Isolation and Characterization of a Novel Coactivator Protein, NCoA-62, Involved in Vitamin D-mediated Transcription*

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The vitamin D receptor (VDR) forms a heterodimeric complex with retinoid X receptor (RXR) and binds to vitamin D-responsive promoter elements to regulate the transcription of specific genes or gene networks. The precise mechanism of transcriptional regulation by the VDR-RXR heterodimer is not well understood, but it may involve interactions of VDR-RXR with transcriptional coactivator or corepressor proteins. Here, a yeast two-hybrid strategy was used to isolate proteins that selectively interacted with VDR and other nuclear receptors. One cDNA clone designated NCoA-62, encoded a 62,000-Da protein that is highly related to BX42, a Drosophila melanogaster nuclear protein involved in ecdysone-stimulated gene expression. Yeast two-hybrid studies and in vitro protein-protein interaction assays using glutathione S-transferase fusion proteins demonstrated that NCoA-62 formed a direct protein-protein contact with the ligand binding domain of VDR. Coexpression of NCoA-62 in a vitamin D-responsive transient gene expression system augmented 1,25-dihydroxyvitamin D$_3$-activated transcription, but it had little or no effect on basal transcription or gal4-VP16-activated transcription. NCoA-62 also interacted with retinoid receptors, and its expression enhanced retinoic acid-, estrogen-, and glucocorticoid-mediated gene expression. These data indicate that NCoA-62 may be classified into an emerging set of transcriptional coactivator proteins that function to facilitate vitamin D- and other nuclear receptor-mediated transcriptional pathways.

Biological responsiveness to 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) is mediated through an intracellular receptor termed the vitamin D receptor (VDR). VDR is a member of the superfamily of nuclear receptors for steroid hormones, and it acts as a ligand-induced transcription factor that binds to specific DNA response elements in the promoter region of vitamin D-responsive genes (1–3). Vitamin D response elements (VDREs) consist of either exact or imperfect direct repeats of the hexanucleotide sequence, GGTTGA, generally separated by a three-nucleotide spacer. High affinity binding of VDR to VDREs requires an additional nuclear factor that is most likely retinoid X receptor (RXR), the nuclear receptor for 9-cis-retinoic acid (4–6). Thus, VDR and RXR heterodimerize to form a complex that binds with high affinity to VDREs, and it is the VDR-RXR heterodimer that may be the functional transcription factor in vitamin D-mediated gene expression.

The mechanism that links the heterodimeric receptor complex bound at the DNA response element to the transcriptional complex is not well understood, but it is presumed to involve protein-protein interactions between the heterodimer and other transcriptional coactivator proteins. Recently, a number of putative coactivator and corepressor proteins have been described for several members of the nuclear receptor superfamily (7). A general property of these transcriptional cofactors is their ability to selectively interact with liganded nuclear receptors and modulate their transcriptional activity. Putative coactivators include steroid receptor coactivator 1 (SRC-1) (8), receptor-interacting protein 140 (9), receptor-associated coactivator 3 (RAC3) (10), a novel nuclear receptor coactivator (ACTR) (11), CREB-binding protein (CBP) (12), and glucocorticoid receptor-interacting protein (GRIP-1) (13), also termed TIF2 (14, 15). Although the precise mechanism is unclear, these coactivators are proposed to function as bridging proteins that link the receptor complex to RNA-polymerase II and the basal transcription machinery. Alternatively, the CBP and SRC-1 coactivators possess intrinsic histone acetyltransferase activity and also interact with histone acetyltransferases, suggesting that they function by altering chromatin structure within hormone-responsive promoters to affect the transcriptional response (16–19).

In the present study, we report the isolation of a cDNA that encodes a 62,000-Da protein that interacts with VDR and several other nuclear receptors. This VDR-interactive protein exhibited striking homology to a Drosophila melanogaster nuclear protein that is implicated in ecdysone-mediated transcription (20). Importantly, expression of this cDNA in mammalian cells strongly augmented vitamin D-, retinoic acid-, estrogen-, and glucocorticoid-activated transcription, but it had little or no effect on basal or gal4-VP16-activated transcription. Based on its interaction with nuclear receptors and its ability to selectively augment nuclear receptor-mediated transcription, this novel protein was designated NCoA-62 (for nuclear receptor coactivator; 62,000 Da).

EXPERIMENTAL PROCEDURES

Yeast Transformation and Library Screening by the Two-hybrid System—All plasmid constructs used the pAS1 and pGAD-GH yeast expression vectors (21, 22). A HeLa cell cDNA library (in pGAD-GH) was

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1 The abbreviations used are: 1,25-(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; VDR, vitamin D receptor; VDRE, vitamin D response elements; SRC-1, steroid receptor coactivator 1; RAC3, receptor-associated coactivator 3; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; GRIP-1, glucocorticoid receptor-interacting protein; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; bp, base pair(s); WT, wild type; RXR, retinoid X receptor; RAR, retinoic acid receptor.

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cotransformed with pAS1-VDR-(93–427) into the HTc7 strain of yeast as described previously (23). Transformants were plated onto media deficient in leucine, tryptophan, and histidine and containing 25 µm 5-aminoleucine, 1,4-triazole (3-AT). Colonies were assayed for β-galactosidase expression using a colony lift filter assay (21).

5′-Rapid Amplification of cDNA Ends Strategy—First strand cDNA was synthesized using SuperScript II reverse transcriptase, 1 µg of poly(A)+ RNA obtained from HeLa cells, and a gene-specific primer (GSP1; ATCTTGAACCTTGGAGGC), which is complementary to nucleotides 657–668 in NCoA-62. After first strand cDNA synthesis, the original mRNA template was destroyed with RNase H. Unincorporated dNTPs, GSP1, and proteins were separated from the cDNA using a GlassMAX spin cartridge. An anchor sequence was then added to the 3′-end of the cDNA using TdT and dCTP. PCR amplification was accomplished using Taq DNA polymerase, an anchor primer, and a nested gene-specific primer (GSP2; GGAAACGAGTCTAGGTAT), which is located approximately 360 base pairs into the cDNA. Following amplification, the 5′-rapid amplification of cDNA ends product was cloned into the pCR II vector (Invitrogen, CA). An insert was excised from pCR II with EcoRI and MscI restriction endonucleases. The EcoRI site was from the pCR II vector, and the MscI site was from the 3′-end of the NCoA-62 PCR product. This fragment was then subcloned into pSG5 ( Stratagene, CA) together with NCoA-62 cDNA from the MscI site to the end of the clone in order to generate the full-length NCoA-62 expression vector.

In Vitro Protein Interaction Assay—The ligand binding domain of human VDR from leucine 116 to serine 427 was inserted into the pGEX-KT expression vector (24). GST-VDR was then expressed in the DH5α strain of E. coli and purified by glutathione-agarose affinity chromatography as described previously (25). 35S-Labeled NCoA-62 was generated using the TNT-coupled transcription-translation system as described by the manufacturer (Promega, Madison, WI). GST or GST-VDR was bound to glutathione-agarose (Sigma) and equilibrated with 1× GBB (20 mM Tris, pH 7.6, 50 mM NaCl, 5 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.2% Nonidet P-40, and protease inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 4.0 µg/ml aprotinin, 2.0 µg/ml leupeptin, and 1 µg/ml pepstatin A). Equivalent volumes of [35S]methionine-labeled, NCoA-62 proteins were incubated with the immobilized GST fusion proteins in 100 µl of 1× GBB for 1 h at 4 °C. The beads were washed three times with 0.5 ml with 1× GBB and with 1 ml 50 mM Tris (pH 8.0) buffer. Bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris buffer. Eluted proteins were resolved by SDS-PAGE and visualized by autoradiography.

Baculovirus-mediated Expression of NCoA-62—Human NCoA-62 cDNA was subcloned into the pVL-1392 polyhedrin transfer plasmid by PCR amplification containing a polyhistidine tag and a protein kinase A consensus site at the N terminus. Recombinant baculovirus was isolated and plaque-puriﬁed by standard methods (26). A 50-ml culture of Sf9 cells (1 × 10^6 cells/ml) was infected for 48 h with the NCoA-62-expressing recombinant baculovirus (multiplicity of infection = 5). Whole cell extracts were prepared, and recombinant NCoA-62 was purified by nickel affinity chromatography.

For Western Blot—Whole cell extracts and Ni2+–nitrilotriacetic acid-puriﬁed, baculovirus expressed proteins from S9 cells or bacterial expressed GST fusion proteins were subjected to SDS-PAGE and electrothermally transffered to a Protran BA-85 nitrocellulose membrane (Schleicher & Schuell). Denaturation and renaturation of the protein blots was performed as described (27, 28). The renatured blot was incubated in HB buffer (25 mM HEPES, pH 7.7, 25 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol) containing 5% nonfat dried milk and then in HB buffer containing 1% milk, 0.05% Nonidet P-40, and a 32P-labeled VDR probe. The VDR probe was prepared by combining puriﬁed, baculovirus-expressed His6 protein kinase A-tagged VDR (300 pmol), 12.5 µl of [γ-32P]ATP (3000 Ci/mol), and 50 units of the catalytic subunit of protein kinase A (Sigma) in HMK buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 12 mM MgCl2, 1 mM dithiothreitol) for 1 h at room temperature. The 32P-labeled VDR probe was puriﬁed on Ni2+–nitrilotriacetic acid-agarose and incubated with the membrane at approximately 500,000 cpm/ml in buffer H (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 0.05% Nonidet P-40, 1% milk, 1 mM dithiothreitol) overnight at 4 °C. After three washes with buffer H, the protein blot was washed on the blotting chamber for 2 h at room temperature.

Plasmids and Transient Transfection Studies—The vitamin D-responsive (VDR)-4′-TK-GH growth hormone reporter plasmid contains four copies of the rat osteocalcin VDRE upstream of the viral thymidine kinase promoter. The pSG5VDR expression plasmid was described previously (29). Full-length NCoA-62 cDNA was subcloned into the mammalian expression vector, pSG5 (Stratagene). All cells were cultured in Dulbecco’s modiﬁed Eagle’s medium containing 10% charcol- stripped, heat-inactivated serum prior to transfection. COS-7 cells were transfected by standard calcium phosphate precipitation procedures as described previously (6). Carrier DNA (pTZ18u) was added to bring the total DNA content to 10 µg. Following a 16-h incubation, the precipitate was removed with two washes of phosphate-buffered saline, and the cells were replenished with fresh media containing 10% charcoal-stripped, heat-inactivated serum. The cells were treated with ligand or vehicle for 24 h, and the amount of secreted growth hormone was determined with an immunoassay kit (Nichols Institute, San Juan Capistrano, CA).

Liquid β-Galactosidase Assay—The indicated plasmid pairs were transformed into HTc7 yeast, plated on media lacking leucine and tryptophan, and incubated at 30 °C for 4 days. Triplicate colonies were harvested and cultured overnight in liquid media deﬁcient in leucine and tryptophan. Cells were assayed for β-galactosidase activity as described previously (23).

Northern Blot Analysis of NCoA-62 Transcript—A multiple tissue Northern blot was obtained from CLONTECH Laboratories. Each lane of the blot contained 2 µg of poly(A)+ RNA puriﬁed from several human tissues. Total RNA isolated from rat osteosarcoma cells (ROS 17/2.8) and from primary rat osteoblasts obtained from newborn calvaria (30) was subjected to Northern analysis as well. A cDNA probe for NCoA-62 was labeled with [α-32P]dCTP in a random primer reaction with the Klenow fragment of DNA polymerase. The blots were hybridized with the labeled probe in ExpressHybridization solution (CLONTECH Laboratories) and were processed according to the manufacturer’s instructions. Autoradiographs were scanned, and the images were quantitated using ImageQuant version 3.0 software from Molecular Dynamics.

RESULTS

Isolation of NCoA-62 cDNA Encoding a VDR-interactive Protein—We used a yeast two-hybrid system to identify cDNA clones that code for proteins that interact with the vitamin D receptor (23). The pAS1-VDR-(93–427) plasmid was used to screen a HeLa cell cDNA library that was constructed in the pGAD-GH plasmid, and positive clones were selected by growth on histidine-deﬁcient media and for the expression of β-galactosidase activity. Speciﬁcity controls included testing each clone against pAS1-RAR, pAS1-RXR, pAS1-p53, pAS1-G, pAS1-VDR, and pAS1. The growth properties of one clone, designated NCoA-62, are illustrated in Fig. 1. As evidenced by growth on histidine-deﬁcient media in the two-hybrid system, NCoA-62 interacted with the VDR hybrid protein (area 1), the RAR hybrid protein (area 3), and the RXR hybrid protein (area 5). In contrast, no interaction was detected with unrelated bait vectors including the pAS1 parent vector (area 2), pAS1-p53...
Characteristics of the NCoA-62 cDNA, Transcript, and Expressed Protein—The NCoA-62 cDNA insert was sequenced and was found to be highly similar to BX42, a nuclear protein from *D. melanogaster* that is involved in ecdysone-stimulated gene expression (Fig. 2) (20). Other related proteins from *Caenorhabditis elegans* and *Schizosaccharomyces pombe* are also illustrated in Fig. 2. The NCoA-62 cDNA insert was 2113 bp. However, based on the BX42 sequence, the original NCoA-62 clone did not contain an obvious initiator methionine with a strong consensus Kozak sequence (31). Therefore, a 5' rapid amplification of cDNA ends strategy (Life Technologies, Inc.) was used with mRNA isolated from HeLa cells, and a single product was obtained that contained an additional 30 bp of 5' sequence, 27 of which constituted a short untranslated region followed by an in frame ATG with a perfect Kozak sequence (data not shown). Thus, the full-length NCoA-62 cDNA is 2146 bp consisting of a noncoding leader sequence of 27 bp, a 1611-bp open reading frame, and 508 bp of 3' noncoding sequence. The size of this cDNA is consistent with Northern blot analysis of mRNA from HeLa cells, which indicated a single 2.2-kilobase pair transcript for NCoA-62 (data not shown). The tissue distribution of the NCoA-62 transcript was determined by Northern blot analysis using mRNA obtained from eight different human tissues (Fig. 3 A). NCoA-62 exhibited a wide expression pattern, being present in all of the various tissues examined. The NCoA-62 mRNA transcript was also observed in a Northern blot analysis of mRNA obtained from an osteoblast-like cell line, ROS 17/2.8, and in primary osteoblasts obtained from newborn rat calvaria (Fig. 3 B). In rat osteoblasts, the
NCoA-62 transcript was a single 2.4-kilobase pair transcript that was not regulated by vitamin D treatment over a 24-h time course (Fig. 3B). In osteoblasts obtained from newborn calvaria (Fig. 3B, right panel), osteocalcin transcripts were up-regulated by 1,25-(OH)2D3 in the differentiated and mineralizing osteoblasts obtained from newborn calvaria in the proliferation (P), differentiation (D), and mineralization (M) stages (right) was hybridized with a 32P-labeled NCoA-62 cDNA probe. The blots were stripped and reprobed with [32P]actin cDNA (left) or [32P]labeled osteocalcin cDNA. The various transcripts are indicated by arrows.

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His-protein kinase A tag, there is agreement between the apparent Mr by SDS-PAGE and the predicted Mr of NCoA-62 based on the primary sequence. Moreover, the estimated molecular mass of the untagged NCoA-62 protein produced by in vitro transcription/translation was approximately 62,000 daltons as assessed by SDS-PAGE analysis (see Fig. 6).

Mapping the Interaction Domains of NCoA-62 and VDR—To identify the regions of VDR that are important for interaction with NCoA-62, several amino- and carboxyl-terminal deletion mutants of pAS1-VDR were examined in the two-hybrid system (Fig. 5A). Nuclear receptors are characterized by an N-terminal DNA-binding domain, which targets the receptor to specific DNA response elements and a large COOH-terminal domain that binds the hydrophobic ligand. At the extreme COOH-terminal end of the ligand binding domain of these receptors is a conserved motif, designated AF-2, which is crucial for the transactivation function of many nuclear receptors. Indeed, several of the putative coactivators described thus far bind to nuclear receptors, in part, through this AF-2 domain (8–10, 32, 33). Comparison of the pAS1-VDR-(3–427) full-length construct and the pAS1-VDR-(116–427) construct clearly demonstrated that the NH2-terminal DNA binding domain of VDR is not required for VDR-NCoA-62 interactions. Furthermore, removal of the AF-2 motif located at the extreme C terminus of VDR (residues 403–427) was not detrimental to the interaction. However, removal of additional residues between 386 and 402 eliminated VDR interaction with NCoA-62. Furthermore, this COOH-terminal domain alone was sufficient to observe modest interaction with NCoA-62. For example, the pAS1-VDR-(373–427) construct, which contains only 55 amino acids from the extreme C terminus of VDR, was capable of interacting with the NCoA-62-GAD fusion in the two-hybrid system. These data strongly suggest a role for the COOH-terminal domain between amino acid residues 373 and 403 of VDR in contacting NCoA-62. A second potential interaction surface may reside between Leu116 and Ser166, since deletion of this domain also ablates VDR-NCoA-62 complex formation. However, this deletion also disrupts ligand binding, RXR interaction, and transcription factor IID interaction as well as interaction between VDR and a number of other clones isolated in our screen.2 Thus, it is also likely that this deletion alters the

2 T. A. Baudino, D. M. Kraichely, S. C. Jefcoat, Jr., and P. N. MacDonald, unpublished data.
FIG. 5. Deletion analysis of VDR and NCoA-62 interaction in the two-hybrid system. A, interaction of various VDR deletion mutants with NCoA-62. Amino- and carboxy-terminal deletion mutants of AS1-VDR were tested against GAD-NCoA-62 in the yeast two-hybrid system. Relative growth on His-deficient plates was assessed after 4 days, and β-galactosidase expression was quantitated in liquid cultures. Results are presented as the mean ± S.D. of triplicate cultures. The box labeled DNA represents the DNA binding domain of VDR, and the solid black box represents the COOH-terminal AF-2 domain. B, deletion analysis of NCoA-62. Deletion mutants of NCoA-62 were constructed in the GAD-GH vector. Each NCoA-62 construct was examined for interaction with AS1-VDR-(93–427) by monitoring expression of the lacZ and HIS3 reporter genes in the two-hybrid system as described for A.

In Vitro Interaction of NCoA-62 with VDR—Far Western analysis was used to show a physical interaction of NCoA-62 with VDR (Fig. 6A). Crude cell extracts obtained from baculovirus-infected Sf9 cells were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with a 35S-labeled VDR probe. Fig. 6A shows that crude extracts obtained from infected cells (lane 4) or from cells infected with the wild-type ACMPV virus (lane 3) lacked proteins that showed significant interaction with the VDR probe. However, a distinct protein of 65,000 Da interacted with the 35S-labeled VDR probe in the extract obtained from cells infected with the NCoA-62 recombinant virus (lane 2). This protein comigrated precisely with purified NCoA-62 protein (lane 1).

NCoA-62 also showed significant in vitro interaction with a GST-VDR fusion protein. As illustrated in Fig. 6B, 35S-labeled NCoA-62 interacted with GST-VDR in a concentration-dependent manner (lanes 5–7), while no interaction was observed with GST alone (lanes 2–4). A 2-fold effect of the 1,25-(OH)2D3 ligand on VDR-NCoA-62 complex formation was noted (Fig. 6C, lanes 7 and 8), while 10−8 M 1,25-(OH)2D3 enhanced VDR-RXR heterodimer formation by 4-fold (Fig. 6C, lanes 4 and 5). We did not observe an effect of 1,25-(OH)2D3 on VDR-NCoA-62 interaction in the two-hybrid system (data not shown). Finally, two COOH-terminal truncation mutants of NCoA-62 (R438STOP or R488STOP) also showed substantial, albeit somewhat re-
duced, interaction with GST-VDR in this system (Fig. 6D), and, as previously stated, these same COOH-terminal truncations also interacted with VDR in the two-hybrid system (Fig. 5B).

**NCoA-62 Enhances VDR-mediated Gene Transcription**—Having demonstrated an interaction between VDR and NCoA-62 both in vivo and in vitro, the effect of NCoA-62 on VDR-mediated gene expression was then examined. Transient expression studies were conducted in COS-7 cells using the (VDRE)4-TK-GH reporter construct and SG5-hVDR (6). As illustrated in Fig. 7A, COS-7 cells cotransfected with these plasmids and then treated with 10⁻⁸ M 1,25-(OH)₂D₃ exhibited a dose-dependent increase in growth hormone reporter expression in the presence of increasing amounts of NCoA-62 expression plasmid. The activation increased from 7-fold in the absence of NCoA-62 to 14-, 19-, and 38-fold with cotransfection of 100, 333, and 1000 ng of the NCoA-62 expression plasmid, respectively. Western analysis revealed equivalent expression of VDR in this assay, demonstrating that alterations in receptor expression did not account for the observed enhancement (Fig. 7A). The transcriptional enhancement observed with NCoA-62 expression required its interaction with the VDR, since deletions within the first 200 NH₂-terminal residues of NCoA-62 retained strong interaction with VDR (NCoA-62-(87–536) and NCoA-62-(220–536) in Fig. 5B), and both deletion constructs were highly active in VDR-mediated transcription (Fig. 7B). In contrast, the NH₂-terminal deletion that eliminated interaction with VDR (NCoA-62-(388–536) in Fig. 5B) was significantly impaired in its ability to augment 1,25-(OH)₂D₃-activated transcription compared with intact NCoA-62 (Fig. 7B). The modest activity observed with this mutant (34-fold activation versus 26-fold with the SG5 control vector) may be due to a weak interaction of NCoA-62-(388–536) with VDR or with RXR in the system (data not shown). Two COOH-terminal deletion mutants of NCoA-62 that retain interaction with VDR either abolished the coactivator activity (NCoA-62 (R488STOP)) or actually inhibited vitamin D-acti-
uated transcription in this system (NCoA-62 (R438STOP)). As shown in Fig. 7B, cells transfected with the SG5-NCoA-62 (WT) expression vector showed 70-fold activation of reporter gene expression by 1,25-(OH)2D3, while coexpression of NCoA-62 (R488STOP) yielded only 21-fold activation, which was not significantly different from the pSG5 parent vector control (26-fold). Furthermore, NCoA-62 (R438STOP) expression reduced 1,25-(OH)2D3-activated transcription to 6-fold (a 77% decrease compared with the SG5 control). Since both NCoA-62 deletion mutants still interact with VDR (Figs. 5B and 6D), these data implicate a functional role for the COOH-terminal region of NCoA-62 in the transactivation effect.

Having observed NCoA-62 interaction with other nuclear receptors in the yeast two-hybrid system (Fig. 1), the effect of NCoA-62 expression on other nuclear receptor pathways was also examined. Fig. 7C illustrates that coexpression of NCoA-62 enhanced retinoic acid-, estrogen-, and glucocorticoid-responsive reporter gene assays to an extent similar to that observed with 1,25-(OH)2D3-mediated transactivation. All systems were enhanced 2–4-fold by NCoA-62 expression in COS-7 cells. Importantly, little if any effect of NCoA-62 was observed on basal transcription or on gal4-VP16-activated transcription, indicating that NCoA-62, through its interaction with various receptors, selectively augments nuclear receptor-mediated transcriptional pathways.

**DISCUSSION**

Nuclear receptor coactivators, such as SRC-1 (8) and GRIP-1 (13), interact with nuclear receptors and modulate the transcriptional responsiveness to ligand. The nuclear receptor coactivators described thus far act promiscuously to augment transactivation by a variety of nuclear receptors, and putative transactivation domains have been identified in these proteins. These coactivators generally interact in a ligand-dependent manner, and the AF-2 transactivation domain of the nuclear receptors plays a central role in this interaction process. Several signature LXXLL motifs within the coactivator primary sequence are necessary and sufficient for coactivator interaction with the receptor AF-2 helix (34). Although the precise mechanism of action is unclear, it has been proposed that these coactivators function as bridging proteins that link the nuclear receptors to the preinitiation complex. Alternatively, several coactivators, including SRC-1 and CBP/P300, have potent histone acetyltransferase activity, suggesting the possibility that nuclear receptors recruit enzymes that modify chromatin structure in the promoters of hormone-responsive genes (16–19).

NCoA-62 interacts with VDR and other nuclear receptors to augment hormone- or ligand-activated transcription. Based on our initial structure/function analysis, the physical interaction with VDR is central to NCoA-62 effects on 1,25-(OH)2D3-activated transcription. For example, an NCoA-62 deletion mutant that does not interact with VDR (NCoA-62(388–536)) exhibited little effect on VDR-activated transcription (Fig. 7B). This suggests that recruitment of NCoA-62 to the promoter through interaction with VDR is required for the observed transcriptional effect. Moreover, in vitro studies indicate that the VDR-NCoA-62 interaction is modestly enhanced by 1,25-(OH)2D3 ligand (Fig. 6C). These two fundamental properties (i.e. ligand-dependent interaction and transactivation) justify NCoA-62’s classification as a bona fide nuclear receptor coactivator. However, in a number of respects, NCoA-62 is distinct from the GRIP1/SRC-1 family of coactivators described thus far. One key difference is that NCoA-62 interaction with VDR is relatively independent of the AF-2 domain of VDR (Fig. 5A). This raises the intriguing possibility that NCoA-62 and distinct AF-2-interacting coactivators may work in concert through different domains of VDR to influence nuclear receptor-mediated transcription. NCoA-62 also does not contain any of the signature LXXLL motifs of the SRC-1 coactivator family (34). The LXXLL motif mediates coactivator interaction predominantly with the AF-2 domain of nuclear receptors. Since NCoA-62 interacts with a surface outside of the VDR AF-2 domain, it is not surprising that NCoA-62 interactions with VDR might be mediated through a distinct interaction surface. Finally, both the CBP and SRC-1 coactivators activate transcriptional processes mediated by enhancers unrelated to the nuclear receptor family including the CREB, p53, gal4-VP16 and SP-1 transcription factors (8, 12). Here we show that NCoA-62 does not affect gal4-VP16-activated transcription (Fig. 7C). Thus, while SRC-1 and CBP/P300 proteins may be more general coactivator proteins involved in a number of enhancer-mediated transcriptional processes, it appears that NCoA-62 exhibits some degree of nuclear receptor selectivity. Obviously, a more extensive survey of enhancer factor activities will be required to confirm this initial observation.

The deduced amino acid sequence of NCoA-62 is 75% identical to a D. melanogaster nuclear protein designated BX42 (20). BX42 was identified as a nuclear protein in D. melanogaster that is tightly associated with nucleosomal chromatin (35). It is widely expressed in Drosophila tissues and is localized to specific, condensed sites on polytene chromosomes. Interestingly, this pattern of chromosomal localization changes in response to the steroid hormone 20-hydroxyecdysone (35). These data suggest that BX42 may play a role in steroid hormone-regulated transcription in D. melanogaster, and this implies a similar important role for NCoA-62 in nuclear receptor-mediated transcription in mammals. Based on sequence comparisons (Fig. 2), NCoA-62 can be divided into three potentially important domains. The central region contains a SNW domain between amino acids 174 and 339, and a portion of this domain is proline-rich (residues 219–233). There is an Src homology 2-like domain between residues 373–453 as well as a highly charged COOH-terminal tail between residues 430 and 536. Functional studies indicate an important role for at least two of these domains in NCoA-62 activity. First, our deletion analysis (Fig. 5) indicates that the highly conserved midregion of NCoA-62 (residues 220–388) is involved in VDR interaction and quite possibly in the interaction of NCoA-62 with other nuclear receptors. A second crucial domain is located in the COOH-terminal region of the molecule. The COOH-terminal 50 amino acid region, which is rich in charged residues (50%), is clearly involved in the transactivation process, since its elimination abrogates NCoA-62 transcriptional effects without compromising VDR-NCoA-62 interaction. Furthermore, R438STOP (elimination of the COOH-terminal 100 residues) also interacts with VDR, but it does not augment VDR-mediated transactivation. Instead, expression of this mutant interferes with VDR-mediated transcription, indicating that NCoA-62 (R438STOP) blocks native NCoA-62 activity and thus may function as a dominant-negative inhibitor of this process (Fig. 7B). It is possible that the elimination of the COOH-terminal 100 amino acids of NCoA-62 may expose crucial residues in the Src homology 2-like domain that regulate NCoA-62 activity, perhaps through additional protein-protein contacts. Clearly, future studies will be directed at characterizing this COOH-terminal, highly charged domain, which is essential for NCoA-62 bioactivity.

In summary, we have demonstrated a direct interaction of NCoA-62 with the ligand binding domain of VDR, and our data demonstrate that this interaction is essential for coactivator activity. The exact mechanism of action of NCoA-62 in VDR-mediated transcription is unknown at the present time. NCoA-62 may be functioning as a bridge between the nuclear
receptor complex and the basal machinery allowing for activated transcription. Perhaps the COOH-terminal activation domain of NCoA-62 is essential for this bridging activity as has been suggested for other transactivation domains. Further characterization of the function and identification of proteins that contact this COOH-terminal domain will provide insight into these possibilities.

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Note Added in Proof—The GenBank™ accession number for the NCoA-62 cDNA is AF045184. It is virtually identical to accession number U51432, a human cDNA termed SKIP.

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