of the inhibition of histone deacetylation by TSA in SF-1 transactivation in COS-1 cells, cotransfections were performed with an SF-1 wild-type plasmid and a reporter plasmid pTS1-4CRS2LUC. The transfected cells were incubated with TSA at various concentrations for 12 h. After 40 h of transfection, cells were lysed, and the luciferase activity was determined. The results in the Fig. 4D indicate that SF-1 activates the pTS1-4CRS2LUC reporter plasmid, and TSA increased SF-1 transactivation in a dose-dependent manner. The addition of TSA to the cells transfected with the KGFFK mutant had shown no effect on the reduced transcriptional activity; a further increase in transcriptional activity was observed with the RFQKK mutant (data not shown).

SF-1 Protein Is Stabilized by Inhibition of Deacetylation—The expression of SF-1 protein in transfected COS-1 cells, in the presence or absence of TSA, was analyzed on a 10% SDS-PAGE, and SF-1 was detected by immunoblotting using polyclonal antibodies specific for SF-1. The addition of TSA resulted in a significant increase of SF-1 protein levels (Fig. 5A).

To demonstrate that the increased protein level is the result of stabilization of the SF-1 protein, SF-1 protein expression was detected by autoradiography (Fig. 5B). In COS-1 cells the amount of SF-1 protein was reduced to 71.4% after 1 h compared with time point zero hour, which was set to 100%. After 3 h the amount of protein measured was 44.8%. The cells treated with TSA, the SF-1 amount of protein in 1 h was 96.7%, and after 3 h it decreased to 65.1%. Increased half-life of SF-1 is observed in the cells treated with TSA (Fig. 5C).

Nuclear Export of SF-1 Protein—Because TSA increased SF-1 protein levels we checked the localization of the endogenously expressed SF-1 in Y-1 cells treated with TSA. The addition of TSA to Y-1 cells increases transactivation (not shown). The localization of endogenous SF-1 in Y-1 cells is revealed by immunofluorescence analysis. Nuclear localization of endogenous SF-1 in the steroidogenic mouse MA-10 Leydig cells and in human adrenocortical H295R cells has been shown previously (42). In Y-1 cells SF-1 is localized in the nucleus (Fig. 6). After treatment with TSA, both cytoplasmic and nuclear localization of SF-1 is observed in Y-1 cells. The same distribution was found in COS-1 cells transfected with an SF-1 expression plasmid (not shown). By using confocal microscopy, punctuated appearance of SF-1 in the cytoplasm is revealed. To show that the cytoplasmic localization of SF-1 is a consequence of inhibition of deacetylation, Y-1 cells were treated with forskolin, which stimulates adenyl cyclase in different cell types. Even though forskolin increased SF-1 transactivation in Y-1 cells (35), the addition of forskolin did not affect the nuclear localization.

DISCUSSION

The recruitment of coactivators and corepressors at the target gene promoter is an important process during transactivation by nuclear receptors (25, 43). Coactivators with HAT ac-
inactive in the presence of 0.5 μM TSA for 12 h. 20 μg of whole cell extracts was used in each lane and analyzed by 10% SDS-PAGE. SF-1 protein was detected by Western blot using antibodies against SF-1. The expression of SF-1 protein in the absence of TSA (lanes 1–3, three independent parallel experiments) and presence of TSA (lanes 4–6, three independent parallel experiments) is demonstrated. Panel B, determination of the protein half-life of SF-1 protein, COS-1 cells were transfected with SF-1 plasmid and incubated in the presence or absence of 0.5 μM TSA for 12 h. The cells were labeled in a methionine-free medium containing 0.25 Ci/ml [35S]methionine for 1 h. Chase was performed in medium supplemented with a 10-fold excess of cold methionine for the time periods indicated. The immunoprecipitated proteins were separated by 10% SDS-PAGE and autoradiographed. Panel C, the intensity of the 35S-labeled SF-1 protein was measured densitometrically. The amount of protein at the time point zero was set to 100%. The figure shows mean values from two independent experiments.

**FIG. 6.** Translocation of SF-1 protein from nucleus to cytoplasm. Y-1 cells, which express endogenous SF-1, were transferred to glass coverslips and treated with 0.5 μM TSA or with 1.0 μM forskolin for 12 h. As a control experiment nontreated cells are shown.

The present study demonstrates that SF-1 is acetylated in vivo. The acetylation of mutant SF-1 proteins might be caused by multiple lysines being acetylated in vivo by different HAT enzymes. In p53, the lysines were acetylated by CBP, and p300/CBP-associated factor are different (31, 41). Among the mammalian nuclear cofactors with HAT activity, human GCN5 has been well characterized (44). The GCN5 HAT enzyme is a part of a multimeric complex with the other components, such as ADA2, which are necessary for its activity (45, 46). The use of immunoprecipitated GCN5 for acetylation experiments provides the additional components important for acetylation. In vitro acetylation of SF-1 by GCN5 shows that GCN5 can be one of the potential enzymes involved in acetylation in vivo. Hypoacetylation of mutants of SF-1 by GCN5 further proves that the lysine motifs C-terminal to the zinc finger domains are the targets of acetylation.

**In vitro** acetylation of SF-1 by GCN5 led us to analyze the consequences of acetylation in vivo. Overexpression of GCN5 increased the transcriptional activity of SF-1. Enhanced transcriptional activity by coactivator GCN5 is consistent with acetylation being a necessary modification used in SF-1 transactivation. Several acetylation-regulated protein-protein interactions have been reported recently. For example, activator of thyroid and retinoic acid receptor association with nuclear receptors is inhibited by acetylation (28). Enhanced protein interactions as a result of acetylation are shown in transcription factors c-Myb and HNF-4 for CBP (47, 48). In the present study recruitment of SF-1 by GCN5 demonstrates in vivo interaction of SF-1 with GCN5. This association of acetylated SF-1 with this HAT protein suggests direct modification of SF-1 by GCN5 in vivo and might be important for the ultimate recruitment of other proteins to the transcriptional complex. Mutations of the lysine residues C-terminal to the zinc finger domains also affect transactivation. These lysines might be necessary for regulation of the SF-1 activity.

Histone deacetylases are involved in transcriptional regulation by deacetylation of histones (49, 50) and transcription factors (51). Histone deacetylase enzyme activities are inhibited by TSA through direct interaction of TSA with histone deacetylases (52, 53). TSA increases the acetylation level and transcriptional activity in p53 (51). The addition of TSA enhanced SF-1-mediated transactivation. Another consequence of the TSA treatment of cells is an increased expression level of SF-1 and its half-life. The mechanism of protection from degradation by inhibition of deacetylation is unknown. Pulse-chase analyses of SF-1 reveal that its turnover is strikingly rapid. Rapid degradation might be necessary for modulating the transcriptional activity of steroid receptors. In glucocorticoid receptors, rapid exchange of the receptor bound to chromatin in the presence of hormones has been demonstrated recently by using photobleaching techniques (54). Continuous exchange of the receptors bound to chromatin takes place even in the presence of hormones or ligands in the cells. Rapid exchange of the receptors might increase the availability of receptors for modifications such as phosphorylation and acetylation.

Histone deacetylase inhibitors are potent antitumor agents, involved in induction of cell differentiation and apoptosis (55, 56). Addition of the deacetylase inhibitor TSA led to cytoplasmic localization of SF-1 protein in Y-1 cells. The punctate clustering appearance of SF-1 in the cytoplasm might be that SF-1 is localized to organelles or associated with other structures in the cytoplasm. Recent reports suggest that cellular localization affects ubiquitin-mediated proteolysis of proteins. Components involved in ubiquitin-mediated degradation are not distributed evenly inside the cell (57). The active transport of SF-1 protein into the cytoplasm might be the reason for the increased half-life of SF-1 in the presence of TSA.
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