0888-8809/01/$03.00/0
Printed in U.S.A.

Molecular Endocrinology 15(8):1318–1328

The Orphan Nuclear Receptor TR2 Interacts Directly with Both Class I and Class II Histone Deacetylases

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A combination of *in vivo* and *in vitro* assays was employed to describe the ligand-independent interaction of the orphan nuclear receptor TR2 and histone deacetylase proteins. The repressive effect of TR2 on transcription of a luciferase reporter driven by a promoter containing a direct repeat-5 (DR5) derived from the human RARβ gene was suppressed by the addition of the histone deacetylase inhibitor trichostatin A. Immunoprecipitation with FLAG-epitope (MDYKDDDDK)-tagged histone deacetylase proteins was used to demonstrate that TR2 and histone deacetylases 3 or 4 are present in the same immunoprecipitated complex. Deacetylase activity was demonstrated for these coimmunoprecipitates, further confirming the *in vivo* interaction of TR2 and histone deacetylases. Immunoprecipitation with anti-TR2 antibody was used to demonstrate interaction of TR2 with endogenously expressed histone deacetylases 3 and 4 in COS-1 cells. Dissection of TR2 domains showed that the DNA binding domain of the receptor was responsible for interaction with both histone deacetylases 3 and 4 in glutathione-*S*-transferase pull-down assays, while the ligand binding domain did not interact. The pull-down data were confirmed with far Western blots that also showed a direct interaction between labeled histone deacetylase proteins and TR2. It is suggested that repression mediated by unliganded TR2 is mediated, in part, by a direct interaction of this receptor with histone deacetylase proteins. (*Molecular Endocrinology* 15: 1318–1328, 2001)

NUCLEAR RECEPTORS COMPRISE a superfamily of transcription factors that regulate gene expression by binding to DNA target sequences through a zinc-finger type DNA binding domain (DBD) (1–5). While some of these nuclear receptors are known hormone receptors, a large number of cloned nuclear receptors remain as orphan members. Despite the lack of identified ligands, several orphan receptors have been shown to play important roles in animal physiology as demonstrated in genetic knockout studies (6–9).

The mouse orphan receptor TR2, isolated from an E8.5 embryonic cDNA library (10) is the mouse homolog of human TR2 that was cloned from a human prostrate cDNA library (11). The biological activity of TR2 was demonstrated as repressive in several heterologous reporter systems. These included reporters driven by a DR4 hormone response element derived from the mouse cellular retinoic acid binding protein I gene promoter (12), a DR1 type retinoic acid response element (RARE) derived from the cellular retinol binding protein II gene promoter (13), and a DR5 derived from the RARβ2 promoter (14). As TR2 was able to bind to these promoters with high affinity, it was proposed that the suppressive activity of TR2 in these reporter systems is mediated by competition with other receptors at DNA binding sites (14–16). By using a DR5 reporter as a model system, the functional characteristics of TR2 suppression have been examined. The molecular features of TR2 required for full suppressive activity included the DBD, the ability to dimerize, the ligand binding domain (LBD), as well as two adjacent glutamate residues (positions 553/554), and three adjacent leucine residues (positions 537–539) that are required for efficient DNA binding (14). In addition, TR2 was able to heterodimerize with orphan receptor TR4 (17) and to recruit nuclear receptor interacting protein 140 (18).

Recently, the regulatory activity of nuclear receptors on gene promoters has been demonstrated to be mediated by a large number of associate proteins called coactivators, corepressors, or coregulators (19–23). Current models of nuclear receptor action involve chromatin modification such as alteration in the acetylation status of histone proteins brought about by associated proteins of nuclear receptors (24). The repressive activity of apo-receptors is mediated by interaction with corepressors such as nuclear receptor corepressor/silencing mediator of retinoid and thyroid hormone receptor that recruit histone deacetylases (HDACs) to nuclear receptor complexes, thereby deacetylating histone proteins in the regulatory region of target genes (25). Upon ligand binding to the LBD, nuclear receptors undergo a conformational change causing the release of corepressors. The holo-recep-

Abbreviations: CMV, cytomegalovirus; DBD, DNA binding domain; FLAG-epitope, MDYKDDDDK; GST, glutathione-*S*-transferase; HDAC, histone deacetylase; LBD, ligand binding domain; PVDF; polyvinylidene difluoride; RA, retinoic acid; RARE, retinoic acid response element; RLU, relative light unit; TSA, trichostatin A.

tors are then able to interact with coactivator proteins, mainly the p160 family, which encode intrinsic histone acetyltransferase activity. The resulting acetylation of histones at target genes is believed to relax chromatin structure such that the transcription machinery is able to efficiently activate gene expression (26). Two major classes of HDACs have been cloned in higher eukaryotes. The yeast Rpd-3 homologs belong to class I HDACs (HDACs 1–3, and 8) and the yeast Hda1 homologs belong to class II HDACs (HDACs 4–7) (27–30).

In the current study, we examine whether the ability of the orphan nuclear receptor TR2 to act as a repressor involves recruitment of HDACs. We chose HDAC3 as a representative of class I HDACs and HDAC4 as a representative of class II HDACs. We have found that TR2 possesses the ability to interact directly with both of these HDAC proteins. This is a property that has not been reported for other orphan receptors or hormone receptors such as the RARs. The HDAC interacting domain was localized to the DBD portion of the receptor that encompasses the two zinc fingers present in the receptor. The repressive activity of TR2 was also found to be suppressed by the HDAC inhibitor trichostatin A (TSA). In addition, immunoprecipitation of both HDAC3 and HDAC4 yielded complexes that included TR2, and immunocomplexes precipitated with anti-TR2 antibody encoded HDAC activity. The combination of *in vivo* and *in vitro* data supporting a direct interaction between TR2 and HDACs suggests a role for HDACs in mediating TR2 repression.

RESULTS

In Vivo Association of TR2 and HDAC

Coimmunoprecipitation. Previously we, and others have demonstrated a strongly suppressive activity of TR2 on reporter gene expression driven by several direct repeat sequences (*e.g*. DR1, DR4, and DR5) (12–14). To investigate whether HDACs, enzymes responsible for deacetylating histone proteins, and the TR2 suppressive activity are related, we first examined the possibility that TR2 and HDAC proteins are present in the same immunocomplex. COS-1 cells were cotransfected with TR2 and FLAG-epitope (MDYKDDDDK)-tagged HDAC3 or FLAG-tagged HDAC4. An anti-FLAG monoclonal antibody (Sigma, St. Louis, MO) was then used to immunoprecipitate the transfected HDAC and associated proteins from the cotransfected cells. The anti-FLAG immunoprecipitates were resolved using SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then probed with anti-TR2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to detect the presence of TR2 in the HDAC immunoprecipitates. A 70-kDa band corresponding to TR2 is clearly shown for COS-1 cells cotransfected with TR2 and either FLAG-HDAC3 or FLAG-HDAC4 (Fig. 1A, lanes 7 and 9). In contrast, cells transfected with TR2, FLAG-HDAC3, or FLAG-HDAC4 alone showed no TR2 band (Fig. 1A, lanes 5, 6, and 8, respectively) when precipitation was done with the anti-FLAG antibody. In addition, as a negative control, a VP16-TR2 fusion construct containing only the TR2 LBD (18) was cotransfected with FLAG-HDAC3. In the anti-FLAG precipitated immunocomplex from these cells, the TR2-LBD could not be detected with the anti-TR2 antibody (Fig. 1A, lane 10). To ensure that transiently transfected cells were efficiently expressing TR2 or the TR2-LBD, lysate from cells transfected with TR2 alone (Fig. 1A, lane 1), TR2 and FLAG-HDAC3 (Fig. 1A, lane 2), TR2 and FLAG-HDAC4 (Fig. 1A, lane 3), or TR2-LBD and FLAG-HDAC3 (Fig. 1A, lane 4) were probed with an anti-TR2 antibody. We were able to detect TR2 or the TR2-LBD in all of these samples. These results indicated that TR2 can be coprecipitated with a member of class I and class II HDAC proteins.

Coimmunoprecipitation of TR2 and Endogenous HDACs. To strengthen the data showing an interaction between TR2 and HDACs, we showed the presence of endogenous HDAC3 and HDAC4 in COS-1 cells and subsequently detected an interaction between TR2 and these proteins. To show the presence of endogenous HDACs, COS-1 cells were transfected with TR2 and cell lysates were collected. These lysates were analyzed by Western blot using antibodies directed against either HDAC3 or HDAC4. The first column of Fig. 1B shows strong bands for HDAC3 (*top panel*) and HDAC4 (*lower panel*) indicating good levels of endogenous HDAC expression in COS-1 cells. To then demonstrate that TR2 interacts with these endogenous HDACs, lysates from the same TR2 transfected sample were immunoprecipitated with anti-TR2 antibody followed by probing of the precipitated immunocomplex with antibodies against HDAC3 or HDAC4. The second column of Fig. 1B shows that the immunoprecipitated TR2 complex contains bands corresponding to both HDAC3 (*top panel*) and HDAC4 (*lower panel*).

Inhibition of TR2-Mediated Repression by the HDAC Inhibitor Trichostatin A

In previous studies we have demonstrated that TR2 is able to repress activity of reporters containing a DR5 type RARE from the human RARβ promoter (14). In this study, we have used the DR5 reporter (RARE-tk-luc), which responds well to retinoic acid (RA) induction, to test whether TR2-mediated repression from this reporter can be blocked by the HDAC inhibitor TSA. If TR2 repression involves the recruitment of HDACs, either directly or indirectly, it is expected that TSA should ameliorate this repression to some extent. In the experiment of Fig. 2, COS-1 cells were transiently transfected with an internal LacZ control and the RARE-tk-luc reporter. The addition of RA (1 μM) to these cells activates endogenous RAR and RXR to drive expression from the luciferase reporter. Each column in Fig. 2A represents the ratio of normalized

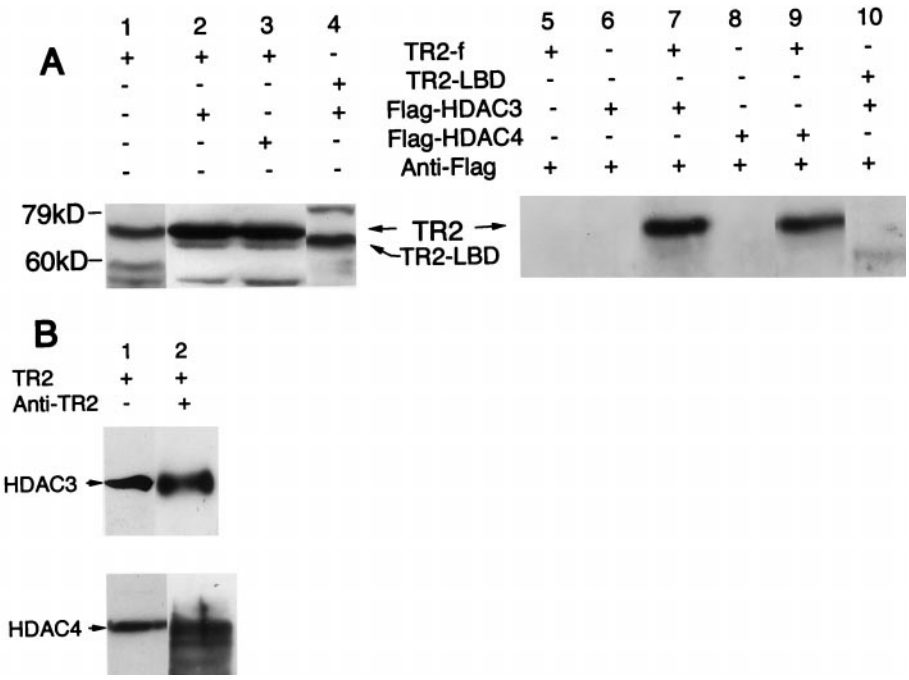

Fig. 1. *In Vivo* Interaction of TR2 and HDACs

A, COS-1 cells were transfected with either hemagglutinin-TR2 (lanes 1 and 5), FLAG-HDAC3 (lane 6), FLAG-HDAC3 and TR2 (lanes 2 and 7), FLAG-HDAC4 (lane 8), FLAG-HDAC4 and TR2 (lanes 3 and 9), or TR2-LBD and FLAG-HDAC-3 (lanes 4 and 10). Whole cell lysates were immunoprecipitated with anti-FLAG monoclonal antibody (lanes 5–10) and pulled out with Protein-G beads. Samples were then separated on 10% SDS-PAGE and immunoblotted with anti-TR2 antibodies. Lanes 1–4 show whole-cell lysates immunoblotted with anti-TR2 antibody to indicate the input level of TR2 and TR2-LBD for the immunoprecipitated samples. B, To test for interaction of TR2 with endogenously expressed HDACs, COS-1 cells were transfected with TR2. Whole-cell lysates were then immunoblotted with antibody against HDAC3 or HDAC4 (lane 1). Samples of the same lysates were immunoprecipitated with TR2 antibody followed by immunoblotting with antibody against HDAC3 or HDAC4 to test whether TR2 coprecipitates the endogenously expressed HDAC proteins (lane 2).

luciferase activity in the presence of RA to the normalized luciferase activity in the absence of RA (see *Materials and Methods*), reported as the fold-RA induction. Column 1 shows that addition of RA caused an 11-fold increase in normalized luciferase activity. However, when TR2 was cotransfected with the reporter, this activation is repressed by 51% (Fig. 2A, compare columns 1 and 2). If the TR2-mediated repression involves recruitment of HDACs, either directly or indirectly, it is expected that TSA should block this repression to some extent. To test this notion, the experiment was repeated with the addition of 100 nM TSA (Fig. 2A, columns 3 and 4) or 200 nM TSA (Fig. 2A, columns 5 and 6). The addition of TSA in the absence of TR2 was found to cause a reduction in the fold-RA induction (Fig. 2A, compare column 1 with columns 3 and 5; see below for explanation). When both TSA and TR2 are present in the COS-1 cells, the repression of reporter activity mediated by TR2 is reduced significantly. Comparing the difference in fold-RA induction between columns 1 and 2 with the difference between columns 3 and 4 of Fig. 2A, it appears that TR2-mediated repression is reduced from 51% with no TSA, to 27% with 100 nM TSA (Fig. 2B). When the concentration of TSA is increased to 200 nM, TR2 repression is almost absent at just 5% (compare the difference between columns 1 and 2 to the difference between columns 4 and 5 in Fig. 2A). The amelioration of TR2 repression caused by the addition of TSA suggests that the repressive activity of TR2 involves recruitment of HDAC. When these experiments were repeated with exogenously added RAR and RXR, similar results were obtained (data not shown).

The reduction in fold-RA induction with the addition of TSA is explained by an increase in RARE-tk-luc reporter activity in the absence of RA (data not shown), caused presumably by a general relief of transcriptional repression. However, TSA effects on the reporter in the presence of RA were less dramatic. It is possible that histone acetylation has already contributed to the induction of the RARE-tk-luc reporter in the presence of RA, and inhibition of histone deacetylation, by TSA, does not afford further enhancement of RA induction.

Measurement of Deacetylase Activity in TR2/HDAC Immunocomplexes

To confirm the *in vivo* interaction between TR2 and HDACs, and to determine enzymatic activities of these complexes, the TR2/HDAC cotransfected cell lysates were immunoprecipitated with anti-TR2 antibody and

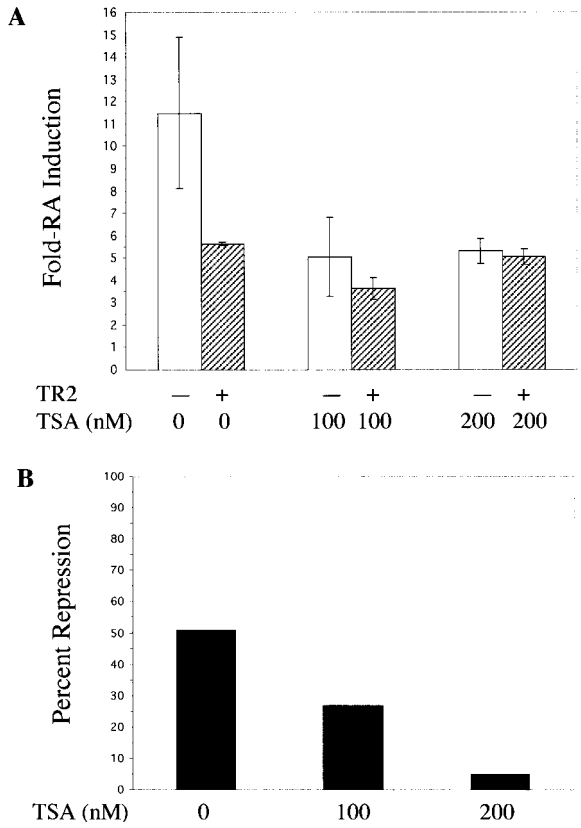

Fig. 2. TSA Blocks TR2-Mediated Repression of RA Induction of the RARE-tk-luc Reporter

A, The RA induction of the RARE-tk-luc reporter in COS-1 cells was assayed in the absence and presence of TR2 with 0, 100 nM, and 200 nM TSA present in the media as described in *Materials and Methods*. The fold-RA induction was determined by comparing activity in the presence of RA to activity without added RA. B, Percent TR2- mediated repression of fold-RA induction of the RARE-tk-luc reporter in COS-1 cells calculated from the data in panel A.

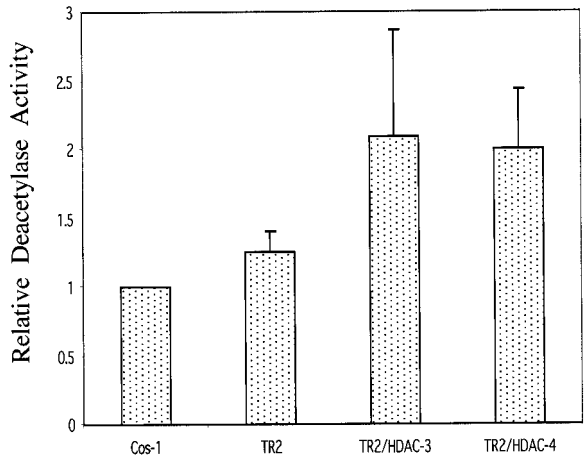

Fig. 3. HDAC Activity Is Associated with TR2 Protein Complex *in Vivo*

COS-1 cells were transfected as described in Fig. 1. COS-1 cells were transfected with TR2 (column 2), HDAC3 and TR2 (column 3), or HDAC4 and TR2 (column 4), and cell lysates were immunoprecipitated with anti-TR2 antibody. The immunoprecipitates were then bound to Protein-G beads and assayed directly for deacetylase activity. Deacetylase activity is expressed as counts per min of ^{3}H-acetic acid released. The deacetylase activity was normalized to the activity found in untransfected COS-1 cells precipitated with the TR2 antibody (column 1). The values for deacetylase activity are the average of five separate experiments with duplicate measurements done for each experiment.

assayed for deacetylase activity. Anti-TR2 antibody was incubated with COS-1 cell lysates to pull out TR2 and any associated proteins. The anti-TR2 complex was then bound to Protein-G beads, washed extensively with HDAC buffer, and incubated with ^{3}H-labeled H4 histone peptide. The released ^{3}H-acetic acid was extracted with ethyl acetate and quantitated using liquid scintillation. For basal level controls, untransfected (Fig. 3, column 1) and TR2-transfected (Fig. 3, column 2) COS-1 cells were precipitated with anti-TR2 antibody and tested for deacetylase activity. The activity detected in the TR2-transfected cells (Fig. 3, column 2) above the activity found in untransfected cells (Fig. 3, column 1) is attributed to the presence of endogenously expressed HDACs pulled down with TR2 (see Fig. 1B, lane 2). A low but significant increase in deacetylase activity above that found in the control samples was detected in the immunoprecipitates of cells that had been cotransfected with TR2 and HDACs (Fig. 3, columns 3 and 4). The demonstration of deacetylase activity in the TR2 coimmunoprecipitates provides evidence for a biologically active complex that includes TR2 and HDAC enzymes.

Mammalian Two-Hybrid Test

As a further test of the interaction of HDAC3 and TR2, a mammalian version of the two-hybrid test was done. In this experiment, the TR2-f (full-length) and the TR2-t (containing an intact DBD but lacking the LBD, Fig. 4, *upper panel*) clones were fused to the VP16 activation domain, and HDAC3 was fused to the GAL4 binding domain. The reporter construct was a tk-Luciferase reporter containing five copies of the GAL4 binding domain (Fig. 4, *upper panel*) (17). COS-1 cells were cotransfected pairwise with the GAL4-BD-HDAC3 fusion and the VP16-TR2 fusions, together with the reporter and a cytomegalovirus (CMV)-LacZ internal control. A basal activity level was measured by testing GAL4-BD-HDAC3 with VP16 (Fig. 4, column 1). The relative luciferase activity was calculated by normalizing luciferase units to LacZ activity. The (-fold) relative luciferase activity was then calculated by normalizing to the basal level of activity found for the GAL4-BD-HDAC3/VP16 cotransfection. Figure 4 shows interaction between HDAC3 and full-length TR2 results in an approximately 3-fold higher reporter activity (column 2), whereas interaction with TR2-t resulted in approximately 2.5-fold higher reporter activity (column 3). Although this test did not show a dramatic increase in

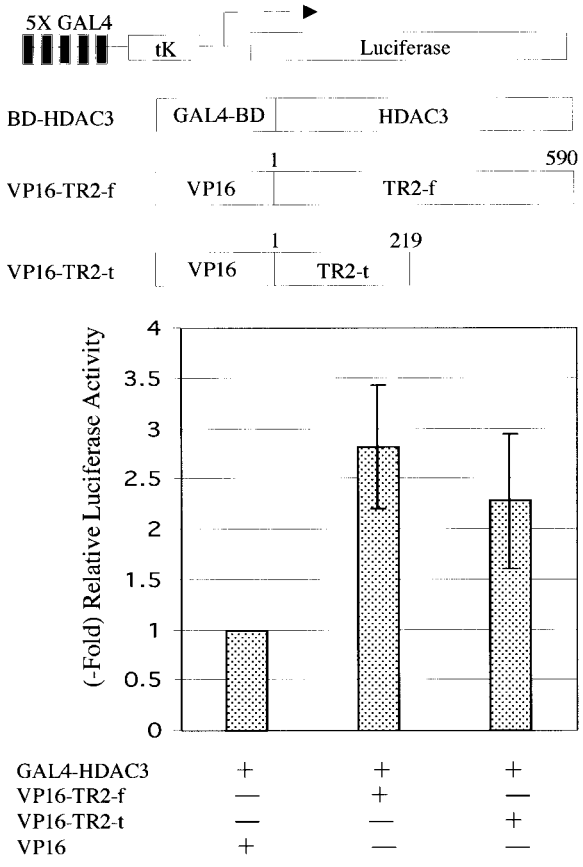

Fig. 4. Interaction of HDAC3 and TR2 in Mammalian Cells

A mammalian version of the two-hybrid system was used to examine interaction between HDAC3 and TR2 in COS-1 cells. The *upper panel* shows a luciferase reporter with five copies of the GAL4 binding site and the expression vectors for HDAC3 and TR2 clones. The GAL4-BD-HDAC3 fusion and VP16-TR2 fusions were cotransfected pairwise in COS-1 cells along with the reporter and a CMV-LacZ internal control. Thirty to 40 h after transfection, cells were harvested and luciferase and LacZ activities were determined. Relative RLU was calculated as described in *Materials and Methods*.

reporter activity, it should be considered that recruitment of HDAC3 to the reporter might attenuate a more vigorous response. In any case, the two-hybrid test showed a low but significant increase in luciferase reporter activity for both the TR2-full length and truncated constructs in this assay. These results suggest that an *in vivo* interaction between TR2 and HDAC3 exists that does not require the TR2 LBD.

In Vitro Interaction of TR2 and HDACs

Glutatione-*S*-Transferase (GST) Pull-Down Assay. To test the interaction between class I and class II HDACs and the mouse orphan receptor TR2, a GST pull-down assay was employed. Initially, the full-length TR2 receptor, the TR2-LBD, and the TR2-t coding regions were ligated to a GST expression vector, expressed in *Escherichia coli*, and bound to glutathione agarose beads. The bound GST-TR2 samples were then incubated with *in vitro* translated ^{35}S-labeled HDAC3 (class I) or HDAC4 (class II). Interaction with HDACs was observed with full-length TR2 (Fig. 5B, TR2-f lane) and a region that includes the TR2 DBD (Fig. 5B, TR2-t lane) but not the LBD region of the receptor (Fig. 5B, LBD lane). The N-terminal and DBD regions of the protein were further dissected by construction of several additional recombinant GST-TR2 fusion proteins (Fig. 5A). To verify the binding of GST-TR2 fusion proteins to the glutathione agarose beads, samples of beads from the binding of each recombinant protein were resolved using SDS-PAGE, and the gel was stained with Coomassie blue (Fig. 5C). The overlapping region between the original full-length and TR2-t constructs, amino acids 166–219 (Fig. 5B, TR2-D lane), was found to be negative for interaction with both HDACs, as was the A domain, which is comprised of amino acids 1–50 (Fig. 5B, A lane). The C domain of the TR2 receptor (amino acids 99–166), which contains two zinc finger motifs located between amino acids 99–133 (ZF-1) and 133–166 (ZF-2), showed interaction with both HDACs (Fig. 5B, lane C). When each zinc finger was expressed separately, both were also found to pull down HDAC3 and HDAC4 (Fig. 5B, lanes ZF-1 and ZF-2). In addition, those constructs that contain only a portion of the C domain, but encompass either zinc finger, showed interaction (Fig. 5B, lanes A/B/C′, B/C′, and ZF-2/D).

Far Western. To verify a direct interaction between TR2 and HDACs, a far Western assay was employed. In this assay the GST-TR2 fusion clones including TR2-f, TR2-LBD, TR2-t, and TR2-C/D were partially purified using glutathione agarose beads, resolved on a 10% polyacrylamide gel using SDS-PAGE, and transferred to PVDF membranes. The membranes were then probed with *in vitro* translated, ^{35}S-labeled HDAC3 or HDAC4. If the HDAC proteins interact with TR2, autoradiography should produce bands of the appropriate size for the various GST-TR2 fusion proteins. Figure 6 shows the results of the far Western blot. Of the clones tested, bands of appropriate size were detected for the full-length TR2, TR2-t, and TR2-C/D clones (Fig. 6, A and B, lanes 2, 3, and 5, respectively). No bands were detected for the negative control (GST sample) or the TR2-LBD (Fig. 6, A and B, lanes 1 and 4, respectively). This result is in agreement with the GST pull-down assay that showed the LBD of TR2 did not interact with either HDAC3 or HDAC4 and confirms that the TR2 DBD domain mediates HDAC interaction.

Interaction of the TR2 DBD and HDAC Requires Zinc

After identifying the TR2 DBD as the region of TR2 that interacts with HDACs, it was of interest to determine whether the three-dimensional structure of this domain was required to promote HDAC binding to TR2.

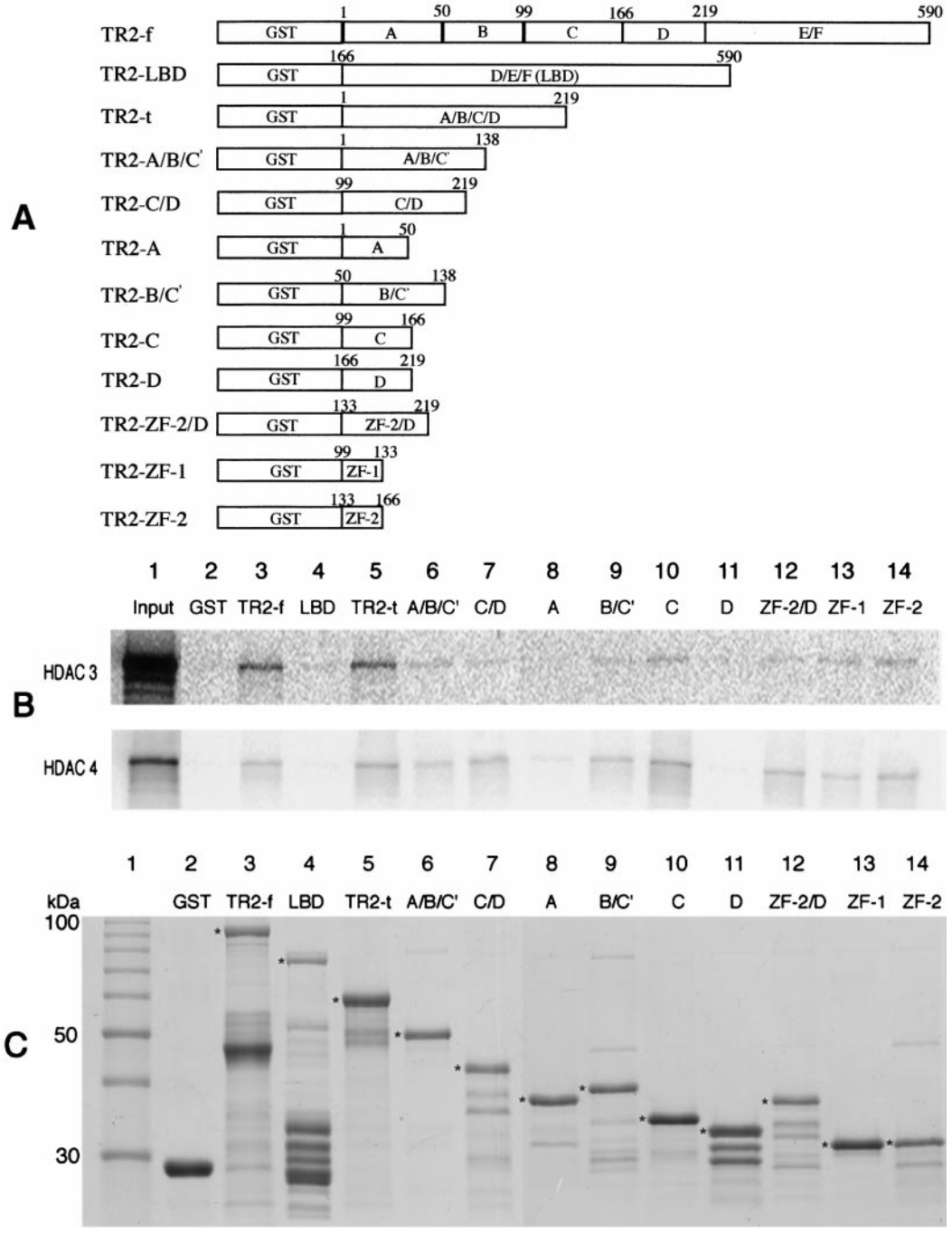

Fig. 5. Recombinant GST-TR2 Constructs and HDAC Pull-Down Data

A, Schematic representation of GST-TR2 recombinant protein constructs. The full-length TR2 construct, TR2-f, includes delineation of nuclear receptor domains. *Numbers on the left* represent the first amino acid encoded by TR2 in each construct and *numbers on the right* represent the last TR2 amino acid. B, GST-TR2 recombinant proteins pull down HDAC3 and HDAC4. Recombinant GST-TR2 protein samples were bound to glutathione agarose and incubated with ^{35}S-labeled HDAC3 or HDAC4. After extensive washing, specific interacting protein was eluted and analyzed by SDS-PAGE and autoradiography. Input control (lane 1), GST control (lane 2), TR2-f (lane 3), TR2-LBD (lane 4), TR2-t (lane 5), TR2-A/B/C′ (lane 6), TR2-C/D (lane 7), TR2-A (lane 8), TR2-B/C′ (lane 9), TR2-C (lane 10), TR2-D (lane 11), TR2-ZF-2/D (lane 12), TR2-ZF-1 (lane 13), and TR2-ZF-2 (lane 14). C, Protein sample input for GST pull down. Recombinant GST-TR2 protein samples were bound to glutathione agarose, washed, separated using SDS-PAGE (10% acrylamide gel), and stained with Coomassie brilliant blue. The same amount of sample material used in the pull-down experiments was loaded on this gel. Samples include protein marker in 10-kDa increments (lane 1); lanes 2–14 are as described in panel B. *Asterisks* mark the GST-TR2 bands of interest.

To investigate this possibility, the GST-TR2-C clone, which contains the two zinc fingers present in TR2 (Fig. 5A), was used in a pull-down assay after chelation of zinc. In the experiment of Fig. 7, GST-TR2-C was bound to glutathione agarose followed by extensive washing and overnight incubation in the presence of 100 mM EDTA. GST alone was included as a negative control. After chelation of zinc with EDTA, the bound TR2-C was then incubated with *in vitro* translated ^{35}S-labeled HDAC3 or HDAC4 in the presence or absence of 100 mM $ZnCl_2$. In the case of HDAC3 the chelation of zinc caused a near complete disruption of binding that was restored with the addition of zinc (Fig. 7A, compare lanes 4 and 5, *upper panel*). For HDAC4, binding to TR2-C still occurred in the absence of zinc, but there was a distinct enhancement of this binding with the addition of zinc (Fig. 7A, compare lanes 4 and 5, *lower panel*). The binding signals of HDAC3 and HDAC4 in the presence and absence of zinc observed in Fig. 7 were consistent over several trials of the experiment. The addition of zinc to bound GST did not enhance interaction with HDACs (Fig. 7A, compare lanes 2 and 3). These results support the notion that the TR2 DBD is the domain that contacts HDACs and indicate that proper folding of the zinc finger domains of TR2 is required for interaction with HDAC3 and enhances interaction of TR2 with HDAC4.

DISCUSSION

Recently, identification of HDAC proteins has provided support for the long held belief that the acetylation status of histones influences transcriptional activity (28, 31–36). Histone deacetylation is thought to repress transcription by inducing changes in chromatin structure that disrupt transcription, or by blocking assembly of the transcription initiation complex (25). Consistent with a role for HDACs in repression, corepressor complexes have been found to include HDAC proteins (20, 23, 34–37). A direct link between HDAC activity and transcriptional repression was recently established when point mutations were introduced in the deacetylation domains of HDAC 5 and 7 (38). The resulting HDAC mutants lost the ability to deacetylate histones, no longer could interact with silencing mediator of retinoid and thyroid hormone receptor, and could no longer efficiently repress basal transcription from a heterologous promoter.

In the present study we addressed the questions of whether TR2-mediated repression involves HDACs, and whether there is a direct interaction between this orphan receptor and the HDAC3 and HDAC4 proteins. A functional connection between TR2-mediated repression and HDAC activity was implied when we were able to demonstrate that the HDAC inhibitor TSA blocks TR2 repression. We were then able to show that TR2 and HDAC3 or HDAC4 are present in the same immunocomplex. When a TR2 antibody was used to immunoprecipitate protein complexes from cells cotransfected with TR2 and either of these HDACs, HDAC activity could be demonstrated. These results gave a clear indication that TR2 and HDACs can be found in the same immunocomplex and that these HDACs are active. Further evidence for interaction was provided by showing that TR2 coprecipitates endogenous HDAC3 and HDAC4 from COS-1 cells.

After demonstrating an *in vivo* association of TR2 and HDAC3 and HDAC4, we then tested the possibility that these proteins interact directly. GST pull-downs showed that both HDAC3 and HDAC4 were able to interact with the wild-type TR2 receptor. Further dissection of TR2 revealed that the interaction domain includes the DBD but excludes the LBD of the receptor. To verify that a direct interaction between HDACs and TR2 was mediated by the DBD, we used a far Western blot. The far Western has the advantage of showing interaction specifically with bands corresponding to the TR2 constructs. This helped us to rule out the possibility that impurities in the GST-TR2 fusion protein preparations were responsible for interaction with labeled HDACs in the GST pull-down assay. The results of the far Western blots were consistent with those of the pull-downs. The DBD of TR2 is a region highly conserved among nuclear receptors, which contains two zinc finger binding motifs. The three-dimensional structure of these domains within TR2 appears to be important for interaction with HDACs as chelation of zinc from these domains completely disrupted the interaction with HDAC3 and to a lesser extent with HDAC4.

We conclude that repression mediated by the mouse orphan receptor TR2 involves recruitment of both class I (HDAC3) and class II (HDAC4) HDACs. While it is likely that this deacetylase activity can be exerted through binding of a corepressor complex, we have provided evidence for a second level of transcriptional regulation through direct interaction of TR2 and HDAC proteins. As a conserved region of nuclear receptors mediates the direct interaction of HDACs and TR2, it is possible that this type of interaction is common to other receptors as well. In support of this notion, the TRβ receptor has been shown to bind HDAC-2 directly and that this binding is mediated by the TRβ DBD (39). Presently, we are testing the DBD of several nuclear receptors for direct interaction with both classes of HDAC proteins.

MATERIALS AND METHODS

Construction of Expression Vectors

To construct GST-TR2 expression vectors, coding regions from TR2 were subcloned into the pGEX-2T (Pharmacia Biotech, Piscataway, NJ) vector such that the fusion proteins produced would have GST fused to the amino-terminal end of the various TR2 constructs. The full-length TR2 construct (TR2-f, amino acids 1–590) was made by releasing a *Bgl*II/*Xba*I fragment from the TR2 cDNA (40) and ligating to the *Bam*HI/*Xba*I sites of pGEX-2T. The TR2-LBD region (amino acids 166–590) was released from the pBD-DEF construct (18) with *Eco*RI/*Xba*I and ligated to the same sites in pGEX-2T. The TR2-t (amino acids 1–219) clone was released by digestion with *Eco*RI and *Xho*I of a cDNA encoding a truncated isoform of TR2 (16) that includes an intact DBD but

lacks the LBD. This fragment was then ligated to the *Eco*RI/*Sal*I sites of the pGEX-2T expression vector. TR2-A/B/C′ (amino acids 1–138) and TR2-A (amino acids 1–50) constructs were created by releasing *Eco*RI/*Hin*dIII fragments from pM vector constructs containing these coding regions (41). These fragments were then subcloned into the *Eco*RI/*Hin*dIII sites of pGEX-2T. The TR2-B/C′ (amino acids 50–138) coding region was released from the full-length cDNA using *Bam*HI and subcloned into the same site in pGEX-2T. The remaining TR2 constructs: TR2-C (amino acids 99–166), TR2-D (amino acids 166–219), TR2- C/D (amino acids 99–219), TR2-ZF-1 (amino acids 99–133), TR2-ZF-2 (amino acids 133–166), and TR2-ZF-2/D (amino acids 133–219) were PCR amplified from the TR2 cDNA such that *Eco*RI and *Hin*dIII sites were introduced at the 5′- and 3′-ends of the PCR products, respectively. These TR2 fragments were then ligated to the *Eco*RI/*Hin*dIII sites of pGEX-2T.

The RARE-tk-luc reporter construct, containing a DR5, was kindly provided by Dr. R. Evans of the Salk Institute, San Diego, CA (42). The FLAG-epitope-tagged HDAC3 (37) and FLAG-tagged HDAC4 (28) vectors were as described. For the mammalian two-hybrid test, HDAC3 was fused to the GAL4 DBD by cloning into the pM vector (CLONTECH Laboratories, Inc.), and full-length TR2 (TR2-f clone) and the isoform TR2-t (lacking the LBD) were fused to the VP16 activation domain by cloning into the pVP16 vector (CLONTECH Laboratories, Inc.). The GAL4-tk-lucerifase reporter was as described previously (17).

Preparation of Protein Samples

GST and GST-TR2 fusion proteins were produced in the *E. coli* BL21(DE3)/pLysS strain. Cells were grown at 37 C to an OD_{600} of 0.5–0.6, followed by induction with 0.1 mM isopropyl β-D-thiogalactopyranoside for 3–5 h. Cells were harvested by centrifugation, washed once in PBS, and suspended in lysis buffer [50 mM Tris-Cl (pH 8), 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% β-mercaptoethanol, and protease inhibitor cocktail). Cells were sonicated three times for 30 sec followed by two passes in a French Pressure cell at 16,000 psi. Samples were then centrifuged at 20,000 × *g* for 1 h. The supernatant was separated from insoluble material, passed through a 0.22-μm filter, and used as the source of fusion protein. The TR2-f and TR2-t constructs were not soluble and had to be extracted from the insoluble material. Sample pellets for these constructs were solubilized in extraction buffer [10 mM Tris-Cl (pH 7.5), 8 M guanidine-HCl, 5 mM EDTA, 5 mM DTT] for 1 h on ice. The solubilized samples were then dialyzed for 16 h against a urea buffer [1 M urea, 50 mM Tris-Cl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail]. Samples were then centrifuged at 12,000 × *g* for 30 min and passed through a 0.22 μm filter. All samples were stored at −80 C.

TR2 Repression Assay

COS-1 cells were maintained in DMEM supplemented with 10% FBS treated with charcoal. To determine the effect of TSA on the suppressive activity of TR2, the reporter RARE-tk-luc, the receptor vector, and an internal control (CMV-LacZ) were cotransfected into COS-1 cells by calcium phosphate coprecipitation. For cotransfection, 0.1 μg of TR2 expression vector, 0.3 μg of the reporter, and 0.05 μg of internal control plasmid were used. For control transfections in which TR2 DNA was not added, the concentration of DNA was made up with sheared salmon sperm DNA. Cells were plated at a density of 5×10^4/well in 24-well plates and incubated overnight before transfection. For induction, RA (1 μM) was added to the medium 6–8 h following transfection. TSA was added (0–200 nM) to the samples at the same time as RA addition. Thirty to 40 h after transfection, total cell extracts were collected and assayed for luciferase activity, LacZ activity, and total protein concentration. Luciferase activity, determined with a commercial assay system (Promega Corp., Madison, WI), was normalized to the internal control LacZ activity determined using orthonitrophenyl-β-D-galactopyranoside (Sigma, St. Louis, MO) as the substrate, and represented as relative luciferase unit (RLU). The reporter activity was normalized to total cell protein in each sample by calculating the ratio of RLU/mg total protein. This was done to correct for cell death caused by expression of TR2 and addition of TSA. All transfections were done in the presence and absence of RA, and the ratio of [RLU/mg protein + RA]/[RLU/mg protein − RA] is reported as the fold RA-induction. Two independent experiments were carried out with three replicate cultures measured for each condition.

Mammalian Two-Hybrid Test

COS-1 cells were maintained in DMEM supplemented with 10% FBS at 37 C in 5% CO_2. To test the interaction of TR2 and HDAC3, cells were plated at a density of 5×10^4/well in 24-well plates and cotransfected with pM-HDAC3 (0.1 μg) and pVP16-TR2-f (0.1 μg) or pVP16-TR2-t (0.1 μg), along with a GAL4-tk-luc (0.5 μg) reporter and a CMV-LacZ internal control (0.05 μg). Thirty to 40 h after transfection, total cell extracts were collected and tested for luciferase and LacZ activity. RLU was calculated as described in the preceding paragraph. The fold-relative luciferase activity was calculated by normalizing RLU activity found in experimental samples to the basal level RLU activity found in the pM-HDAC3/pVP16 control cotransfection. Reported values are an average of three experiments with triplicate measurements taken in each experiment.

Coimmunoprecipitation

COS-1 cells were maintained in DMEM supplemented with 10% FBS at 37 C in 5% CO_2. Cells were plated at a density of 1×10^5/10-cm dish and cotransfected with hemagglutinin (HA)-tagged TR2 (7.5 μg) and FLAG-tagged HDAC3 (7.5 μg) or FLAG-tagged HDAC-4 (7.5 μg) expression vectors or TR2-LBD (7.5 μg) and FLAG-tagged HDAC-3 (7.5 μg). Forty eight hours after transfection, cells were harvested and resuspended in lysis buffer [20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 2 μg PMSF, 10% glycerol, protease inhibitor cocktail]. Cells were sonicated twice in 20-sec pulses on ice. Lysates were clarified by centrifugation at 10,000 × *g* for 10 min, and supernatant was used as the whole cell extract. For coimmunoprecipitation, 150–200 μl of cell extract were incubated with anti-FLAG monoclonal antibody (Sigma) at 4 C for 3 h, followed by addition of 20 μl of Protein-G agarose resin (Sigma). The samples were rocked for 1 h at 4 C, followed by extensive washing with lysis buffer to remove unbound proteins. The beads were then suspended in SDS-PAGE buffer for Western blot analysis. Samples were separated on a 10% gel and transferred on to PVDF membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). The blot was incubated with rabbit anti-TR2 antibody for 2 h at 4 C, followed by washing in 1× PBS, 0.1% Tween-20. Secondary antibody, antirabbit horseradish peroxidase, was then added to the blot for an additional 1.5 h. The signal was then detected with enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

For determining the interaction of TR2 with endogenous HDAC3 or HDAC4 in COS-1 cells, lysates of TR2-transfected cells were incubated with anti-TR2 antibody. The immunocomplex was precipitated by adding Protein-G beads, separated on 10% SDS-PAGE and immunoblotted with rabbit anti-HDAC3 or HDAC4 antibodies as detailed above. The anti-HDAC3 and -HDAC4 antibodies were also used to show

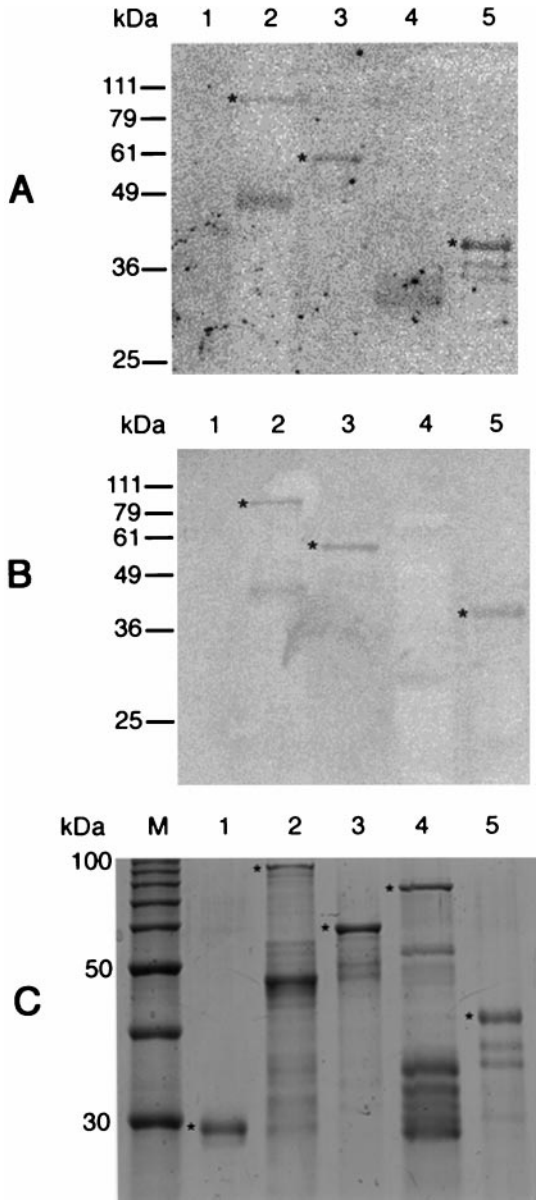

Fig. 6. Far Western Blot Showing Direct Interaction between TR2 and HDACs

GST-TR2 fusion proteins were partially purified by binding to glutathione agarose. After extensive washing, bound protein was eluted with glutathione buffer. Protein samples were subjected to SDS-PAGE and transferred to PVDF membrane. Membranes were probed with ^{35}S-labeled HDAC3 or ^{35}S-labeled HDAC4. A, Autoradiogram showing interaction of TR2 and HDAC3. GST control (lane 1), TR2-f (lane 2), TR2-t (lane 3), TR2-LBD (lane 4), TR-C/D (lane 5). *Asterisks* indicate the interacting TR2 bands. B, Autoradiogram showing interaction of TR2 and HDAC4. Lanes 1–5 are as described in panel A. C, Coomassie-stained gel showing input for far Western samples in panels A and B. Protein marker in 10-kDa increments (lane M); lanes 1–5 are as described in panel A. *Asterisks* mark the bands for GST (lane 1) and TR2 samples (lanes 2–5).

the presence of endogenously expressed HDAC3 and HDAC4 in the whole-cell lysate of TR2-transfected COS-1 cells by Western blot analysis.

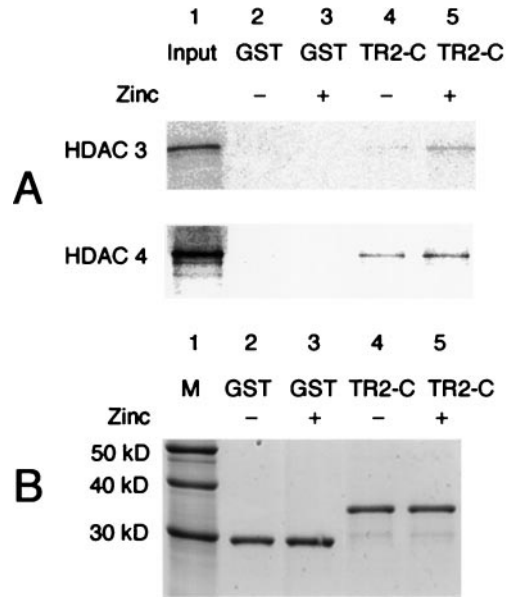

Fig. 7. GST Pull-Down of HDACs after Chelation of Zinc

GST and the GST-TR2-C recombinant clone, containing the two zinc fingers of the TR2 DBD, were bound to glutathione agarose, washed extensively, and incubated overnight in the presence of 100 mM EDTA. The bound proteins were then incubated with ^{35}S-labeled HDAC3 or HDAC4 in the presence and absence of 100 mM $ZnCl_2$. After extensive washing, interacting protein was eluted and analyzed by SDS-PAGE and autoradiography. Input control (lane 1), GST (lane 2), GST + $ZnCl_2$ (lane 3), TR2-C (lane 4), TR2-C + $ZnCl_2$ (lane 5). B, Protein sample input for GST pull down. GST and recombinant TR2-C protein samples were bound to glutathione agarose, washed, and separated using SDS-PAGE (10% acrylamide gel) and stained with Coomassie brilliant blue. The same amount of sample material used in the pull-down experiments was loaded on this gel. Protein marker in 10-kDa increments (lane 1); lanes 2–5 are as described in panel A.

HDAC Activity

Deacetylase activity was measured in the samples using a kit (Upstate Biotechnology, Inc., Lake Placid, NY) according to the manufacturer's instructions.

GST Pull-Down Assay

GST and GST fusion proteins were partially purified by binding to 60 μl of glutathione-agarose beads (Sigma). Due to differences in binding affinity for the various GST-TR2 constructs, preliminary binding studies were done to determine the amount of each sample preparation that would yield bands of approximately equal intensity on a Coomassie-stained SDS-PAGE (10% gel). After sample binding, the beads were washed twice with 20 vol of 1× PBS and once with binding buffer [20 mM HEPES (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 10% Glycerol]. ^{35}S-labeled HDAC3 or HDAC4 (2 μl per GST-TR2 fusion sample) prepared in TNT reactions (Promega Corp.) was then added to each GST-TR2 sample in 300 μl of binding buffer supplemented with protease inhibitor cocktail. The samples were allowed to incubate at 4 C for 90 min followed by three washes with 20-bead volumes of binding buffer to remove unbound proteins. The beads were collected by centrifugation and suspended in binding buffer (20 μl) and 4× SDS

sample buffer (20 μl). Binding to GST was included as a negative control. Samples were divided, and an equal amount was resolved using SDS-PAGE (10% gel) on two separate gels. One gel was stained with Coomassie and the second gel was fixed, dried, and exposed to a PhosphoImager screen (Molecular Dynamics, Inc., Sunnyvale, CA) overnight to detect labeled HDAC proteins.

Zinc Chelation with GST Pull Down

The pull-down procedure described above was repeated using GST (negative control) and the GST-TR2-C clone with the following modifications. After sample binding, beads were washed three times with 20 vol of 1× PBS, 0.1% NP-40 (Sigma), followed by two washes with the same buffer containing 100 mM EDTA. Beads were then washed overnight in the 1× PBS, 0.1% NP-40, 100 mM EDTA buffer to chelate zinc from the two zinc finger domains present in the TR2-C construct. The samples were then washed three more times in 1× PBS, 0.1% NP-40 and once in binding buffer. ^{35}S-labeled HDAC3 or HDAC4 (2 μl per sample) was then added to each sample in 300 μl of binding buffer (containing 10 mM EDTA) in the presence or absence of 100 mM $ZnCl_2$. To remove unbound proteins, samples were washed three times with binding buffer lacking EDTA. The beads were collected and suspended in 20 μl of 4× SDS sample buffer. Samples were analyzed as described in the preceding paragraph.

Far Western

GST-TR2 fusion protein samples were partially purified by binding to glutathione agarose as described above, followed by extensive washing with PBS to remove unbound protein. Bound protein was then eluted from the beads with a glutathione buffer (10 mM Tris-Cl, 10 mM reduced glutathione). Protein samples (10–30 μg) were resolved on a 10% polyacrylamide gel using SDS-PAGE and subsequently transferred to PVDF membrane. The far Western protocol was modified from that of Guichet *et al.* (43) omitting the denaturation/renaturation steps. Briefly, after transfer, membranes were washed twice in PBS and once in binding buffer followed by overnight blocking in binding buffer/5% BSA. A solution composed of binding buffer/5% BSA/1 mM DTT and ^{35}S-labeled HDAC3 or HDAC4 prepared in TNT reactions was then used to probe the membranes for 3–5 h. GST (50 μg) was added to the probe mix to block nonspecific binding. Membranes were then washed once with binding buffer/5% BSA/1 mM DTT and five times with binding buffer. All washing and probe steps were carried out at 4 C. The membranes were dried and exposed to a PhosphoImager screen.

Acknowledgments

The HDAC4 cDNA was kindly provided by S. L. Schreiber (Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA). We thank S.C. Tsai (Molecular Oncology Program, H. Lee Moffit Cancer Center and Research Institute, University of South Florida, Tampa, FL) for construction of the HDAC4 expression vectors.

Received October 19, 2000. Accepted May 8, 2001.

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This work was supported by grants from the NIH (DK-54733), the United States Department of Agriculture (98-35200-6264), and the American Cancer Society (RPG-99-237).

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