Transcriptional regulation by the repressor of estrogen receptor activity via recruitment of histone deacetylases

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The abbreviations used are: HDAC, histone deacetylase; REA, repressor of estrogen receptor activity; GST, glutathione-S-transferase; COUP-TF, chicken ovalbumin upstream binding-transcription factor; bp, base pair(s); TSA, trichostatin A; HBS, HEPES buffered saline; ER, estrogen receptor; ERE, estrogen responsive element; DBD, DNA binding domain; RXR, retinoic X receptor; TR, thyroid hormone receptor; SRC-1, steroid receptor co-activator 1; GFP, green fluorescent protein

The mouse REA cDNA sequence has been deposited in the GenBank database under the accession number AY211613.
SUMMARY

Histone acetyltransferases and deacetylases are recruited by transcription factors and adapter proteins to regulate specific subsets of target genes. We were interested to identify interaction partners of histone deacetylase 1 (HDAC1), which might be involved in conferring target or substrate specificity. Using the yeast two-hybrid system, we isolated the repressor of estrogen receptor activity (REA) as a novel HDAC1-associated protein. We demonstrated the in vivo interaction of REA with HDAC1 and characterized the respective domains required for their interaction in vitro. In addition, we found that REA also associates with the class II histone deacetylase HDAC5. In luciferase reporter assays, REA decreased transcription and this repression was sensitive to the deacetylase inhibitor trichostatin A. Finally, we showed that REA specifically interacts with the chicken ovalbumin upstream binding-transcription factors (COUP-TF) I and II. The nuclear receptor COUP-TFI was found to cooperate with REA and histone deacetylases in the repression of target genes. We therefore propose a novel function for REA as a mediator of transcriptional repression by nuclear hormone receptors via recruitment of histone deacetylases.
INTRODUCTION

Eukaryotic DNA is packaged into highly dynamic chromatin, the basic unit of which is the nucleosome. This consists of a central “hub”, comprising two of each of the core histone proteins H2A, H2B, H3 and H4, around which are wrapped 146 bp of DNA.

The highly conserved histone proteins are subject to several post-translational modifications, including acetylation, phosphorylation, methylation and sumoylation. Extensive studies have forged a link between the acetylation state of chromatin and its transcriptional status. Dynamic acetylation and deacetylation of lysine residues within the N-terminal tails of core histones seem to be necessary for a series of crucial nuclear events such as silencing, replication and correct DNA repair.

Initial research into the relevance of histone acetylation aimed at identifying and characterizing the enzymes involved, that is the histone acetyltransferases and their antagonists, the histone deacetylases (HDACs). Class I HDACs, which include HDAC1, HDAC2, HDAC3, HDAC8 and HDAC11, have been implicated in the regulation of cell cycle progression, differentiation and development (1), and a series of studies have revealed a potential role for HDACs in carcinogenesis (reviewed in (2)).

The prototypical histone deacetylase HDAC1, a homologue of the *S. cerevisiae* transcriptional modulator Rpd3 (3), was first identified using a trapoxin affinity matrix (4). An important aspect of HDAC1 function is its target specificity, determined predominantly by the precise composition of HDAC1-containing complexes and the nature of HDAC1 associated proteins. Apart from the well
characterized SIN3 and NuRD complexes ((1,5) and references therein), an additional HDAC1-containing complex, named CoREST, has been isolated from human cells (6,7). HDAC2, the second member of the class I deacetylases, is commonly found within the same complexes as HDAC1 (1,5-7).

The third class I deacetylase HDAC3 is generally found to be associated with the corepressors SMRT and N-CoR, as well as with the class II deacetylase enzymes HDAC4 and HDAC5 (reviewed in (8)). As yet, little is known about the function of the two other members of the class I histone deacetylase family, the recently described HDAC8 (9-11) and HDAC11 (12). Class II enzymes, comprising HDACs 4-7 and HDAC9-10, are generally linked to the regulation of differentiation-related genes, and, in contrast to the strictly nuclear class I deacetylases HDAC1 and HDAC2 are found in both the nucleus and cytoplasm of cells (13). Class III deacetylases have been implicated in gene silencing and control of aging (14,15).

A large number of transcription factors regulate gene expression through the recruitment of histone deacetylase complexes. Nuclear hormone receptors are well-characterized examples of HDAC-recruiting proteins. Initially, the retinoic acid receptors and thyroid receptors were shown to interact indirectly with HDACs, through their interaction with N-CoR and SMRT in the absence of ligand (16,17). Recruitment of HDAC2 through N-CoR and SMRT was also described for steroid receptors in the presence of partial antihormones (18). Similarly, HDACs associate both in vitro and in vivo with different transcription cofactors that bind nuclear receptors in the presence of agonists (19-21). More recently, a direct association between HDACs and nuclear receptors was reported (22), thus increasing the complexity of the interaction between these two types of partners.
HDAC1 is certainly the best-studied mammalian histone deacetylase. The enzyme is not only essential for mouse development, but also for normal proliferation of mouse embryos and embryonic stem cells (23). HDAC1 expression has to be tightly regulated since both overexpression and loss of HDAC1 result in severe disturbance of proliferation and cell cycle progression (23,24). Transcription of the murine HDAC1 gene is induced by cooperative phosphorylation and acetylation signals, allowing both growth factor-dependent activation and feedback regulation (25,26). In addition, post-translational modifications seem to be important for the biological function of the enzyme and the regulation of its activity by other factors (27-29).

HDAC1 has been shown to form homodimers and to associate with HDAC2 (30,31). This has already led us to propose dimerization as a pivotal event for HDAC1 enzymatic activity (30). Apart from this, specific recruiting factors are required to confer high target specificity upon HDAC1. To this aim, we employed the yeast two-hybrid system to isolate hitherto unknown interaction partners of HDAC1. One of the candidate proteins isolated was the repressor of estrogen receptor activity (REA) (32). By co-immunoprecipitation experiments and glutathione-S-transferase (GST) pull-down assays, we established the interaction between REA and both HDAC1 and HDAC5. Moreover, we demonstrated that REA specifically interacts not only with estrogen receptor, but also with the orphan nuclear receptors chicken ovalbumin upstream binding-transcription factor (COUP-TF) I and II. Our data identify REA as novel HDAC-interacting protein that modulates the activity of a defined subset of nuclear hormone receptors.
EXPERIMENTAL PROCEDURES

Chemicals and materials. 17ß-estradiol and 4-hydroxy-tamoxifen were obtained from Sigma-Aldrich and resuspended in 96% ethanol. Custom oligonucleotides were purchased from VBC-Genomics, Vienna, Austria.

Cell culture and cell transfection. U2OS, HeLa, 293, Swiss 3T3 and MCF7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. Embryonic stem cells were grown as previously described (23). DNA transfection was carried out according to the previously published polyethylenimine transfection procedure (33).

Plasmid construction. The mBAP37 cDNA was kindly provided by M. Lamers (34). Deletions were constructed using standard PCR-based methods. Specific information regarding oligonucleotide sequences is available upon request. The bait plasmid used in two-hybrid screening was constructed by inserting a PCR fragment corresponding to aa 299 to 482 of mouse HDAC1 into pEG202, downstream of the region encoding the LexA DNA binding domain (DBD). Prey libraries of HeLa cDNA or mouse embryonic brain cDNA were kindly provided by R. Brent and W. Stockinger, respectively. The REA cDNA fragment encoding aa 89 to 299 was PCR-amplified and subcloned via BamHI/XmaI into pQE30, for expression of 6xHis-tagged REA in the E. coli BL21 DE3 pLysS strain. Correct reading frames were verified by sequencing. REA deletion constructs were PCR-amplified and cloned into adequately digested pGEX4T1 vectors for expression as a GST fusion proteins. GST-HDAC1 constructs have been described elsewhere (30). The mHDA5 expression vector was
kindly provided by S. Khochbin. Expression plasmids for COUP-TFI and II were obtained from M.G. Parker and the GFP-tagged COUP-TFI plasmid was obtained from F. Pakdel. C-terminal and N-terminal deletion mutants of COUP-TFII were obtained by digestion with EagI and BamHI, respectively.

**Yeast two-hybrid screening.** Plasmids and yeast strains used in this study were adapted from the system of R. Brent. The yeast strain EGY191, which contains a chromosomally integrated auxotrophic marker (LEU2) and an ectopic lacZ reporter plasmid (pSH18-34), was sequentially transformed with the bait-encoding pEG202-HDAC1(299-482) plasmid and either the pJG4-5 mouse embryonic brain cDNA or the HeLa cDNA prey libraries. Transformants were plated on media lacking leucine. Galactose inducible LEU+ colonies exhibiting lacZ activity, as determined by filter-lift assays, were further characterized. Colonies harboring interacting prey proteins were used to quantify β-galactosidase activity by an o-nitrophenyl-beta-D-galactopyranoside-based liquid assay.

**Northern blot analysis.** mRNA expression was analyzed using the Northern blot sandwich method as described previously (35).

**Antibodies.** To raise a specific antibody directed against mouse REA, the 6xHis-tagged fragment (aa 89 to 299) expressed in *E. coli* BL21 DE3 pLysS was used to immunize rabbits by standard methods. Antibodies were affinity-purified by overnight incubation on nitrocellulose strips loaded with recombinant His-REA. After washing with 10 mM Tris-HCl pH 8.0 and 10 mM Tris-HCl pH 8.0, 0.5 M NaCl, bound antibodies were eluted by sequential incubation with 100 mM glycine pH 2.5 and 100
mM triethylamine pH 10.0. Eluates were neutralized by addition of 1 M Tris-HCl pH 8.0 and tested for specificity by Western blot analysis. The REA antibody described above, as well the HDAC1 monoclonal antibody 10E2 and the RbAp48 monoclonal antibody 13D10 are commercially available at Upstate USA. The estrogen receptor alpha antibody H-184, the Gal4 DNA-BD monoclonal antibody and the MTA1 antibody C-17 were purchased from Santa Cruz. Other antibodies used in this study were the HA-specific monoclonal antibodies 12CA5 and 16B12 and the GFP monoclonal antibody from Clontech.

**Glutathione-S-transferase pull-down assays.** GST-fusion proteins were expressed in *E. coli* BL21 DE3 pLysS. GST-fusion proteins with HDAC1, HDAC2 and HDAC3 have been described previously (30). GST-SRC-1 and expression vectors for RXRα and TRα were obtained from M.G. Parker. Large-scale preparation, purification of the recombinant proteins and binding to the glutathione beads were performed as previously described (36). Beads coated with GST-fusion proteins were incubated with *in vitro* translated, 35S-methionine labeled full-length REA or HDAC1 protein for two hours in lysis buffer (20 mM Tris-HCl pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5 % NP-40; Complete Protease Inhibitor Cocktail). Beads were washed three times in lysis buffer, one time in RIPA buffer (150 mM NaCl; 1% (v/v) NP-40; 0.5% (w/v) sodium deoxycholate; 0.1% (w/v) SDS, 50 mM Tris-HCl pH 8.0) and boiled in SDS-PAGE sample buffer to elute bound proteins. Eluates were then resolved by SDS-PAGE and visualized by autoradiography. To control normalized amounts of immobilized GST and GST-fusion proteins, duplicate inputs were resolved by SDS-PAGE and subsequently stained with Coomassie Brilliant Blue solution.
**Protein isolation and immunoprecipitation.** Whole-cell protein extraction and immunoprecipitation experiments were performed as previously described (24,37). Briefly, equal amounts of protein were incubated with specific antibodies for one hour at 4°C in lysis buffer (20 mM Tris-HCl pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5 % NP-40; Complete Protease Inhibitor Cocktail). Then, 20 µl of 50 % v/v proteinA-coupled sepharose bead slurry was added and incubated under gentle agitation overnight at 4°C. After three washes in lysis buffer, bound proteins were resolved by SDS-PAGE and analyzed on Western blots. Alternatively, one half of the immunoprecipitates was used directly to assay deacetylase activity, whereas the second half was mixed with SDS-PAGE sample buffer and analyzed on Western blots.

**Isolation of cytoplasmic and nuclear protein fractions.** To separate nuclear and cytoplasmic proteins, cells were lysed in ice-cold hypotonic buffer (10 mM HEPES pH 7.9; 10 mM KCl; 1.5 mM MgCl₂; 0.3 mM PMSF; 0.5 mM DTT). Following homogenization by passing the suspension through a 20-gauge syringe needle and control of efficiency by phase contrast microscopy, nuclei were pelleted by centrifugation at 2000 g for 8 minutes at 4°C. The supernatant cytoplasmic extract was transferred to a fresh tube. To obtain nuclear proteins, nuclei were resuspended in 1 volume lysis buffer (20 mM Tris-HCl pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5 % NP-40; Complete Protease Inhibitor Cocktail) and incubated at 4°C for 30 minutes under agitation. The nuclear extract was then cleared from debris by centrifugation at 16000 g for 30 minutes at 4°C.
**Histone deacetylase activity assays.** Histone deacetylase activity was measured as previously described (24, 38). In short, protein extracts or immunoprecipitated proteins were incubated with 5 µl of ³H-acetate-labeled chicken erythrocyte histones in a total volume of 30 µl lysis buffer (20 mM Tris-HCl pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5 % NP-40; Complete Protease Inhibitor Cocktail) for one hour at 30°C. The reaction was stopped by addition of 36 µl of 1 M HCl, 0.4 M acetate and released acetate was extracted with 800 µl ethyl acetate. After centrifugation at 8400 g for 5 minutes, 600 µl of the organic phase were counted in 3 ml of toluol liquid scintillation cocktail.

**Western blot analysis and indirect immunofluorescence.** Protein extracts or immunoprecipitated proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher & Schuell). After incubation with specific antibodies, the proteins were detected with the ECL kit from NEN. Subcellular localization of HDAC1 and REA was determined by indirect immunofluorescence microscopy (Zeiss Axiovert 135TV microscope and Leica TCS NT confocal microscope) as previously described (30). Nuclear DNA was visualized with 4',6-diamidino-2-phenylindole (DAPI) or TOTO-3 (Molecular Probes Inc.).

**Luciferase reporter assays.** SV40 TK minimal promoter/Gal4-luciferase reporter constructs and Gal4-DBD fusion mammalian expression vectors were a generous gift from D. Eberhard and have been described previously (39). For luciferase reporter assays cells were grown and transfected in 6-well tissue culture dishes. 48 hours after transfection, cells were lysed by incubation in 200 µl luciferase assay buffer (25 mM Tricine pH 7.8; 0.5 mM EDTA; 0.54 mM Na-tripolyphosphate; 16.3 mM


Luciferase levels were measured using a Mediators Photoluminometer. Transfection efficiency was assessed by measuring β-galactosidase levels in an 80 µl aliquot of the cell lysate using an o-nitrophenyl-beta-D-galactopyranoside-based liquid assay. Additionally, an aliquot of each extract was analyzed on Western blots to evaluate the levels of co-expressed proteins.
RESULTS

REA interacts with HDAC1 in the yeast two-hybrid system

The class I enzyme HDAC1 is present within three large multi-protein complexes, referred to as the SIN3, NuRD (reviewed in (5)) and CoREST complexes (7). Specificity for target genes may be conferred by the composition of the HDAC containing complexes. For this reason, we were interested in identifying additional HDAC1-interacting proteins.

Pilot experiments indicated that high expression of full length HDAC1 protein fused to a DNA-binding domain impaired significantly the viability of yeast (J. Taplick, V. Kurtev and C. Seiser, unpublished data). Therefore, we performed a yeast two-hybrid screen using a truncated mouse HDAC1 protein (aa 299-482) as bait with cDNA libraries from human HeLa cells and mouse embryos. Yeast colonies harboring HDAC1 interacting proteins were identified by their inducible viability on media lacking leucine and increased transcription from the lacZ gene. In this manner, we repeatedly identified the repressor of estrogen receptor activity (REA) (32) as an HDAC1 interacting protein in both the human and the murine cDNA libraries. Sequencing of the corresponding cDNAs revealed that they encode truncated versions of REA encompassing amino acids 89-299 and 103-299 of the human and mouse proteins, respectively. Mouse REA was first identified as the murine B-cell receptor associated protein 37 (mBAP37), a protein originally isolated through its physical association with the B lymphocyte IgM antigen receptor (34). Complete sequencing of the murine ORF revealed that the encoded mouse REA displays 100% identity with the human REA protein (data not shown).
The interaction of HDAC1 with REA was confirmed by backcrossing. Freshly transformed *S. cerevisiae* strain EGY191 expressing REA (aa 89-299) as bait and HDAC1 (aa 299-482) as prey showed inducible viability on media lacking leucine (Fig. 1A). In contrast, transformants expressing an unrelated bait protein (LexA-bicoid) or the LexA DNA binding domain alone were not viable under these conditions, thus confirming the specificity of the HDAC1/REA interaction. These results were complemented by the analysis of *lacZ* transcription levels (Fig. 1B), which showed an approximately 6-fold induction of β-galactosidase activity in strains co-expressing REA(89-299) and LexA-HDAC1(299-482) when compared to the LexA DNA binding domain or the unrelated control LexA-bicoid.

**Expression and intracellular localization of REA**

We first looked at the expression pattern of REA in a variety of tissues and cell lines. 

$^{32}$P-labeled REA cDNA was hybridized to a Clontech Northern blot of poly(A)$^+$ RNA isolated from different mouse tissues. REA mRNA was detectable in several tested tissues, with very high expression in heart, liver, kidney and, to a lower extent, testis (Fig. 1C). HDAC1 mRNA was detected at high levels in testis and kidney, and at lower levels in all other tissues tested with the exception of skeletal muscle.

To analyze REA protein expression, we raised a REA-specific polyclonal antiserum using bacterially expressed, purified 6xHis-tagged REA protein (aa 89-299). Specificity of the REA antiserum was confirmed by Western blot analysis and further enhanced by affinity purification against the antigen (data not shown). This affinity-purified antibody detected a single band corresponding to a 37 kDa protein in Western blots of whole cell protein extracts from a variety of established human and mouse cell lines (Fig. 1D). Among those tested were the human osteosarcoma cell line U2OS.
and the human mammary carcinoma cell line MCF-7. Mouse cell lines analyzed for REA expression were the IL2-dependent T cell line B6.1 and primary erythroblasts, as well as embryonic stem cells derived from HDAC1 wildtype and homozygous mutant mice. In addition, a signal at 37 kDa was also detected in whole cell protein extracts from rat embryonic fibroblasts, indicating reactivity of the antibody for human, mouse and rat species. HDAC1 protein was present in all cell lines tested, with the exception of the HDAC1 -/- ES cells.

To establish further the connection between REA and the nuclear enzyme HDAC1, we next determined the subcellular localization of the REA protein. A putative nuclear localization sequence (aa 86-89) has been found through analysis of the polypeptide sequence (32); however, to our knowledge, a direct evidence for the presence of REA within the nucleus is lacking. Using the affinity purified REA antibody, we analyzed the subcellular localization of REA in logarithmically growing MCF-7 cells by confocal microscopy. REA was present in a distinct speckled pattern throughout the cell, with denser signals within the area of the nucleus (Fig. 2A, upper panel). A similar REA distribution pattern was observed in Swiss 3T3 fibroblasts (Fig. 2A, lower panel) and HeLa cells (data not shown). Our data indicate that a subpopulation of REA shares its subcellular localization with the strictly nuclear HDAC1 (24,30). REA was reported to display increased association with estrogen receptor α (ERα) in the presence of the antiestrogen 4-hydroxy-tamoxifen (32). We were therefore interested to know whether treatment of MCF-7 cells with the hormone would lead to a re-distribution of REA within the cell. Confocal immunofluorescence studies of MCF-7 cells treated for various periods of time with $10^{-6}$M 17ß-estradiol or $10^{-6}$M 4-hydroxy-tamoxifen showed that REA signal
intensity and distribution remained unaffected by the presence of ligand (data not shown).

In a separate approach, we analyzed the intracellular localization of REA by Western blot analysis of biochemically fractionated cell extracts. Logarithmically growing MCF-7 cells were harvested and used to prepare cytoplasmic versus nuclear extracts (as described in the Experimental Procedures). Sequential probing with antibodies specific for REA and HDAC1 confirmed that a significant portion of REA was present in the nuclear fraction, coincident with the localization of HDAC1 (Fig. 2B). Probing with a tubulin-specific antibody indicated only minimal contamination of nuclear fractions with cytoplasmic proteins.

**In vivo association of REA with HDAC1**

To demonstrate the *in vivo* interaction between REA and HDAC1, we decided to precipitate endogenous REA and analyze co-precipitated proteins for the presence of HDAC1. Whole cell protein extracts prepared from logarithmically growing MCF-7 cells were used for immunoprecipitation with REA antibody or, as a negative control, with the corresponding pre-immune serum. Precipitated proteins were analyzed for associated HDAC1 by Western blotting (Fig. 3A). Duplicate immunoprecipitations were used for HDAC activity assays (Fig. 3B).

As illustrated in Fig. 3A, REA was precipitated by the REA specific antibody, but not by the pre-immune serum. Furthermore, HDAC1 was associated with immunoprecipitated REA, as determined by probing of the Western blot with HDAC1 antibody. Consistent with this finding, HDAC assays using immunoprecipitated fractions showed that considerable levels of HDAC activity were associated with REA (Fig. 3B). These were comparable to activities measured in RbAp48
immunoprecipitations (data not shown). Since RbAp48 is widely regarded as an
integral part of the HDAC1 enzyme complex (40), these data indicate efficient
recruitment of deacetylases by the REA protein.

Conversely, to analyze REA association with immunoprecipitated HDAC1, whole
cell protein extracts were subjected to immunoprecipitation with the HDAC1
monoclonal antibody 10E2 and, as control, with an unrelated monoclonal antibody.
Precipitated proteins were then examined by Western blot analysis. As shown in Fig.
3C, REA specifically co-immunoprecipitated with the HDAC1 protein, confirming an
in vivo interaction between REA and HDAC1.

In vitro interaction between HDAC1 and REA

To define the HDAC1-REA interaction domains within both proteins, we performed
glutathione-S-transferase (GST) pull-down experiments. To ensure comparable
inputs, duplicates of all GST-fusion proteins were separated by SDS-PAGE and
stained with Coomassie Brilliant Blue (Supplementary data Fig. S1). Full length
HDAC1 and different portions of mouse HDAC1 fused to GST (depicted in Fig. 4A)
were bound to glutathione beads and incubated with in vitro translated $^{35}$S-
radiolabeled REA.

As shown in Fig. 4B, full length HDAC1 associated with REA, suggesting a direct
interaction between these proteins. The region of HDAC1 corresponding to aa 1-303
(delA) exhibited only negligible binding, similar to the signal obtained with GST
alone. In contrast, a GST-fusion protein comprising the C-terminal 179 aa of HDAC1
was sufficient to mediate interaction with REA (data not shown), comparable to the
interaction seen for a full-length GST-HDAC1 fusion protein. This was in accordance
with the fact that, in the two-hybrid screen, REA was identified using the C-terminal
half of the HDAC1 protein as bait. Further subdivision of the C-terminus into two parts (delB and delC) resulted in polypeptides which retained their binding capacity for REA, suggesting the existence of two distinct REA binding domains (Fig. 4B).

To define the HDAC binding region within REA, the REA polypeptide was subdivided into a series of complementary fragments and fused to GST (represented schematically in Fig. 4A). Pull-down experiments were performed with these constructs or full-length REA fused to GST using radiolabeled full-length HDAC1. As can be seen in Fig. 4C, GST-REA interacted with HDAC1, while the unrelated control protein LR8 showed no interaction. GST-REAdel9 (aa 185-299) was sufficient for interaction with HDAC1. However, in addition to this carboxy-terminal association region, REA bound HDAC1 via a second domain located within the N-terminal part, witnessed by the binding ability of GST-REAdel1 and GST-REAdel2 (aa 1-85 and aa 86-146, respectively). The N- and C-terminal interaction domains recruited HDAC1 independently. Binding affinity of the individual domains was markedly lower than that of the full-length protein, indicating that under physiological conditions there might be cooperativity between these two regions for recruitment of HDAC1. GST-del3 (aa 147-184) and GST-del8 (aa 117-184) showed no specific binding, indicating that the central portion of the REA protein (aa 117-184) was expendable for interaction with HDAC1. Furthermore, HDAC1-REA binding was not mediated by DNA, since addition of ethidium bromide (100 µg/ml) to the pull-down reactions had no effect upon HDAC1 precipitation (data not shown).

Analysis of REA-associated deacetylase activity in HDAC1-deficient embryonic stem cells (23) by immunoprecipitation experiments revealed significant HDAC activity associated with REA in the absence of HDAC1 (Supplementary data: Fig. S2). These data suggested the interaction of other members of the deacetylase family with REA.
Therefore, we analyzed the interaction of other deacetylases with the REA protein. As shown in Fig. 4D, both HDAC2 and HDAC3 GST fusion proteins interacted with REA.

Interestingly, members of the HDAC class II family, such as HDAC5, also bound to REA in GST pull-down assays (Fig. 4E). When compared to HDAC1, HDAC5 showed in part different specificity with respect to the interaction domains within the REA protein. HDAC5 interacted predominantly with the N-terminus of REA (del1), while HDAC1 associated with two REA domains (see above). To test whether HDAC5 interacted with REA in an *in vivo* assay, we expressed Flag-tagged HDAC5 alone or together with HA-REA in 293 cells. Epitope tagged REA was precipitated with HA antibody and the immunoprecipitate was analyzed for presence of REA with REA specific antibody. HDAC5 was present in the HA precipitate in the presence of HA-REA, but not in the absence of the protein, indicating a specific *in vivo* interaction between REA and HDAC5.

**REA acts as a transcriptional repressor**

Although REA was initially described as a repressor of estrogen receptor activity (32), there is, to our knowledge, only indirect evidence for a repressive function of REA. The prevailing model for the REA mode of function is based upon the competition for binding to ER between REA and the SRC-1 co-activator. Our data showing an association between REA and histone deacetylases indicated that REA could additionally act as a repressor on its own. We therefore decided to assess the possible repressive function of this protein using mammalian *in vivo* reporter gene assays. We cloned full-length REA as a C-terminal fusion to the Gal4 DNA binding domain (DBD). This construct was transiently transfected into U2OS cells, together
with a plasmid carrying a luciferase reporter cassette under the control of a SV40 TK promoter preceded by four Gal4 binding sites (39).

Luciferase levels upon co-transfection of the Gal4 DBD alone were arbitrarily set to 100%. Transfection of the Gal4DBD-REA expressing construct led to a reduction of luciferase activity to 40% of the control value (Fig. 5A, gray bars). Based upon the interaction of REA with HDACs, we asked the question whether the observed repression could be alleviated by treatment with an HDAC inhibitor. Indeed, REA-mediated repression of the reporter cassette was nearly completely abolished by treatment with the deacetylase inhibitor trichostatin A (TSA) (Fig. 5A, black bars), a known specific inhibitor of histone deacetylases. Western blot analysis of Gal4DBD-REA expression levels confirmed that the reduced repressor function of REA in response to TSA treatment was not due to decreased expression of the Gal4DBD-REA fusion protein (Fig. 5B). A fusion construct between Gal4 DBD and GRG4 (39), a member of the mammalian Groucho family known to be independent of deacetylases for its transcriptional repression, was used as control (Fig. 5A). In contrast to REA, GRG4 mediated repression was not affected by treatment with TSA.

A subset of nuclear hormone receptors interacts with REA

Next, we examined the specificity of the association between REA and ERα by GST pull-down assays. Consistent with the initial reports characterizing REA as a repressor of estrogen receptor activity (32,41,42), GST-REA precipitated radiolabeled ERα in a ligand-independent manner, but did not interact with retinoic acid receptor RXRα or thyroid hormone receptor TRα (Fig. 6A). In contrast, the co-activator SRC-1 associated with all three nuclear receptors in the presence of the respective ligand (Fig. 6A).
In addition, we tested the orphan nuclear hormone receptors COUP-TF I and II for interaction with REA. These nuclear receptors have been implicated in the shut-off of MHC I transcription (43) and play important roles in embryonic development (reviewed in (44)). Interestingly, GST-REA had a very high affinity for COUP-TFI and COUP-TFII (Fig. 6B). While ER-α interacted with both the N-terminal and the C-terminal portion of REA, COUP-TFII preferentially associated with the N-terminal portion of REA (Fig. 6C). In GST pull-down assays, full length COUP-TFII and a mutant lacking the N-terminus (COUP-TFII(56-414)) efficiently associate with \textit{in vitro} translated REA (Fig. 6D). In contrast, the N-terminal part of COUP-TF did not interact with REA, suggesting that the C-terminal part of the receptor is sufficient for association with the REA protein.

Next, we asked whether REA associates \textit{in vivo} with nuclear hormone receptors. While a functional relationship between estrogen receptor and REA was convincingly established (32,42), evidence for a physical \textit{in vivo} interaction of nuclear receptors with REA was still missing. To analyze the association of HDAC1 and REA with ERα, we prepared whole cell protein extracts from ER-positive MCF-7 cells. Using an ERα-specific polyclonal antibody, we immunoprecipitated ERα and analyzed associated proteins and co-immunoprecipitated HDAC enzymatic activity. Sequential probing of Western blots with the respective antibodies (Fig. 7A) revealed that ERα was precipitated very efficiently. Furthermore, both REA and HDAC1 were specifically co-precipitated with the ERα-antibody, but not with the control antibody. In parallel, we tested the ERα immunoprecipitates for HDAC activity and found that ERα was associated with significant deacetylase activity (Fig. 7B).

To examine a potential \textit{in vivo} association of the COUP-TF orphan receptor with REA, we expressed HA-REA together with GFP-COUP-TFI or GFP in HeLa cells.
As shown in Fig. 7C, immunoprecipitated REA protein was associated with GFP-COUP-TFI, but not with GFP alone. Taken together, these data show a specific in vivo interaction of REA with ERα and COUP-TF, suggesting that these nuclear receptors could be specifically targeted by the REA-HDAC complexes.

**REA acts as a transcriptional co-repressor of the orphan receptor COUP-TFI**

Next, we tested the effect of REA on the transcriptional regulator activity of COUP-TFI. COUP-TFs can bind to a wide spectrum of response elements exhibiting variations in the AGGTCA core motif. This allows COUP-TFs to interact with a variety of hormone response elements recognized by other members of the nuclear receptor superfamily. In particular, COUP-TFs were shown to bind EREs (45,46) and to act as competitive repressors for ER-mediated transactivation (47-49).

Therefore we examined the influence of REA and COUP-TF on an estrogen-responsive ERE-TK-luciferase reporter cassette (50) in ER-negative HeLa cells (Fig. 7D). Co-expression of REA with COUP-TFI led to significant down-regulation of luciferase activity of the reporter. Additional expression of HDAC5 further increased the repressor effect of COUP-TFI and REA, while co-expression of HDAC1 had no enhancing effect. The difference in the effects of co-expressed HDAC1 and HDAC5 is most probably due to the high expression levels of endogenous HDAC1 in HeLa cells. A similar repression upon transfection of REA and HDAC5 was obtained in MCF-7 cells on a cathepsin D reporter construct (data not shown). Together with the findings on REA-HDAC interaction, these results indicate that REA acts as a co-repressor of the orphan receptor COUP-TFI by recruiting histone deacetylases.
DISCUSSION

REA as an HDAC-associated protein

The specificity of histone deacetylases as transcriptional repressors is defined by the recruitment of these enzymes by transcription factors or co-repressor proteins. In this study, we have identified the repressor of estrogen receptor activity (REA) as an HDAC1-associated factor and we have characterized the interaction of REA with histone deacetylases.

The HDAC1 protein contains several functional domains (see Fig. 4A). The central part of the HDAC1 protein encompasses the catalytic domain called HDAC consensus motif that is common to all class I and class III deacetylases (51). The HDAC association domain (HAD) within the HDAC1 N-terminal part was previously shown to be required for homodimerization and for interaction with HDAC2, as well as with other components of HDAC1 complexes such as Sin3A/Sin3B and RbAp48 (30).

The C-terminal part of HDAC1 contains at least two important motifs: The IACCE motif required for interaction with the tumor suppressor Rb (52,53) and the functional nuclear localization signal KKAKRVK (30). In addition, the C-terminal part of HDAC1 is a target for modification by sumoylation and phosphorylation (27,28).

Sumoylation of lysine residues seems to be required for the transcriptional repressor function of HDAC1, but not for the binding of the adapter protein Sin3A (28). The data presented in this study indicate that HDAC1 interacts with the co-repressor REA through its C-terminal domain, suggesting that the HDAC1 protein contains specific interaction surfaces for different adapter proteins.
REA most probably interacts directly with HDAC1 via two independent domains located in the N-terminus (aa 1-117) and the C-terminus (aa 185-299), respectively. The fact that binding activity of the full-length protein is higher than that of the individual domains suggests a cooperativity of these two domains. Interestingly, although isolated in this study as interacting with HDAC1, REA can form stable interactions with other members of the histone deacetylase family, intriguingly even with class II deacetylases, e.g. HDAC5. Notably, the interaction with HDAC5 requires only the N-terminal part of REA. This N-terminal domain, that is also involved in the interaction with HDAC1, contains RD1, one of the two domains previously shown to possess transcriptional repression activity (32). Consistent with these findings, REA immunoprecipitates show remarkably robust deacetylase activity in murine and human cell lines. Taken together, these data indicate that the REA protein has to be considered as a co-repressor that can recruit several members of the HDAC family.

The REA protein was originally identified as BAP37, a factor characterized as a B-cell receptor associated protein (34,54). As shown in this study, REA mRNA and protein are expressed in a large variety of tissues and cell lines, arguing against a B-cell restricted function. A significant portion of REA molecules shows nuclear localization and is found in association with the nuclear deacetylase HDAC1.

**REA as transcriptional co-repressor of nuclear hormone receptors**

Several proteins, including N-CoR, SMRT, SHP and REA, have been identified as transcriptional co-repressors of ER (55). REA was first characterized as an antagonist-dependent inhibitor of estrogen receptor transcriptional activity (32) that exerts its function through competition with co-activators for binding to ER. Subsequent studies reported also a repressive effect of REA upon ligand-bound ER, i.e. association with
an estrogen receptor, thereby dampening the estrogen-induced transcriptional activation (42). We propose that REA acts as co-regulator of ERα by recruiting HDACs to nuclear receptor target genes.

As shown by immunoprecipitation experiments, endogenous ERα interacts with REA and HDAC1. Targeting of REA to luciferase reporter constructs confirms an inherent repressive function, partially reversible by treatment with TSA. RD2, the second repression domain of REA, is not required for the interaction with deacetylases and may account for the HDAC-independent repressive activity of REA. Similar HDAC-dependent and independent repressor activities were also observed for other transcriptional regulators, such as Sin3 and Rb (53,56).

REA was shown to bind to ER in the presence of estrogen and to counteract the ligand-induced activity of the receptor (32,42). In this respect, REA is different to other co-repressors of nuclear hormone receptors, namely N-CoR and SMRT (reviewed in (55)). REA was shown to compete with the co-activator SRC1 for binding to ER, and this effect seems to depend on the abundance of REA and additional regulatory factors, including prothymosin α (32,41,42). In an in-depth study of cofactor dynamics at estrogen target promoters, Shang and colleagues (57) showed cyclic recruitment of co-activators in response to the hormone. The temporal presence of acetyltransferases such as CBP and pCAF induced a corresponding cyclic hyperacetylation separated by a transient wave of deacetylation. These results strongly suggest that histone deacetylases are present at ER target promoters even under inducing conditions in the presence of ligands. Several co-regulators of ER, including LCoR, MTA1 and RIP140, recruit histone deacetylases in the presence of estrogen (19,21,58,59).
Our experiments show an interaction of REA not only with ERα, but also with other members of the nuclear receptor family, namely COUP-TFI and COUP-TFII. COUP-TFs bind DNA either as homodimers or as heterodimers with RXR and have been shown to negatively regulate the activation function of other nuclear hormone receptors (reviewed in (60)). A variety of co-factors such as NCoR/SMRT (61), CTIP1 and 2 (62,63) and CIP-2 (64) have previously been shown to be involved in the repressive activity of COUP-TFs. In agreement with a repressor function, COUP-TFs associate directly or indirectly with deacetylases, including HDAC1 (65), HDAC5 (this study) and SIRT1 (63). Our data showing physical and functional interaction between REA and COUP-TFs suggest that REA acts as a more general co-repressor protein, which interacts with a subset of nuclear hormone receptors.

**The REA/prohibitin family of co-repressors**

Homologues of REA have been found in rodents, *C. elegans* and *S. cerevisiae*, as well as in the cyanobacterium *Synechocystis* (data not shown), suggesting important cellular functions for these proteins. The mammalian REA homologue prohibitin was identified as a growth inhibitory protein that represses E2F-dependent transcription by binding to the Rb protein (66). Strikingly, the transcriptional regulator function of prohibitin is also linked to the recruitment of HDACs (67). These findings, together with the data reported in the present study, suggest that REA and prohibitin belong to a family of co-repressor proteins that mediate their function by recruiting deacetylases to distinct sets of transcription factors.

**ACKNOWLEDGEMENTS**

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REFERENCES


FIGURE LEGENDS:

**Figure 1.** REA aa 89-299 interact with the C-terminus of HDAC1 in the yeast LexA-based two-hybrid system. (A) Streaking of transformants on media lacking leucine and containing galactose as the sole carbon source led to induction of REA prey protein expression. Interaction, as documented by viability, can be observed for strains expressing LexA-HDAC1(aa 299-482) (HDAC1) as bait but not for strains expressing the control baits LexA DNA binding domain alone (LexA) and LexA-bicoid homeodomain (bicoid). (B) Transformants were also analyzed for activation of \( \text{lacZ} \) transcription upon shifting from liquid media containing glucose to liquid media containing galactose as the sole carbon source. (C) Clontech Northern blot with poly(A)+ RNA isolated from different mouse tissues was analyzed by sequential hybridization with \( ^{32}\)P-labeled cDNA probes for REA and HDAC1. As control, the blot was hybridized with a \( \beta \)-actin probe. (D) Western blot analysis of whole cell protein extracts from the indicated cell lines of rat, mouse and human origin with the polyclonal REA antibody reveals a single signal corresponding to a 37 kDa protein. HDAC1 is co-expressed in all cell lines except the HDAC1 -/- ES cells.

**Figure 2.** Endogenous REA is localized in the cytosol and the nuclear compartment. (A) The REA protein was detected in logarithmically growing MCF-7 cells (upper panel) and Swiss 3T3 cells (lower panel) using a polyclonal REA antibody and Texas red-conjugated anti-rabbit immunoglobulin G. Nuclear DNA was counterstained with DAPI, or with TOTO following RNaseA treatment. (B) Nuclear (nuc) and cytoplasmic (cyt) extracts were prepared from logarithmically growing MCF-7 cells as described in Experimental Procedures. 30µg total protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes and analyzed by sequential probing with antibodies for REA, HDAC1 and \( \beta \)-tubulin as control.

**Figure 3.** *In vivo* association of HDAC1 and REA. (A) Endogenous REA was immunoprecipitated from MCF-7 whole cell protein extracts. The pre-immune serum was used as control. Precipitated proteins were identified by sequential Western blot analyses with antibodies specific for REA and HDAC1. (B) Duplicate REA immunoprecipitations were used to quantify associated HDAC enzymatic activity.
The data shown are representative of three independent experiments. (C) Whole cell extracts were prepared from Swiss 3T3 cells and used for immunoprecipitation with HDAC1 antibody. Cross-linked HA antibody was used as control. Western blots were probed with HDAC1 and REA-specific antibodies.

Figure 4. Interaction between REA and histone deacetylases. (A) Schematic representation of GST-HDAC1 and GST-REA fusion proteins used to map the interaction domains for HDAC1/REA interaction. HAD, HDAC association domain; NLD, nuclear localization domain, R1 and R2, repression domains 1 and 2; ERI, estrogen receptor interaction domain. (B) Radiolabeled full-length REA was incubated with GST-HDAC1 fusion protein or truncation mutants of HDAC1 fused to GST. Protein complexes were analyzed by SDS-PAGE. GST protein was used as negative control. (C) GST pull-down experiments were performed with radiolabeled full-length HDAC1 and GST-REA or deletion mutants of REA fused to GST. A GST-lipoprotein receptor 8 (LR8) fusion protein was included as a negative control. (D) In vitro interaction of class I deacetylases with REA. GST pull-down experiments were performed with GST-HDAC1, GST-HDAC2 or GST-HDAC3 and radiolabeled REA. GST protein was used as negative control. (E) REA associates in vitro with HDAC5. Radiolabeled HDAC5 was incubated with GST-REA or deletion mutants of REA fused to GST. (F) In vivo interaction of REA and HDAC5. FLAG-tagged HDAC5 was expressed without or with HA-tagged REA in 293 cells. HDAC5 and REA proteins were analyzed in input extracts and HA-immunoprecipitates by sequential probing with FLAG antibody and REA antibody. HA-tagged REA is indicated by the gray arrow, endogenous REA protein by the black arrow.

Figure 5. Transcriptional repression by REA is sensitive to the deacetylase inhibitor trichostatin A. (A) U2OS cells were transiently co-transfected with a reporter plasmid carrying a luciferase gene under the control of the SV40 thymidine kinase promoter (SV40 TK) preceded by four copies of a Gal4 binding site (depicted schematically) and plasmids encoding either Gal4 DNA binding domain (DBD) alone, Gal4DBD-REA or Gal4DBD-GRG4 as a positive control for repression. Gal4DBD-REA leads to 2-3 fold repression in comparison to the Gal4DBD alone (gray bars). Treatment of transfected cells with 100 ng/ml trichostatin A (TSA) for 20 hours prior to harvest, leads to a partial reversion of REA-mediated repression (black bars). Values are the
means +/− SD of at least three independent transfections performed in triplicate. (B) U2OS cells were transiently transfected with the Gal4 reporter plasmid and the Gal4DBD-REA plasmid and left untreated or treated with TSA as described above. Protein extracts were prepared and analyzed for expression of Gal4DBD-REA and as control for endogenous REA.

**Figure 6.** REA interacts with a subset of nuclear hormone receptors. (A) GST-REA and GST-SRC-1 fusion proteins were used in pull-down assays to precipitate radiolabeled, *in vitro* translated nuclear hormone receptors in the presence and absence of ligands (17β-estradiol for ERα, 9-cis retinoic acid for RXR and T3 for TRα). In contrast to GST-SRC1, GST-REA interacts with ERα, but not with RXRα or the thyroid receptor TRα. (B) Radiolabeled COUP-TFI and COUP-TFII were analyzed in pull-down assays with GST-REA and GST. (C) REA interaction domains for nuclear hormone receptors were mapped by incubating REA deletion constructs fused to GST with radiolabeled ERα and COUP-TFII. Input of GST-fusion proteins was controlled by Coomassie staining of duplicate SDS-PAGE gels (Supplementary data Fig. S1). (D) Upper panel: Schematic drawing of the COUP-TFII protein with the autonomous functional domains A-E and the transcriptional activation domain AF-2 (68). Lower panel: COUP-TFII deletion mutants fused to GST were analyzed for interaction with radiolabeled REA.

**Figure 7.** *In vivo* interaction of REA with ERα and COUP-TF. (A) and (B) ERα complexes were immunoprecipitated from whole cell protein extracts of MCF-7 cells by use of a specific ERα antibody. An unrelated polyclonal rabbit serum was used as control. Immunoprecipitates were split and one third was analyzed by Western blotting and subsequent probing with the indicated antibodies (panel A), while the remaining two thirds were used to determine associated HDAC enzymatic activity (panel (B)). (C) COUP-TFI interacts with REA. REA was expressed together with GFP-COUP-TFI or GFP in HeLa cells. Input extracts and immunoprecipitates obtained with a GFP antibody were analyzed for presence of GFP-COUP-TFI, GFP and REA. (D+E) Cooperativity of REA and COUP-TF1 in the repression of target promoters. HeLa cells were transiently transfected with ERE-TK-luciferase reporter construct together with different combinations of expression vectors for COUP-TF1,
REA, HDAC1 and HDAC5. (D) Relative luciferase reporter activity is depicted with the COUP-TF1 single transfection arbitrarily set as 100%. Values are shown for one representative series of transfections of experiments performed in triplicate. (E) In parallel, protein extracts from transfected cells were prepared and analyzed for expression levels of COUPTF-GFP, HA-REA, HDAC1-myc and HDAC5-HA.
A Prey: REA_{89-299}

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B

C

heart brain spleen lung liver skeletal muscle kidney testis

REA HDAC1 ß-actin

D

REF52 U2OS MCF-7 B6.1 wt ES HDAC1 + ES Epithelial Skies 3T3 rat human mouse

REA HDAC1
Figure 2

A

MCF7

RE A

DAPI

overlay

Swiss 3T3

RE A

TOTO

overlay

B

cyt nuc

REA

HDAC1

tubulin
Figure 3

A

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B

HDAC activity (cpm)

Control IP | REA IP
0 | 5000
1000 | 4000
2000 | 3000
3000 | 2000
4000 | 1000
5000 |

C

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Figure 5

**A**

Diagram showing the SV40 TK luciferase reporter system with Gal4 binding sites.

**B**

Western blot analysis showing untreated and 20h TSA treated conditions for Gal4REA and REA proteins.
Figure 7

A

B

C

D

E

HDAC activity (cpm)

0
1000
2000
3000
4000
5000
6000
7000

input
control IP
ER\(\alpha\) IP

GFP-COUP-TF
GFP
GFP-COUP-TF

GFP
REA

HA-REA
HDAC1-myc
HDAC5-HA

HDAC activity (cpm)

0
1000
2000
3000
4000
5000
6000
7000

input
control IP
ER\(\alpha\) IP

GFP-COUP-TF
GFP
GFP-COUP-TF

GFP
REA

HA-REA
HDAC1-myc
HDAC5-HA

Luciferase activity

0
20
40
60
80
100

+ + + + + + COUPTF-GFP
HA-REA
HDAC1-myc
HDAC5-HA

+ + + + + + COUPTF-GFP
HA-REA
HDAC1-myc
HDAC5-HA
Figure S1. Loading controls for GST pull-down experiments. To ensure comparable inputs for the pull-down experiments, duplicates of immobilized GST-fusion proteins used in the precipitation reactions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue.
Figure S2. REA recruits HDAC1-independent HDAC activity. Whole cell protein extracts were prepared from mouse HDAC1 and -/- embryonic stem (ES) cells. (A) The extracts were analyzed for presence of HDAC1 and REA wt (left panel) and histone deacetylase activity (right panel). (B) The extracts were subjected to immunoprecipitation with polyclonal REA antibody. One third of immunoprecipitates was analyzed by SDS/PAGE and Western blotting for precipitated REA and associated proteins (left panel). The remaining two thirds were used to determine associated HDAC enzymatic activity (right panel).
Transcriptional regulation by the repressor of estrogen receptor activity via recruitment of histone deacetylases
Vladislav Kurtev, Raphael Margueron, Karin Kroboth, Egon Ogris, Vincent Cavailles and Christian Seiser

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