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RIF-1, a Novel Nuclear Receptor Corepressor That Associates With the Nuclear Matrix

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Abstract The retinoic acid receptors (RARs) are ligand-dependent transcription factors that play critical roles in cell differentiation, embryonic development, and tumor suppression. RAR transcriptional activities are mediated by a growing family of nuclear receptor (NR) coregulators. Here we report the cloning and characterization of a novel protein RIF1 (receptor interacting factor) that interacts with RARα in vivo and in vitro. RIF1 encodes a novel 739 amino acid protein that is ubiquitously expressed in a variety of tissues and cell lines. GST-pull down assays show that RIF1 also interacts with a number of other NRs. The interaction domain of RIF1 for RARα is located at the C-terminal region of RIF1, between amino acids 512 and 674. RIF1 is localized exclusively in the cell nucleus and specifically to the nuclear matrix. Mutation of the nuclear localization signal abolishes this nuclear localization and causes RIF1 to appear in the cytoplasm. Co-transfection of RIF1 with RAR causes RAR to localize to the nuclear matrix. RIF1 contains a strong transcriptional repression domain that robustly inhibits ligand-dependent transcriptional activation by RARα. This domain is located to the distal C-terminal 100 amino acids, distinct from the RARα-interaction and nuclear matrix-targeting domains. The transcriptional repression activity of RIF1 is mediated at least in part through direct recruitment of histone deacetylases. This study identifies RIF1 as a novel nuclear matrix transcription repressor, and suggests a potential role of RIF1 that regulates NR transcriptional activity. J. Cell. Biochem. 102: 1021–1035, 2007.

Key words: nuclear receptor; nuclear matrix protein; corepressor; transcription

The steroid/nuclear receptor (NR) superfamily is a large class of ligand-dependent transcription factors involved in the regulation of genes critical in a variety of biological processes, including development, reproduction, and homeostasis. This NR superfamily can be subdivided into three classes: steroid receptors, non-steroid receptors and orphan nuclear receptors (For review, see Mangelsdorf et al. [1995]). Steroid receptors include receptors for estrogens (ER), progestins (PR), androgens (AR), glucocorticoid (GR), and mineralcorticoids (MR). They form homodimers to bind to their DNA response elements. The non-steroid receptors include receptors for *all-trans* retinoic acid (atRA) (RARs), thyroid hormone (TR), vitamin D (VDR), peroxisome proliferators (PPAR) and liver X receptor (LXR). They form heterodimers with retinoid-X receptor (RXR), and the heterodimers bind constitutively to DNA response elements consisting of direct repeats. The third class of NRs is the orphan receptors, so-called because endogenous ligands for these proteins are currently unknown.

Most members of the nuclear receptor superfamily share similar structure: the N-terminus contains a weak variable ligand-independent activation function 1 (AF-1) domain, followed by a conserved DNA-binding domain (DBD) and a hinge region; the C-terminus of nuclear receptors contains a strong ligand-dependent transactivation domain, which includes ligand-binding domain (LBD) and the ligand-dependent activation function-2 (AF-2) domain. In the absence of ligand, many NRs are able to repress basal transcription via direct interactions with the NR corepressors SMRT and N-CoR [Chen

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and Evans, 1995; Horlein et al., 1995; Park et al., 1999]. SMRT and N-CoR directly recruit other corepressor proteins including mSin3 and histone deacetylases (HDACs), suggesting that the mechanisms of transcriptional repression by NRs involve histone deacetylation [Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997]. Ligand binding triggers the release of corepressors and subsequent recruitment of coactivators: the ligand-dependent activation functional domain (AF-2) undergoes a conformational change upon ligand binding to facilitate interactions with coactivators [Darimont et al., 1998; Feng et al., 1998; Nolte et al., 1998; Shiau et al., 1998]. The coactivators thus enhance ligand dependent transcription of nuclear receptors by further recruiting general coactivators such as CBP/p300 to interact with RNA polymerase II [Kamei et al., 1996; Li and Chen, 1998].

The NR coregulators are proteins that interact with nuclear receptors and mediate transcriptional activation or repression. To date, over 200 NR coregulators have been identified (For review, see Lonard and O'Malley [2006]). Coactivators are the coregulators that mediate NR ligand-dependent activation. Many of them either possess histone acetylase/methylase activities such as p160 SRC family, CARM and CBP/p300 to modify the chromatin template; or alter the high order chromatin structure such as Swi/Snf family of chromatin remodeling complex; or interact directly with the general transcriptional machinery to initiate transcription such as TRAP/DRIP mediator complex. Contrary to coactivators, not many corepressors have been identified. SMRT/NcoR are the first corepressors identified: they repress transcription through directly interaction with unliganded nuclear receptors and the recruitment of histone deacetylase complexes.

In addition to the above NR corepressors and coactivators, there is also an interesting class of NR-interacting proteins that appear to interact with liganded NRs, and inhibit ligand-dependent transcriptional activation. These include ER interacting proteins, RIP140 [Cavaillès et al., 1995; Treuter et al., 1998; Windahl et al., 1999; Wei et al., 2000, 2001], REA [Montano et al., 1999; Delage-Mourroux et al., 2000; Martini et al., 2000; Simon et al., 2000], SAFB1 [Oesterreich, 2003; Townson et al., 2003; Townson et al., 2004; Debril et al., 2005; Ivanova et al., 2005; Jiang et al., 2006] and MRF1 [Georgescu et al., 2005]. RIP140 was originally isolated as a transcriptional coactivator based on its ligand-dependent interaction with ERα. It was later found that RIP140 also interacts with RAR and GR and inhibits ligand-dependent transcriptional activation through recruitment of histone deacetylases to the liganded receptors [Windahl et al., 1999; Wei et al., 2000, 2001]. Gene ablation experiments in mice demonstrated that RIP140 is essential for ovulation and female fertility [White et al., 2000]. REA was isolated as an estrogen receptor-selective coregulator that potentiated the effectiveness of antiestrogens and repressed the activity of estrogens [Montano et al., 1999]. REA and the coactivator SRC-1 were involved in a functional competition for regulation of ER transcriptional activity [Delage-Mourroux et al., 2000] and the expression of REA varies among breast tumors and is correlated with known treatment response markers [Simon et al., 2000]. SAFB1 is a nuclear matrix scaffold attachment factor. It interacts with estrogen receptor (ER) independent of estradiol and represses ER transcriptional activation [Oesterreich et al., 2000]. Modulator Recognition Factor 1 (MRF1) highly enriched in muscle and liver cells, interacts with ERα independent of estrogen. It functions as a potent corepressor for Estrogen-ERα mediated transactivation [Georgescu et al., 2005].

The identification and cloning of NR interacting cofactors are crucial steps towards elucidating the molecular mechanisms of transcriptional regulation by NRs. Recent advances in studying the molecular mechanisms for transcriptional activation of nuclear receptors have established that transcriptional activation is a sequential and dynamic process involving cycling of different sets of coregulators onto NR target promoters [Shang et al., 2000]. However, fewer coregulators have been identified that mediate transcriptional repression of NRs. To characterize the molecular basis of transcriptional regulation by NRs, we have used the yeast two-hybrid system to search for potential RAR interacting proteins. This study identifies a novel RAR-interacting protein we have designated nuclear receptor-interacting factor 1 (RIF1). We show that RIF1 is able to repress the basal transcriptional activity and to inhibit RA-induced transcriptional activation of RARα. We further demonstrate that RIF1 is a nuclear matrix protein.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen

A Gal4 DBD fusion of the full-length human RARα was constructed and used as bait for yeast two-hybrid (y2h) screening. A human B-cell cDNA library was screened in the absence of ligand as described [Durfee et al., 1993]. After selection on synthetic dropout plates lacking Tryptophan, Leucine, and Histidine, and supplemented with 50 mM 3-Aminotriazole, we isolated 20 colonies for further analysis of β-galactosidase expression as described [Chen et al., 1996]. The library plasmids were rescued and retransformed into yeast cells together with the original bait or other constructs to test the specificity of the protein–protein interaction.

Isolation and Construction of Full-Length RIF1

The cDNA insert of RIF1 y2h clone was labeled with ^{32}P-dATP by DECAprime II DNA labeling kit (Ambion), and used to screen a λ ZAP bone marrow cDNA library using RIF1 as probe (Stratagene, La Jolla, CA). One clone with a 3.5-kb insert was isolated and found to encode the full-length RIF1. The full-length RIF1 cDNA was ligated into the pCMX expression vector [Umesono et al., 1991] with an HA epitope linked to the N-terminus. Site-directed mutagenesis was carried out using the QuikChange® kit as described by the manufacturer (Stratagene).

Far-Western Assay

Far-Western assay was carried out as described [Li et al., 1997]. Briefly, GST fusion proteins were expressed in DH5α' cells and purified with glutathione agarose beads (Sigma). Purified proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membrane. Proteins were denatured with 6 M guanidine hydrochloride (GnHCl) and renatured by stepwise dilution of GnHCl. Membranes were then blocked and hybridized overnight with ^{35}S-labeled hRARα generated by in vitro transcription/translation reaction in reticulocyte lysate (Promega). The membrane was washed and bound proteins were detected by autoradiography.

GST-Pull Down Assay

Approximately 0.2 μg of purified GST fusion protein was incubated with 5 μl of in vitro translated ^{35}S-labeled receptors with moderate shaking at 4°C overnight in binding buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 1 μg/ml bovine serum albumin). The bound protein was washed five times with binding buffer, and the beads were collected by centrifugation at 3,000 rpm. The bound protein was eluted into SDS sample buffer, subjected to SDS–PAGE, and detected by autoradiography.

Northern Blot

The ^{32}P-dCTP labeled C-terminal 940-bp or N-terminal 1.2 kbp RIF1 cDNA was used as a probe to hybridize with a human 12-lane Multiple Tissue Northern Blot (BD Clontech, Palo Alto, CA) or human cell line message strip (Stratagene) according to the manufacture's instructions. Each lane in the blot contains approximately 1 μg of poly-A+ RNA, and equal amount of β-actin mRNA is present in all lanes.

Cell Culture and Transient Transfection

Cells were maintained in Dulbecco's modified eagle's media with 10% fetal bovine serum and penicillin–streptomycin. For transactivation-reporter assays, cells were seeded in 12-well plates at 20,000 cells per well one day prior to transfection. The culture media were changed to phenol red-free DMEM media supplemented with 10% Charcoal-stripped Fetal Bovine Serum (Invitrogen/Gibco, Carlsbad, CA) after the cells attached well to the culture plate. A DNA mixture containing 0.5 μg of expression vector, 0.1 μg of internal control pCMX-β-Gal, 1 μg of luciferase reporter, and 0.9 μg of carrier DNA (pGEM), was prepared in a final volume of 30 μl. The DNA solution was mixed dropwise with one volume of 0.5 M CaCl2 and two volumes of 2× BBS (50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES; Calbiochem), 280 mM NaCl, 1.5 mM Na2HPO4, pH 6.95). The DNA precipitates were allowed to form at room temperature for 10 min and applied evenly to the culture cells. Transfection was allowed to continue for 12 h, and the precipitates were removed by washing cells twice with phosphate buffered saline (PBS). Transfected cells were treated with fresh media containing vehicle alone or vehicle plus indicated ligands and the cells were harvested 24–36 h after treatment.

Luciferase and β-Galactosidase Assay

Transfected cells in each well were lysed in 120 μl of cell lysis solution (Promega), and processed for luciferase and β-galactosidase assay. Fifty microliters of lysed cells were transferred into 96-well microtiter plates for luciferase assay and 96-well microtiter plates for β-galactosidase assay as described [Chen et al., 1996]. The luciferase activities were determined with a MLX microtiter plate luminometer (Dynex) using 100 μl of assay solution (100 mM KPO4, 5 mM ATP, 10 mM MgCl2) and 100 μl of luciferin solution (10 mM D-luciferin in 100 mM KPO4, pH 7.8). The luciferase activities were normalized to the β-galactosidase activity expressed from the cotransfected pCMX-β-Gal plasmid.

Indirect Immunofluorescence Microscopy

HeLa or COS7 cells were grown on coverglasses in 12-well plate 24 h prior to transfection as described above. Immunostaining was performed as described [Li et al., 2000]. Briefly, cells were washed twice with PBS and fixed in methanol/acetone (1:1) for 1 min on dry ice. They were subsequently overlaid with mα HA (Santa Cruz Biotechnologies, Santa Cruz, CA) for 2 h. After extensive washing, a rhodamine-conjugated goat anti-mouse secondary antibody was added and the cell nuclei were later counterstained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride hydrate) (Sigma). The coverglasses were mounted on microscopy slides and imaged on an epi-fluorescence microscope (Axiovert 200) with an Axiocam CCD camera and Axiovision software (Carl Zeiss, Inc.).

Co-Precipitation Assay

Gal4-tagged RIF1 (Gal-RIF1) and HA-tagged RARα (HA-RAR) were transfected into COS7 cells separately or in combination by standard Calcium phosphate precipitation method as described above. Twenty-four hours after transfection, the cells were lysed in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% NP-40, 1 mM DTT and protease inhibitor cocktail (Roche Pharmaceutical, Inc.)). Following centrifugation at 13 krpm for 10 min in cold room, both supernatant and pellet were dissolved in 2× SDS sample buffer. Equal volume percentage of pellet and supernatant were resolved on 8.5% SDS–PAGE, electro-transferred to nitrocellulose membrane and subjected for western blotting analysis.

Western Blot Analysis

Western blot assays were performed using standard protocols with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). In brief, the transferred blots were incubated overnight with blocking buffer (TBST + 2% non-fat dry milk). After extensive washing with TBST, the blots were hybridized with primary antibodies (1:1,000 dilutions for the mouse anti-Gal DBD monoclonal antibody (Sigma-Aldrich, San Louis, MO); 1:500 dilutions for anti-HA polyclonal or monoclonal antibody (Santa Cruz)) followed by incubation with 1:2,000 dilution of HRPT-conjugated secondary antibodies against mouse or rabbit IgG (Sigma-Aldrich, San Louis, MO) accordingly. The proteins were detected using enhanced chemiluminescence as recommended by the manufacturer (Amersham Biosciences). The other antibodies used in this reports were mαflag (Sigma), rαlaminA/C (Cell Signaling), mαGAPDH (Calbiochem), rαRARα and mαβ-actin (Santa Cruz Biotechnology).

Biochemical Fractionation

Cells were biochemically fractionated according to a protocol previously described [Merriman et al., 1995], with minor modifications. MCF7 cells grown on 100 mm plates were transfected with 10 μg of empty vector or pCMX-HA-RIF1(472–739) construct and subcellular fractions were prepared 40 h post transfection. For subcellular fractionation, cells were collected in ice-cold 1× PBS containing 1× complete protease inhibitors (Roche Molecular Biochemicals). Cell pellets were resuspended in 300 μl CSK buffer (100 mM NaCl, 0.3 M sucrose, 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, pH 6.8) for 10 min on ice, before subjected to centrifugation at 600*g* for 5 min. The supernatant containing cytosolic proteins was removed and stored in aliquots at −70°C. The nuclei pellet was extracted with 300 μl digestion buffer (50 mM NaCl, 0.3 M sucrose, 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, pH 6.8) containing 400 U DNase I (Roche Molecular Biochemicals) for 30 min at room temperature. Two molar ammonium sulfate was then added to the mixture to a final concentration of 0.25 M, incubated for 5 min at room temperature and centrifuged at 600*g* for

5 min. The soluble nuclear proteins (chromatin fraction) were removed and insoluble nuclear-matrix-intermediate filament fraction (NM-IF) was then boiled in 300 μl of direct lysis buffer containing 2% SDS for 5 min. The same volume percentage of each fraction was analyzed by 10% SDS–PAGE.

RESULTS

Isolation of RAR-Interacting Proteins by Yeast Two-Hybrid Screening

We performed a yeast two-hybrid screen of a human B cell cDNA library using the hRARα as bait and identified several positive clones. The RIF1 clone was the strongest hRARα interacting protein among the isolated clones. RIF1 was tested again with both RARα and another member of nuclear receptor RXRα as baits in the yeast two-hybrid assay. In initial studies, β-galactosidase assays demonstrated that coexpression of Gal4 AD-RIF1 with Gal4 DBD-RARα fusion proteins strongly activated the reporter gene expression in the absence of ligand (data not shown). In contrast, co-expression of Gal4 AD-RIF1 with Gal4 DBD-hRXRα did not activate reporter gene expression, suggesting that in the absence of ligand RIF1 interacts specifically with RARα but not with RXRα.

To determine if the RIF1-RAR association in yeast is a result of a direct physical interaction, we conducted a Far-Western assay using ^{35}S-labeled hRARα and immobilized GST or GST-RIF1 yeast two hybrid fusion protein (GST-RIF1y2h). Coomassie blue staining detected an intact GST-RIF1 fusion protein of about 65 kDa along with several degradation products (Fig. 1A). Far-Western assay using ^{35}S-labeled hRARα showed that RARα bound to intact GST-RIF1 fusion protein, but not to the GST alone or any of the apparent degradation products (Fig. 1A). These data suggest that RIF1 directly interacts with RARα in vitro. We tested the in vitro interaction further using a GST-pull down assay (Fig. 1B) and found that GST-RIF1y2h, but not GST alone, strongly bound to RARα. In contrast, GST-RIF1y2h did not pull down RXRα in this assay (Fig. 1B), consistent with the yeast two-hybrid interaction results. Addition of hormones (atRA for RARα and 9-cis RA for RXR) did not alter the interaction of RIF1 with RAR or RXR in these GST pull-down assays (data not shown). Taken together, these

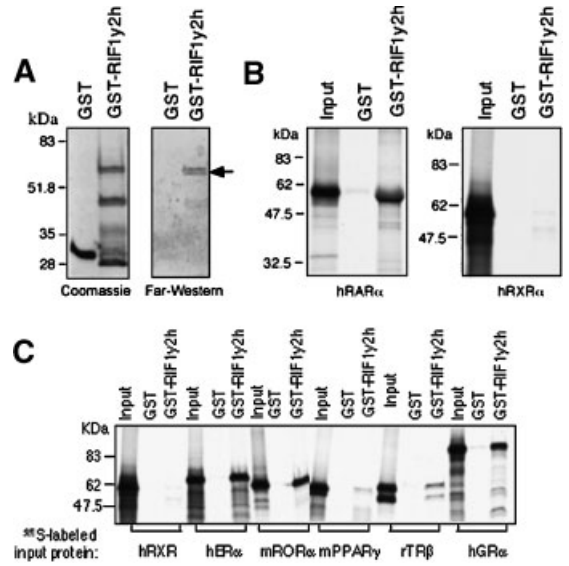

Fig. 1. Interaction of RIF1 with RARα in vitro. **A**: Far-Western assay was conducted with GST and GST-RIF1y2h proteins hybridizing with in vitro translated ^{35}S-hRARα. The arrow points to the intact GST-RIF1y2h fusion protein that binds to ^{35}S-hRARα. The amount of purified GST and GST-RIF1 fusion proteins used in the assay are shown by Coomassie blue stain on the left. **B**: GST pulldown assays were performed using in vitro translated ^{35}S-hRARα (left) or ^{35}S-hRXRα (right) as probe incubated with either GST or GST-RIF1y2h fusion protein. Input represents 20% of the total probes used in each pull down reaction. **C**: RIF1 interacts with members of nuclear receptor superfamily in GST-pull down assay. A number of ^{35}S radiolabeled nuclear receptor proteins including ER, RORα, PPARγ, TRβ, GRα, and RXR were translated in vitro and incubated with GST or GST-RIF1y2h in GST pull down assay.

data indicate that RIF1 interacts directly with RARα in a yeast two hybrid assay and in vitro in a hormone-independent manner.

We then tested whether RIF1 interacts with other members of the nuclear receptor superfamily. GST-pull down assays using GST-RIF1y2h and a number of ^{35}S-labeled nuclear receptors showed that RIF1 interacts strongly with ERα, mRORα and GRα, weakly with TRα and PPARγ and not at all with RXR (Fig. 1C). Addition of cognate hormones for these various receptors did not alter their interactions with RIF1 (data not shown). These results demonstrate that RIF1 interacts with multiple nuclear receptors, albeit with potentially different affinities, in a hormone-independent manner.

Determination of the Amino Acid Sequence and the Expression Pattern of RIF1

The full-length RIF1 cDNA was next isolated by a combination of cDNA library screening and

analysis of existing EST sequences in the GenBank. cDNA library screening using the RIF1 y2h DNA fragment as a probe resulted in the isolation of a 3.5-kb clone. Sequence analysis indicated that this 3.5-kb clone encoded for a protein of 739 a.a. residues, corresponding to full-length RIF1 product (Fig. 2A). The original RIF1 y2h clone was then designated as RIF1 (472–739), which encodes the C-terminal 267 amino acids of the full length RIF1 (Fig. 2A). Assembly of the overlapping RIF1 EST clones resulted in the prediction of an open reading frame of 739 amino acids as well. In vitro translation of RIF1 produced a single polypeptide of about 85 kDa (data not shown), consistent with the estimated molecular weight of 81.2 kDa predicted from the RIF1 amino acid sequence. Blastp search of the protein database revealed similarity of RIF1 to only several hypothetical proteins such as the FLJ11269 (GenBank accession number NP_060842), which maps to the chromosome 1p12 region. Analysis of the RIF1 amino acid sequence through PSORT (http://psort.nibb.ac.jp) revealed a putative bipartite nuclear localization signal at residues 598–615 (Fig. 2A, underlined). In addition, a short sequence containing a potential hydrophobic heptad repeat was found at the C-terminus of RIF1 (Fig. 2A, boxed residues). The heptad repeat region is predicted

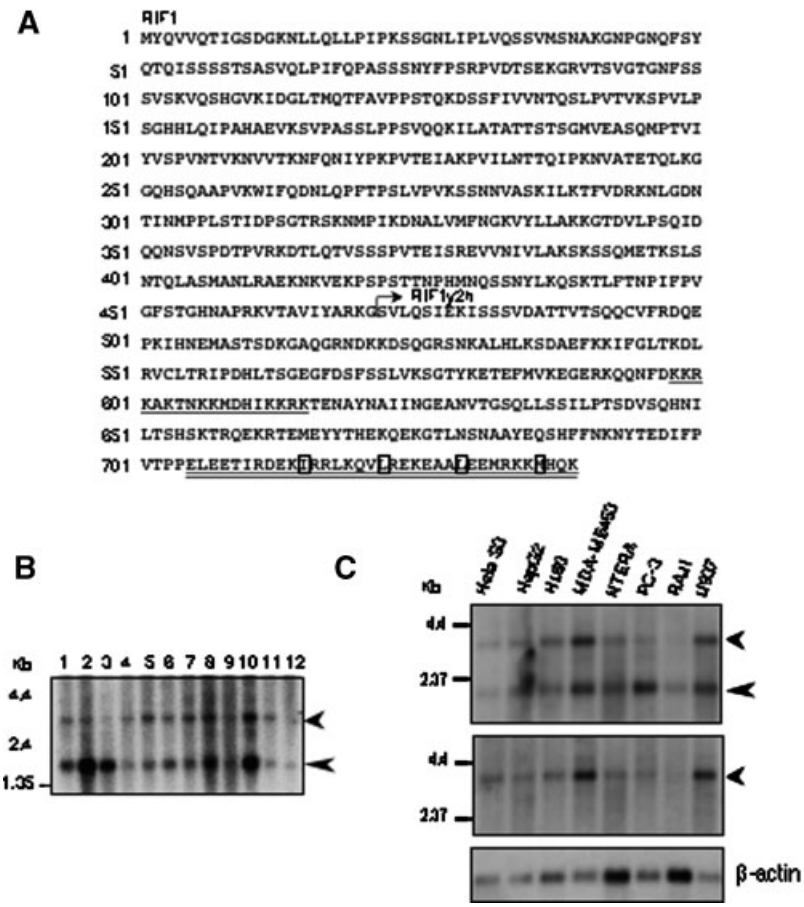

Fig. 2. Amino acid sequence and expression of RIF1. **A**: Amino acid sequence of RIF1. The full-length RIF1 encodes a 739-amino acid protein with an estimated molecular weight of 81,238 Da. The RIF1 yeast two-hybrid (RIF1y2h) clone starts from residue 472 to the C-terminal end. The potential bipartite nuclear localization signal is underlined. The four hydrophobic residues in a hydrophobic heptad repeat are shown in boxes with the predicted helical region double underlined. **B**: Northern blot analysis detected RIF1 expression in human tissues as two distinct transcripts (3.5-kb and 1.8-kb respectively) using a 800 bp RIF1 fragment encoding from a.a. 472 to 739 as a probe. Lanes 1, brain, 2; heart, 3; skeletal muscle, 4; colon, 5; thymus, 6; spleen, 7; kidney, 8; liver, 9; small intestine, 10; placenta, 11; lung, 12; peripheral blood leukocytes. Each line contains equal amount of β-actin signal. **C**: RIF1 is expressed in multiple cancer cell lines as indicated in Northern blot assays using RIF1. cDNA probes corresponding either to the C-terminus RIF1 a.a. 472–739 (top panel) or to the N-terminus a.a. 1–470 (middle panel). The bottom panel showed the expression of β-actin in each lane as control for the loading.

to form an amphipathic alpha helical wheel starting from amino acid 705 to the C-terminal end (Fig. 2A, double underlined). Such amphipathic alpha helical wheels have been shown to act as protein–protein interaction domains in other proteins.

The expression profile of RIF1 was analyzed in multiple human tissues by Northern blot analysis using a ^{32}P labeled RIF1 C-terminal DNA fragment as a probe. RIF1 is expressed in most human tissues (Fig. 2B), with the highest expression levels in heart, liver, and placenta. Two distinct RIF1 transcripts of 3.5 and 1.8-kb were observed in most tissues. The 3.5-kb message (Fig. 2B, arrow head) is less abundant in skeletal muscle, whereas the 1.8-kb message is very limited in lung and colon (Fig. 2B, narrow arrow head). The existence of two RIF1 transcripts detected on the Multiple Tissue Northern blot was further confirmed in Northern blot analysis of expression of RIF1 in multiple human cell lines using RIF1 C-terminal or N-terminal cDNA fragments as a probe. The C-terminal RIF1 probe detected both of the distinct RIF1 transcripts of 3.5 and 1.8-kb in these cell lines (Fig. 2C, top panel). When using an N-terminal cDNA fragment (encoding a.a. 1–470) as a probe in the Northern blot assay, only the full length RIF1 transcript was detected (Fig. 2C, arrowhead in middle panel).

Interaction of RIF1 With RARα Through the C-Terminal Domain

The yeast two hybrid (y2h) RIF1 clone RIF1 (472–739) fragment was isolated as an RAR interacting protein, indicating that the RAR interacting domain of RIF1 resides at its C-terminus. To determine more specifically the amino acid sequence of RIF1 involved in the interaction with RARα, we generated various GST-RIF1 deletional fusion proteins. GST pull down assays showed that the GST-RIF1 (472–739) interacted with RARα in the absence or presence of its ligand atRA. Secondary domain structure predictions revealed that the C-terminus of RIF1 contains a potential hydrophobic heptad repeat located at region from a.a. 705 to 739. Since hydrophobic heptad repeats often mediated protein–protein interactions, we determined whether the potential heptad repeat of RIF1 is important for its interaction with RARα. To our surprise, the C-terminal domain of RIF1 (amino acid 637–739) containing the heptad repeats failed to interact with

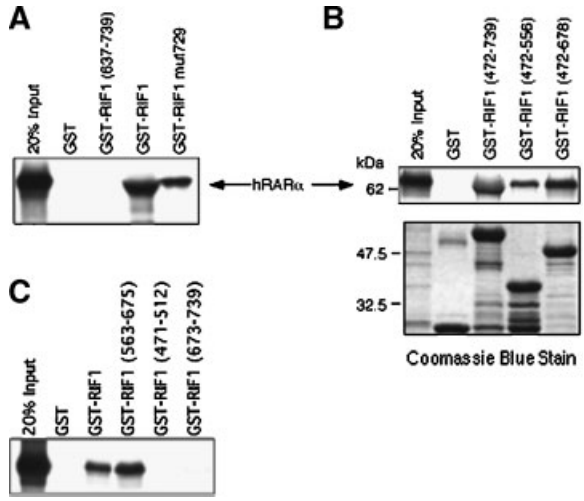

Fig. 3. The interacting domain of RIF1 with RAR is located at C-terminus. **A**: GST-pull down assays were conducted by incubating in vitro translated ^{35}S-labeled RARα with GST or GST fusion of RIF1 mutants: GST-RIF1 (472-739), GST-RIF1 (637-739) that covers the region that contains potential helical wheel, and GST-RIF1mut729 is GST-RIF1 (472–739) with a mutation of Leu to Pro change at position. **B**,**C**: GST or GST fusions of RIF1 deletion mutant proteins were used to pull down the in vitro translated ^{35}S-labeled hRARα in GST pull down assays. The bottom panel of (B) is Coomassie blue stain showing equal GST protein loading.

RARα (Fig. 3A). In addition, mutation of the Leucine at position 729 to a Proline, (RIF1mL729P), predicted to disrupt the helical structure of this peptide, did not affect the interaction of RIF1 with RARα. These data support that the putative C-terminal leucine heptad repeats are not involved in mediating RIF1 interaction with RARα. To further define the RIF1-hRARα interaction domain, we generated two additional GST-RIF1 fusion proteins: GST-RIF1 (472–556) and GST-RIF1 (472–678). As shown in Figure 3B, both of these GST-fusion proteins bound hRARα. These results suggest that the interaction domain of RIF1 resides in the region from amino acid 472–678. Two additional smaller GST-RIF1 mutants were then generated: GST-RIF1 (471–512) and GST-RIF1 (507–674). GST-RIF1 (507–674) showed a strong interaction with RARα; while GST-RIF1 (471–512) failed to interact with RARα at all, supporting that the domain in RIF1 interacting with RARα is located in the region between amino acids 512 and 674.

RIF1 is a Nuclear Protein

RIF1 is predicted to be a nuclear protein with 86% confidence using the PSORT program,

consistent with the finding of a potential bipartite nuclear localization signal within the RIF1 amino acid sequence. To assess whether RIF1 is indeed a nuclear protein, and to determine the importance of the potential nuclear localization signal in nuclear localization, we analyzed the subcellular distribution of RIF1 by indirect immunofluorescence following transient expression of RIF1 in HeLa cells. Figure 4 shows that the wild-type (full length) RIF1 protein is localized exclusively in the nuclei of transfected HeLa cells, and excluded from nucleoli, confirming that RIF1 is a nuclear protein. The importance of the nuclear localization signal was then investigated by creating site-directed mutations of the two KKRK basic motifs in the bipartite NLS predicted between a.a. 598 and 615 into the sequence AAAK, either separately or in combination. Mutant proteins were expressed in HeLa cells and visualized by indirect immunofluorescence. We found that disruption of the N-terminal KKRK motif (m598) had a very modest effect on RIF1 nuclear localization, leaving the majority of the expressed RIF1 mutant protein still in the nucleus. However, when compared with the wild type protein, a higher amount of cytoplasmic staining was observed, suggesting the N-terminal basic sequence makes at least a modest contribution to RIF1 nuclear localization. In contrast, disruption of the C-terminal KKRK motif (m612) abolished RIF1 nuclear localization completely such that the transfected mutant RIF1 protein was uniformly distributed throughout the cells. As expected, mutations of both motifs (m598/m612) also abolished the nuclear localization of RIF1 completely. These data demonstrate that the predicted bipartite its nuclear localization signal in RIF1 is responsible for its nuclear localization, and that the C-terminal KKRK motif (a.a. 612–615) is substantially more important than the N-terminal motif KKRK for RIF1 nuclear localization.

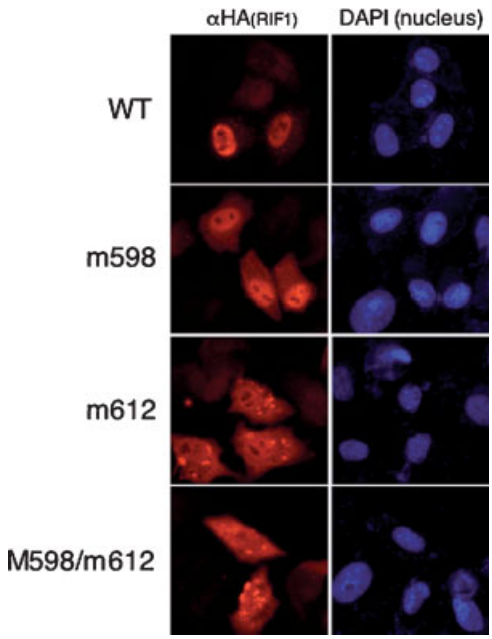

Fig. 4. RIF1 is a nuclear protein. The HA-tagged wild type RIF1 (WT) and the three nuclear localization mutants (m598, m612, and m598/m612 double mutant) in mammalian expression vector were transfected into HeLa cells. The distribution of RIF1 proteins were analyzed by indirect immunofluorescence staining with anti-HA monoclonal antibodies and rhodamine-conjugated secondary antibodies (red color). The cell nuclei were stained with DAPI (blue color). Please note that in each panel there were non-transfected cells with background HA staining.

RIF1 is a Nuclear Matrix Associate Protein That Interacts With RARα In Vivo

Preliminary cell fractionation studies in a variety of buffer conditions supported a strong association between the original y2h clone RIF1 (472–739) and the nuclear pellet; thus led us to speculate that RIF1 might be a nuclear matrix protein. To explore this further, we prepared cytosolic, chromatin and nuclear matrix fractions from MCF7 cells transfected with vector alone or with epitope-tagged-RIF1 (472–739) using an established sequential high salt extraction procedure [Merriman et al., 1995]. RARα protein is highly enriched in MCF7 cells. To confirm the fidelity of this extraction procedure, we analyzed the distributions of compartment-specific proteins such as lamin A/C, GAPDH and RARα in the various fractions by Western blot analysis (Fig. 5). Control studies showed that when MCF7 cells were transfected with vector alone, lamin A/C was detected exclusively in the nuclear matrix fraction, and GAPDH was found exclusively in the cytosolic fraction. Consistent with previous reports [Barrett and Spelsberg, 1999; DeFranco and Guerrero, 2000], RARα was detected in all three fractions, with the highest abundance in the soluble chromatin fraction. Using this approach, we observed RIF1 (472–739) to be localized exclusively to the nuclear matrix fraction when transfected into MCF7 cells (Fig. 5). Intriguingly, overexpression of RIF1 (472–739) altered the distribution of RARα such that the large majority of RARα shifted from the

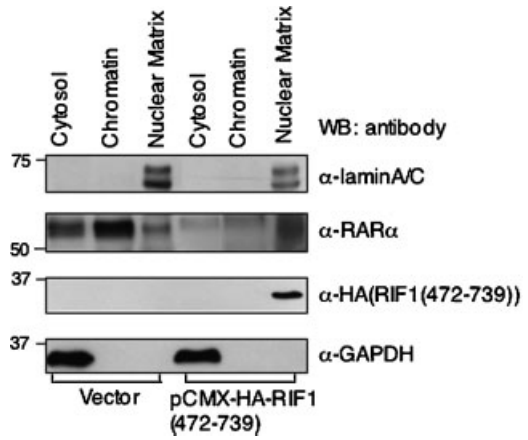

Fig. 5. RIF1 is a nuclear matrix protein. MCF7 cells transfected with vector alone or pCMX-HA-RIF1 (472–739) were biochemically fractionated into cytosol, chromatin and nuclear matrix fractions. Equal volume percentage of each fraction was analyzed by 8.5% SDS–PAGE and subjected to Western blotting with antibodies against Lamin A/C, RARα, HA, and GAPDH, respectively.

cytosolic and chromatin fractions to the nuclear matrix fraction (Fig. 5, HA-RIF1 (472–739) lanes). These results support that RIF1 is a nuclear matrix associated protein and that RIF1 can recruit RARα to the nuclear matrix fraction through their identified protein–protein association.

The recruitment of RAR to nuclear matrix fraction by RIF1 was further explored in co-precipitation assays. Gal4-tagged RIF1 (472–739) and HA-tagged RARα (HA-RARα) were transfected in COS7 cells alone or in combination. The relative amount of Gal4-RIF1 (472–739) and HA-RARα in the supernatant versus pellet fractions was determined by Western blotting with anti-Gal4 and anti-HA antibodies, respectively. As illustrated in Figure 6A, most RIF1 (472–739) protein was found in the pellet portion of the cell extracts, while RARα proteins were mainly in the supernatant when expressed in the absence of RIF1. When HA-RARα was co-transfected with RIF1 (472–739), a significant amount of RARα shifted to the pellet. Addition of the RARα ligand atRA in the cultured cells did not change the distribution pattern (data not shown). However, RIF1 failed to recruit RXR into the pellet (Fig. 6B), consistent with the yeast two-hybrid and in vitro GST pull down assays above.

Interactions between RIF1 and RARα were further confirmed by immunofluorence assay in transfected COS7 cells. COS7 cells were transfected with mammalian expression plasmids

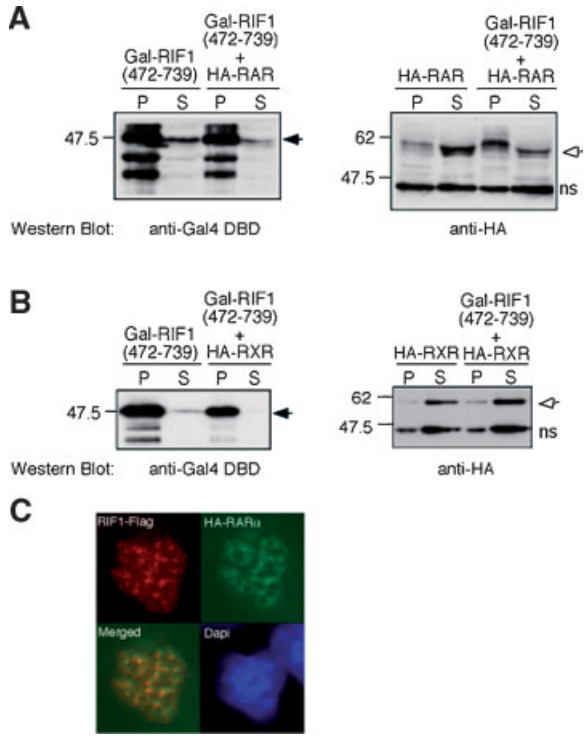

Fig. 6. RIF1 interacts with hRARα in vivo. **A**: COS7 cells transfected with Gal-tagged RIF1 (472–739) and/or HA epitoped hRARα were lysed in lysis buffer and ''co-precipitated'' as described in Materials and Methods. The pellet and the supernatant were dissolved in 2× SDS sample buffer. Equal volume percentage of pellet and supernatant were loaded on to 8.5% SDS–PAGE. RIF1 (472–739) and RARα proteins were detected by Western blotting with Gal-DBD antibody (left panel) or HA antibody (right panel). P indicates pellet portion and S indicates supernatant portion of the lysis. ''ns'' stands for non-specific band as a natural loading control. The solid arrow indicated the position of RIF1 (472–739) protein while the open arrowhead pointed the position of RARα. **B**: Co-precipitation experiment was performed by centrifugation of COS7 cell lysates transfected with Gal-tagged RIF1 (472–739) and HA-tagged RXR. RIF1 (472–739) and RXR proteins were detected by Western blot analysis with anti-Gal-DBD (left) and anti-HA (right) antibodies. The open arrowhead here indicated the position of RXRα. **C**: Immunofluorences microscopic analysis of RIF1/RAR expression pattern in COS7 cells. CMX-RIF1-flag and CMX-HA-RARα plasmids were cotransfected into COS7 cells. The RIF1 and RAR proteins were detected with mouse anti-flag and rabbit anti HA, and visualized with Cy3-conjugated anti-mouse (red) and FITC-conjugated anti-rabbit (green), respectively. The colocalization of RIF1 with RAR was shown as ''merged'' (yellow). Dapi stains for the position of nuclei.

expressing flag-tagged RIF1 and HA-tagged RARα. Both RIF1 and RARα showed nuclear localization: the majority of transfected cells showed near homogenous nuclear expression, while about 10% of transfected cells showed speckled patterning (Fig. 6C). When merged, RIF1 colocalized with RARα in the nucleus of

those cells. The colocalization of full length RIF1 with RARα was also observed in transient transfection assays when tagged RIF1 and RARα were overexpressed in COS7 cells (data not shown).

RIF1 Possesses an Intrinsic Transcriptional Repression Activity Located at C-terminus

Since RIF1 is a nuclear protein that interacts with RARα, we speculated that RIF1 might regulate the transcriptional activity of RARα. We first linked RIF1 to a GAL4 DNA binding domain in a mammalian expression vector and examined the effect of RIF1 on a Gal4-responsive reporter. As shown in Figure 7A, full length RIF1 possesses strong transcriptional repression activity. RIF1 repression activity increased in a dose-dependent fashion, with maximal levels of repression of 60- to 70-fold in this assay. We further defined the RIF1 repression domain by fusing different fragments of RIF1 to the Gal4 DBD and testing repression activity in this assay (Fig. 7B). Full length RIF1 repressed 95% of basal DBD activity, while the RIF1 domain a.a. 472–739 showed 63% reduction of basal DBD activity. Gal-RIF1 (1–512), which represents the N-terminus of RIF1, exhibited around 20% reduction in activity. These results indicate that the RIF1 repression domain resides mainly at the C-terminus of RIF1. We next divided RIF1 (472–739) into two two fragments: RIF1 (472–634) and RIF1 (638–739), and tested the repression activities of these two fragments of RIF1. RIF1 (472–634) did not repress RIF1 basal activity, while RIF1 (638–739) repressed 85% of the basal activity in this Gal4/Gal4-DBD-luciferase reporter transactivation assay, supporting that the main repression domain of RIF1 is localized to the C terminal region of the protein from a.a. 638 to 739.

Histone deacetylation is the best-characterized mechanism underlying transcriptional repression by corepressors [Xu et al., 1999]. We therefore examined whether Trichostatin A (TSA), a potent histone deacetylase inhibitor, had any effect on the RIF1 repression activities. As shown in Figure 7C, addition of TSA partially relieved the repression activity of Gal-RIF1 (638–739). The activity of RIF1 (638–739) in presence of 100 nM TSA increased 4.4-fold as compared to control and to domains of RIF1 lacking repression activity (Fig. 7C). These results support that histone deacetylation accounts at least in part for RIF1-mediated repression.

The involvement of histone deacetylation in RIF1 repression was further explored in GST-pull down assays with different classes of HDACs. Three different HDACs, representing two classes of HDACs (HDAC2/HDAC3 in class I, HDAC4 in class II) were studied. As shown in Figure 7D, RIF1 (472–739) showed strong interaction with all three HDACs tested, while no interactions between GST alone and the in vitro translated HDAC2, HDAC3 and HDAC4 proteins were detected. Together, these data suggested that RIF1 possesses a strong transcriptional repression domain located at the distal C-terminal 100 amino acids (a.a. 638 to 739) and that RIF1 repression is mediated in part through recruitment of HDACs.

RIF1 Inhibits Transcriptional Activation by RARα

The ability of RIF1 to repress transcription and to interact with RARα led us to predict that RIF1 might function as a transcriptional repressor for RARα. To test this possibility, we assayed the ligand-dependent transcriptional activity of RARα on a consensus RAR response element-driven reporter in the presence or absence of RIF1 (Fig. 8A). Co-expression of RIF1 dramatically inhibited ligand-induced transcriptional activation by RARα in a dose-dependent manner, while RIF1 alone had no significant effect on the basal promoter activity in the absence of ligand (data not shown). The decrease in RARα transcriptional activation was not caused by decreased expression of RARα protein (Fig. 8A, bottom). The observation that RIF1 repressed ligand dependent RARα transactivation was specific, as RIF1 had little effect on the transcriptional activation of VP16 (Fig. 8B). Taken together, these data support that RIF1 functions as a transcriptional inhibitory factor for RARα.

DISCUSSION

Transcriptional activities of nuclear receptors are mediated by recruiting coregulators through direct protein–protein interactions. Various modifications including ubiquitination, sumoylation, acetylation, methylation, and phosphorylation have been implicated in regulating NR signaling, either through modifying

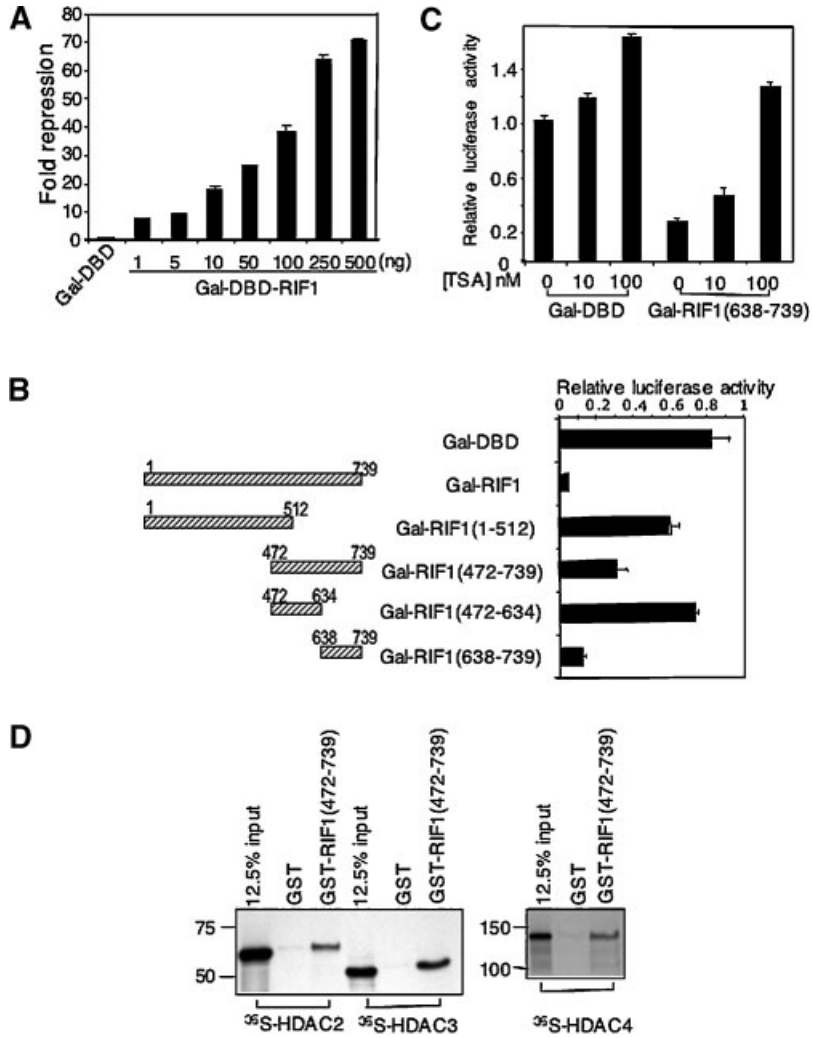

Fig. 7. RIF1 represses basal transcription. **A**: Luciferase reporter activities was determined in 293T cells transfected with plasmids encoding Gal4-RIF1 (full length RIF1 fused to GAL-DBD), GAL4 DBD, along with the MH100-TK-Luc reporter construct. The amounts of plasmid DNAs used in each transfection were as indicated (in ng). Data presented was a representative of three independent experiments. Values are expressed as the fold repression in luciferase activity in the presence of GAL-RIF1 expression plasmids to that of Gal-DBD. **B**: RIF1 domain from amino acid residues 638–739 repressed DBD basal transcriptional activity as determined by reporter gene analysis. Left panel is the schematic representation of RIF1 deletion mutants that fused with Gal-DBD in mammalian expression vector. The number represented the start and end of amino acids of each RIF1 fragment. Fifty nanograms of CMX-Gal-RIF1 deletion constructs were transiently transfected into 293T cells on 12-well plate, together with 400 ng of MH100-tk-Luc reporter construct and 100 ng RSV-β-gal as internal control. **C**: TSA partially reversed RIF1 repression activities on Gal-DBD reporter gene activities transfected in 293T cells. The concentration of TSA used in cultured cells was 0, 10, and 100 nM. The repression domain fragment from amino acids 638 to 739 was fused to GAL-DBD domain in mammalian expression vector under CMV promoter (CMX-Gal-RIF1(638-739)). CMX-Gal-DBD plasmid was used as a control. **D**: RIF1 interacted with various HDACs in GST-pull down assay. HDAC2, HDAC3 and HDAC4 were ^{35}S-labeled and translated in vitro. GST or GST-RIF1 (472–739) was incubated with respective HDACs in the binding buffer and bound proteins were resolved in 8.5% SDS–PAGE and detected by autoradiography.

chromatin structure or NR coregulator protein stability and/or cytoplasmic-nuclear shuttling (for reviews see [Baek and Rosenfeld, 2004; Wu et al., 2005]). Here, we report the cloning and characterization of a novel nuclear matrix-associated protein RIF1 that has intrinsic repression activity and that can repress RARα-mediated transcriptional activation.

The nuclear matrix is a filamentous nuclear structure composed of lamins (A/C and B),

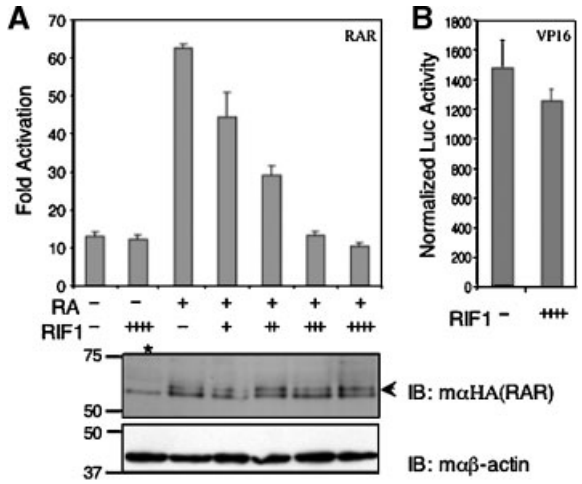

Fig. 8. RIF1 inhibits transcriptional activation by RARα. **A**: Reporter gene activities were analyzed for HEK293T cells transfected with pCMX-HA-RARα, a RARE-driven luciferase reporter and increasing amount of pCMX-RIF1α. The "-" signs indicate no RA treatment or no pCMX-RIF1α transfected. The RA concentrations used were 1 μM (+). The amounts of pCMX-RIF1α used were: +, 0.01 μg; ++, 0.1 μg; +++, 0.5 μg; and ++++, 0.9 μg. The bottom panel is the Western blot analysis of the expression of HA-RARα and β-actin in lanes corresponding to each transfected cells (except the first lane with *). The first lane marked with * represented cells with no pCMX-HA-RARα transfection as a control. β-actin expression was used to show equal protein loading. Arrow indicated the position of HA-RARα. **B**: RIF1 has minimal effect on reporter transcriptional activation by VP16. The VP16 AD was fused with Gal4 DBD and transfected into 293T cells together with MH100-TK-Luc reporter. The relative luciferase activity was determined from three independent transfections. The amount of pCMX-RIF1α used was the same as in (A).

intermediate filament proteins, ribonucleoproteins and a variety of nuclear matrix associated proteins. The function of this intranuclear macromolecular scaffold has been linked to chromatin modification, DNA replication and repair, gene transcription and RNA splicing [Bode et al., 2000; Stein et al., 2000]. A variety of transcription factors, including nuclear hormone receptors, SatB1, ATF, OCT-1, Pit-1, RunX/AML, and AP-1 have been shown to be associated with the nuclear matrix dynamically, depending on cell growth/differentiation status. We identified a novel nuclear matrix protein RIF1 as a RAR interacting protein via yeast two hybrid screen. Amino acid sequence analysis indicates that RIF1 is a highly charged basic protein with an isoelectric point of 10.4. When cells were lysed in lysis buffer, with a pH of 7.5, RIF1 protein would readily form an aggregate in the insoluble portion (precipitate), even at high salt conditions of 400 mM of NaCl. Because of the nature of the RIF1 protein, we failed to demonstrate the interaction of RIF1 with RARα in the soluble lysates using coimmunoprecipitation analysis. However, we observed an increased amount of RARα protein presented in the insoluble portion when coexpressed with RIF1 in mammalian cells. RIF1 could not bring RXR into the insoluble precipitate, suggesting that the in vivo association of RIF1 with RARα is specific. Further biochemical fractionation experiments demonstrated that RIF1 is associated with the nuclear matrix, and overexpression of RIF1 recruited RARα from a predominantly chromatin fraction to nuclear matrix fraction (Fig. 5). Protein motif search did not reveal homology of RIF1 to the known nuclear matrix targeting sequence (NMTS). However, NMTS is not well defined and many nuclear matrix associate proteins do not contain this NMTS. We have mapped RIF1 nuclear matrix targeting domain to the C-terminal region, which is separate from the receptor interacting domain and the transcriptional repression domain (data not shown).

The y2h RIF1 protein fragment (RIF1 (472–739)) was easily detected with predominant nuclear staining in immunofluorence microscopic assays when pCMX-HA-RIF1 (472–739) was overexpressed in COS7 cells with over 70% transfection efficiency (data not shown). However when full length RIF1 (pCMX-HA-RIF1 (1–739)) was transfected in COS7 cells with comparable transfection efficiency, less then 5% cells showed weak nuclear staining with a speckled pattern (data not shown). We were unable to detect the full-length HA-tagged RIF1 protein by Western blot using whole cell lysates from transfected COS7 cells lysed directly in SDS-sample buffer. However, when cells tranfected with RIF1 full length plasmid were pretreated with a proteasome inhibitor, MG132, HA-RIF1 full length protein could be detected as an 83 kDa protein (data not shown). These results suggest that the full-length RIF1 might be tightly regulated by ubiquitin/proteasome pathway. A group of important labile transcription factors have been reported, including p53, snail, p160 SRC3, cMyc, and HIF-1 [Muratani and Tansey, 2003; Shao et al., 2004; Zhou et al., 2004; Li et al., 2006]. The unstable character of these proteins is critical in the control of cellular homeostasis. We have define the region that convene the instability of RIF1 to its N-terminal domain from a.a. 1 to 512; as RIF1 (1–512)

protein can barely be detected while the RIF1 (472–739) protein expresses well when over-expressed in mammalian cells (data not shown), suggesting a function for RIF1 N-terminus in regulating RIF1 stability.

RIF1 contains strong intrinsic repression activity. We have mapped the repression domain to the last 100 amino acid sequence including a putative leucine heptad repeat structure. We determined that the repression domain of RIF1 interacts with histone deacetylases (HDACs). In addition, the specific HDAC inhibitor TSA was able to relieve partially RIF1 repression in cultured cells, suggesting chromatin modification contributes to RIF1 repression mechanism. However, the involvement of histone deacetylases does not exclude the existence of other possible repression mechanisms.

We have demonstrated that RIF1 recruiting HDACs via direct protein–protein interaction contributed to its repression mechanism. However, multiple possible mechanisms can be involved in RIF1 repression of liganded nuclear receptor activities. Our preliminary studies showed that RIF1 interacts with ER-DBD domain (179–285) (unpublished data). It is possible that RIF1 binding to the DBD domain affects NR binding to its targeting promoter and sequesters NRs to the nuclear matrix. It is also possible that the interaction of RIF1 with liganded RARα might trigger the proteolysis of the receptor, thereby decrease the effective concentration of RARα. The possibility is supported by the experiment described in Figures 5 and 8. Although increased concentrations of RIF1 did not affect the exogenous over-expression levels of the RARα protein levels in the western blot assay using the whole cell lysates (Fig. 8), we observed a more degraded endogenous RARα in nuclear matrix fraction after biochemical fractionation assays when RIF1 was transfected in MCF7 cells that contain high levels of endogenous RARα (Fig. 5). This observation supports the hypothesis that RIF1 may induce RAR degradation to reduce RAR-mediated transcriptional activity. We also cannot exclude the possibility that RIF1 binding to the liganded NR, which results in the distortion of the NR structure and thus is unfavorable for recruiting nuclear receptor coactivators for the activation.

RIF1 interacts with liganded nuclear receptors including RARα, GR, and ER (Fig. 1C). Our studies indicate that RIF1 can inhibit ligand-dependent transcriptional activation of RARα (see Fig. 8) and ERα (unpublished observation). Ligand induced cell differentiation processes involve not only activation of differentiation specific genes, but also repression of genes involved in cell proliferation. Transcriptional repression is an important mechanism in regulating growth and development [Frasor et al., 2003; Weston et al., 2003; Privalsky, 2004; Kawai-Kowase and Owens, 2006]. Therefore, identification of corepressors for ligand bound NRs provides insights into hormone regulated transcriptional repression mechanisms. More evidence documents that transcriptional activation is a dynamic process, involving many cofactors cycling on the target promoter in a temporal and sequential manner [Hager et al., 2004; Ismail and Nawaz, 2005]. However, not many studies have been done in liganded nuclear receptor-mediated transcriptional repression. It is equally possible that RIF1 belongs to a growing family of corepressors that mediate transcriptional repression in a sequential and dynamic cycling fashion.

In summary, we have identified and characterized a novel nuclear matrix protein that inhibits the ligand-induced transcriptional activity of RARα. RIF1 contains three important characteristics that qualify it as a transcriptional inhibitory factor. First, RIF1 interacts directly with liganded RARα; Second, RIF1 contains intrinsic transcriptional repression activity; and third, RIF1 inhibits transcriptional activation by liganded RARα. Furthermore, we demonstrate that RIF1 represses RARα activity via direct recruitment of HDACs. The discovery of RIF1 as a nuclear matrix protein support our hypothesis that, under environment stimuli, nuclear matrix protein RIF1 recruits RARα and HDAC to create a microenvironment/scaffold of a higher organization of chromatin to repress NR-targeted promoter activation.

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to M.M. Data deposition: The GenBank accession number of the RIF1 sequence: AY190122.

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