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Ligand-Dependent Nuclear Receptor Corepressor LCoR Functions by Histone Deacetylase-Dependent and -Independent Mechanisms

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Summary

LCoR (ligand-dependent corepressor) is a transcriptional corepressor widely expressed in fetal and adult tissues that is recruited to agonist-bound nuclear receptors through a single LXXLL motif. LCoR binding to estrogen receptor α depends in part on residues in the coactivator binding pocket distinct from those bound by TIF-2. Repression by LCoR is abolished by histone deacetylase inhibitor trichostatin A in a receptor-dependent fashion, indicating HDAC-dependent and -independent modes of action. LCoR binds directly to specific HDACs in vitro and in vivo. Moreover, LCoR functions by recruiting C-terminal binding protein corepressors through two consensus binding motifs and colocalizes with CtBPs in the nucleus. LCoR represents a class of corepressor that attenuates agonist-activated nuclear receptor signaling by multiple mechanisms.

Introduction

Nuclear receptors are ligand-regulated transcription factors whose activities are controlled by a range of lipophilic extracellular signals. They directly regulate transcription of genes whose products control many aspects of physiology and metabolism (Chawla et al., 2001). Receptors are composed of a series of conserved domains, A–F. Many N-terminal A/B regions contain transactivation domains (activating function-1; AF-1), which can cooperate with AF-2, located in the C-terminal ligand binding domain (LBD) (Tora et al., 1989; Robyr et al., 2000). Crystal structures of agonist- and antagonist-bound LBDs have revealed conserved α helical structures (Bourget et al., 1995; Renaud et al., 1995; Wagner et al., 1995; Brzozowski et al., 1997). Agonist binding induces conformational changes that reorient the C-terminal AF-2 helix (helix 12) to create a binding pocket recognized by coactivators.

Several coregulatory proteins control nuclear receptor function (Robyr et al., 2000; Glass and Rosenfeld, 2000; Dilworth and Chambon, 2001; Rosenfeld and Glass, 2001). Their diversity suggests that transcriptional activation by receptors occurs through recruitment of multiple factors acting sequentially or combinatorially. Coactivators of the p160 family, SRC1/NCoA1, TIF-2/GRIP-1, and pCIP/AIB1/RAC3/ACTR/TRAM-1 (Onate et al., 1995; Chakravarti et al., 1996; Hong et al., 1996; Voegel et al., 1996; Anzick et al., 1997; Chen et al., 1997), which interact with ligand-bound receptors through LXXLL motifs or NR boxes (Voegel et al., 1996; Heery et al., 1997). Cocrystallographic studies of ligand-bound receptors revealed α-helical NR boxes oriented within a hydrophobic pocket containing helix 12 held by a charge clamp composed of conserved residues in helices 3 and 12 (Darimont et al., 1998; Feng et al., 1998; Nolte et al., 1998; Shiau et al., 1998). P160 coactivators recruit other proteins essential for transactivation, including CREB binding protein (CBP) and its homolog p300 (Kamei et al., 1996; Chen et al., 1997; Torchia et al., 1997). Several coactivators including CBP/p300 and associated factor p/CAF possess histone acetyltransferase activity, required for chromatin remodeling (Ogryzko et al., 1996; Yang et al., 1996; Chen et al., 1997; Kurokawa et al., 1998) and subsequent access of the transcriptional machinery to promoters.

Corepressors NCoR and SMRT mediate ligand-independent repression by thyroid and retinoic acid receptors (Horlein et al., 1995; Chen and Evans, 1995; Perissi et al., 1999) and recruit multiprotein complexes implicated in transcriptional repression and histone deacetylation (Alland et al., 1997; Hassig et al., 1997; Heinzel et al., 1997; Kadosh and Struhl, 1997; Laherty et al., 1997; Nagy et al., 1997; Pazin and Kadonaga, 1997). Histone deacetylases (HDACs) fall into three classes based on homology, domain structure, subcellular localization, and catalytic properties (Khochbin et al., 2001; Ng and Bird, 2001; Wade, 2001). NCoR and SMRT are components of several different complexes containing distinct combinations of ancillary proteins and class I or class II HDACs (Rosenfeld and Glass, 2001), suggesting that their function depends on cell type, combinations of transcription factors bound to specific promoters, and phase of the cell cycle.

Here, we have identified a ligand-dependent corepressor (LCoR) that interacts with ERα and other class I and class II nuclear receptors through a single NR box. LCoR, which is expressed from the earliest stages of mammalian development, functions in an HDAC-dependent and -independent manner through interactions with multiple cofactors. LCoR represents a distinct class of

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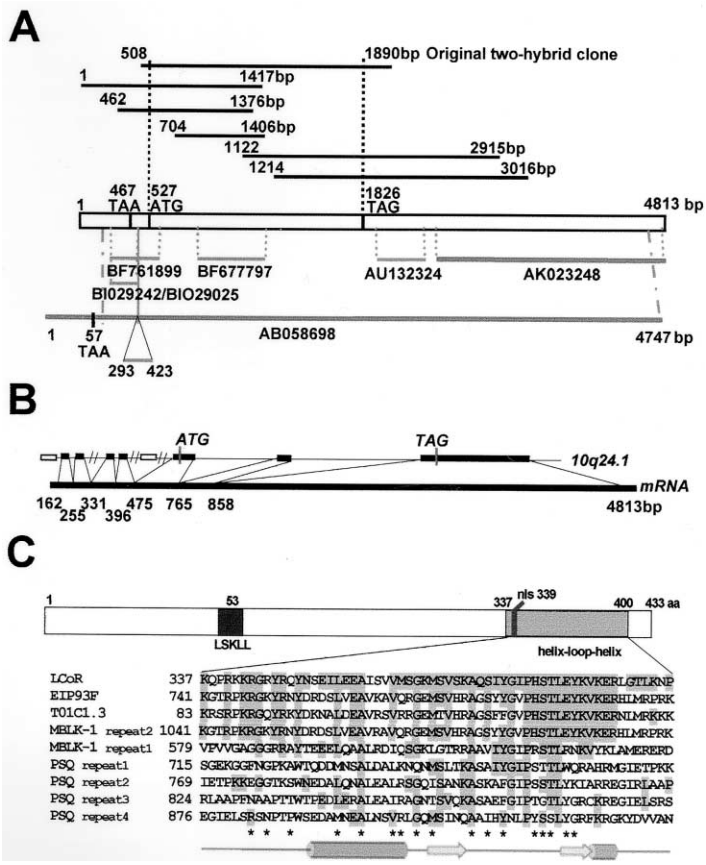

Figure 1. LCoR Gene, Transcript, and Protein Structure

(A) The LCoR two-hybrid cDNA clone (top) and clones isolated from a prostate cDNA library (below) are shown. LCoR ESTs are shown below the composite 4813 bp cDNA sequence (white bar). The open reading frame of LCoR is indicated by the start codon and the downstream stop codon. The first upstream in-frame stop codons are also indicated. Human ESTs were identified using the INFOBIOGEN site (http://www.infobiogen.fr/services/analyseq/cgi-bin/blast2_in.pl). ESTs BF761899, BF677797, AU132324, AK023248, and BI029242/B1029025 are from adult colon, adult prostate, NT2 teratocarcinoma cell line, and adult marrow cDNA libraries, respectively. A 4747 bp cDNA (AB058698) identified from a human brain library (Nagase et al., 2001) containing an extra 5′UTR exon is indicated at the bottom.

(B) Structure of the LCoR gene deduced using the Human Genome Browser (http://genome.ucsc.edu/cgi-bin/hggateway). The extra 5′UTR exons present in the human brain cDNA AB058698 are indicated as white bars. Intron sizes are indicated where known.

(C) Schematic representation of LCoR protein. The NR box LSKLL, nuclear localization signal (NLS), and putative helix-loop-helix (HLH) domain are indicated. The homologies of the HLH with other proteins are shown, with asterisks indicating positions of amino acid similarity. Existence of the HLH was predicted using Psired (http://bioinf.cs.ucl.ac.uk) and Network Protein Sequence Analysis (http://pbil.ibcp.fr).

nuclear receptor corepressor that acts to attenuate signaling by agonist-bound receptors.

Results

Identification of LCoR

LCoR was isolated from a yeast two-hybrid library as a cDNA containing a 1299 bp open reading frame (433 amino acids; 47,006 kDa; Figures 1A and 1D) encoding a protein that interacted with the ERα LBD in an estradiol-dependent manner. Additional cDNAs were obtained from a human prostate cDNA library, and several expressed sequence tags (ESTs; Figure 1A). Human sequences were also highly homologous (~95%) to several mouse ESTs, including multiple clones from a two-cell embryo library (data not shown), indicating that LCoR is expressed from the earliest stages of mammalian development. The 4.8 kb of cDNA sequence encompasses seven exons on chromosome 10q24.1, including four short 5′UTR exons that contain several in-frame stop codons (Figure 1B and data not shown). A human brain EST (Nagase et al., 2001) contains a single exon insert that lengthens the 5′UTR without extending the open reading frame and contains an upstream stop codon (Figures 1A and 1B). The initiator ATG of LCoR lies within a consensus Kozak sequence RNNatgY (Kozak, 1996).

LCoR bears only limited resemblance to known coregulators. There is a single LXXLL motif (NR box) at amino acid 53 and a PRKKRGR motif at amino acid 339 that is homologous to a simple nuclear localization signal (NLS) of the SV40 large T antigen-type. The NLS lies at the N terminus of a putative helix-loop-helix domain (Figure 1C and see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/11/1/139/DC1 for LCoR sequence), which is 48%, 48%, and 43% homologous to motifs encoded by the Eip93F, T01C1.3, and MBLK-1 genes of *Drosophila*, *C. elegans*, and Honeybee (*Apis mellifera*; Takeuchi et al., 2001), respectively (Figure 1C). The domain also bears 35% homology to the pipsqueak motif (PSQ) repeated four times in the DNA binding domain of the *Drosophila* transcription factor pipsqueak (Lehmann et al., 1998).

LCoR Is Widely Expressed in Fetal and Adult Tissues

LCoR transcripts are broadly expressed at varying levels in human adult and fetal tissues, with highest expression observed in placenta, the cerebellum, and corpus callosum of the brain, adult kidney and a number of fetal tissues (see Supplemental Figure S2 at http://www.molecule.org/cgi/content/full/11/1/139/DC1). LCoR transcripts were also detected in a wide variety of human cell lines (Figure 2A), with highest levels of expression observed in intestinal Caco-2 cells and embryonic HEK293 kidney cells. While LCoR transcripts were abundant in MDA-MB361 breast carcinoma cells, expression was weaker in MDA-MB231 and MCF-7 breast cancer lines (Figure 2A). Along with the EST data cited above, these results indicate that LCoR transcripts are widely

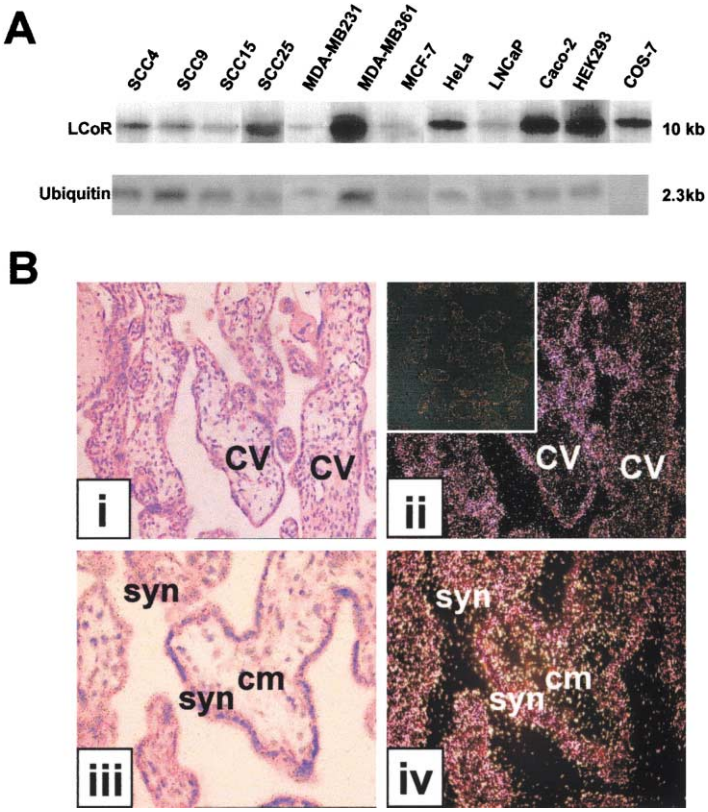

Figure 2. LCoR Transcripts Are Widely Expressed

(A) Northern blot of 15 μg of total RNA isolated from the cell lines indicated with LCoR or ubiquitin probes. SCC4, SCC9, SCC15, and SCC25 are human head and neck squamous carcinoma lines; MDA-MB231, MDA-MB361, and MCF-7 are human breast carcinoma cell lines; HeLa, LNCaP, and CaCo-2 are human cervical, prostate, and colon carcinoma lines, respectively. HEK293 cells are derived from human embryonic kidney and COS-7 from monkey kidney.

(B) In situ hybridization analysis of LCoR expression in human placenta. (i and ii) Bright and dark field photomicrographs of the chorionic villi (CV) of a near term placenta (36 weeks) probed with a 443 b ^{35}S-labeled LCoR antisense probe. Magnification, 20×. (ii) (inset) Dark field photomicrograph of a section probed with a control LCoR sense probe. (iii and iv) As in (i) and (ii), except at 40× magnification. Syn, syncytiotrophoblast; cm, chorionic mesoderm.

expressed throughout fetal development and in the adult.

Given the robust expression of LCoR transcripts in placenta and the complex placental steroid physiology, LCoR expression was investigated further by in situ hybridization analysis of a section of human placenta (Figure 2B). The results reveal that LCoR is predominantly expressed in the syncytiotrophoblast layer of terminally differentiated cells, which acts as a barrier between maternal circulation and the fetus whose function is critical for controlling maternal hormonal signals that modulate fetal metabolism and development (Pepe and Albrecht, 1995).

Agonist-Dependent Interaction of LCoR and ERα In Vivo

An affinity-purified antibody developed against an LCoR peptide detected a protein of approximately 50 kDa in MCF-7, HEK293, and COS-7 cell extracts (Figure 3A), in excellent agreement with cDNA cloning data. The antibody also specifically detected several LCoR fusion proteins and deletion mutants (data not shown). Immunocytochemical studies with the antibody in all three lines revealed a nuclear protein (data not shown and see below). Consistent with two-hybrid cloning, endogenous LCoR coimmunoprecipitated with endogenous ERα in an estradiol-dependent manner from MCF-7 cell extracts (Figure 3B). No immunoprecipitation of ERα or LCoR was observed when anti-ERα antibody was replaced by control IgG (Figure 3B). Note that reduced ERα expression after estradiol treatment is consistent with enhanced turnover of the receptor observed in hormone-treated MCF-7 cells (Wijayaratne and McDonnell, 2001).

Interaction of ERα and LCoR in vivo was further tested by bioluminescence resonance energy transfer (BRET) in living COS-7 cells transiently cotransfected with plasmids expressing ERα-EYFP and LCoR-rluc fusion proteins. BRET and its variant fluorescence resonance energy transfer (FRET) have been used in the past to study receptor-coregulator interactions (Llopis et al., 2000). Treatment with estradiol or diethylstilbestrol (DES) enhanced BRET ratios 2.5- to 3-fold (Figure 3C), consistent with agonist-dependent interaction of LCoR and ERα, whereas treatment with antiestrogens 4-hydroxytamoxifen (OHT) or raloxifene had no significant effect. Moreover, mutation of the NR box of LCoR to LSKAA largely disrupted hormone-dependent interaction and reduced hormone-independent interaction of the two proteins by approximately 2-fold (Figure 3C), indicating that the LCoR LXXLL motif is essential for ligand-dependent interaction with ERα.

Interaction of LCoR with Nuclear Receptor Ligand Binding Domains In Vitro

In vitro translated LCoR selectively bound to the ERα LBD fused to GST (GST-ERα-LBD) in a partially estrogen-dependent manner (Figure 4A). Consistent with BRET analyses, antiestrogens OHT, raloxifene, or ICI 164,384 did not induce interaction of LCoR with ERα (Figure 4A), and hormone-dependent binding of ERα was abolished by mutation of the LCoR NR box (LSKAA; Figure 4B). Similar results were obtained with GST-ERα fusions and in vitro translated LCoR-LSKAA (data not

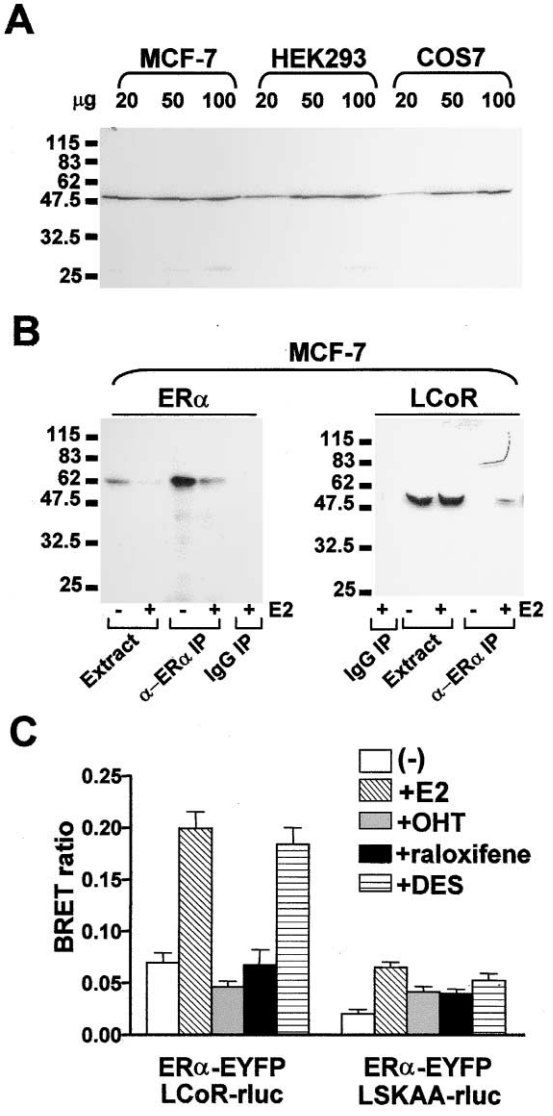

Figure 3. Interaction of LCoR and ERα In Vivo

(A) Western analysis of LCoR in 20, 50, or 100 μg of extract from MCF-7, HEK293, and COS-7 cells using a rabbit polyclonal antipeptide antibody.

(B) Coimmunoprecipitation of LCoR with ERα. Western blots (WB) of ERα (left) and LCoR (right) in immunoprecipitates of ERα with control mouse IgG or mouse monoclonal anti-ERα antibody from extracts of MCF-7 cells treated for 4 hr with vehicle (−) or estradiol (E2).

(C) Bioluminescence resonance energy transfer (BRET) assays on COS-7 cells transiently cotransfected with plasmids expressing EYFP-ERα and rluc-LCoR or rluc-LCoR-LSKAA fusion proteins and treated with 10^{-7} M β-estradiol (E2), hydroxytamoxifen (OHT), raloxifene, diethylstilbestrol (DES), or ethanol (−). BRET ratios were calculated as described in the Experimental Procedures. The data shown represent the mean ± SEM of three experiments.

shown). Double point mutation of ERα helix 12 (L539A, L540A; mAF-2) abolished ligand-dependent binding of LCoR (Figure 4C), demonstrating the importance of the AF-2 domain. ERα was also truncated to amino acid 378 (ER378), leaving regions A–D and the N-terminal third of the LBD (Figure 4D), or to amino acid 282 in region D (HE15) or 180, which encodes the A/B domain (data not shown). While ER378 bound specifically to GST-

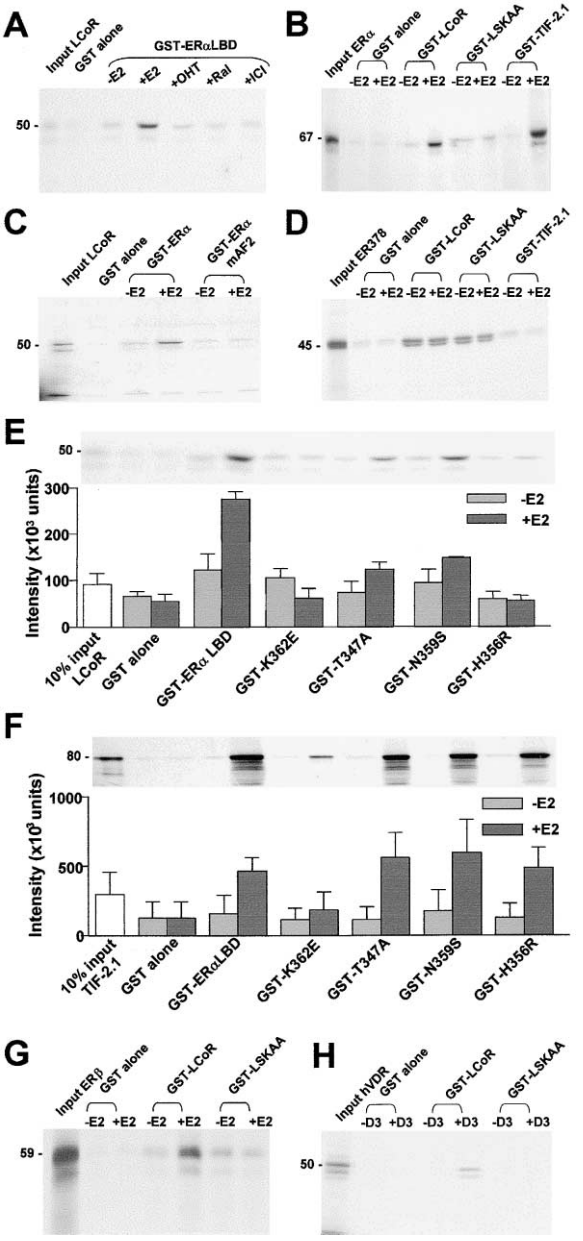

Figure 4. Characterization of LCoR Interaction In Vitro with ERα, ERβ, and VDR by GST Pull-Down Assay

Estradiol (E2), hydroxytamoxifen (OHT), raloxifene (Ral), and ICI164,384 (ICI), vitamin D3 (D3) were added to 10^{-6} M as indicated. Inputs (lanes 1) represent 10% of the amount of labeled protein used in assays.

(A) Ligand-dependent interaction of in vitro translated LCoR with GST-ERα LBD.

(B and D) Interaction of in vitro translated ERα (HEG0; [B]) or ER378 (D) with GST fused to LCoR, LCoR-LSKAA, or TIF2.1 as indicated.

(C) Interaction of LCoR with GST-ERα or a helix 12 mutant (ERα-mAF-2).

(E and F) Interaction of GST fusions of wild-type ERα LBD or LBD mutants T347A, H356R, N359S, and K362A with LCoR (E) or TIF-2.1 (F). Histograms of results of triplicate experiments are shown. Bands were quantitated using the FluorChem digital imaging system and AlphaEaseFC software (Alpha Innotech Corp, San Leandro, CA).

(G and H) Interaction of ERβ (G) and VDR (H) with GST-LCoR and GST-LSKAA.

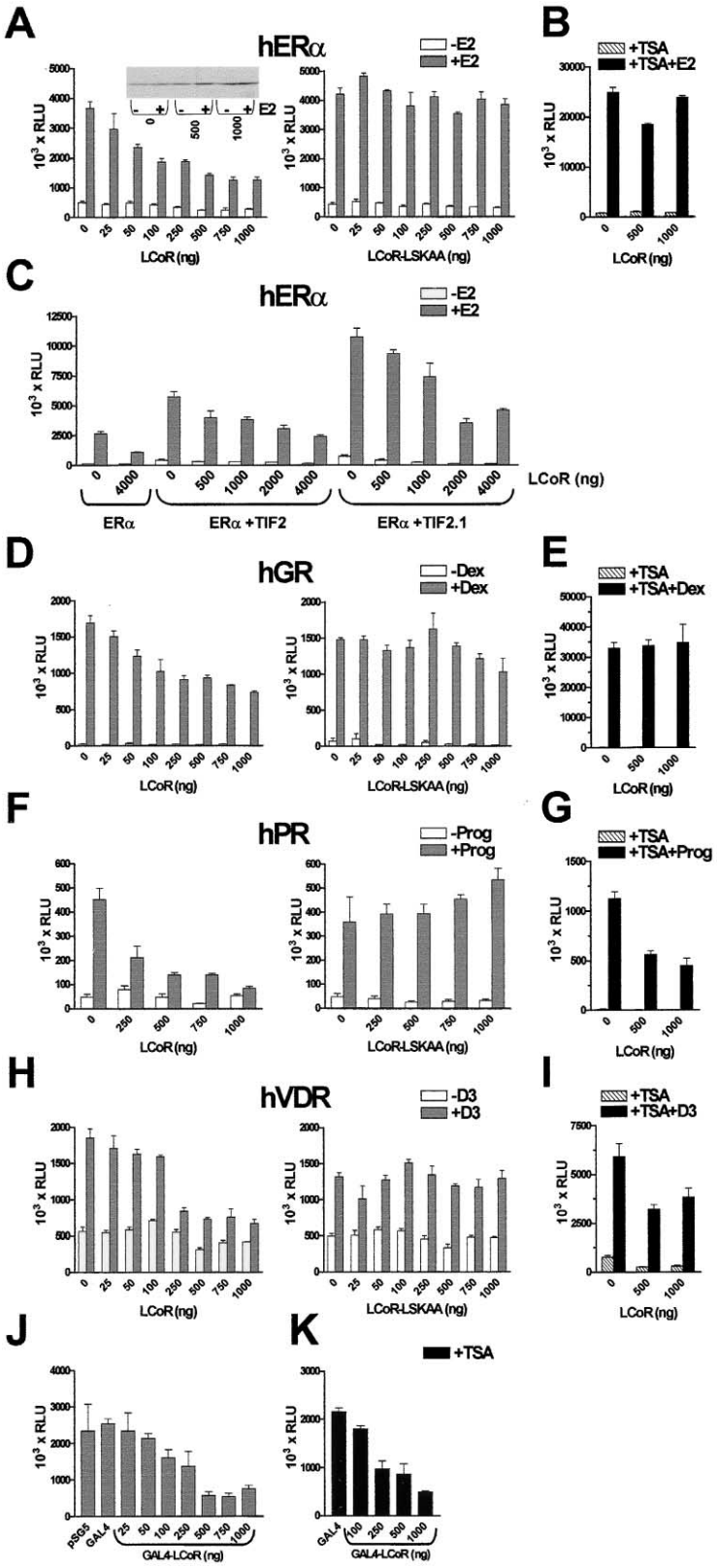

Figure 5. LCoR Is a Nuclear Receptor Corepressor

(A, C, D, F, and H) LCoR represses ERα-, GR-, PR- and VDR-dependent transactivation. COS-7 cells were cotransfected with expression vectors for ERα HEG0 (A and C), GR (D), PR (F), or VDR (H), ERE3-TATA-pXP2 (A and C), GRE5/pXP2 (D and F), or VDRE3tk/pXP2 (H) luciferase reporter vectors, pCMV-β-gal as internal control, and LCoR/pSG5 or LSKAA/pSG5 expression vectors as indicated. Cells were treated with 10^{-7} M of hormones (solid bars) or vehicle (open bars). Normalized luciferase activities (RLU) are the means ± SEM from at least three experiments. (A) (inset) Control Western blot of ERα from extracts of COS-7 cells transfected with ERα HEG0 and 0, 500, or 1000 ng of LCoR/pSG5 in the absence or presence of estradiol. (C) LCoR represses TIF-2 coactivation of ERα. Cells were transfected as in (A) with LCoR, TIF-2, or TIF2.1 as indicated. (J) A GAL4-LCoR fusion protein represses transactivation. COS-7 cells were transfected with 750 ng of 17-mer-5tk/pXp2, with indicated amounts of GAL4-LCoR/pSG5, 1000 ng of pSG5, or GAL4/pSG5. Normalized luciferase activities (RLU) are the means ± SEM from at least three experiments. (B, E, G, I, and K). Differing effects of HDAC inhibitor TSA on repression by LCoR. Transfections were performed as in the left-hand panels except that TSA (3 μM) was added.

LCoR but not TIF-2.1 in a hormone-independent manner (Figure 4D), no such interaction was observed with HE15 or the A/B domain (data not shown), suggesting that residues contributing to ligand-independent interaction with LCoR are located between ERα amino acids 283 and 377.

Interaction of LCoR with helix 3 was further probed using GST fusions of ERα point mutants T347A, H356R, N359S, and K362E. Helix 3 forms a critical part of the static region of the coactivator binding pocket (Shiau et al., 1998), and the integrity of lysine 362 at the C terminus of helix 3 (Brzozowski et al., 1997) is essential for ligand-dependent binding of p160 coactivators (Henttu et al., 1997). While the K362A mutation disrupted both TIF-2.1 and LCoR binding, mutations T347A, H356R, and N359S had a minimal effect on interaction of TIF-2.1, but partially or completely abolished binding of LCoR (Figures 4E and 4F). The above data indicate that LCoR and TIF-2.1 recognize overlapping binding sites, although LCoR interacts with residues on helix 3 that are distinct from those recognized by TIF-2.1.

Binding of LCoR to other nuclear receptors was also analyzed by GST pull-down assays, which showed that LCoR also bound LBDs of ERβ, VDR, RARs α, β, and γ, and RXRα in a ligand-dependent manner (Figures 4G and 4H, and data not shown). Taken together, the above results indicate that LCoR binds to the LBDs of several nuclear receptors in a hormone-dependent or partially hormone-dependent manner, and the interaction of LCoR with the static portion (helix 3) of the coactivator binding pocket of ERα differs from than that of TIF-2.1.

LCoR Is a Repressor of Ligand-Dependent Transcription Induced by Class I and Class II Nuclear Receptors

The effects of LCoR on transactivation by nuclear receptors were tested by transient transfection in COS-7 cells (Figure 5), which revealed that LCoR is a repressor of ligand-dependent transcription of class I and II receptors. Coexpression of LCoR produced a dose-dependent repression of hormone-dependent transactivation by ERα which was abolished by mutation of the NR box, as the LSKAA mutant had no effect on ERα function (Figure 5A). Repression of estrogen-dependent gene expression was not due to downregulation of ERα protein in cells cotransfected with LCoR (Figure 5A, inset). Similar results were obtained in MCF-7 and HEK293 cells (data not shown). Consistent with LCoR and TIF-2 recognizing overlapping binding sites on ERα, LCoR repressed estrogen-dependent expression coactivated by TIF2 or TIF2.1 (Figure 5C). Repressive effects of 1 μg of transfected LCoR on ligand-activated transcription on the order of 2.2- to 5-fold were observed in experiments with the glucocorticoid, progesterone, and vitamin D receptors (Figures 5D, 5F, and 5H). In each case, mutation of the NR box disrupted transcriptional repression. Moreover, GAL4-LCoR fusion repressed the activity of the 5 × 17-mer-tk promoter in a dose-dependent manner by 4-fold (Figure 5J), whereas free LCoR had no effect on the 5 × 17-mer-tk promoter (data not shown). The mechanism of action of LCoR was investigated by analyzing the effect of the HDAC inhibitor trichostatin A (TSA) on repression of ligand-dependent transcription. Remarkably, while TSA completely abolished LCoR-dependent repression of ERα and GR function (Figures 7B and 7E), it had little or no effect on repression of PR or VDR, or on repression by GAL-LCoR (Figures 5G, 5I, and 5K). This suggests that LCoR may function by HDAC-dependent and -independent mechanisms.

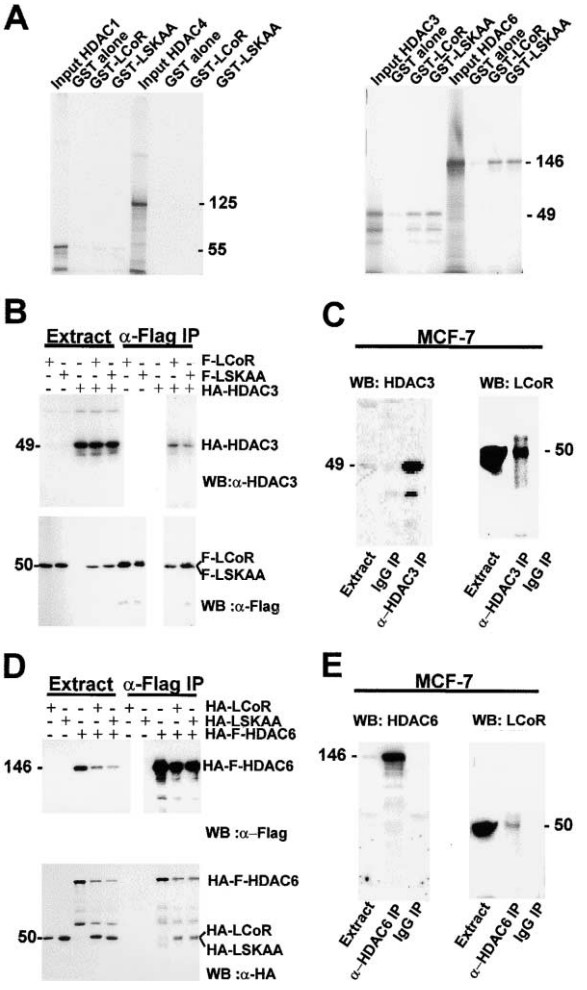

Figure 6. LCoR Interacts Directly with Specific HDACs

(A) HDACs 1, 3, 4, and 6 were in vitro translated and incubated with GST alone or with GST-LCoR or GST-LSKAA fusion proteins. The input (lane 1) represents 10% of the amount of labeled protein used in the assays.

(B) Association of tagged LCoR or LCoR-LSKAA with HDAC3. Lysates from COS-7 cells transiently transfected with HA-HDAC3 and Flag-LCoR or Flag-LSKAA were precipitated with anti-Flag antibody. Cell extract and immunocomplexes were analyzed by Western blotting with anti-HDAC3 or anti-Flag.

(C) Endogenous LCoR coimmunoprecipitates with endogenous HDAC3. Immunoprecipitations from MCF-7 cell extracts were performed with either rabbit control IgG or anti-HDAC3 antibody, and immunoprecipitates were probed for HDAC3 or LCoR as indicated.

(D) Association of LCoR and LCoR-LSKAA with HDAC6. Lysates from COS-7 cells transiently cotransfected with HA-Flag-HDAC6 and HA-LCoR or HA-LSKAA were precipitated with anti-Flag antibody, and the immunocomplexes were analyzed by Western blotting with anti-HA or anti-Flag.

(E) Endogenous LCoR coimmunoprecipitates with endogenous HDAC6. Immunoprecipitations from MCF-7 cell extracts were performed with either rabbit control IgG or anti-HDAC6 antibody, and immunoprecipitates were probed for HDAC6 or LCoR as indicated.

LCoR Interacts Selectively with Histone Deacetylases

Pull-down assays performed with GST-LCoR and GST-LSKAA to screen for potential interactions with class I HDACs 1 and 3, and class II HDACs 4 and 6 revealed that both LCoR proteins interacted with HDACs 3 and

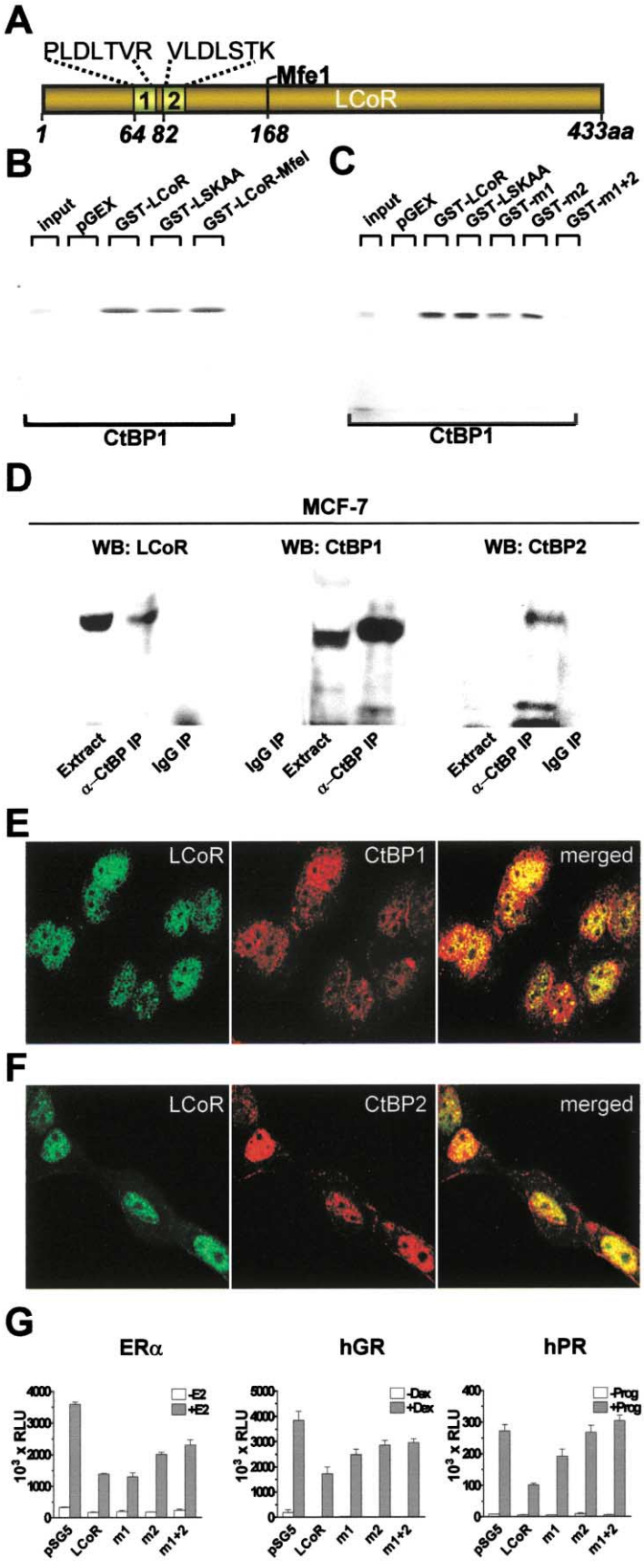

Figure 7. LCoR Interacts with C-Terminal Binding Proteins

(A) Schematic representation of LCoR showing CtBP binding sites 1 and 2, and the position of the Mfe1 site used to create C-terminally truncated LCoR.

(B) GST pull-down assays were performed with in vitro translated CtBP1, and GST control (pGEX) or fusions with LCoR, LCoR-LSKAA, or LCoR-Mfe1 deletion mutant.

(C) GST pull-down assays were performed with in vitro translated CtBP1, and GST control (pGEX) or fusions with LCoR, LCoR-LSKAA, or LCoR mutated in CtBP binding sites 1 (m1), 2 (m2), or 1 and 2 (m1+2). All GST fusion proteins were expressed at similar levels (data not shown).

(D) LCoR coimmunoprecipitates with CtBPs. Extracts of MCF-7 cells were immunoprecipitated with rabbit control IgG or with a rabbit polyclonal anti-CtBP antibody, and immunoprecipitates were probed for CtBP1, CtBP2, or LCoR.

(E and F) Colocalization of LCoR and CtBP1 (E) or CtBP2 (F) by confocal microscopy (see Experimental Procedures for details).

(G) Mutation of CtBP binding motifs attenuates repression by LCoR. COS-7 cells were cotransfected with expression vectors for ERα or GR or PR as indicated, along with ERE3-TATA-pXP2 or GRE5/pXP2 as appropriate, and either wild-type LCoR or LCoR mutated in CtBP binding motifs 1 or 2 as indicated.

6, but not with HDACs 1 and 4 (Figure 6A). Reciprocal coimmunoprecipitation experiments revealed an interaction between epitope-tagged LCoR or LCoR-LSKAA and HDAC3 (Figure 6B and data not shown). Moreover, interaction between endogenous LCoR and HDAC3 was confirmed by coimmunoprecipitation with an anti-HDAC3 antibody from extracts of MCF-7 cells (Figure 6C). Identical results were obtained in extracts of HEK293 cells (data not shown). Similarly, HA-LCoR and HA-LCoR-LSKAA were coimmunoprecipitated with HA-Flag-HDAC6 by an anti-Flag antibody (Figure 6D), and endogenous LCoR coimmunoprecipitated with HDAC6 from extracts of MCF-7 cells (Figure 6E). Taken together, these results indicate that LCoR can function to couple specific HDACs to ligand-activated nuclear receptors.

LCoR Interacts with C-Terminal Binding Protein (CtBP) Corepressors

Analysis of LCoR sequence (Figure 7A) revealed PLDLTVR (aa 64) and VLDLSTK (aa 82) motifs that are homologous to the PLDLS/TXR/K sequence defined as a binding site for the corepressor CtBP1 (Vo et al., 2001). CtBP1, which was originally found as a protein that interacts with the C terminus of E1A, functions by HDAC-dependent and -independent mechanisms (Chinnadurai, 2002) and is highly homologous to CtBP2 (Sewalt et al., 1999). GST pull-down assays revealed an interaction between CtBP1 and wild-type LCoR, the LSKAA mutant, and an LCoR mutant lacking the C-terminal half of the protein (LCoR-Mfe1). CtBP1 binding was abolished only when both binding sites in LCoR were mutated (m1+2; Figure 7C). While NADH can modulate CtBP function (Zhang et al., 2002), no effect of NADH was seen on its interaction with LCoR in vitro (data not shown).

CtBP1 and 2 are most efficiently immunoprecipitated with an antibody that recognizes both proteins. Western analysis suggested that the immunoprecipitates of MCF-7 cells contained mostly CtBP1 (Figure 7D). Significantly, LCoR was coimmunoprecipitated with CtBP proteins under these conditions (Figure 7D). A similar coimmunoprecipitation of LCoR was observed from extracts of HEK293 cells (data not shown). In addition, immunocytochemical analysis of LCoR and CtBP1 expression in MCF-7 cells revealed a strongly overlapping expression pattern of the two proteins in discrete nuclear bodies (Figure 7E). Similarly, the expression patterns of LCoR and CtBP2 overlapped in MCF-7 cell nuclei (Figure 7F). Note that no fluorescence signal was seen in control experiments where specific antibody was removed or replaced with control IgG (data not shown). Mutation of CtBP binding sites partially reduced the capacity of LCoR to repress ligand-dependent transcription by ERα and the GR (Figure 7G), and consistent with the effect on the wild-type protein, TSA completely abolished the residual repression of ERα by LCoR mutated in both binding sites (data not shown). Significantly, mutation of site 2 or both sites largely abolished repression of PR-dependent transactivation. Taken together, the above data shows that binding of CtBPs contributes to transcriptional repression by LCoR. Moreover, the greater dependence on the CtBP binding sites of LCoR for repression of progesterone-induced transactivation would be consistent with CtBP and its associated factors contributing to the TSA-insensitive repression of the PR observed above.

Discussion

We have identified LCoR, a corepressor that is widely expressed in human adult and fetal tissues and cell lines. LCoR function differs from those of NCoR and SMRT as it is recruited to receptors through an NR box in the presence of agonist. Highly homologous murine LCoR is expressed in two-cell embryos, suggesting that it functions from the earliest stages of embryonic development. LCoR is most highly expressed in the placenta and at near term is predominantly present in syncytiotrophoblasts. Receptors for estrogen, progesterone, and glucocorticoids are expressed in the syncytiotrophoblast layer, which represents a barrier between the maternal and the fetal circulation and is a critical site of steroid hormone signaling, biosynthesis, and catabolism (Pepe and Albrecht, 1995; Whittle et al., 2001). The function of LCoR as an attenuator of nuclear receptor signaling suggests that it may be an important modulator of steroid hormone signaling in syncytiotrophoblasts.

LCoR contains a putative helix-loop-helix domain. Multiple repeats of an HLH domain are required for high-affinity site-specific DNA binding of *Drosophila* pipsqueak proteins (Lehmann et al., 1998). Similarly, mutation of one of the two HLH motifs in the MBLK-1 gene strongly reduced site-specific DNA binding (Takeuchi et al., 2001). The pipsqueak domain is homologous to motifs found once in a number of prokaryotic and eukaryotic proteins that interact with DNA, such as recombinases (Lehmann et al., 1998; Sigmund and Lehmann, 2002), suggesting that LCoR itself may interact with DNA.

Analysis of the interaction of LCoR with nuclear receptors by BRET, coimmunoprecipitation, and GST pull-down assays indicates that LCoR binds to receptor LBDs in a ligand-dependent or partially ligand-dependent manner. Moreover, the dependence of LCoR binding to ERα on the integrity of its LXXLL motif and the integrity of ERα helix 12 indicates that LCoR associates with the same hydrophobic pocket in the LBD as p160 coactivators. However, while mutation of K362 (helix 3) disrupted binding of both LCoR and TIF-2.1, LCoR binding was more sensitive to mutation of other helix 3 amino acids than TIF-2.1. Of particular note, LCoR binding was sensitive to the integrity of residue 347 of ERα, which lies outside binding groove residues 354–362 recognized by the NR box II peptide of TIF-2 (GRIP1; Shiau et al., 1998), suggesting that LCoR recognizes an extended region of helix 3. LCoR residues outside the LXXLL motif may thus contact the ERα LBD.

LCoR inhibited ligand-dependent transactivation by nuclear receptors in a dose-dependent manner up to 5-fold and functioned as a repressor when coupled to the GAL4 DNA binding domain. While LCoR and p160 coactivators both bind in an agonist-dependent manner to coactivator binding pockets, several results indicate that the repression observed by LCoR was not simply a result of blockage of p160 recruitment. Rather, LCoR recruits multiple factors that act to repress transcription. While the HDAC inhibitor TSA abolished repression

by LCoR of estrogen- and glucocorticoid-dependent transcription, the compound had little or no effect on repression of progesterone- or vitamin D-dependent transcription or repression by GAL-LCoR, indicating HDAC-dependent and -independent modes of action.

LCoR interacted with HDACs 3 and 6 but not HDAC1 or HDAC4, in vitro, and interactions with HDACs 3 and 6 were confirmed in coimmunoprecipitations. Preliminary experiments indicate that HDACs 3 and 6 interact with distinct regions of LCoR in the C-terminal half of the protein (our unpublished data). HDACs 3 and 6 are class I and II enzymes, respectively. Unlike other class II enzymes, HDAC6 contains two catalytic domains (Bertos et al., 2001; Khochbin et al., 2001) and has not previously been associated with nuclear receptor corepressor complexes. HDAC6 is both cytoplasmic and nuclear, and recent studies have revealed its capacity to deacetylate tubulin (Hubbert et al., 2002), suggesting that it may have broad substrate specificity.

Several biochemical studies to date have characterized different corepressor complexes associated with nuclear receptors, which include different HDACs (Glass and Rosenfeld, 2000; Rosenfeld and Glass, 2001). Using SMRT affinity chromatography, HDAC3 was identified as a component of a multiprotein complex that also contained transducin β-like protein, TBL1, a homolog of the groucho corepressor (Guenther et al., 2000). NCoR was also found to be part of a large complex purified by HDAC3 affinity chromatography (Wen et al., 2000). Whether LCoR is also a component of these complexes or different complex(es) remains to be seen. Studies to date suggest that NCoR and SMRT may interact with varying stability with distinct corepressor complexes that include multiple HDACs, indicating that compositions of individual corepressor complexes are not fixed.

Significantly, we also found that LCoR interacts with the corepressor CtBP1 through tandem consensus CtBP-interaction motifs. Like LCoR, the sensitivity of repression by CtBPs to TSA is dependent on the promoter tested, indicative of HDAC-dependent and -independent modes of action (Chinnadurai, 2002). CtBP proteins interact with several different transcriptional repressors, including the nuclear receptor corepressor RIP140 (Vo et al., 2001). The TSA-sensitive and -insensitive actions of LCoR are analogous to another CtBP-interacting repressor Ikaros, which is composed of distinct domains mediating repression by HDAC-dependent and -independent mechanisms (Koipally and Georgopoulos, 2002a, 2002b). CtBP binding to Ikaros contributes to its HDAC-independent mode of action (Koipally and Georgopoulos, 2002a). CtBPs also associate with specific polycomb group (PcG) repressor complexes (Sewalt et al., 1999), and HDAC-independent repression of transcription by CtBP has been linked to its association with PcG complexes (Dahiya et al., 2001). Our initial experiments indicate that LCoR also associates with components of PcG complexes (our unpublished data).

Our studies have suggested that LCoR can act as a corepressor for several receptors. However, it will be essential to verify the effects of LCoR on regulation of endogenous nuclear receptor target genes using chromatin immunoprecipitation assays. In addition, overexpression/knockdown experiments will determine whether LCoR acts gene specifically or is a general attenuator of ligand-dependent transactivation. The action of corepressors such as LCoR that recognize agonist-bound receptors is perhaps counterintuitive. However, their existence suggests that there exist signals that act to attenuate the consequences of hormone-induced receptor function. Such effects would provide a counterbalance to signaling that augments hormone-induced transactivation; for example, the stimulatory effects of MAP kinase signaling on ERα function (Kato et al., 1995).

One of the keys to understanding the function of LCoR will be to determine the mechanisms modulating its agonist-dependent interaction with nuclear receptors. If LCoR acts to attenuate the function of agonist-bound receptors, then it is likely that posttranslational modification or LCoR and/or receptors will affect the relative affinities of LCoR and p160s for coactivator binding pockets. LCoR contains several putative phosphorylation motifs, including a number of MAP kinase sites in the region of the NR box, as well as potential sites for protein kinases A and C, raising the possibility that its interaction with ligand-bound nuclear receptors may be modulated by phosphorylation. In addition, LCoR contains a consensus leptomycin B-sensitive nuclear export signal ($LX_3LX_3LXIX_3L$; aa149–164), suggesting that its access to receptors may be regulated by nuclear export under some conditions. Such a mechanism would be analogous to a recent study showing that NCoR corepression of NF-κB signaling can be attenuated by nuclear export (Baek et al., 2002).

In summary, we have identified a nuclear receptor corepressor LCoR, which is widely expressed throughout mammalian development and represses ligand-dependent nuclear receptor transactivation by recruitment of multiple factors. Our studies suggest that LCoR is an important attenuator of nuclear receptor signaling during fetal development and in the adult.

Experimental Procedures

Note that descriptions of antibodies, plasmid constructions, Northern blotting, and transfections are provided in the supplemental data at http://www.molecule.org/cgi/content/full/11/1/139/DC1.

Isolation of LCoR cDNA Sequences

A yeast two-hybrid screen (2×10^6 transformants; Clontech human fetal kidney cDNA Matchmaker library PT1020-1; Palo Alto, CA) with an ERα-LBD bait in the presence of 10^{-6} M estradiol yielded 10 $His^+/LacZ^+$ colonies, of which six were dependent on estradiol for LacZ expression. Three clones contained 1.2 kb inserts identical to coactivator AIB-1 (Anzick et al., 1997), and one contained an insert of 1.3 kb of LCoR sequence. 1.6×10^6 human λgt11 prostate cDNA clones (Clontech, HL1131b) were screened for more LCoR sequence, yielding five clones containing LCoR sequences 1–1417, 462–1376, 704–1406, 1122–2915, and 1214–3016. Multiple alignment of the different cDNA clones was performed (CAP program; INFOBIOGEN site http://www.infobiogen.fr). Homologies to ESTs and proteins were found using BLAST2 and PSI-BLAST, respectively, employing standard parameters and matrices.

Immunocytochemistry and In Situ Hybridization

MCF-7 cells were cultivated on collagen IV-treated microscope slides in 6-well plates, fixed with 2% paraformaldehyde for 15 min at room temperature, washed (3×) with PBS, and permeabilized with 0.2% Triton X100, 5% BSA, 10% horse serum in PBS. Cells were then incubated with α-LCoR (1:500), and αCtBP1 or αCtBP2 (1:50) in buffer B (0.2% Triton X100, 5% BSA in PBS) for 1 hr at

room temperature. Cells were washed (3×) with PBS and incubated with goat anti-rabbit-Cy2 and donkey anti-goat Cy3 (1:300) in buffer B for 1 hr at room temperature. Slides were mounted with Immuno-Fluore Mounting Medium (ICN, Aurora, OH) and visualized using a Zeiss LSM 510 confocal microscope at 63× magnification. In situ hybridization was carried out (Han et al., 1996) using 443 bp sense and antisense LCoR probes, and a hybridization temperature of 60°C and maximum wash conditions of 0.1× SSC at 65°C.

GST Pull-Down Assays and Immunoprecipitations

GST pull-down assays were performed as described (Eng et al., 1998), with the exception that assays performed with in vitro translated ER378 included two more washes made with the GST buffer containing 150 mM NaCl. For immunoprecipitations of tagged proteins, COS-7 cells in 100 mm dishes were transfected with 6 μg of HA-LCoR and/or 6 μg of HA-Flag-HDAC6 or with 6 μg of Flag-LCoR and/or 6 μg of HA-HDAC3 and pSG5 carrier. Forty-eight hours after transfection, cells were lysed 30 min at 4°C in 1 ml of JLB (20 mM Tris-HCl [pH 8], 150 mM KCl, 10% glycerol, 0.1% IGEPAL CA-630, and complete protease inhibitor cocktail; Boehringer-Mannheim, Laval, Quebec, Canada). Cell debris were pelleted by centrifugation (14,000 rpm, 5 min), and proteins were immunoprecipitated from 600 μl of supernatant by incubation for 1 hr at 4°C with 4 μg of α-Flag M2 antibody or polyclonal anti-HDAC3, followed by overnight incubation with protein A+G agarose or protein-A agarose beads for anti-Flag and anti-HDAC3, respectively. Beads were washed (3×) with JLB. Bound immunocomplexes were boiled in Laemmli buffer, separated by 10% SDS-PAGE, and blotted on PVDF membrane with α-Flag M2-peroxidase, α-HDAC3, α-HA-peroxidase (1:500), and detected by enhanced chemiluminescence (NEN Life Science Products, Boston, MA). For immunoprecipitation of endogenous HDAC3 or HDAC6, MCF-7 cells in 150 mm dishes were lysed in 2 ml of JLB. Supernatants were cleared, incubated with 4 μg of αHDAC6 or αHDAC3 or control rabbit IgG in the presence of protein A agarose, and Western blotted as above. For ERα or CtBP, MCF-7 cells were lysed in 2 ml of 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.2 mM Na orthovanadate, 1 mM EDTA, 1 mM EGTA, 1% Triton-100X, 0.5% IGEPAL CA-630, protease inhibitor cocktail, and immunoprecipitated as above with 4 μg of αCtBP or αERα antibodies, or corresponding control IgG in the presence of protein A or protein A+G agarose, respectively. Dilutions of specific antibodies used for Western blotting were: LCoR, HDAC3, and HDAC6 (1:1000); CtBP1, CtBP2, and ERα (1:100).

BRET Assays

COS-7 cells in 6-well plates were transfected with 250 ng of LCoR-rluc alone or with 2.5 μg of ERα-EYFP, and treated 24 hr later with 10^{-7} M estradiol or OHT for 18 hr. Cells were washed (2×) with PBS and harvested with 500 μl of PBS, 5 mM EDTA. Twenty thousand cells (90 μl) were incubated with 5 μM final of coelenterazine H in 96-well microplates (3610, Costar, Blainville, Quebec, Canada) as recommended (Angers et al., 2000). Luminescence and fluorescence signals were quantified with a 1420 VICTOR^2-multilabel counter (Wallac-Perkin Elmer, Boston, MA), allowing sequential integration of signals detected at 470 and 595 nm. Readings were started immediately after coelenterazine H addition, and ten repeated measures were taken. The BRET ratio was defined as [(emission at 595)−(emission at 470) × Cf]/(emission 470), where Cf corresponded to (emission at 470/emission at 595) for the rluc-LCoR expressed alone in the same experiments.

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