Receptor-interacting protein 140 (RIP140) Directly Recruits Histone Deacetylases for Gene Silencing*

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Li-Na Wei‡, Xinli Hu‡, Dhyana Chandra‡, Edward Seta¥, and Maria Farooqui‡

From the §Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455 and the ¶Molecular Oncology Program, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

Receptor-interacting protein 140 (RIP140) encodes a histone deacetylase (HDAC) inhibitor-sensitive repressive activity. Direct interaction of RIP140 with HDAC1 and HDAC3 occurs in vitro and in vivo as demonstrated in co-immunoprecipitation and glutathione S-transferase pull-down experiments. The HDAC-interacting domain of RIP140 is mapped to its N-terminal domain, between amino acids 78 and 303 based upon glutathione S-transferase pull-down experiments. In chromatin immunoprecipitation assays, it is demonstrated that histone deacetylation occurs at the chromatin region of the Gal4 binding sites as a result of Gal4 DNA binding domain-tethered RIP expression. The immunocomplexes of RIP140 from cells transfected with RIP140 and HDAC are able to deacetylate histone proteins in vitro. This study presents the first evidence for RIP140 as a negative coregulator for nuclear receptor actions by directly recruiting histone deacetylases and categorizes RIP140 as a novel negative coregulator that is able to directly interact with HDACs.

Nuclear receptors play important roles in gene regulation. In most cases, they regulate target gene expression by binding to their cognate DNA response elements and recruiting associate proteins to the transcription machinery (1, 2). A large number of associate proteins of nuclear receptors have been found and demonstrated as co-activators or co-repressors (3). One mechanism of co-repressor and co-activator action has been involved in the recruitment of such acetylation of chromosomal histone proteins (4–6). For instance, corepressors such as N-CoR/SMRT interact with apo-receptors in their hinge region and recruit histone deacetylases (HDACs) directly through the Sin3 complexes (4, 7). However, recent studies have shown that co-repressors bind to the same cavity formed by helices 3–5 and 12 in the ligand binding domain of nuclear receptors as do co-activators (8–10). Upon ligand binding, the ligand binding domain undergoes a conformational change, releases corepressors, and recruits coactivators by re-positioning helix 12 (AF-2 domain). Several coactivators, mainly the p160 family, have been identified, including SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, and pCIP/RAC3/ACTR/AIB1 (11–16). The SRC-1, ACTR, and CBP/p300 have been shown to encode intrinsic histone acetyl transferase activities (5, 6, 17).

Two major classes of HDACs have been cloned in higher eukaryotes. Class 1 HDACs (HDAC 1–3) are homologous to the yeast Rpd-3 (18–20), and class 2 HDACs (HDAC 4–7) are homologues of the yeast Hda1 (21). It has been demonstrated that disruption of either Rpd3 or Hda1 in yeast resulted in global hyperacetylation of histones (19). The acetylation of chromosomal histones is correlated strongly with active gene transcription; conversely, hypoacetylation of a gene regulatory region is usually indicative of gene silencing (22–25). Whereas acetylation of histones is not sufficient to dictate the efficiency of transcription, an overwhelmingly large volume of convincing evidence has supported the notion that the acetylation/deacetylation state of histones affects chromatin structures, thereby modulating the efficiency of transcription machinery.

The human RIP140 was first demonstrated as a co-activator in a chimeric estrogen receptor system (26). The mouse RIP140 was cloned in this lab by screening mouse embryonic and testis libraries with the ligand binding domain of orphan nuclear receptor TR2 and was shown to be a potent co-repressor for TR2 in the absence of putative ligands (27). Recently, we have demonstrated that mouse RIP140 interacts, in a ligand-dependent manner, with retinoic acid receptor (RAR) and retinoid receptor X (RXR). However, in contrast to the cases of classical co-activators that interact with ligand-bound hormone receptors to activate target gene expression, RIP140 interaction with holo-nuclear receptors results in suppressed target gene expression (28). Furthermore, the ligand-dependent receptor-interacting motif of RIP140 is mapped to its C-terminal domain (28). In most recent studies, RIP140 has also been shown as a co-repressor for other hormone nuclear receptors and orphan receptors (29–32). Despite the widely documented modulatory role of RIP140 in nuclear receptor actions, the actual function of RIP140 remains unclear. Moreover, none of these studies has provided a mechanism for the function of RIP140 in gene regulation.

This study aims to address the enigmatic action of RIP140 by asking whether RIP140 action involves histone modification, and if so, how this may occur. It is demonstrated that the co-repressive and trans-repressive activities of RIP140 are sensitive to a HDAC inhibitor. A direct interaction of RIP140 with HDAC1 and HDAC3 is demonstrated using different in vitro and in vivo protein interaction tests, and the HDAC-interacting domain of RIP140 is mapped to amino acids 78–303 of its N-terminal domain. Subsequently, it is demonstrated that hi-
stone deacetylation of the Gal4 DNA target sequences occurs as a result of expressing a Gal4 DNA binding domain (BD)-tethered RIP140 in the cells. Finally, the in vitro evidence for HDAC activities encoded by immunocomplexes precipitated with anti-RIP140 is provided.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors and Mammalian Cell Transfection—The full-length RIP140 fused to the Gal4 DNA binding domain for testing trans-repressive activities, and full-length RIP140, RAR, RXR expression vectors for testing co-repressive activities, as well as Gal4- and DR5-thymidine kinase (TK)-luc reporters have been described (27, 28). The techniques for culturing COS-1 cells, transfection, and luciferase and lacZ assays are as described (27). All cultures were maintained in Dulbecco's modified Eagle's medium containing dextran charcoal-treated serum (DCC medium). For retinoic acid (RA) induction experiments, all-trans RA was added at a final concentration of 5 × 10⁻⁶ M for 24 h. Each experiment was carried out in triplicate. At least three independent experiments were conducted to obtain the mean and the standard error of the mean (S.E.).

Chromatin Immunoprecipitation Assay (ChIP)—COS-1 cells were transfected with Gal4 reporter and either Gal4 BD-RIP or Gal4-BD. The ChIP assay (33) was performed according to the manufacturer's recommendation (Upstate Biotechnology, Lake Placid, NY). Histone was cross-linked to DNA by adding formaldehyde to a final concentration of 1%. Precipitated chromatin was incubated with an anti-acetylated histone 3 antibody (Upstate Biotechnology) overnight at 4 °C. The immunocomplex was treated with proteinase K, and DNA was purified by phenol extraction for PCR detection with primers flanking the tk promoter region of the Gal4-tk-luc reporter (5′ AGCGGTCGCTGCAG 3′ and 5′ TTAAAGCGGGTGCGCTGCAG 3′). Amplified fragments (130 bp) were analyzed on a 1.2% agarose gel. The amplified internal control SV40 promoter fragment is 300 bp in length.

Protein Interaction Tests—The GST pull-down assay was conducted as described (27). Various RIP140 domains were cloned by fusing restriction fragments of RIP140 to the GST vector. The N-terminal amino acid (aa) 1–496 domain (RIP-N), central aa 333–1007 domain (RIP-Cent), and C-terminal aa 977–1161 domain (RIP-C) were obtained as EcoRI, NcoI/SmaI, and EcoRI fragments, respectively. The smaller N-terminal fragments, RIP-B, RIP-Na, RIP-Nc, and RIP-Sp, were obtained by BamHI, NcoI, NcoI, and SphI digestion, respectively, from RIP-N. Escherichia coli BL21 transformed with the GST fusion vectors were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h, and fusion proteins were partially purified from glutathione-Sepharose column. The partially purified GST-RIP fusion protein was incubated with [35S]Met-labeled HDAC1 or HDAC3 for pull-down reaction at 4 h, and fusion proteins were partially purified from glutathione-Sepharose column. The partially purified GST-RIP fusion protein was incubated with [35S]Met-labeled HDAC1 or HDAC3 for pull-down reaction as described (27). The full-length HDAC1 and HDAC3 (20, 21) were expressed from T7 vectors and labeled with [35S]Met using a TNT kit (Promega, Madison, WI).

Co-immunoprecipitation was conducted as described (27). HEK293 cells (ATCC CRL-1573) were co-transfected with RIP140 plus an empty vector or a flag-HDAC3 vector (21). Reciprocal experiments were conducted by co-transfecting cells with HDAC3 plus an empty vector or a flag-RIP vector. The flag-RIP was constructed by ligating the flag epitope to the C terminus of RIP. Cell lysates were incubated with anti-flag (Santa Cruz Biotechnology, Santa Cruz, CA), followed by Western blot analyses of immunocomplexes with an anti-RIP antibody (Santa Cruz Biotechnology) or an anti-HDAC3 antibody (Upstate Biotechnology) for the reciprocal experiments.

HDAC Enzyme Assay—The histone deacetylase assay was modified from the protocol of Kolle et al. (34). Calf thymus histone (Sigma) was acetylated in vitro with [3H]acetyl-CoA (12 Ci/mmol, Amersham Pharmacia Biotech) with histone acetylase pCAF (35) expressed in E. coli (36). Deacetylase activity was determined by incubating 1.5 μg of [3H]acetyl histone with RIP140 immunocomplexes in a total volume of 100 μl of HDAC buffer (15 mM Tris, pH 7.9, 10 mM NaCl, 0.25 mM EDTA, 10 mM β-2-mercaptoethanol, and 10% glycerol) for 2 h at 37 °C. Reaction mixtures were analyzed on 15% SDS-polyacrylamide gel electrophoresis, and gels were soaked in 1 M sodium salicylate (37), dried, and exposed to films.

RESULTS

RIP140 Suppresses RA-induced DR5 Reporter Expression, a Trichostatin A (TSA)-sensitive Activity—The biological activity of RIP140 in hormone receptor actions has been controversial. In the RAR/RXR system, we have demonstrated a ligand-induced interaction of RIP140 with RAR and RXR, and this interaction results in suppressed reporter activities (27). To determine whether this effect of RIP140 involves histone deacetylases, co-transfection experiments were performed to test the effect of HDAC inhibitor, TSA, in a DR5-driven RA reporter system as shown in Fig. 1A. A typical RA induction of this reporter is an approximately 50–100-fold-induction (Fig. 1A, bars 1 and 4). However, RIP140 suppresses RA induction to an approximately 10-fold-induction (Fig. 1A, bars 2 and 5). In the presence of TSA, the suppression can be partially released (to an approximately 30-fold-induction) (Fig. 1A, bars 3 and 6), suggesting a role of HDACs in the suppression of RA induction by RIP140.

Trans-repressive Activity of RIP140 Is Sensitive to TSA—The biological activity of RIP140 was further addressed by directly tethering this molecule to the BD of Gal4 and assessing its effect on a Gal4 reporter. This Gal4 BD-RIP140 fusion protein exerts a trans-repressive activity on the Gal4 reporter approximately 5–6 folds as shown in Fig. 1B. Consistent with the results obtained from the DR5 reporter system, this trans-repressive activity can also be partially reversed by TSA in a dose-dependent manner (25–250 nM). Therefore, RIP140 sup-
presses RA-induced reporter gene expression and encodes a transferable repressive activity. Additionally, these biological activities of RIP140 may involve the action of HDACs.

Direct Interaction of RIP140 with HDACs—To determine whether RIP140 can recruit HDACs, several protein interaction tests were performed. Because of the intrinsic suppressive activity of both RIP140 and HDACs on gene activities, which would complicate reporter assays, the two-hybrid-based protein interaction tests were not suitable for the studies of RIP140 interaction with HDACs. Therefore, we used several in vivo and in vitro approaches that did not rely on reporter assays. We first utilized a co-immunoprecipitation method to investigate immunocomplexes precipitated through either the RIP140 or the HDAC moiety. The HEK293 cells were cotransfected with RIP140 plus either a flag-tagged HDAC3 (lanes 2 and 4) or an empty vector (lanes 1 and 3). The cell lysate was precipitated with anti-flag, and the immunoprecipitate was analyzed with an anti-RIP140 antibody (lanes 3 and 4). On the same gel, the total lysate was monitored for the expression of RIP140 (lanes 1 and 2). The arrow points at the RIP140 band in the total lysate of each culture (lanes 1 and 2) as well as in the immunoprecipitate (lane 4). A set of control experiments was conducted by utilizing a nonspecific rabbit antiserum in immunoprecipitation (lanes 5 and 6). B, reciprocal co-immunoprecipitation experiment using flag-RIP and HDAC3. The cell lysate was precipitated with anti-flag, and the immunoprecipitate was analyzed with an anti-HDAC3 antibody (lanes 3 and 4). The total lysate was monitored for the expression of HDAC3 (lanes 1 and 2). The arrow points at the HDAC3 band in the total lysate of each culture (lanes 1 and 2) as well as in the immunoprecipitate (lane 4). C, maps of the different portions of RIP140 used in GST pull-down assays. The numbers above the map indicate the aa positions of restriction sites used to generate these deletion mutants of the mouse RIP140. Restriction sites are labeled as follows: SphI (Sp), NcoI (Nc), NaeI (Na), BamHI (B), EcoRI (R), and SmaI (S). RIP-F, full-length RIP140. D, GST pull-down assay. HDACs 1 and 3 were each expressed in T7 vectors, labeled with [35S]Met, and analyzed for their ability to interact with various GST-RIP140 fusion proteins (upper panels). The labeled input was monitored as shown in lane 1. A negative control of GST alone is shown in lane 10. The bottom panel shows a Coomassie Blue-stained protein gel resolving one half of the pull-down complexes in each reaction. Asterisks indicate partially purified GST fusion proteins in the complexes. C, C-terminal aa 977–1161 domain; Cent, central as 333–1007 domain; Sp, SphI; Na, NaeI; Nc, NcoI; B, BamHI; N, N-terminal as 1–496 domain; F, full-length; M, marker.
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HDACs, an complexes. To map the HDAC-interacting domain as shown in Fig. 2, the full-length RIP140 was first dissected into three overlapping fragments and several smaller fragments to map the HDAC-interacting domain. The region between aa 78 and aa 303 of HDAC1 and HDAC3 was observed (Fig. 2). To map the interaction domain, the full-length RIP140 was first dissected into three overlapping fragments and several smaller fragments to map the HDAC-interacting domain as shown in Fig. 2C. Fig. 2D shows the results of GST pull-down assays. The upper panel shows the phosphorimage of the [35S]Met label, approximately 52 kDa in the precipitate of the flag-RIP plus HDAC3 complex. To map the HDAC-interacting domain of RIP140, the critical HDAC-interacting motif of RIP140 is mapped to its N-terminal domain. The region between aa 78 and aa 303 of RIP140 is critical for HDAC interaction, based upon the GST pull-down assay.

Gal4 BD-RIP Renders Histone Deacetylation on the Chromatin Spanning the Gal4 Binding Site—To determine whether RIP140 can interact directly with HDACs, an in vitro protein interaction test, a GST pull-down assay, was conducted. A strong interaction of RIP140 with HDAC1 and HDAC3 was observed (see Fig. 2). To map the interaction domain, the full-length RIP140 was first dissected into three overlapping fragments and several smaller fragments to map the HDAC-interacting domain. The region between aa 78 and aa 303 of RIP140 is critical for HDAC interaction, based upon the GST pull-down assay.

Fig. 3. ChIP assay to demonstrate histone deacetylation as a result of RIP140 expression. Input DNA (1 μl from a 20× dilution) before immunoprecipitation (lanes 5 and 6) and plasmid DNA (P, lane 8) were used as positive controls for PCR. Reactions with a nonspecific rabbit antiserum are shown in lanes 3 and 4, and the negative control (water) is shown in lane 7. In the immunoprecipitated chromatin, histone deacetylation occurs as a result of BD-RIP140 expression (lane 2) as compared with the sample expressing BD alone (lane 1). The top panel shows the Gal4-specific fragment (130 bp) amplified in PCR reactions; the bottom panel shows the internal control of SV40 promoter (300 bp) amplified in the PCR. H3, histone 3; ctrl, control; pm-RIP, pm-RIP fusion.

This study demonstrates, for the first time, that RIP140 complexes precipitated from cell lysates encode HDAC activities, cells (one 10-cm plate of HEK293 cells) were co-transfected with RIP140 and flag-HDAC3 to overexpress these proteins. Lysates were collected and immunoprecipitated with anti-RIP. The immunocomplex was resuspended in 100 μl, and 50 μl was used in each HDAC assay. As shown on the upper panel of Fig. 4, RIP140 immunocomplexes from cultures cotransfected with RIP140 and flag-HDAC3 (lane 3) encode HDAC activities. Fig. 4, lane 2 shows a positive control where a lysate of cells transfected with flag-HDAC3 alone was immunoprecipitated with anti-flag. One negative control that was transfected with flag-HDAC3 (lane 4) alone and precipitated with anti-RIP140 shows very low activity. To monitor the expression of flag-HDAC3 in transfected cultures, the total lysate of all the transfected cultures (Fig. 4, lanes 2–4) was detected with anti-flag as shown on the lower panel. Therefore, immunocomplexes of RIP140 from RIP140- and HDAC-overexpressing cells encode HDAC enzyme activities higher than the background level, indicating that RIP140 is able to recruit HDACs to form more active enzyme complexes in the precipitates.

**DISCUSSION**

This study demonstrates, for the first time, that RIP140 encodes a TSA-sensitive trans-repressive activity and that RIP140 is able to directly interact with HDACs in vitro and in vivo. The HDAC-interacting domain of RIP140 is mapped to its N-terminal domain, between amino acids 78 and 303. By using the Gal4 BD-RIP and Gal4 reporter system in ChIP assays, we demonstrate histone deacetylation on the chromatin of the Gal4 target site as a result of Gal4-RIP140 expression. Fur-
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Fig. 4. Determination of the HDAC enzyme activity of RIP140 immunocomplexes. The HDAC enzyme assay of the immunocomplexes (upper panel) was conducted as described under “Experimental Procedures.” Samples are as follows: control-labeled histones (lane 1), anti-flag-precipitated flag-HDAC3-transfected cells (lane 2), and anti-RIP140-precipitated cells transfected with RIP140 plus flag-HDAC3 (lane 3) or flag-HDAC3 alone (lane 4). The lower panel shows the flag-HDAC detected by anti-flag antibody in the total lysate of untransfected cells (lane 1) and transfected cultures (lanes 2–4).

thermore, the immunocomplexes of RIP140 from cells cotransfected with RIP140 and HDAC expression vectors are able to deacetylate histone proteins in vitro. This study presents the first evidence for RIP140 as a negative regulator for nuclear receptor actions by directly recruiting histone deacetylases and categorizes RIP140 as a novel negative coregulator that is able to directly interact with HDACs.

The actions of RIP140 have been disputed over different experimental systems, partly because these studies are based upon reporter activities in cotransfection experiments. Therefore, the mechanism underlying the actions of RIP140 cannot be elucidated. The current study takes a direct approach and provides biochemical evidence for the direct association of RIP140 with HDACs. However, as shown in Fig. 1, TSA can only partially block the action of RIP140. It is quite possible that RIP140 also involves other pathways to modulate gene activities. Another interesting observation is that whereas cells expressing RIP140 and HDAC3 show HDAC activities in an in vitro assay (Fig. 4, lane 3), the endogenous RIP140 does not seem to be in sufficient quantity to precipitate overexpressed HDAC3 for an in vitro assay (Fig. 4, lane 4). In our previous study, we have shown that RIP140 is expressed in many cell types but at a low level (27). It is possible that the expression level or local concentration of RIP140 is also critical for its ability to recruit HDACs. Given the fact that RIP140 can interact with many nuclear factors, it will be important to investigate the dynamics and kinetics of RIP140 interaction with these nuclear factors in the future.

Unlike typical nuclear receptor corepressors that exert repressive effects by recruiting HDACs through Sin3 complexes (4, 7), RIP140 is able to directly interact with HDACs. The HDAC-interacting motif is mapped to aa 78–303 based upon GST pull-down results (Fig. 2B). In our preliminary studies, RIP140 was also shown to be able to interact with HDAC4, a class II HDAC (data not shown). The HDAC-interacting property of RIP140 would categorize RIP140 in a distinct family of nuclear receptor coregulators that directly associate with chromatin remodeling complexes. We are currently investigating the molecular mechanisms underlying the interaction between RIP140 and HDACs.

In addition to this unique property, RIP140 exhibits three other interesting features. First, for hormone receptors, ligand induces an enhanced interaction of receptors with RIP140. However, unlike coactivator interaction with holo-receptors, which usually involves an interacting motif (LXXLL) (38, 39), RIP140 interaction with various nuclear receptors utilizes different portions of this molecule (27). Most interestingly, its interaction with holo-RAR and holo-RXR is mediated through its C-terminal domain, which lacks a LXXLL motif (28). Second, RIP140 is able to interact with a large number of nuclear receptors in both ligand-dependent and -independent manners (27–32). For instance, the TR2 orphan receptor-interacting domain of RIP140 has been mapped to the N-terminal and central portions of this molecule (27); yet RA-induced interaction of RAR/RXR utilizes the C-terminal domain (28). Third, interaction of RIP140 with wild type nuclear receptors mostly results in suppressed gene activities, both for ligand-bound receptors and apo-receptors (27–32). Controversial studies have shown some activation function of RIP140 in nuclear receptor-mediated gene expression (40, 41). However, these studies also demonstrate some unusual features of RIP140. For instance, RIP140 is able to interact with antagonist-occupied receptors such as ICI182,780-occupied ER (40). In another study, RIP140 suppresses E2-dependent ER activation but activates the E2-dependent ER mutant K366A (41). In the VDR system, RIP140 also interacts with VDR in the presence of 1,25-(OH)2D3 (42). These features are quite distinct from that suggested for a typical coactivator or corepressor based upon the current working model. Therefore, RIP140 may be categorized under a distinct family of coregulators that interact extensively with various factors to affect transcription efficiency. Among these, HDAC families are of significant interest and may account for, at least partially, the widely documented suppressed activities of RIP140 in different experimental systems. Questions remain as to how RIP140 may differentially interact with both apo- and holo-nuclear receptors and what other transcription components may be affected by RIP140.

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