

Nuclear Receptor Coactivator ACTR Is a Novel Histone Acetyltransferase and Forms a Multimeric Activation Complex with P/CAF and CBP/p300

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Summary

We report here the identification of a novel cofactor, ACTR, that directly binds nuclear receptors and stimulates their transcriptional activities in a hormone-dependent fashion. ACTR also recruits two other nuclear factors, CBP and P/CAF, and thus plays a central role in creating a multisubunit coactivator complex. In addition, and unexpectedly, we show that purified ACTR is a potent histone acetyltransferase and appears to define a distinct evolutionary branch to this recently described family. Thus, hormonal activation by nuclear receptors involves the mutual recruitment of at least three classes of histone acetyltransferases that may act cooperatively as an enzymatic unit to reverse the effects of histone deacetylase shown to be part of the nuclear receptor corepressor complex.

Introduction

Nuclear hormone receptors for steroids (such as GR and ER), retinoids (RARs and RXRs), thyroid hormone (TRs), vitamin D₃ (VDR), and prostanoids (PPARs) comprise a large family of sequence-specific transcription factors. They play diverse roles in the control of cell growth and differentiation, development, and homeostasis by stimulating or repressing target gene expression (Mangelsdorf et al., 1995). Nuclear receptors bind to their response elements on the target gene promoter as either homodimers, heterodimers, or monomers. In the case of heterodimers, RXR has been shown to be the common partner for receptors such as RARs, TRs, and VDRs (for review, see Mangelsdorf et al., 1995; Glass, 1994; Mangelsdorf and Evans, 1995). Structure

and function analysis indicates that the C-terminal region of receptors is functionally complex and contains activities for ligand binding, receptor dimerization, hormone-dependent transactivation, and in the case of TR and RAR, hormone-independent repression. Further studies showed that the integrity of a well-conserved amphipathic α helix (AF2/ τ c domain) located in the extreme C termini of receptors is required for both the ligand-induced conformational change and the hormone-dependent transactivation activity (Baretino et al., 1994; Durand et al., 1994; Baniahmad et al., 1995; Leng et al., 1995; Schulman et al., 1996).

The mechanism of transcriptional regulation by nuclear receptors has been a focus of intense study. The demonstration of direct interaction of receptor with the basal transcription factors, such as TFIIB and TBP (Ing et al., 1992; Baniahmad et al., 1993; Fondell et al., 1993; Blanco et al., 1995; Schulman et al., 1995), suggested that liganded receptor may directly influence the function of basal transcription machinery. However, these direct interaction models do not explain transcriptional squelching between receptors (Meyer et al., 1989; Baretino et al., 1994) or the role of receptor-associated cofactors (for review, see Horwitz et al., 1996). In order to understand the molecular mechanism of transcription repression mediated by nuclear hormone receptors, we and others have isolated a family of proteins including SMRT/TRAC and N-CoR/RIP13 (Chen and Evans, 1995; Horlein et al., 1995; Soel et al., 1995; Sande and Privalsky, 1996) that interact with receptors in the absence of hormone and act as transcriptional corepressors for TR and RAR. Recently, the continuing search for cofactors that mediate ligand-dependent transactivation function of the nuclear hormone receptors led to the finding that CBP and its homolog p300 can interact with several nuclear receptors and potentiate their transactivation activity (Chakravarti et al., 1996; Hanstein et al., 1996; Kamei et al., 1996; Yao et al., 1996). In addition, it was also shown that CBP and p300 can form a complex with SRC-1, a recently cloned transcriptional coactivator for steroid receptors (Onate et al., 1995; Hanstein et al., 1996; Kamei et al., 1996; Yao et al., 1996). These recent findings suggest that bridging protein factors exist and function to transmit the signal of ligand-induced conformational change to the basal transcription machinery.

CBP and p300 are large nuclear proteins and have been demonstrated to interact functionally with a number of sequence-specific transcriptional activators (for review, see Janknecht and Hunter, 1996), including CREB (Chrivia et al., 1993; Arany et al., 1994; Kwok et al., 1994), bHLH factors (Eckner et al., 1996), Stat proteins (Bhattacharya et al., 1996; Zhang et al., 1996), AP1 (Arias et al., 1994; Bannister and Kouzarides, 1995), NF- κ B (Perkins et al., 1997) and nuclear receptors (referenced as above) as well as general transcription factors TFIIB and TBP, the oncoprotein E1A, and a human histone acetyltransferase protein P/CAF (Yang et al., 1996). Recently, it was found that CBP and p300 themselves have intrinsic histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996).

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Therefore, it appears that CBP/p300 and its associated protein P/CAF play a pivotal role in the control of cell growth and differentiation by integrating diverse signals for gene expression and function through direct contacting of the transcription machinery and/or targeted modification of the chromatin structure.

Although initially isolated as a protein interacting with progesterone receptor, SRC-1 was also found to be able to interact with several members of the nuclear receptor family in a hormone-dependent manner (Onate et al., 1995; Hanstein et al., 1996; Kamei et al., 1996; Yao et al., 1996). It was demonstrated to enhance the ligand-dependent transactivation activity of PR, GR, ER, TR, and RXR (Onate et al., 1995). Through interaction screening, genes encoding proteins sharing sequence homology with SRC-1 were also cloned and designated as either GRIP1 (Hong et al., 1996) or TIF2 (Voegel et al., 1996). They were shown to associate with members of the nuclear receptors family and augment the trans-activation activity of steroid receptors such as GR and ER (Voegel et al., 1996). In any case, however, the mechanism by which those cofactors function in mediating hormone signaling by receptors is still largely unknown. In addition, the functional domains of those cofactors have not been defined.

Targeted chromatin structure change has been postulated to associate with the regulation of gene expression by nuclear receptors (Zaret and Yamamoto, 1984; Truss et al., 1995; Wong et al., 1995). Recent biochemical and genetic studies support the notion that hyperacetylation of core histones is a characteristic of gene activation, and, conversely, histone deacetylation is involved in transcriptional repression (reviewed by Brownell and Allis, 1996; Wolffe and Pruss, 1996). One striking example is the demonstration made by us and others that nuclear receptor corepressors SMRT and N-CoR form complexes with Sin3 and histone deacetylase proteins, suggesting that chromatin remodeling by histone deacetylation is a possible mechanism for receptor-mediated repression (Heinzel et al., 1997; Nagy et al., 1997; reviewed by Pazin and Kadonaga, 1997; Wolffe, 1997). In order to understand the processes of hormone-dependent transactivation by nuclear receptors, we have performed functional analysis of a novel coactivator for nuclear hormone receptors, designated as ACTR. We found that not only can ACTR function by recruiting HAT proteins CBP/p300 and P/CAF, but by itself it is a HAT. ACTR is able to acetylate both free histones and nucleosome histones with substrate preference on histones H3 and H4. These results suggest that nuclear receptors target at least three different and self-interacting HAT activities (including ACTR or related proteins, CBP/p300, and P/CAF) to promoters, indicating a critical role for chromatin remodeling in hormonal signaling.

Results

Isolation of ACTR, a Novel Member of the Receptor Coactivator Family That Enhances the Transactivation Function of RAR in Yeast

The yeast one-hybrid selection scheme was employed to identify proteins that interact with nuclear receptors.

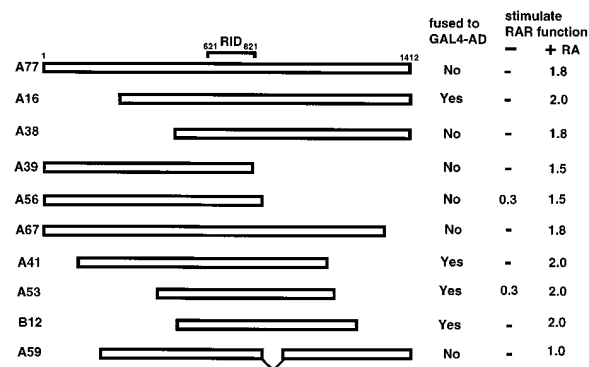


Figure 1. ACTR Stimulates RAR Function in Yeast

Yeast strain RH4XRE constitutively expressing hRAR β was transformed by different clones of ACTR isolated from the cDNA library screening. Three individual transformants from each clone were streaked on the selection medium (see Experimental Procedures) with or without 5 μ M atRA. After incubation for 3 days at 30°C, sizes (mm) of individual colonies were scored as indicated. Individual transformants from each clone gave the same results. Schematics of various ACTR isolates fused to the Gal4 activation domain present in pGAD10 vector (Clontech) are shown. Clone A77 is the full-length clone of ACTR. Other clones are aligned relative to A77. Clone A59 harbors a 65-amino-acid naturally occurring deletion (Figure 2B).

We constructed a yeast strain (RH4xRE) in which two chromosomally integrated reporter genes (*HIS3* and *lacZ*) are under the control of retinoic acid response elements (RARE). The yeast strain constitutively expressing full-length hRAR β was then transformed with a human GAL4 activation-domain fusion cDNA library. The transformants were plated on selection medium containing 5 μ M all-*trans* retinoic acid (atRA). Among the positive colonies analyzed, we identified cDNAs encoding partial or full-length proteins for RXR α , RIP140, and SRC-1. However, most other clones are repeated isolates and share overlapping sequences (Figure 1). One of them (clone A77) has a 6.7 kb cDNA insert that encodes an ORF of 1412 amino acids with predicted MW 154 kDa. This cDNA clone contains an in-frame stop codon upstream of its first methionine and downstream of the 3' end of the coding sequences for GAL4 activation domain in the vector and therefore is considered to be a full-length clone. Interestingly, more than half of the receptor-interacting clones analyzed lack an in-frame GAL4 activation domain (AD) (Figure 1), suggesting that they encode peptides that are able to interact with receptors and stimulate transcription independent of Gal4-AD in yeast. Reintroducing these clones into yeast strain RH4xRE harboring expression vectors for hRAR β confers strong hormone- (atRA-) dependent growth. In contrast, yeast expressing only RAR or any of the clones alone fail to grow on the selection plates (Figure 1 and data not shown). These results indicate that the peptides encoded by these clones are able to stimulate the transactivation activity of liganded receptors in yeast.

The deduced amino acid sequence of the full-length clone (ACTR) is shown in Figure 2A. It shows regional sequence similarities to SRC-1 and TIF2/GRIP1 (Figure 2B). The most prominent region sharing high homology (~80% similarity) among the three members is the



Sequencing analysis of some of the clones isolated revealed multiple potential alternative splicing variants of ACTR (Figure 2A), including a 65-amino-acid deletion (clone A59, Figure 1), which is also observed in TIF2 (Voegel et al., 1996). Like mSRC-1 (Kamei et al., 1996), ACTR was also found to have variants resulting from potential alternative splicing at its N terminus (Figure 2A and data not shown). These similarities further establish that ACTR, SRC-1, and TIF2/GRIP1 define a coactivator gene family and raise the possibility that distinct roles may be played by these splicing isoforms.

The mRNA level of ACTR varied highly among human tissues examined by Northern analysis (Figure 2Ca). The transcript with apparent length of ~ 9.2 kb is abundant in heart, skeletal muscle, pancreas, and placenta and

Figure 2. Sequence and Expression of ACTR. (A) Amino acid sequences of ACTR. Closed triangle indicates the point from which an N-terminal variant clone A94 (data not shown) differs from ACTR (A77). Open triangles indicate the endpoints of a fragment that is missing in clone A59. The initiation codon of clone A38 is indicated by an arrow. The potential receptor interaction motifs are underlined. (B) Schematic alignment of ACTR, hSRC-1 (full-length), and hTIF2. Regions in TIF2 and SRC-1 with significant homology are aligned with ACTR, and percentages of amino acids identical to ACTR are indicated. Functional domains or regions identified in this study and others are also indicated. (C) Northern blot analysis of ACTR in different human tissues (a) and cancer cell lines (b). The human cell lines are as follows: HL60, promyelocytic leukemia cell line; Hela, cervical carcinoma cell line (S3); K562, chronic myelogenous leukemia cell line; Molt4, lymphoblastic leukemia cell line; Raji, Burkitt's lymphoma cell line; SW480, colorectal adenocarcinoma cell line; A549, lung carcinoma cell line; G361, melanoma cell line. The relative positions of RNA markers (kb) are at the left side of each panel.

is relatively low in brain, very low in lung and liver, and barely detectable in kidney. Shorter transcripts could also be detected in different tissues with lower abundance. Similar to the tissue expression profile, human cancer cell lines examined also showed restricted expression of ACTR (Figure 2Cb).

ACTR Enhances the Transactivation Function of Nuclear Receptors in Mammalian Cells

The ability of ACTR to stimulate receptor function in yeast prompted us to test whether similar activities can be detected in mammalian cells. Transient transfection was initially performed in A549 human lung carcinoma cell line in which low level of ACTR expression was detected (Figure 2C). As shown in Figure 3A, cotransfection of these cells with expression vectors for ACTR or an N-terminal deletion variant (A38, Figure 2A) significantly increased RAR-mediated induction of the β RARE-tk-luc reporter gene activity (compare Figure 3A, lanes 3 and 4 to lane 2). Similar results were obtained in CV-1 cells (data not shown). To determine whether ACTR stimulates other nuclear hormone receptors, similar transfection experiments were carried out in CV-1 and HeLa cells as shown in Figures 3B, 3C, and 3D. Introducing only a hTR β expression plasmid into CV-1 cells led to a modest increase in reporter DR4-tk-luc activity in the presence of T3 (Figure 3B, lane 2). When ACTR and its derivative A38 are cotransfected with TR, the reporter activity is remarkably increased (Figure 3B, lanes 3 and 4). The strongest effects were seen with RXR on the ApoA1 RXRE tk-luc reporter (~ 4 -fold) (Figure 3C, lanes 2 and 3). We have also performed the cotransfection experiments with VDR, GR, and ER and observed similar effects in each case (Figure 3D and data not shown). These results indicate that ACTR is indeed a nuclear receptor coactivator.

ACTR Interacts with Nuclear Hormone Receptors in an Agonist- and AF2/ τ c-Dependent Manner

To test whether ACTR interacts with receptors directly, we first performed series of experiments using glutathione-S-transferase (GST) pull-down assays (Figures 4A and 4B). ACTR protein (aa 493 to C terminus) was expressed using baculovirus system and purified as GST fusion. As shown in Figure 4A, in the presence of their cognate ligands, a large portion (approximately 20% to 40% of the input) of 35 S-labeled receptor proteins were specifically retained by the GST-ACTR fusion protein immobilized on glutathione-agarose beads (lanes 4, 8, and 12), while no significant binding was observed either in the absence of ligands or by GST alone (lanes 2, 3, 6, 7, 10, and 11). Thus, ACTR is able to interact with several members of the receptor superfamily in a ligand-dependent manner in vitro. We next tested if mutations in C-terminal activation domain (AF2/ τ c) affect ACTR binding. As shown in Figure 4B, wild-type RXR interacts with ACTR in the presence of its agonist LG69 (lane 4), while point mutations at positions 450 (F450P) or 451 (L451A) either abolished or severely diminished this association (lanes 6 and 8).

GAL4-DBD fusion of ACTR was used to demonstrate in vivo interaction with receptors in transiently trans-

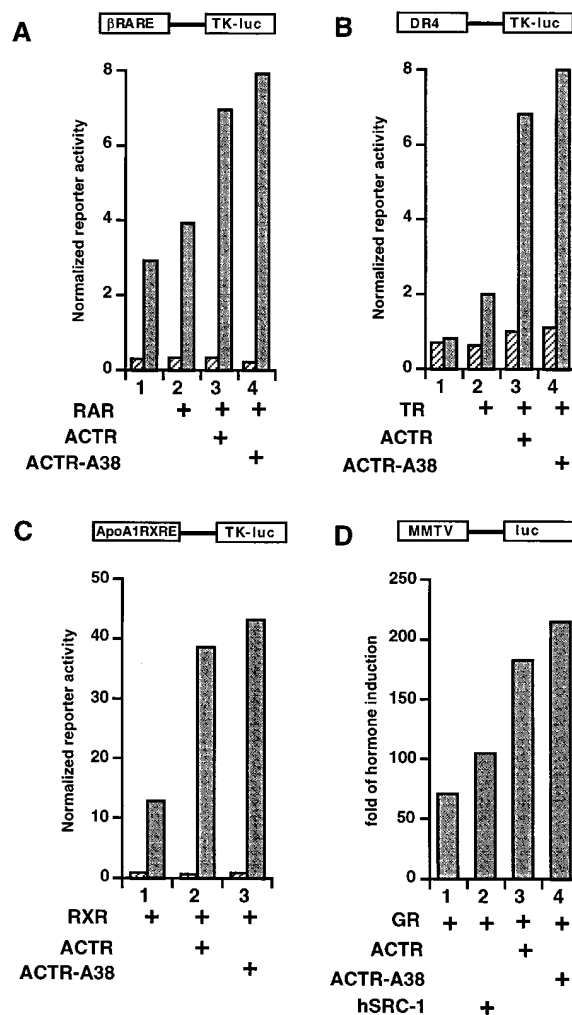


Figure 3. ACTR Stimulates the Transactivation by Nuclear Receptors in Mammalian Cells

Luciferase reporter gene activities under the control of β RARE-tk (A), DR4-tk (B), ApoA1-RXRE-tk (C), and MMTV (D) promoters were measured (see Experimental Procedures) from extracts of A549 lung carcinoma cells (A), CV-1 cells (B and C) and HeLa cells (D) after the cells were transiently transfected by the corresponding reporter and expression plasmids. Relative luciferase activities in the presence (solid bars) or absence (hatched bars) are presented in (A), (B), and (C) after being normalized by the internal control β -gal activities. The fold of hormone induction in (D) is derived from the normalized luciferase activity in the presence of dexamethasone divided by the activity in the absence of hormone, which was essentially not affected by the introduction of ACTR or hSRC-1 expression plasmids. The ligands and their final concentrations used in the transfection are as follows: (A), atRA (100 nM); (B), T3 (100 nM); (C), LG69 (100 nM); and (D), Dex (25 nM). ACTR-A38 is a shorter version of full-length ACTR (see Figures 1 and 2 for comparison).

fecting cells. As shown in Figure 4C, lane 1, GAL4-DBD-ACTR alone displays significant reporter activity due to the presence of intrinsic activation functions (see below). Nonetheless, cotransfection with VP16-receptor ligand-binding domains (LBDs) dramatically increased the reporter gene activity in the presence of T3 (lane 2), LG69 (lane 4) and 1,25-(OH) $_2$ -D $_3$ (lane 6), although in the cases of RAR and ER, the increase of reporter gene activity is relatively low (lanes 3 and 5). Taken together,

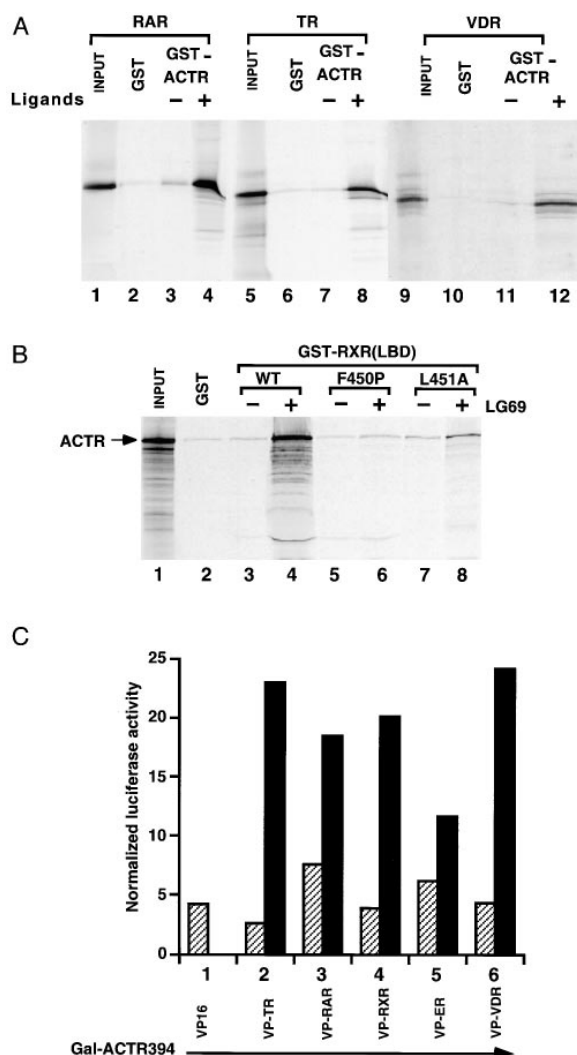


Figure 4. ACTR Association with Nuclear Receptors Is Ligand and AF2 Dependent

(A) In vitro translated and 35 S-labeled full-length receptor (hRAR α , rTR α , and hVDR) products were incubated with GST or GST-ACTR (493 to C terminus) immobilized on the glutathione beads in the presence or absence of 1 μ M of corresponding ligands (Am580, lane 4; T3, lane 8; and 1,25-(OH) $_2$ -D $_3$, lane 12). Labeled proteins retained on the beads after extensive washing were analyzed by SDS-PAGE and autoradiography, together with 20% of the translated products used in each incubation (INPUT).

(B) In vitro translated full-length ACTR was incubated with GST or GST fusions of wt or mutant hRXR α LBD.

(C) The interaction of ACTR (394 to C terminus) with receptor LBDs in vivo was measured as luciferase reporter activities in the presence (solid bars) or absence (hatched bars) of the corresponding hormones (100 nM T3, lane 2; 100 nM Am580, lane 3; 100 nM LG69, lane 4; 50 nM β -estradiol, lane 5; and 100 nM 1,25-(OH) $_2$ -D $_3$, lane 6) by the mammalian two-hybrid assay performed in CV-1 cells. The MH100x4-tk-luc reporter used has four GAL4-binding sites. Note that GAL4-DBD-ACTR fusion exhibits significant level of reporter activity (lane 1) due to the transactivation function of ACTR as described in the text.

the results from both the in vitro and in vivo experiments reveal ACTR directly binds nuclear receptor LBDs in an agonist- and AF2/ τ c-dependent manner.

ACTR Contains Multiple Receptor Interaction Domains

To map the receptor-interaction domain (RID) in ACTR, deletion constructs were generated and used in mammalian two-hybrid assay as described above. Results shown in Figure 5A indicate that the central region (aa 596 to 833) of ACTR is able to associate with nuclear hormone receptors. This is in agreement with the data published for SRC-1 (Yao et al., 1996) and TIF2 (Voegel et al., 1996) and with our results of library screening where all the clones isolated share the central region (Figure 1). To further map the RID, more deletions were made within the central fragment (aa 596–833), and a 200-amino-acid fragment (aa 621–821) was found to contain all the activity to interact with different receptors tested (Figure 5Ba) and therefore named as ACTR_{RID}. Interestingly, when this 200-amino-acid fragment (aa 621–821) was subcloned as two smaller fragments (aa 621–728, N-RID and aa 729–821, C-RID), each retained the ability to interact with RAR, RXR, and TR in hormone-dependent fashion (Figures 5Bb and 5Bc). To confirm the above transfection-based observations, individual RID fragments were made as GST fusion proteins and tested in the in vitro GST pull-down assay. As shown in Figure 5C, in the presence of hormones, both N-RID and C-RID are able to bind independently to the RAR. Similar results were obtained for RXR and TR (data not shown). We thus conclude that ACTR has at least two separable RIDs located in its central region.

ACTR Associates with Receptors on Hormone Response Elements

To characterize further interaction between ACTR and receptor homo- and heterodimers, we have performed gel mobility shift assays using purified proteins. Results shown in Figure 5D indicate that, in the absence of ligands, no significant association of ACTR is detected with RXR/RAR and RXR/TR heterodimers or RXR homodimers (lane 5 in Figure 5Da, lanes 3 and 7 in Figure 5Db, and lane 6 in Figure 5Dc). In contrast, in the presence of agonist, receptor–DNA–ACTR complexes are readily observed (lanes 6 and 7 of Figure 5Da with atRA and RAR synthetic agonist TTNPB; lanes 4 and 8 of Figure 5Db with T3; lanes 7 and 8 with 9-*cis* RA and RXR specific agonist LG69). Similar results were obtained by using larger ACTR fragments (aa 493 to C terminus) without the GST moiety (data not shown). Recently, a novel RXR homodimer antagonist (LG100754) was found to be able to activate specifically RAR/RXR heterodimer (Lala et al., 1996). As predicted, LG100754 did not promote the association of ACTR with RXR homodimers (lane 9 of Figure 5Dc) but did induce ACTR binding to the heterodimer (lane 8 of Figure 5Da). It should be noted that in the gel-shift assays, the recruitment of ACTR to receptor–DNA complex does not incur significant change in the affinity of receptors to hormone response elements (Figure 5D; compare the receptor–DNA complex formation in the presence and absence of ACTR), suggesting that ACTR probably does not function through stimulating receptor DNA binding.

CBP/p300 is an ACTR Cofactor

To gain insight into its mechanism of activation, GAL4-DBD fusions of ACTR fragments were used to map putative activation domains (Figure 6A). Truncation analysis

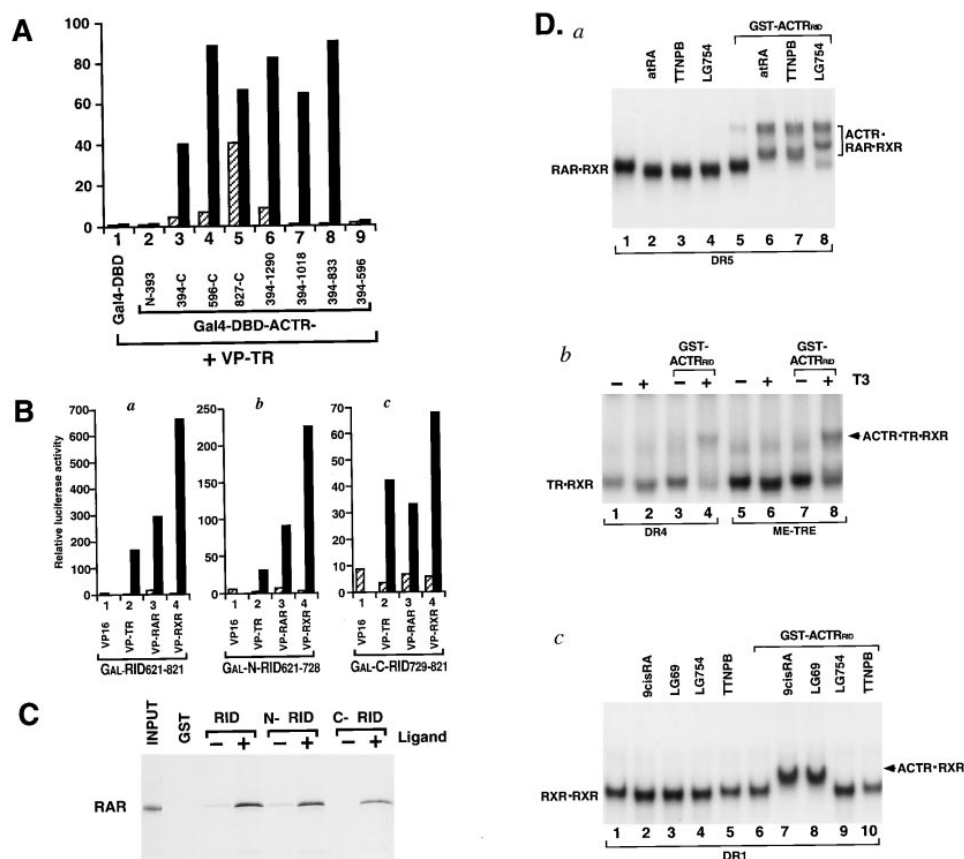


Figure 5. ACTR Has Two Receptor-Interaction Domains and Associates with Receptors on DNA Elements

Mammalian two-hybrid assay was used (A) to assess the ability of ACTR derivatives to interact with TR in the presence (solid bars) and absence (hatched bars) of 1 μ M T3 and (B) to measure the interaction of the receptor-interaction domain (RID, [a]; and its subdomains (N-RID, [b]; C-RID, [c]) of ACTR with different receptors in the presence (solid bars) or absence (hatched bars) of 1 μ M ligands (T3 for TR β , Am580 for RAR α , and LG69 for RXR α). Note that data from the same experiment is plotted at three different scales. (C) In vitro translated hRAR α was incubated with GST or GST-ACTR-RIDs immobilized on glutathione beads in the presence or absence of 1 μ M of Am580. Retained proteins were analyzed by SDS-PAGE and autoradiography, together with 20% of the translated products (INPUT). (D) Gel mobility shift assays were performed with purified GST-ACTR_{RID} (aa 621–821) and full-length nuclear receptors in the presence or absence of 1 μ M ligands as indicated. The positions of nuclear receptor–DNA complexes and ACTR–receptor–DNA complexes are indicated. Free probes as indicated at the bottom of each panel are omitted.

reveals the region to be localized within the C-terminal half of ACTR (aa 827 to C terminus) (Figure 6A, lane 6). Through further N- and C-terminal deletion analysis, two separable activation domains (AD-A and AD-B) were identified. As shown in Figure 6B, additional deletions localize AD-A to a fragment of 50 amino acids (aa 1039–1088). The small size of this fragment suggests it may function as an interaction domain to recruit another coactivator. While AD-A fails to interact with TBP, TFIIB, and the TAF_{II}s (data not shown), a strong interaction with CBP was observed (Figure 6C, aa 1018–1290). This interaction is mediated by the glutamine-rich region of CBP (aa 1891 to C terminus) (data not shown). Further analysis indicates that the fragment of ACTR (aa 1018–1088) that mediates the interaction with CBP is the same region that harbors the strong autonomous transactivation function of ACTR (Figure 6B, lane 6, and Figure 6D, lanes 9, 11, and 12). In contrast, the less active C-terminal fragment shows no significant interaction with either CBP or TBP, the TAF_{II}s, and TFIIB (Figure

6B, lane 4, Figure 6D, lane 10, and data not shown). Therefore, we have linked the ability of ACTR to activate transcription to apparently recruiting the acetyltransferase CBP/p300. To assess the importance of the CBP interaction, an appropriate deletion mutant (aa 1018–1088) of ACTR was constructed. As shown in Figure 6E, while transient cotransfection of CV-1 cells with wild-type ACTR strongly augments GR induction of the MMTV reporter (compare lanes 2 and 3 with lane 1), the CBP interaction domain (CID) deletion mutant shows no effect above background (lanes 4 and 5). This result strongly supports the notion that CBP is an essential component of the activation complex.

ACTR Recruits P/CAF

As CBP/p300 interact with both ACTR and P/CAF, we tested the potential interaction of ACTR with P/CAF. Flag-tagged P/CAF was expressed and purified on Flag-Ab agarose beads and used in the pull-down experiment. Results shown in Figure 7A indicate that ACTR

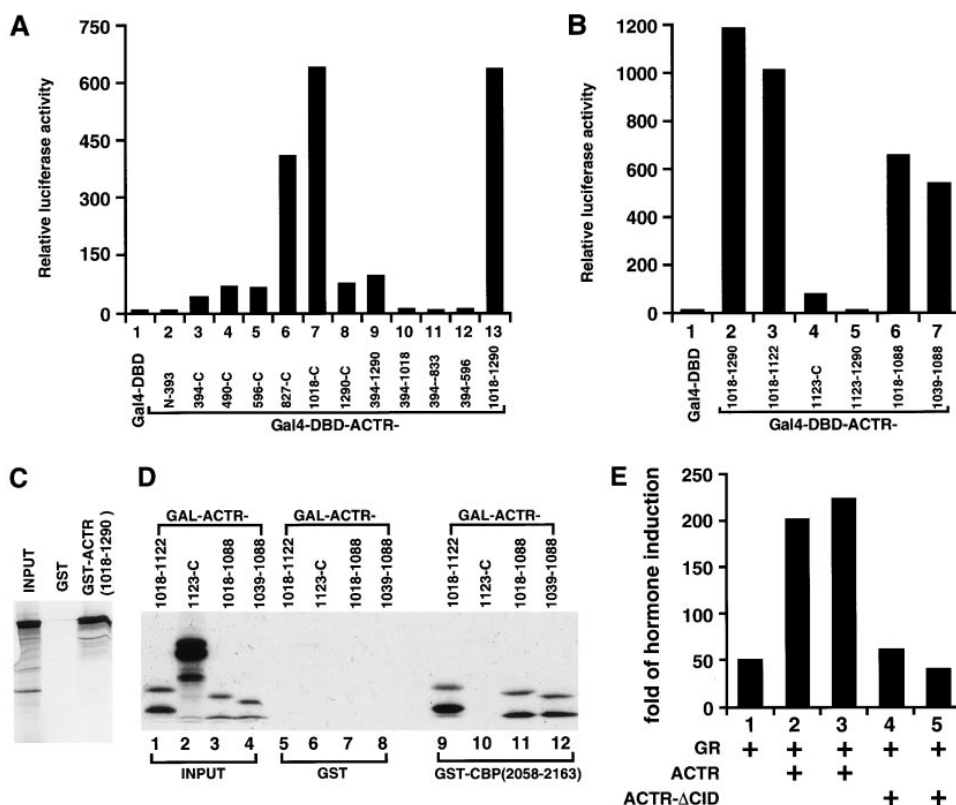


Figure 6. ACTR Transactivation Domain Interacts with CBP

(A and B) The indicated deletions of ACTR were made and fused to Gal4-DBD. These derivatives were then tested on a luciferase reporter under the control of GAL4-binding sites.

(C and D) Interaction of ACTR derivatives with CBP were determined by GST pull-down assay similar to the ones described in Figures 5 and 7 with in vitro translated VP16-CBP (aa 1891 to C terminus) fusion (C) or GAL-ACTR derivatives (D) and GST-ACTR (C) or GST-CBP (D). Note that 40% of the input translation product was loaded to the SDS-PAGE gel in (D).

(E) Transient transfections were performed essentially the same as in Figure 4. Five nanograms (lanes 2 and 4) or 15 ng (lanes 3 and 5) per well of plasmid-encoding ACTR full length (lanes 2 and 3) or ACTR CID deletion (lanes 4 and 5) was used in transfection of 48-well plates of CV-1 cells.

indeed is able to interact with P/CAF in vitro (lane 3). Similar results were obtained by yeast two-hybrid assay, as shown in Figure 7B. Thus, ACTR independently interacts with both CBP/p300 and their associated factor P/CAF, serving as a docking platform to bridge the HAT protein complex to DNA bound nuclear receptors.

ACTR Has Intrinsic HAT Activity

Given that intrinsic HAT activity has been found in various coactivators, including GCN5 (Kuo et al., 1996), CBP/p300 (Ogryzko et al., 1996; Bannister and Kouzarides, 1996), P/CAF (Yang et al., 1996), and TAF_{II} 250 (Mizzen et al., 1996), such activity might be a general feature of other coactivators. Based on this hypothesis, we tested Flag-tagged full-length ACTR protein expressed in a recombinant baculovirus system for histone acetylase activity. Our initial data demonstrated the presence of histone acetylase activity in this preparation of ACTR. In order to determine whether the histone acetylase activity is intrinsic to ACTR, the affinity-purified ACTR was resolved on a 10%–30% glycerol gradient. The gradient fractions were analyzed by SDS-PAGE (Figure 8A) and assayed for histone acetylase activity

(Figure 8B). The data clearly show that the histone acetylase activity cofractionates with ACTR and not with any other proteins. Moreover, the histone acetylase activity also cofractionated with ACTR on a Mono Q column (data not shown). Since only one polypeptide, corresponding to ACTR, chromatographs with histone acetylase activity in two separation methods that rely on totally different polypeptide characteristics, it seems likely that the histone acetylase activity is intrinsic to ACTR itself and not due to the presence of an associated or contaminating histone acetylase activity.

In order to determine which histones are acetylated by ACTR, we performed histone acetylase assays with either free core histones or mononucleosomes and analyzed the products of the reaction by SDS-PAGE followed by autoradiography (Figure 8C). ACTR acetylated primarily histones H3 and H4 on both free histone and mononucleosomes from HeLa cells, while a low level of acetylation of H2b can be detected when free histones are used as substrate (lanes 2 and 4). Control samples where ACTR was replaced with bovine serum albumin demonstrated barely detectable background acetylation of histones (lanes 1 and 3). When histones were

replaced in the reaction by bovine serum albumin or lysozyme, no acetylation of these lysine-rich nonhistone proteins was detected (data not shown). Thus, ACTR appears to define a novel class of histone acetylase with clear activity on both free core histones and nucleosomes.

We have localized the enzymatic activity in ACTR by generating a variety of truncated ACTR constructs that were then expressed and purified from either insect cells or *Escherichia coli*. Constructs 1 to 3 (Figure 8D) show that either Flag- or GST-tagged ACTR variants from baculovirus vectors localize HAT activity to the C terminus of ~400 amino acids. Construct 5 shows the smallest region (aa 1092–1292) retaining HAT activity that deletes both the CID and the extreme C terminus. To rule out the possibility that endogenous insect cell proteins might contribute to the HAT activity, we expressed in *E. coli* a C-terminal segment of ACTR (aa 1018 to C terminus, construct 4) and found that the GST fusion is a potent HAT. In contrast, purified N-terminal or other GST fusions (constructs 6, 7, and 8) are completely inactive. Together, this work establishes that histone acetylase activity is an intrinsic ACTR function.

Discussion

This work describes the isolation and functional analysis of a novel nuclear receptor coactivator (ACTR) and defines a plausible biochemical pathway for its action. ACTR appears to exert its regulatory effects in two distinct biochemical steps. First, ACTR associates with both CBP/p300 and P/CAF and thus functions to recruit two transcriptional cofactors to the nuclear receptor–DNA complex. The ability of ACTR to recruit these cofactors appears to be essential for its positive function and is consistent with previous studies demonstrating the essentiality of CBP in receptor function and the essentiality of P/CAF in CBP function. Secondly, we demonstrate that ACTR possesses intrinsic HAT activity. This

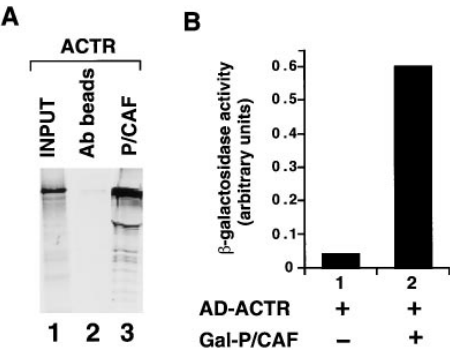


Figure 7. ACTR Interacts with P/CAF
(A) In vitro translated and ³⁵S-labeled ACTR was incubated with M2 Ab agarose beads (lane 2) or Flag-P/CAF immobilized on the beads (lane 3). Labeled peptides retained on the beads after extensive washing were analyzed by SDS-PAGE and autoradiography, together with 10% of the translated products used in the incubation (INPUT).
(B) The interaction of ACTR with P/CAF in yeast was measured as β-galactosidase activity in yeast strain Y190 that was transformed with GAL4 activation domain fused to full-length ACTR and Gal4-DBD (lane 1) or Gal4-DBD fused to full-length P/CAF (lane 2).

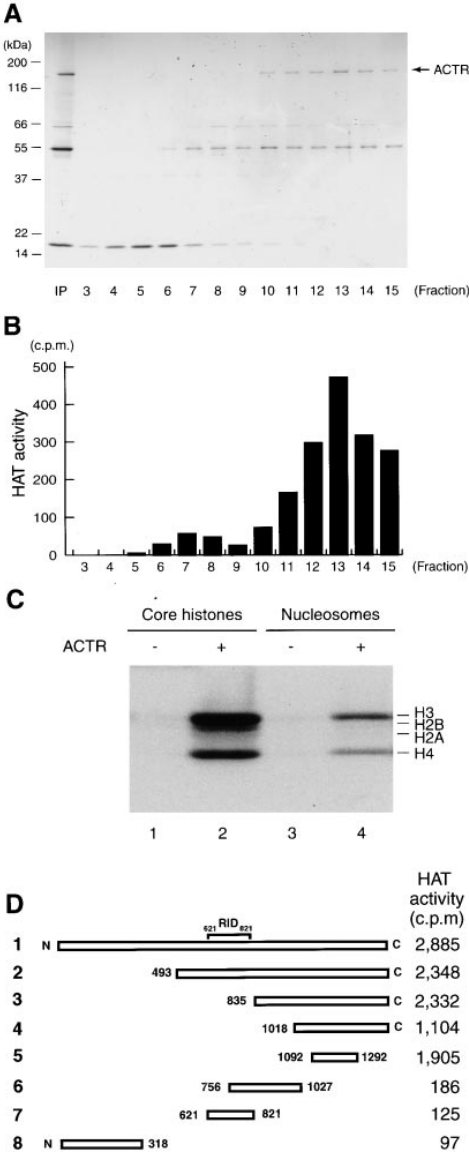


Figure 8. ACTR Is a HAT
(A) SDS-PAGE analysis of glycerol gradient fractions of recombinant ACTR. A 7.5 μl aliquot of the indicated fractions was separated on a 4%–20% SDS-PAGE gel and stained with Coomassie blue. Two microliters of input (IP) was also analyzed. The protein corresponding to full-length ACTR is indicated by an arrow.
(B) Histone acetylase activity of glycerol gradient fractions of ACTR. A 5 μl aliquot of the indicated fractions was assayed for histone acetylase activity in a histone filter-binding assay. The levels of enzyme activity are represented as cpm of radioactive [³H]acetate transferred from acetyl-CoA to histone.
(C) Acetylation profiles of the substrate specificity of ACTR. Core histones (lanes 1 and 2) or mononucleosomes (lanes 3 and 4) were incubated with BSA (lanes 1 and 3) or recombinant ACTR (lanes 2 and 4). [¹⁴C]-acetylated histones were detected by autoradiography after separation by SDS-PAGE.
(D) HAT activity in ACTR is localized in the C-terminal region. Full-length or truncated derivatives of ACTR were expressed as either GST (constructs 2, 4, 6, 7, and 8) or Flag (constructs 1, 3, and 5) fusions and purified from either *E. coli* (constructs 4, 6, 7, and 8) or Sf9 cells (constructs 1, 2, 3, and 5). Liquid HAT assays were performed using 1 mg/ml of calf thymus histones and ~1 μg purified ACTR proteins.

is unexpected, since ACTR does not possess homology to other known HATs and thus apparently defines a novel class of these regulatory enzymes. Thus, ACTR is both a HAT and by virtue of its ability to bind both CBP and P/CAF promotes the formation of a multisubunit activation complex. Interestingly, while all currently identified HATs (including ACTR) are capable of acetylating core histones H3 and H4, ACTR displays an unusual additional activity as an effective acetylator of mononucleosomes, similar only to that observed in CBP/p300. In contrast, neither GCN5 (Kuo et al., 1996), P/CAF (Yang et al., 1996), nor TAF_{II} 250 (Mizzen et al., 1996) are capable of acting on H3 and H4 in mononucleosomes. These results suggest a potential cooperative activity in which distinct histone acetylases with different regulatory capacities mutually recruit each other to provide a concerted regulatory effect. While the individual roles of each HAT is not yet clear, it seems unlikely that they subserve completely redundant functions. Rather, it is possible that they will either modify selective sites on the histone tails or act in an ordered fashion to control different aspects of transcriptional activation.

Previous studies have suggested that liganded nuclear receptors may alter nucleosome structure and that histone acetylation may be sufficient to activate a hormone-responsive promoter (Zaret and Yamamoto, 1984; Fragoso et al., 1995; Wong et al., 1995; Bartsch et al., 1996). The relevance of this pathway is further substantiated by the observation that the recently characterized transcriptional corepressor complex possesses histone deacetylase activity (Nagy et al., 1997). This argument is relevant since both the coactivators (such as CBP, ACTR, and SRC-1) and the corepressors (such as SMRT and N-CoR) were isolated largely based on their ability to interact with nuclear receptors (Chen and Evans, 1995; Horlein et al., 1995). Thus, the demonstration of opposing enzymatic activities of these complexes suggests that control of histone acetylation is indeed a critical feature of hormone signaling.

The sequence homology between ACTR and two related cofactors, SRC-1 and GRIP/TIF2, defines a cofactor gene family and suggests that these other proteins are likely to function as novel histone acetylases. Indeed, as anticipated, we have also demonstrated that SRC-1 exhibits HAT activity (data not shown). Several common features are obvious among this nuclear receptor coactivator family. First, they share a number of functional domains (Figure 2B). One of the highly conserved regions (more than 60% identity within ~280 amino acids) is located at the N-termini of all three members of this family. Although this region does not appear to be required for ACTR and SRC-1 to enhance receptor-mediated transactivation (Figure 4) (Onate et al., 1995), it may still contribute to aspects of ACTR function. In contrast, the central conserved region of ~200 amino acids comprises the RIDs and is essential for function. Although no apparent homology can be found among regions associated with receptor-interaction activity, computer analysis indicates they all contain an amphipathic α -helical structure with conserved sequences LXXLL (Figure 2A). Interestingly, sequences resembling LXXLL were recently identified as the receptor-interaction box

(NR-box) in TIF1 β proteins (Le Douarin et al., 1996). In addition, the well-conserved C-terminal region (CID) interacts strongly with CBP and corresponds to a potent transactivation domain.

A second feature of this gene family is their alternative splicing products detected by cDNA library screening in this study (Figure 2) and others (Kamei et al., 1996; Voegel et al., 1996), which presumably results in the expression of variant coactivator proteins. While SRC-1a was found to have another RID at its C-terminal end (Figure 2B) (Onate et al., 1995; Kamei et al., 1996), in general, the functional differences of these variants are not understood. Nevertheless, the conservation of these splicing events suggests that distinct roles may be played by these isoforms. In light of the recent observation that CBP/p300 is involved in multiple transactivator-mediated signaling pathways, it seems reasonable to speculate that ACTR and its family members, including their splicing variants, are likely to constitute a widely used coactivation complex.

A third common characteristic of the coactivator family is the ability to interact with multiple members of the receptor superfamily with apparently different interaction strengths (this study and references in Introduction). In addition, their tissue expression patterns are distinct, with ACTR being the most restricted (Figure 2C). Thus, it is conceivable that each member of this family could serve as a primary coactivator for a subset of receptors. In a given cell type, however, which coactivator(s) is involved in a particular hormonal signaling pathway would be determined by the expression level of both receptors and coactivators and possibly the interaction of receptors with other proteins such as promoter-specific transcription factors. Therefore, the seemingly redundant functions played by the different coactivators may, in fact, add another level of complexity for cells to control precisely the level of gene expression in response to hormones.

The results of this study suggest that nuclear receptors recruit an enzymatic complex that includes at least three distinct classes of mutually interacting HATs. In addition, multiple additional proteins may be present in the final complex (Fondell et al., 1996). Receptors apparently directly interact with at least CBP and ACTR to nucleate this complex and thus target it to the DNA template. This association is mediated by the LBD and is hormone and AF2 dependent. Intrinsic to this activation process is the reversal of receptor-mediated transcriptional repression. In the repressive state, the receptors associate with silencing proteins such as SMRT and N-CoR. Recent studies demonstrate that SMRT and N-CoR associate with the corepressor mSin3A and the histone deacetylase (HDAC-1) to form a transcriptional repressor complex (Nagy et al., 1997; Heinzel et al., 1997). This repressor complex is dissociated from the receptor following addition of hormone. The resulting allosteric changes then attract the ACTR-CBP-P/CAF activation complex, which is composed of at least three distinct HATs. In this model, we suggest that hormone signaling may function at least in part by controlling the balance of competing enzymatic activities at a target promoter. In this view, hormonal control of targeted chromatin remodeling may be a critical feature of physiologic homeostasis.

Experimental Procedures

Yeast Strains and cDNA Library Screening

The yeast strain RH4XRE was derived from parental strain RH1533 by integrating into its chromosome two reporter genes, *HIS3* and *lacZ*, following the procedure described previously (Gstaiger et al., 1995). The reporter genes were constructed by replacing the UAS sequences in pLI4 and pBM1499 with 4× multimerized β RARE sequence. Full-length hRAR β was constitutively expressed in strain RH4XRE by transforming the yeast with pG1-RAR β (Chen et al., 1993). For library screening, strain RH4XRE carrying pG1-RAR β was transformed with human leukemia GAL4 AD fusion cDNA library (CLONTECH). Approximately 2×10^7 transformants were selected on plates with synthetic medium lacking histidine and leucine and containing 5 μ M atRA and 40 mM 3AT in order to suppress the low level expression of *HIS3* due to the transactivation activity of liganded receptor. About 250 colonies that are 3AT resistant and strongly blue were isolated for subsequent analysis. Among the colonies analyzed, essentially all of them carried plasmids rendering the yeast strain receptor and/or hormone-dependent growth on the selection medium. Yeast liquid β -galactosidase assay was carried out in strain Y190 according to the manufacturer's protocol (Clontech).

Plasmids and Transfection

Expression plasmid for full-length ACTR was constructed by introducing Kozak sequence and an Asp-718 site at the 5' end of clone A77 via PCR and then cloned into pCMX-PL2 vector as Asp-718 and XhoI fragment. ACTR-A38 clone was introduced to pCMX-PL2 via Sall site. Deletions of ACTR were made via PCR amplification (Pfu DNA polymerase, Stratagene) of fragments of clone A77 sequences and subcloning them into pCMX-PL2. Constructs as GAL4-DBD fusion used in mammalian two-hybrid assays were made by cloning PCR-amplified fragments of ACTR either into pM vector (Clontech) via BamHI site or into pCMX-Gal-N via Asp-718 and BamHI. Transient transfection was performed on cells plated in 48-well plates by lipofection. CMX- β -gal plasmid was used as an internal control to normalize the luciferase activity. Each transfection was carried out in either duplicate or triplicate and repeated at least twice.

Pull-Down Assays and Gel Shift Assay

Usually, 50 μ l of 40% GST-protein beads slurry, which contains ~ 0.5 μ g of proteins and was resuspended in the binding buffer (20 mM HEPES [pH 7.9]; 100 or 150 mM NaCl; 1 mM EDTA; 4 mM MgCl₂; 1 mM DTT; 0.02% NP-40; 10% glycerol supplemented before use with 1 mg/ml BSA, 0.5 mM PMSF, and a protease inhibitors cocktail), was mixed with 10 μ l of in vitro translation reactions with [³⁵S]methionine (Promega TNT kit) for 30 min at 4°C. The beads were then washed three times with the binding buffer and resuspended in SDS-PAGE sample buffer. After electrophoresis, radio-labeled polypeptides retained on the beads were visualized by autoradiography. Pull-down experiment using Flag-Ab beads was performed in the same fashion. Gel mobility shift assays were performed essentially as described before (Chen et al., 1993). Ligands dissolved in ethanol were added to final concentration of 1 μ M to the binding buffer containing receptor and ACTR proteins.

The RID (aa 621–821), N-RID (aa 621–728), and C-RID (aa 729–821) of ACTR were cloned to pGEX-KG, expressed, and purified from *E. coli*. The large fragment of ACTR (aa 493 to C terminus) was cloned to pAcGHLT, expressed, and purified from Sf9 cells using the Baculovirus expression system (Pharmingen). Full-length hRXR α was cloned to pAcGHLT, expressed, and purified from Sf9 cells after thrombin cleavage of the GST portion. Human RAR α and TR β proteins were cloned to pVL1393, coexpressed, and purified with GST-hRXR α .

Northern Analysis

Human multiple tissue and cancer cell line Northern blots (Clontech) were hybridized with a radio-labeled 0.75 kb EcoRI fragment (from clone B6 and encompassing aa 703 to 927 of ACTR) and exposed on X-ray film.

Glycerol Gradient Fractionation of Flag-Affinity

Purified ACTR

Full-length ACTR was cloned to pAcSG2 (Pharmingen) via PCR amplification with Flag sequence incorporated into the 5' primer. Sf9 cells infected with the recombinant Flag-ACTR baculovirus were harvested 48 hr postinfection and lysed in BCT 500 (20 mM Tris-HCl [pH 8], 5 mM MgCl₂, 500 mM KCl, 0.1% Tween-20, 1 mM PMSF, and 10 mM β -mercaptoethanol) supplemented with 10% glycerol and complete protease inhibitor cocktail (Boehringer Mannheim). Recombinant protein was purified on M2-agarose (Kodak-IBI) and eluted with Flag peptide according to the manufacturer's protocol. Approximately 5 μ g of recombinant ACTR was applied onto a 4 ml 10%–30% glycerol gradient prepared in BCT 500 and centrifuged in a Beckman SW-60 rotor for 15 hr at 485,000 $\times$ g. The gradient was fractionated into 200 μ l aliquots. Each fraction was analyzed on a 4%–20% SDS-PAGE gel and assayed for histone acetylase activity.

Histone Acetylase Activity Assays

Histone acetylase assays were performed as previously described (Ogryzko et al., 1996) with the following modifications. Filter-binding assays shown in Figure 8B were performed using 66 μ g/ml of HeLa core histones and 12 pmol of [³H]acetyl CoA (4.3 mCi/mmol, Amersham Life Science Inc.). Assays in Figure 8D were performed with a modification that calf thymus histones (Sigma Chemicals) were used as substrates and that GST or Flag-ACTR fusion proteins were used as they were still associated with the agarose beads. The GST-fusion proteins were expressed in and purified from *E. coli* strain BL21(DE3)pLysS. For SDS-PAGE analysis of acetylated histones, 180 pmol of [¹⁴C]acetyl CoA (55 mCi/mmol, Amersham Life Science Inc.) and either 66 μ g/ml of HeLa core histones or mononucleosome core particles were used. HeLa core histones and mononucleosome core particles were prepared as described (Hayes et al., 1994).

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