Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptors, as a Novel Transcriptional Corepressor Molecule of Activating Protein-1, Nuclear Factor-κB, and Serum Response Factor*

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Silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) is known to interact with Sin3 and recruit the histone deacetylases (HDACs) that lead to hypoacetylation of histones and transrepression of target transcription factors. Herein, we found that co-expression of SMRT significantly repressed transactivations by activating protein-1 (AP-1), nuclear factor-κB (NFκB), and serum response factor (SRF) in a dose-dependent manner, but not in the presence of trichostatin A, a specific inhibitor of HDAC. Similarly, coexpression of HDAC1 and mSin3A also showed repressive effects. Consistent with these results, the C-terminal region of SMRT directly interacted with SRF, the AP-1 components c-Jun and c-Fos, and the NFκB components p50 and p65, as demonstrated by the yeast and mammalian two hybrid tests as well as the glutathione S-transferase pull down assays. Thus, we concluded that SMRT serves to recruit Sin3/HDACs to SRF, NFκB, and AP-1 in vivo and modulate their transactivation.

Retinoic acid and thyroid hormone receptor bind to their target genes and repress transcription (for a review see Ref. 1). The silencing mediator of retinoic acid and thyroid hormone receptors (SMRT)1 (2) and the nuclear hormone receptor corepressors (N-CoR) (3) were originally isolated as factors associated with the hinge and ligand binding D/E domains of unliganded receptors. These corepressors interact with Sin3 and recruit the histone deacetylases (HDAC1 or Rpd3-HDAC2) that lead to hypoacetylation of the histones, consistent with the concept that histone hypoacetylation correlates with gene repression (4–7). Two groups have recently reported the isolation of histone deacetylase core complexes (mSin3 and the NuRD complex) that are critically involved in this transcriptional repression (8, 9). A few components of the NuRD complex are also present in the mSin3 complex that consists of seven polypeptides. In particular, SAP30 directly interacts with N-CoR, although N-CoR does not appear to be stably associated with this mSin3 complex. Antibody-blocking experiments and studies with histone deacetylase inhibitors also supported the idea that N-CoR/SMRT may serve as an adapter molecule between the core mSin3 complex and sequence-specific transcriptional repressors such as unliganded nuclear receptors (8, 9). However, recent evidence also showed that N-CoR/SMRT and Sin3 directly interact with the key components of the transcriptional initiation process (TFIIB, and the TBP-associating factors) to inhibit basal transcription, suggesting an alternative repression pathway (10, 11). Interestingly, the N-CoR/Sin3-HDAC complex is also known to mediate transcripational repression from a wide variety of other nonreceptor-mediated pathways. These include MxyD (12), the bHLH-LZ proteins Mad and Mxi that mediate repression of myc activities and tumor suppression (13), E2F-repressive retinoblastoma protein (14), and the oncoproteins PLZF-RAR (15) and LAZ3/BCL6 (16), which are involved in acute promyelocytic leukemia and non-Hodgkin lymphomas, respectively.

Serum response factor (SRF), activating protein-1 (AP-1), and nuclear factor-κB (NFκB) are known to control a surprisingly diverse set of genes. However, these factors have been shown to share at least one common property, i.e. stimulation of cellular proliferation processes. Along with ternary complex factor, SRF binds to and activates the serum response element (SRE). The SRE sequences are found in the upstream regulatory sequences of myogenic genes as well as a number of immediate early genes, including c-fos, which in turn activate genes critical for cell proliferation (for a review see Ref. 17). SRF belongs to the MADS box family of proteins and recognizes a CArG box in the SRE, whereas the ternary complex factor does not bind autonomously to the element but requires the assistance of SRF to efficiently contact the DNA. The AP-1 complex, immediate early response genes, consists of a heterodimer of a Fos family member and a Jun family member (for a review see Ref. 19). This complex binds the consensus DNA sequence (TGAGTCA) (termed AP-1 sites) found in a variety of promoters. The Fos family contains four proteins (c-Fos, Fos-B, Fra-1, and Fra-2), whereas the Jun family is composed of three (c-Jun, Jun-B, and Jun-D). Fos and Jun are members of the basic leucine zipper (bZIP) family of sequence-specific dimeric DNA-binding proteins. The C-terminal half of the bZIP domain is amphipathic, containing a heptad repeat of leucines that is critical for the dimerization of bZIP proteins. The N-terminal half of the long bipartite helix is the basic region that is critical for sequence-specific DNA binding. Finally, NFκB is composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family of polypeptides (for a review see Ref. 19). In vertebrates, this family comprises p50, p65 (RelA), c-Rel, p52

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§§ The abbreviations used are: SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; N-CoR, nuclear hormone receptor corepressors; HDAC, histone deacetylase; SRE, serum response factor; AP-1, activating protein-1; NFκB, nuclear factor-κB; SRE, serum response element; bZIP, basic leucine zipper; GST, glutathione S-transferase; TSA, trichostatin A.

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and RelB. These proteins share a 300-amino acid region, known as the Rel homology domain, which binds to DNA and mediates homo- and heterodimerization. This domain is also a target of the IκB inhibitors, which include IκBα, IκBβ, IκBγ, Bcl-3, p105, and p100. In the majority of cells, NFκB exists in an inactive form in the cytoplasm bound to the inhibitory IκB proteins. Treatment of cells with various inducers results in the degradation of IκB proteins, and the bound NFκB is released and translocates to the nucleus, where it activates appropriate target genes. Interestingly, a naturally occurring splice variant of RelA was shown to transform Rat-1 cells (20), whereas antisense oligonucleotides to RelA were shown to inhibit proliferation and tumorigenicity of several tumor cell lines, including the human breast cancer cell lines MCF7 and T47D (21). In addition, activation of NFκB through the disruption of IκBα regulation was shown to result in malignant transformation (22). From these results, it was suggested that constitutive activation of NFκB contributes to the malignant phenotype of tumor cells.

Herein, we report our findings that SMRT physically interacts with NFκB, AP-1, and NFκB and inhibits their transactivations via recruitment of Sin3-HDACs. Thus, SMRT may play a critical role in the cellular proliferation control or other functions of these transcription factors in vivo.

EXPERIMENTAL PROCEDURES

Hormones and Plasmids—TSA was obtained from Sigma. EGY48 cells, the lexA-galactosidase reporter construct, and the LexA and B42 parental vectors (i.e. pJG4–5 and p202PL) were as reported (23). Mammalian expression vectors encoding Gal4-p65, SMRT, mSin3A, HDAC1, p65, c-Fos, VP16-TR, glutathione S-transferase (GST) fusion vectors for c-Jun, c-Fos, p50, p65, and SRF, T7 vectors for c-Jun, p65, and SRF, and B42 fusion vectors for c-Jun, JunD, JunD2, JunD3, c-Fos, FosA1, FosB, FosB3, SRF, and p50, as well as the reporter constructs Gal4-TK-LUC, (αβ)4-IL-2-LUC, (TRE)5-TK-LUC, SRE-c-fos-LUC, and c-fos-LUC, and the transfection indicator construct pRSV-β-gal were as described (2, 3, 24–28). Expression vectors for Gal4 fusions to c-Fos and SMRT, VP16 fusions to p50, p65, c-Jun, and SRF, and B42 fusions to SRFαC, SRFαN, p65αC, and p65αN were constructed by inserting appropriate polymerase chain reaction fragments into EcoRI and XhoI restriction sites of pCMXGal4/N, pCMXVP16, and pCMXVP16, respectively. Similarly, polymerase chain reaction fragments of the full-length SMRT and its various fragments were cloned into EcoRI and XhoI restriction sites of pcDNAs3, p202PL, and pGEX4T for T7 in vitro transcription, LexA fusions, and GST fusions, respectively.

Yeast Two Hybrid Tests—The cotransformation and β-galactosidase assays in yeast were performed as described (23). For each experiment, at least three independently derived colonies expressing chimeric proteins were tested.

GST-Pull Down Assays—The GST-fusions or GST alone were expressed in Esherichia coli, bound to glutathione-Sepharose-4B beads (Amersham Pharmacia Biotech), and incubated with labeled proteins expressed by in vitro translation by using the TNT-coupled transcription-translation system, with conditions as described by the manufacturer (Promega, Madison, WI). Specifically bound proteins were eluted from beads with 40 mM reduced glutathione in 50 mM Tris (pH 8.0) and analyzed by SDS polyacrylamide gel electrophoresis and autoradiography as described (23).

Cell Culture and Transfection—Various mammalian cells were grown in 24-well plates with medium supplemented with 10% fetal calf serum. After 24 h of incubation, cells were transfected with 100 ng of β-galactosidase expression vector pRSV-β-gal and 100 ng of an indicated reporter gene, along with various mammalian expression vectors. Total amounts of expression vectors were kept constant by adding decreasing amounts of pcDNA3 to transfections. 12 h later, cells were washed and refed with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum either in the presence or absence of 100 nM TSA and harvested 24 h later for luciferase activity. With the serum-response specific SRE-c-fos-LUC and c-fos-LUC reporters, however, the transfected cells were incubated with medium containing 0.5% fetal calf serum for 20 h and washed and refed with Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum either in the presence or absence of 100 nM TSA. Cells were harvested 4 h later and measured for luciferase activity, as described (24). All of the results were normalized to the

![Fig. 1. Effects of SMRT cotransfection on transactivations by Gal4 fusions to p65 and c-Fos.](http://www.jbc.org/)

**RESULTS AND DISCUSSION**

**SMRT Inhibits Transactivation by AP-1, NFκB, and SRF**—In our recent studies of various transcription coactivators and corepressors, we found that coexpression of SMRT consistently inhibited transactivation by a Gal4 fusion to the NFκB component p65 or the AP-1 component c-Fos in a dose-dependent manner (Fig. 1). Similar results were also obtained with a Gal4 fusion to the NFκB component p50 or the AP-1 component c-Jun, whereas SMRT was not able to repress transactivation by Gal4 alone (results not shown). To confirm these results, SMRT-expression vector was cotransfected into CV1 cells along with a reporter construct (αβ)4-IL-2-LUC, which was previously characterized to efficiently mediate the NFκB-dependent transactivations in various cell types and consists of a minimal promoter from the interleukin-2 gene and four upstream xB sites from the interleukin-6 gene (25). Increasing amounts of cotransfected SMRT inhibited the reporter gene expressions in a SMRT dose-dependent manner, with cotransfection of 100 ng of SMRT decreasing the fold activation approximately 5-fold (Fig. 2A). In L929 cells, SMRT also inhibited the tumor necrosis factor α-induced level of transactivations in a SMRT dose-dependent manner (results not shown). In contrast, cotransfection of SMRT did not affect the LacZ reporter expression of the transfection indicator construct pRSV-β-gal either in the presence or absence of tumor necrosis factor α (results not shown). When tested with a reporter construct (TRE)5-TK-LUC, which consists of a minimal promoter from the thymidine kinase gene and four upstream consensus AP-1 sites (26), SMRT inhibited the reporter gene expressions (Fig. 2B). Similarly, SMRT repressed the 12-O-tetradecanoylphorbol-13-acetate-induced level of transactivations in HeLa and CV-1 cells (results now shown). Serum shock with 20% fetal calf serum resulted in an ~10-fold increase in transactivation of a reporter construct, SRE-c-fos-LUC, which consists of a minimal promoter from the c-fos gene and a single upstream consensus SRE (24). Surprisingly, SMRT also repressed this serum-induced level of transactivation by SRF (Fig. 2C). In contrast, SMRT did not affect the TK-LUC reporter expression (Fig. 2D). Overall, these results strongly suggest that SMRT is a novel transcription corepressor molecule of AP-1, NFκB, and SRF.

**Inability of SMRT to Inhibit the AP-1, NFκB, and SRF Transactivations in the Presence of TSA**—SMRT/N-CoR is known to function as an adaptor molecule between HDAC core complexes (mSin3 and the NuRD complex) (8, 9) and target transcription factors such as unliganded nuclear receptors (2, 3). Interestingly,
SMRT was not capable of inhibiting the NFκB, AP-1, and SRF transactivations in the presence of 100 nM TSA, a specific HDAC inhibitor (29) (Fig. 2), whereas TSA was without any significant effect on the basal level of these transcription units (Fig. 2D and results not shown). In addition, coexpression of HDAC-1 and mSin3A also showed strong repressive effects (Fig. 3). In particular, SMRT-mSin3A-HDAC-1 functionally interacted with SRF in the context of a native c-fos full promoter (Fig. 3C). From these results, we propose that SMRT may specifically recruit the HDAC complexes to NFκB, AP-1, and SRF in vivo to repress their transactivation potential. Finally, it is notable that each reporter construct showed a much higher level of gene expressions in the presence of both activating signals and TSA (Fig. 2, A–C), suggesting that the AP-1, NFκB, and SRF transactivation can be intrinsically compromised by the endogenously expressed SMRT/N-CoR. Therefore, the expression level of endogenous SMRT/N-CoR may serve as an important determinant for the overall transcriptional activities of these transcription factors within a given cell type.

**SMRT Binds to SRF, p50, p65, c-Jun, and c-Fos**—To recruit HDACs to AP-1, NFκB, and SRF, SMRT should directly interact with these transcription factors. To examine this possibility, we constructed yeast expression vectors encoding chimeric proteins of the B42 transactivation domain (23) fused to various SMRT fragments (Fig. 4A). SMRT-A, SMRT-B, and SMRT-C did not show any interactions with c-Jun, c-Fos, p50, p65, and SRF in yeast (results now shown). However, SMRT-D consisting of the C-terminal SMRT residues 1060–1495 strongly interacted with c-Jun, c-Fos, SRF, p50, and p65ΔN (i.e. the p65 residues 353–550) (Fig. 4B). Furthermore, SMRT-D specifically interacted with JunΔ3, FosΔ1, FosΔ2, and SRFΔN (i.e. the SRF residues 203–508) but not with JunΔ1, JunΔ2, FosΔ3, SRFΔC (the SRF residues 1–323), and p65ΔC (the p65 residues 1–323). In particular, JunΔ3 and FosΔ2 contain the previously defined bZIP domains (18), which also serve as binding sites for the transcription coactivator molecule Bcl-3 (27). In this regard, it is also noted that the C-terminal, SMRT binding regions of SRF and p65 contain their transactivation function (24, 25). Similar yeast results were also obtained when tested with LexA fusions to various SMRT fragments and B42 fusions to c-Jun, c-Fos, p50, p65, and SRF (results not shown). These interactions were further confirmed in the mammalian two hybrid tests, in which coexpression of VP16 fusions to p50, p65, c-Jun, c-Fos, and SRF showed strong repressive effects (Fig. 5A). As previously shown (3), VP16-TR also showed interactions with Gal4-SMRT-D in the absence of T3.

**Corepression of AP-1, NFκB, and SRF by SMRT**

![Fig. 2 Effects of SMRT cotransfection on the NFκB, AP-1, and SRF transactivations. CV1 cells were transfected with a LacZ expression vector and an increasing amount of SMRT expression vectors along with various reporter genes as indicated either in the presence or absence of 100 nM TSA. Normalized luciferase expressions from triplicate samples are presented relative to the LacZ expressions and the standard deviations are less than 5%. Similar results were also obtained with L929 and HeLa cells.](http://www.jbc.org/)

![Fig. 3 HDACmSin3A inhibits the NFκB, AP-1, and SRF transactivations. CV1 cells were transfected with LacZ expression vector and an increasing amount of SMRT, HDAC1, and mSin3A expression vectors along with various reporter genes as indicated. Normalized luciferase expressions from triplicate samples are presented relative to the LacZ expressions, and the standard deviations are less than 5%. Similar results were also obtained with HeLa cells.](http://www.jbc.org/)
interacted with GST-SMRT-D but not with GST alone or GST-fusions to SMRT-A, SMRT-B, and SMRT-C (Fig. 5B and results now shown). Overall, these results, along with the yeast and mammalian two hybrid results, indicate that the C-terminal subregion of SMRT directly binds to c-Jun, c-Fos, p50, p65, and SRF. In summary, our results indicate that SMRT physically interacts with and can function as a novel transcription corepressor molecule of AP-1, NFκB, and SRF through recruitment of the HDAC complexes. Because AP-1, NFκB, and SRF are potent mitogenic factors (17–19), the SMRT-dependent modulation of these transcription factors may contribute to the cellular proliferation control in vivo. Thus, it is an interesting hypothesis that the expression level of endogenous SMRT, as recently evidenced by differential expression levels of SMRT in various cell types (30–32), could be a key factor determining the overall cellular proliferation capacity of the given cell type. However, SMRT can also modulate other functions of AP-1, NFκB, and SRF (17–19). In particular, it will be interesting to test whether SRF-mediated activation of MyoD in muscle differentiation (33) also becomes subjected to repression by SMRT-HDACs. Finally, it is noteworthy that all the target factors of SMRT-HDACs (8, 9), including MyoD (12), AP-1, NFκB, and SRF, regulate various cellular proliferation and differentiation processes in vivo, nicely corroborating with the recent proposal that HDACs play an important role in these processes (11).

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