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Functional analyses of an LXXLL motif in nuclear receptor corepressor (N-CoR)

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Abstract

Transcriptional repression is a major regulatory mechanism in cell differentiation, organogenesis, and oncogenesis. Two repressors of ligand-dependent transcription factors, nuclear receptor corepressor (N-CoR) and the related protein SMRT were identified as a silencing mediator for thyroid hormone receptor β and as a silencing mediator for retinoic acid and thyroid hormone receptors, respectively. Nuclear receptor coactivators such as steroid receptor coactivator-1 (SRC-1) contain multiple LXXLL motifs, which are essential and sufficient for its ligand-dependent interaction with nuclear receptors. N-CoR also has an LXXLL motif, located between repressor domains 1 and 2, and conserved between mouse and man. In contrast, SMRT lacks this motif.

This paper describes functional implications of the LXXLL motif in N-CoR. A 57-amino acid portion of N-CoR containing the LDNLL sequence (N-CoR$_{LDNLL}$) fused to GST interacted with retinoic acid receptor α (RARα) and thyroid hormone receptor β (TRβ) in vitro. Similarly, [^{35}S-methionine]N-CoR$_{LDNLL}$ interacted with a RARα fusion protein. N-CoR$_{LDNLL}$ also bound to RARα in vivo as determined in mammalian one-hybrid system in transfected CV-1 cells and by two-hybrid assays in bacteria. The interaction with RARα in vitro and in vivo was specific as determined by mutation of the sequence LDNLL to LDNAA. Our data suggest that the LDNLL motif in N-CoR has functional significance because it mediates interaction with nuclear receptors such as RARα and TRβ.

Keywords: Nuclear receptor; Transcriptional regulation; Differentiation; Coactivator; Corepressor

1. Introduction

Transcriptional repression is a major regulatory mechanism in cell differentiation, organogenesis, oncogenesis and homeostasis [1]. The nuclear receptor corepressor (N-CoR) interacts with unliganded nuclear receptors [2,3], thereby recruiting transcriptional repressor complexes to the sites of regulated target genes [4,5]. The interaction of N-CoR and the related silencing mediator of retinoid and thyroid hormone receptors (SMRT) [6] with nuclear receptors occurs via motifs with the consensus sequence L/I-X-X-I/V-I termed CoRNR boxes [7].

Nuclear receptors bind to response elements consisting of two AGGTCA separated by a specific number of bases [8,9]. Both retinoic acid receptor (RAR) and thyroid hormone receptor (TR) bind as heterodimers with retinoid X receptor (RXR) [10,11]. Gene activation occurs when RAR or TR and to some extent RXR bind ligand [12]. This leads to association of coactivators such as steroid receptor coactivator-1 (SRC-1) and CREB binding protein (CBP) to the nuclear receptor at a target gene. Coactivators bind both to liganded nuclear receptors and to other coactivators via a motif (NR

Abbreviations: ATRA, all *trans*-retinoic acid; CBP, CREB binding protein; CoRNR motif, corepressor-nuclear receptor-binding motif; DBD, DNA binding domain; GSH, glutathione; GST, glutathione-S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; N-CoR, nuclear receptor corepressor; NR box, coactivator-nuclear receptor-binding motif (consensus LXXLL); PCR, polymerase chain reaction; RARα, retinoic acid receptor α; RE, response element; RXR, retinoid X receptor; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SMRT, silencing mediator for retinoid and thyroid hormone receptors; SRC-1, steroid receptor coactivator-1; TRβ, thyroid hormone receptor β; TSHβ, thyroid stimulating hormone β subunit, thyrotropin β subunit; TRIAC, 3,3′,5-triiodothyroacetic acid

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box), with the consensus sequence LXXLL [13,14]. The NR box and the CoRNR box bind to overlapping surfaces on nuclear receptors, thus allowing for competition [15].

We now report that a conserved N-CoR motif, which is not present in SMRT, mediates interaction with the nuclear receptors RARα and TRβ.

2. Materials and methods

2.1. Materials

Cell culture media, penicillin–streptomycin, trypsin, culture plates, PCR primers and restriction enzymes were obtained from Invitrogen Life Science, Scotland. TNT Quick Coupled Transcription/Translation Systems Kit from Promega, Madison, WI, ^{35}S-labeled L-methionine and GSH-Sepharose from Amersham Biosciences, Uppsala, Sweden. QuikChange Site-Directed Mutagenesis Kit, BacterioMatch vectors pBT and pTRG, and reporter strain supercompetent bacteria were from Stratagene, XAR film from Kodak Rochester, NY, and tetracycline, chloramphenicol, kanamycin, carbenicillin, ampicillin, LB-broth base and LB-agar from Sigma–Aldrich, St. Louis, MO. D-Luciferin was purchased from Labsystems, Helsinki, Finland.

2.2. Recombinant plasmids

cDNA encoding amino acids 20–462 from the human RARα gene was PCR amplified using forward primer 5′-CACCTCAATGGATCCCCGGTGCCTCC-3′ and reverse primer 5′-GTCCATGTCTCGAGGGCGGTCACGG-3′. The product was inserted into *Bam*HI and *Xho*I restriction sites of the pGEX-KG vector, in frame with the GST gene. pGEX-TRβ was kindly provided by Dr. Riki Kurokawa; pCMX-RARα and peA101 (TRβ expression vector) by Dr. Christopher K. Glass, both at the University of California, San Diego. cDNA encoding amino acids 665–721 of mouse N-CoR (N-CoR$_{LDNLL}$) was amplified by PCR using forward primer 5′-CAGTGCAGAATTCTCTATTTTAAC-3′ and reverse primer 5′-CACTATCCTCGAGATTTTCCTCC-3′. The product was inserted in frame with GST at the *Eco*RI and *Xho*I restriction sites of pGEX-KG or at the *Eco*RI and *Xho*I sites, in frame with a Kozak translation initiation site in pcDNA3. cDNA encoding N-CoR(665–721) was PCR amplified using primers 5′-GTGCGGATCCTTCTATTTTAAC-3′ and 5′-CACTGAATTCTGGATTTTACTCC-3′, and cloned into the *Bam*HI and *Eco*RI restriction sites of pCMV-BD in frame with the DNA binding domain of GAL4. N-CoR$_{LDNAA}$ was obtained by PCR using primers 5′-CGGCATATTC-TTGACAAC**GC**T**GC**GCAGCAACATAAACAGAAAGC-3′ and 5′-GCTTTCTGTTTATGTTGCTGC**GC**A**GC**GTT-GTCAAGAATATGCCG-3′ (altered bases are in bold type) and QuikChange Site-Directed Mutagenesis Kit. cDNA with these mutations was subcloned into pGEX-KG, pcDNA3 and pCMV-BD as described above. pBT-N-CoR$_{LDNLL}$ and pBT-N-CoR$_{LDNAA}$ were similarly constructed by insertion into the *Eco*RI and *Xho*I restriction sites of the pBT vector, in frame with the lambda C1 gene. pTRG-RARα has RARα inserted into the *Bam*HI and *Xho*I cloning sites in frame with the RNAP-α gene of the pTRG vector. The reporter plasmid 5× UAS-tk-luc has a firefly luciferase gene under the control of five copies of the GAL1 gene UAS and a thymidine kinase promoter. Correct insertion was verified in all plasmids by restriction analysis. Predicted sizes of GST fusion proteins and in vitro translated proteins were verified by SDS-PAGE.

2.3. GST fusion proteins

E. coli strain Y1090 was grown to OD_{595} = 0.8 at 37 °C, and GST fusion protein expression was induced with IPTG, 3 mM. After 3 h at room temperature, cells were harvested by centrifugation. Lysates were prepared, all steps on ice, by sonicating the bacteria in TEDG buffer (50 mM Tris (pH 7.4), 1.5 mM EDTA, 0.4 M NaCl, 10% glycerol) and centrifugation at 77,000 × *g* for 30 min. The supernatant was stored as aliquots at −70 °C. Lysates were analyzed by SDS-PAGE to normalize relative amounts of fusion protein in different preparations.

2.4. In vitro translation

pcDNA3-N-CoR$_{LDNLL}$, pcDNA3-N-CoR$_{LDNAA}$, pcDNA3-RARα and peA101 (TRβ) were used as templates for in vitro transcription followed by translation in the presence of ^{35}S-methionine, using the TNT T7 Quick Coupled Transcription/Translation Systems Kit.

2.5. GST pulldown assays

GST fusion protein lysates were incubated with GSH-Sepharose beads in NET-N buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) at 4 °C with rotation and repeated washing with NET-N buffer. When appropriate, the beads were incubated with ligand for 30 min at room temperature with tilting and then with ^{35}S-labeled protein (150,000 cpm per sample), for 1 h at 4 °C with rotation. The beads were then washed repeatedly, and boiled for 3 min in 2× SDS sample buffer, separated by SDS-PAGE and stained with Coomassie Brilliant Blue to verify equal loading of fusion protein. Radioactivity was detected by autoradiography.

2.6. Interaction assay in intact cells

CV-1 cells (10^5 cells/well), were grown for 24 h, then transfected (calcium phosphate method) with 250 ng of the reporter plasmid and 100 ng each of the remaining (see Fig. 2) plasmids. New growth medium was added the next day after washing with PBS. Ligand was added and cells were harvested and assayed for luciferase reporter activity 26–30 h later.

2.7. Interaction assay in intact bacteria

pBT-N-CoR$_{LDNLL}$ (or pBT-N-CoR$_{LDNAA}$) and pTRG-RARα were co-transformed into BacterioMatch reporter strain bacteria (http://www.stratagene.com/manuals/200412.pdf). The number of co-transformed clones was established by manually counting the colonies on LB-agar plates containing tetracycline, chloramphenicol and kanamycin. The same number of co-transformants was also spread on plates containing carbenicillin (250 μg/ml), and the percentage of car-

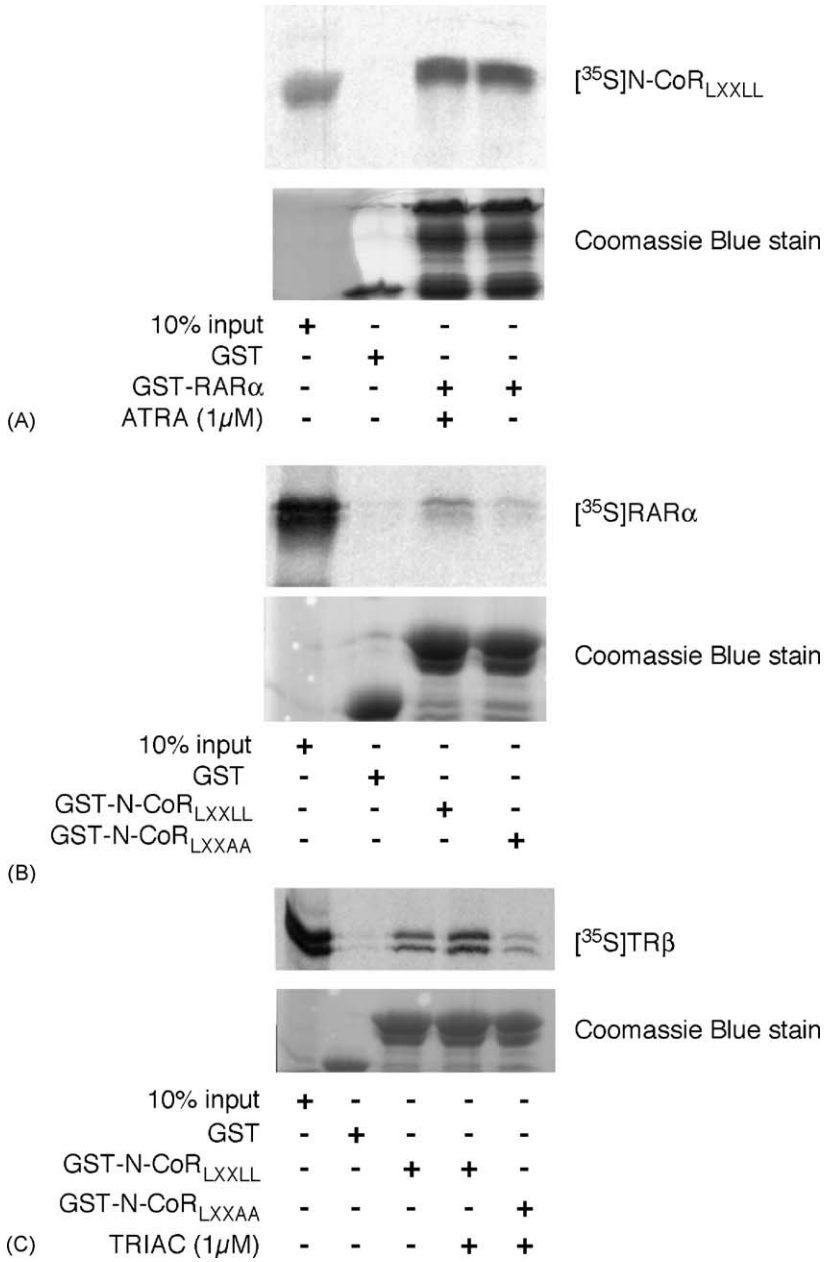

Fig. 1. N-CoR interacts with RARα and TRβ through its LXXLL motif. (A) GST–RARα fusion protein was immobilized on GSH-Sepharose beads and incubated with an ^{35}S-methionine labeled N-CoR fragment containing amino acids 665–721 (N-CoR$_{LDNLL}$). After extensive washing, specifically bound [^{35}S-methionine]N-CoR$_{LDNLL}$ was eluted with 2× SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography. Ten percent of the amount of [^{35}S-methionine]N-CoR$_{LDNLL}$ added to each pulldown assay was applied to the gel as a reference (lane labeled 10% input). Coomassie Blue stain is included to show protein load. (B) [^{35}S-methionine]RARα was incubated with immobilized GST–N-CoR$_{LDNLL}$ and GST–N-CoR$_{LDNAA}$, respectively. After extensive washing, specifically bound [^{35}S-methionine]RARα was released by boiling in 2× SDS sample buffer and then separated on SDS-PAGE, followed by autoradiography. Ten percent of the applied amount of ^{35}S-labeled RARα was applied to the gel as a reference (lane labeled 10% input). GST alone incubated with [^{35}-methionine]RARα served as a negative control. Coomassie Blue stain is included to show protein load. (C) [^{35}S-methionine]TRβ was incubated with immobilized GST–N-CoR$_{LDNLL}$ and GST–N-CoR$_{LDNAA}$ fusion proteins. Specifically bound proteins were separated on SDS-PAGE followed by autoradiography. Ten percent of the applied amount of ^{35}S-labeled TRβ was applied to the gel as a reference (lane labeled 10% input). GST alone incubated with [^{35}-methionine]TRβ served as a negative control. Coomassie Blue stain is included to show protein load.

benicillin resistant co-transformants (due to interaction between pBT-N-CoR$_{LDNLL}$ and pTRG-RARα) was calculated.

3. Results

3.1. N-CoR(665–721) and RARα interact through the LDNLL motif present in N-CoR

The ability of GST–RARα fusion protein to interact with an [^{35}S-methionine]N-CoR fragment (amino acids 665–721) containing the LDNLL motif was tested. The results shown in Fig. 1A, show that N-CoR$_{LDNLL}$ interacted efficiently with GST–RARα. The interaction was not altered by the presence of ligand. To investigate the specificity of the LDNLL motif, this sequence was mutated to LDNAA [14] and subsequently used in GST pulldown experiments, (Fig. 1B). In comparison with GST–N-CoR$_{LDNLL}$ the interaction of GST–N-CoR$_{LDNAA}$ with [^{35}S-methionine]RARα was markedly reduced indicating a specific interaction, dependent on the LDNLL motif, between N-CoR$_{LDNLL}$ and RARα.

3.2. N-CoR(665–721) and TRβ interact through the LDNLL motif present in N-CoR

The ability of [^{35}S-methionine]TRβ to interact with amino acids 665–721 of N-CoR, containing the LDNLL motif, fused to GST was tested. The results shown in Fig. 1C, show that GST–N-CoR$_{LDNLL}$ interacted efficiently with TRβ. The interaction was stronger in the presence of TRβ ligand TRIAC (Fig. 1C). When GST–N-CoR$_{LDNLL}$ was replaced with GST–N-CoR$_{LDNAA}$ (the LDNLL motif mutated to LDNAA) the interaction was nearly abolished (Fig. 1C).

3.3. N-CoR(665–721) and RARα interact in a mammalian one-hybrid system

The ability of N-CoR(665–721) to interact with RARα was next investigated in intact mammalian cells. N-CoR$_{LDNLL}$ was fused in frame with yeast GAL4 DNA binding domain in the expression plasmid pCMV-BD. RARα was transiently transfected into CV-1 cells together with the plasmid encoding GAL4-DBD/N-CoR$_{LDNLL}$ and a luciferase reporter plasmid with a 5× UAS sequence upstream of a thymidine kinase promoter. Reporter gene expression was 100-fold higher than in the absence of RARα (Fig. 2). Addition of ligand further increased the interaction (Fig. 2). If GAL4-DBD/N-CoR$_{LDNLL}$ was replaced with GAL4-DBD/N-CoR$_{LDNAA}$ the reporter gene activity decreased by more than 80% (Fig. 2).

3.4. N-CoR$_{LDNLL}$ is similar to CBP NR box 1

Comparison of the amino acid sequence surrounding the LXXLL motif in N-CoR with that of nuclear receptors and coactivator proteins revealed a close resemblance with NR box 1 in CBP (Fig. 3) particularly the sequence downstream of N-CoR LXXLL compared to that downstream of CBP LXD$_2$ (Fig. 3).

3.5. N-CoR$_{LDNLL}$ interacts with RARα in a bacterial two-hybrid assay

The in vivo interaction between N-CoR$_{LDNLL}$ and RARα was investigated using the BacterioMatch two-hybrid system. pBT-N-CoR$_{LDNLL}$ or pBT-N-CoR$_{LDNAA}$ and pTRG-RARα were co-transformed into BacterioMatch reporter

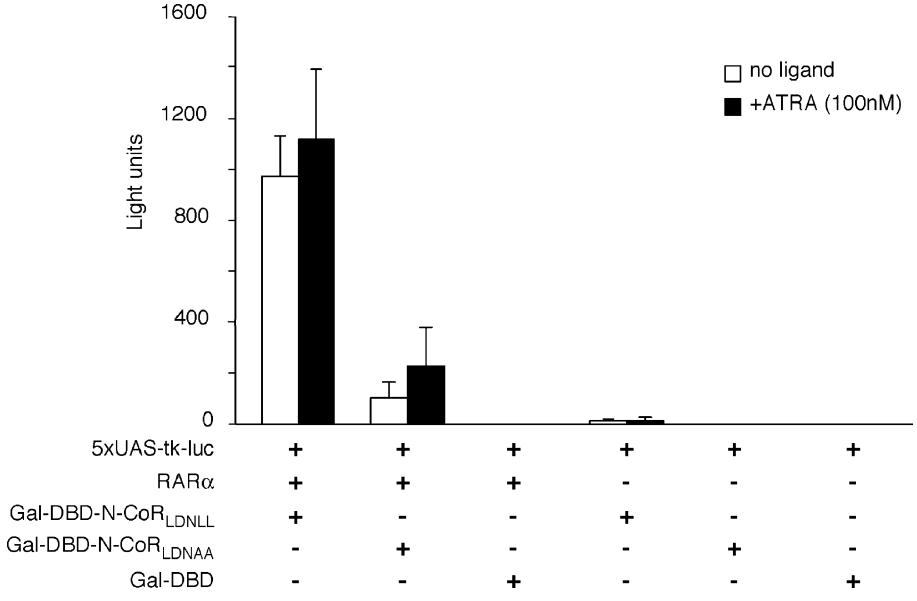

Fig. 2. Interaction between N-CoR$_{LDNLL}$ and RARα in a mammalian one-hybrid system. CV-1 cells were transiently transfected as indicated in the figure, and subsequently grown in the absence (white columns) or presence (black columns) of the RAR specific ligand all *trans*-retinoic acid (ATRA) for 24–30 h, before harvest and assay of luciferase activity. Columns represent mean values and bars represent S.D. (n = 3).

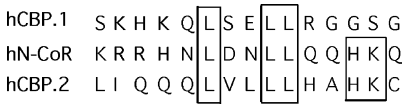

Fig. 3. Comparison of N-CoR and CBP LXXLL motif environments. Alignment of human N-CoR LXXLL motif, human CBP LXD1 (amino acids 72–76) and hCBP LXD2 (amino acids 370–374) with surrounding amino acids. The leucines which constitute the motif are marked with boxes, as are two identical basic amino acids C-terminal of the LXXLL motif (positions +3 and +4 relative to the motif) in hCBP LXD2 and hN-CoR. Three conserved basic amino acids at positions −4, −3 and −2 relative to the motif in hN-CoR and hCBP LXD1 are marked in bold letters.

Table 1
Bacterial two-hybrid interaction between N-CoR and RARα (cfu)

Antibiotics	pTRG-RARα + pBT-N-CoR$_{LXXLL}$	pTRG-RARα + pBT-N-CoR$_{LXXAA}$
TCK	478	4020
CTCK (250 μg/ml)	102 (21%)	516 (13%)

cfu, colony forming unit; TCK, tetracycline, chloramphenicol and kanamycin; CTCK, carbenicillin, tetracycline, chloramphenicol and kanamycin.

strain bacteria. The results demonstrated carbenicillin resistance among 21% of pBT-N-CoR$_{LDNLL}$ plus pTRG-RARα co-transformants. When pBT-N-CoR$_{LDNAA}$ was used instead the level of co-transformants exhibiting carbenicillin resistance was reduced to about 13% (Table 1).

4. Discussion

Nuclear receptors regulate expression of genes with appropriate response elements by recruiting coactivators or corepressors to increase or reduce gene expression. Binding of ligand induces a receptor conformational change which favors recruitment of coactivator via the consensus sequence LXXLL [14,16–20]. Unliganded or (in some cases) antagonist-bound nuclear receptors interact with corepressors such as N-CoR via a L/V-X-X-I/V-I consensus sequence [4,7,21]. Interaction between ERβ and N-CoR through a C-terminal LXXLL-like motif has been demonstrated [22]. Recently, LXXLL-dependent interaction has been reported for corepressors other than N-CoR [23–27]. This paper deals with the functional significance of an LXXLL motif found in human, and mouse N-CoR. Our data clearly showed that N-CoR$_{LDNLL}$ interacted with RARα and TRβ in vitro in a specific manner as judged by mutation of the LXXLL motif to LXXAA (Fig. 1). Supporting results were obtained using a mammalian one-hybrid system demonstrating that N-CoR also interacted with RARα in vivo through its LXXLL motif (Fig. 2).

Interaction between N-CoR and the coactivator ACTR has been reported [28]. This raises an interesting possibility of the formation of a dual functionality complex between N-CoR and SRC-1 with both coactivator and corepressor function. Such a complex might function both as a negative and positive regulator of gene expression. Li et al. showed that N-CoR can facilitate binding of ACTR to unliganded TRβ [28]. The NRIF3 family of coregulators mediates both positive and negative regulation of gene expression through two separate domains: an amino-terminal LXXLL domain and a carboxy-terminal LXXIL domain [24].

In conclusion, we have established that N-CoR can interact with nuclear receptors such as RARα and TRβ through its LXXLL motif. These interactions were demonstrated to occur both in vivo and in vitro.

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