Mapping the Domains of the Interaction of the Vitamin D Receptor and Steroid Receptor Coactivator-1

Rajbir K. Gill, Loretta M. Atkins, Bruce W. Hollis, and Norman H. Bell

Departments of Medicine and Pediatrics
Medical University of South Carolina
Department of Veterans Affairs Medical Center
Charleston, South Carolina 29401-5799

The vitamin D receptor (VDR) binds to the vitamin D response element (VDRE) and mediates the effects of the biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], on gene expression. The VDR binds to the VDRE as a heterodimeric complex with retinoid X receptor. In the present study, we have used a yeast two-hybrid system to clone complementary DNA that codes for VDR-interacting protein(s). We found that the human steroid receptor coactivator-1 (SRC-1) interacts with the VDR in a ligand-dependent manner, as demonstrated by β-galactosidase production. The interaction of the VDR and the SRC-1 takes place at physiological concentrations of 1,25$(OH)_2D_3$. A 48.2-fold stimulation of β-galactosidase activity was observed in the presence of 10^{-10} M 1,25-$(OH)_2D_3$. In addition, a direct interaction between the ligand-activated glutathione-*S*-transferase-VDR and ^{35}S-labeled SRC-1 was observed *in vitro*. Deletion-mutation analysis of the VDR established that the ligand-dependent activation domain (AF-2) of the VDR is required for the interaction with SRC-1. One deletion mutant, pGVDR-(1–418), bound the ligand but failed to interact with the SRC-1, whereas another deletion mutant, pGVDR-(1–423), bound the ligand and interacted with the SRC-1. We demonstrated that all the deletion mutants were expressed as analyzed by a Gal^4 DNA-binding domain antibody. Deletion mutation analysis of the SRC-1 demonstrated that 27 amino acids (DPCNTNPTPMTKATPEEIKLEAQSQFT) of the SRC-1 are essential for interaction with the AF-2 motif of the VDR. (Molecular Endocrinology 12: 57–65, 1998)

0888-8809/98/$3.00/0
Molecular Endocrinology

INTRODUCTION

The vitamin D receptor (VDR) is a member of the nuclear hormone receptor family, which also includes retinoid, thyroid hormone, and steroid hormone receptors. These receptors function as ligand-inducible transcription factors by binding to specific DNA sequences known as hormone response elements in the promoters of the target genes (1–4). The VDR mediates the actions of the biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], by binding to the vitamin D response element (VDRE) and modulates transcription of the target genes, presumably by interacting with the basal transcriptional machinery (5). The VDRE sequences described to date, including those of rat (6–8) and human (9) osteocalcin, mouse osteopontin (10), rat 25-hydroxyvitamin D_3-24-hydroxylase (11), avian integrin β_3 (12), and mouse calbindin-D_{28k} (13), consist of two hexamers arranged in direct tandem repeats and a composite response element as described in c-*fos* (14). Studies with VDR obtained from an overexpression system indicate that the VDR alone binds to VDRE with low affinity and requires an additional nuclear protein(s), termed receptor or nuclear auxiliary factor, for high affinity binding to the VDREs (15–18). Several groups have demonstrated that isoforms of the retinoid X receptor (RXR) function as auxiliary factors, as RXRs mimic receptor auxiliary factor activity (19–21). In addition, involvement of RXR in VDR-mediated transcription is supported by the observation that vitamin D-dependent transcription is augmented by exogenous RXR in the transient expression system (19, 21), and VDR mutants that fail to interact with RXR also fail to activate transcription (22). The role of RXR in VDR-mediated transcription has been confirmed in yeast that does not express any endogenous RXR (23, 24). Thus, transcriptional activation by the VDR appears to require heterodimeric interaction with another nuclear receptor, such as RXR.

Recently a steroid receptor coactivator has been cloned and shown to bind and regulate transcription mediated by progesterone receptor (PR), estrogen receptor (ER), thyroid hormone receptor (TR), and RXR (25, 26). In the current study, we demonstrate that the VDR forms a direct protein-protein interaction with the newly described steroid receptor coactivator-1 (SRC-1) and that the interaction is dependent on the presence of the ligand. In addition, we mapped the regions of the VDR and the SRC-1 that are required for this interaction.

RESULTS

Isolation of SRC-1 from Human Kidney cDNA Library with Gal^4-VDR-(1–427)

In the preliminary experiment, the VDR (1–427 amino acids) expressed as a fusion protein with Gal^4 DNA-binding domain (Gal^{4}DB), was assayed in the two-hybrid system to test for background transcriptional activity. When Gal^{4}DB-VDR alone (Fig. 1, A and B) or coexpressed with the pGAD10 [Gal^4 activation domain (Gal^{4}AD)] plasmid (Fig. 1,C and D), β-galactosidase activity was not observed in the absence or presence of 1,25-$(OH)_2D_3$ (10^{-7} M). Similarly, transcriptional activity was not detected in the presence or absence of 1,25-$(OH)_2D_3$ when the VDR was coexpressed with an unrelated plasmid (data not shown). Thus, we established that the VDR has little if any transcriptional activity in yeast in the presence or absence of ligand. To identify the cDNA coding for the VDR-interacting proteins, we transformed YPB6 cells expressing Gal^4-VDR with a cDNA library constructed in pGAD10 vector [Gal^4 activation domain plasmid (Gal^{4}AD)] and tested the double transformants for β-galactosidase activity and histidine prototrophy in the presence of 10^{-7} M 1,25-$(OH)_2D_3$. Of 2.5×10^7 individual clones examined in the presence of 1,25-$(OH)_2D_3$, 73 of them demonstrated histidine prototrophy and expressed β-galactosidase, as determined by filter assay. The specificity of interaction with Gal^4-VDR of these 73 clones was tested by curing the yeast cells of the bait plasmid and testing them for β-galactosidase activity. In 63 clones, loss of Gal^4-VDR resulted in concordant loss of β-galactosidase activity, demonstrating that β-galactosidase production was due to interaction of the protein and the VDR.

Among the 63 positive clones obtained by screen, 13 of them were identified to be SRC-1 (25, 26) by sequencing and comparison with the GenBank database. Amino acids of SRC-1 coded by different cDNA inserts are as follows: nine clones coded amino acids 1–1260, two clones coded amino acids 342-1440, one clone coded amino acids 711-1160, and one clone coded amino acids 745-1180.

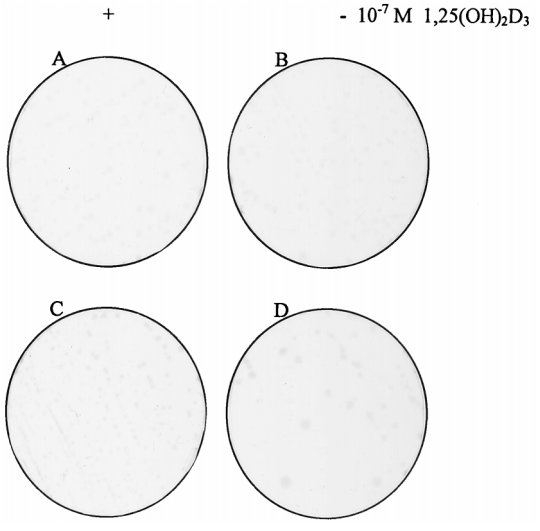

Fig. 1. Demonstration of Lack of Intrinsic Transcriptional Activity of the VDR in the Yeast Two-Hybrid System

Gal^{4}DB-VDR fusion protein does not contain transcriptional activation functions in yeast. YPB6 cells were transformed with pGVDR-(1–427) (Gal^4-VDR) alone (A and B) or cotransformed with pGAD10 (Gal^{4}AD; C and D). Transformed cells were plated on selection medium containing vehicle (A and C) or 10^{-7} M 1,25-$(OH)_2D_3$ (B and D). Colonies were tested for β-galactosidase activity by filter assay.

Requirement of 1,25-$(OH)_2D_3$ for Interaction of VDR and SRC-1

To determine whether the ligand is required for interaction, yeast cells containing both plasmids were plated on Leu$^-$,Trp$^-$ synthetic MEM (SD medium) in the absence or presence of 10^{-7} M 1,25-$(OH)_2D_3$ and assayed for β-galactosidase activity (Fig. 2). Transcriptional activation of β-galactosidase was observed in the presence of ligand, as demonstrated by the development of blue color (Fig. 2, A1 and B). In the absence of ligand, VDR and SRC-1 failed to interact with each other (Fig. 2A2). In addition, we examined the interaction between VDR and SRC-1 *in vitro*. As shown in Fig. 2C, very little binding of ^{35}S-labeled SRC-1 was observed with unactivated glutathione-*S*-transferase (GST)-VDR (in the absence of ligand), and binding was significantly enhanced when GST-VDR was activated with 1,25-$(OH)_2D_3$. These results demonstrated that the VDR undergoes direct interaction with the SRC-1 in a ligand-dependent manner. To determine whether these interactions occur at physiological concentrations of 1,25-$(OH)_2D_3$, the cells containing both plasmids were cultured at concentrations ranging from 10^{-12}-10^{-7} M 1,25-$(OH)_2D_3$, and β-galactosidase activity was determined by liquid assay. Transcriptional activation was dose dependent and reached a maximum level at 10^{-8} M 1,25-$(OH)_2D_3$ (Fig. 2B). A significant stimulation (48.2-fold) of β-galactosidase activity was observed at 10^{-10} M 1,25-$(OH)_2D_3$. These results demonstrate that SRC-1 binds to VDR in a dose-dependent manner.

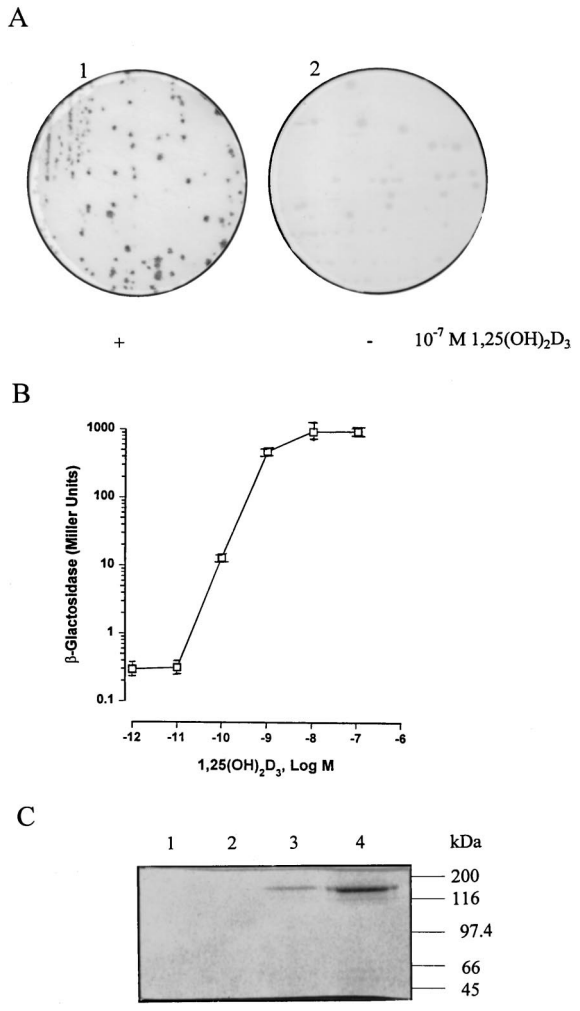

Fig. 2. Interaction of the VDR with the SRC-1 in the Presence of Ligand

A, Stimulation of β-galactosidase activity due to interaction of Gal^{4}DB-VDR (bait plasmid) and Gal^{4}AD-SRC-1 in the absence (A2) and presence of 10^{-7} M 1,25-(OH)$_2$D$_3$ (A1). B, Dose response of β-galactosidase activity stimulation. The pGVDR-(1–427) was cotransformed with Gal^{4}AD-SRC-1. Transformants were cultured in selection medium, and β-galactosidase activity was quantitated by liquid assay. C, *In vitro* interaction of the SRC-1 with the VDR. SRC-1 (3.4 kb) radiolabeled with [^{35}S]methionine was incubated in batch with yeast-expressed GST (lanes 1 and 2) or GST-VDR fusion protein (lanes 3 and 4) in the absence (lanes 1 and 3) or presence of ligand (lanes 2 and 4). Bound SRC-1 was eluted and analyzed on SDS-PAGE.

Requirement for the Ligand-Dependent Activation Domain (AF-2) Region of the VDR for Interaction with the SRC-1

To determine the domains of the VDR required for interaction with the SRC-1, deletion mutant fragments of the VDR were PCR amplified as shown in Fig. 3A. Deletion fragments were cloned to express Gal^4 fusion proteins. These deletion mutant constructs were cotransformed with SRC-1 into YBP6 and cultured onto Leu$^-$,Trp$^-$ SD medium containing 1,25-(OH)$_2$D$_3$. As shown in Fig. 3A, loss of amino acids 1–116 of the VDR had no effect on the interaction or on ligand binding. These results demonstrate that the conserved DNA-binding domain is not essential for ligand binding and interaction between these proteins. Loss of amino acids 117–382 of the VDR resulted in the failure of ligand to bind the mutant VDR (Fig. 3A), and as interaction of VDR and SRC-1 takes place in the presence of ligand, these VDR deletion mutants fail to interact with SRC-1. A deletion of amino acids 419–427 of the VDR resulted in failure to interact with the SRC-1. However, this mutant could bind ligand (Fig. 3A). Deletion of amino acids 423–427 of the VDR had no effect on ligand binding and interaction with the SRC-1 (Fig. 3A).

The failure of interaction between SRC-1 and the VDR deletion mutants could be due to the loss of a binding site in the VDR deletion mutant. Alternatively, loss of interaction could also result from impaired protein synthesis in the deletion mutant or degradation of the synthesized protein. To distinguish between the loss of a binding site in deletion mutants *vs.* the failure of fusion protein synthesis, we analyzed the Gal^4-VDR deletion mutants by immunological assay. As demonstrated in Fig. 3B, analysis with anti-Gal^{4}DB monoclonal antibody revealed that all fusion proteins are expressed, and loss of interaction is due to the loss either of the binding site for SRC-1 in the deletion mutant or of the sequence that is essential for ligand binding. Thus, the lack of β-galactosidase activity is due to the failure of the two proteins to interact, which results in formation of a functional Gal-4 transcription factor, and functional Gal-4 is required for *lacZ* transcription.

These results demonstrate that the AF-2 region of the VDR is required for ligand-dependent interaction with the SRC-1. Amino acids 417–422 of the VDR represent consensus for AF-2, as shown by *bold letters* in Fig. 4B, and are conserved in the nuclear receptor. The main features of this motif are a central acidic amino acid (E) flanked by two hydrophobic amino acids (Fig. 4B).

Identification of the SRC-1 Region That Interacts with the VDR

Deletion mutation analysis was performed to identify the region of the SRC-1 that binds the VDR. We isolated 13 clones in the library screening that coded for 4 SRC-1 peptides (C1–C4; Fig. 5A). Two additional deletion mutants (C5 and C6) were constructed in which amino acids 745–809 and 745–781, respectively, were used as Gal^4 fusion proteins. These deletion mutants were cotransformed with Gal^{4}DB-VDR-(1–427) into YPB6 and analyzed for stimulation of *lacZ* transcription. Proteins coded by C1–C5 bound the VDR, as shown by stimulation of β-galactosidase assay (Fig. 5A), whereas protein coded by C6 failed to

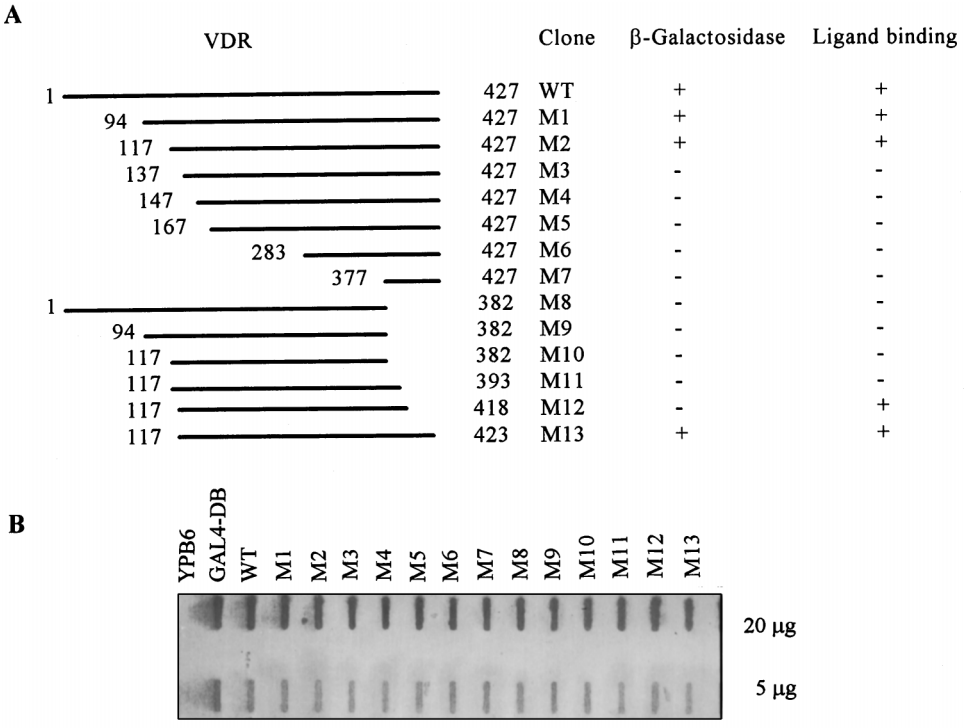

Fig. 3. Identification of the Region of the VDR Required for Interaction with SRC-1

A, Diagrammatic representation of deletion fragments of the VDR that are fused in-frame with Gal^4DB to express them as fusion protein. The VDR deletion mutant construct was cotransformed with SRC-1 into YBP6. Transformants were plated on selection medium containing 10^{-7} M 1,25-$(OH)_2D_3$ and tested for β-galactosidase activity by filter assay. Transformants were cultured in selection medium in the absence of ligand, and whole cell extracts were analyzed for ligand binding. +, The presence of β-galactosidase activity or ligand binding; −, the absence of β-galactosidase activity or ligand binding. B, Expression of VDR deletion mutants. Twenty and 5 μg of total protein were analyzed by immunological assay with Gal-DB monoclonal antibodies.

bind the VDR, as demonstrated by lack of β-galactosidase activity (Fig. 5A). These results show that amino acids 782–809 of SRC-1 (Fig. 5B) are involved in binding the VDR. These residues of SRC-1 form an amphipathic helix with charged and polar residues on one side of the helix, as shown in Fig. 5C.

In summary, six amino acids in the ligand-binding domain of the VDR, which represent AF-2, are involved in the interaction (Fig. 4). Twenty-seven amino acids of the SRC-1 are required to interact with the VDR in the presence of the ligand (Fig. 5).

DISCUSSION

The VDR, like other steroid receptors, functions as a ligand-dependent transcription factor. Enhancement of transcription initiation by sequence-specific DNA binding of these factors and their interaction with specific components of the basal transcriptional machinery are principal mechanisms for regulating transcription (27–29). Steroid receptors may interact with the basal machinery or interact through an adapter. A direct protein-protein interaction of transcription factor IIB (TFIIB) and the VDR both *in vivo* and *in vitro* as well as the role of TFIIB in ligand-dependent transcription regulation have been demonstrated (30, 31).

The present report demonstrates that human SRC-1 interacts with the VDR in a ligand-dependent manner (Fig. 2A). Similar to the VDR, SRC-1 has been shown to interact directly with the PR, ER, retinoic acid receptor (RAR), RXR, and TFIIB in the presence of ligand (25, 26). The interaction between the SRC-1 and the VDR occurs at physiological concentrations of 1,25-$(OH)_2D_3$, as demonstrated by stimulation of *lacZ* transcription (Fig. 2B). A significant stimulation of β-galactosidase activity was observed at 10^{-10} M 1,25-$(OH)_2D_3$, with maximum stimulation at 10^{-8} M. In addition, SRC-1 has been shown to stimulate transcription mediated by PR, ER, TR, RXR, and glucocorticoid receptor by their respective ligands (25).

In addition to SRC-1, several other potential factors have been demonstrated to interact with the steroid hormone receptors in the presence of ligand. These include mouse bromodomain-containing protein, known as TIF-1, Trip-1 and other TR-associated proteins, and the ER-associated proteins, p^{160}, RIP160 (receptor interacting protein-160), and RIP80 (32–35). Even though these proteins interact with steroid receptors, their roles as potential coactivator need to be

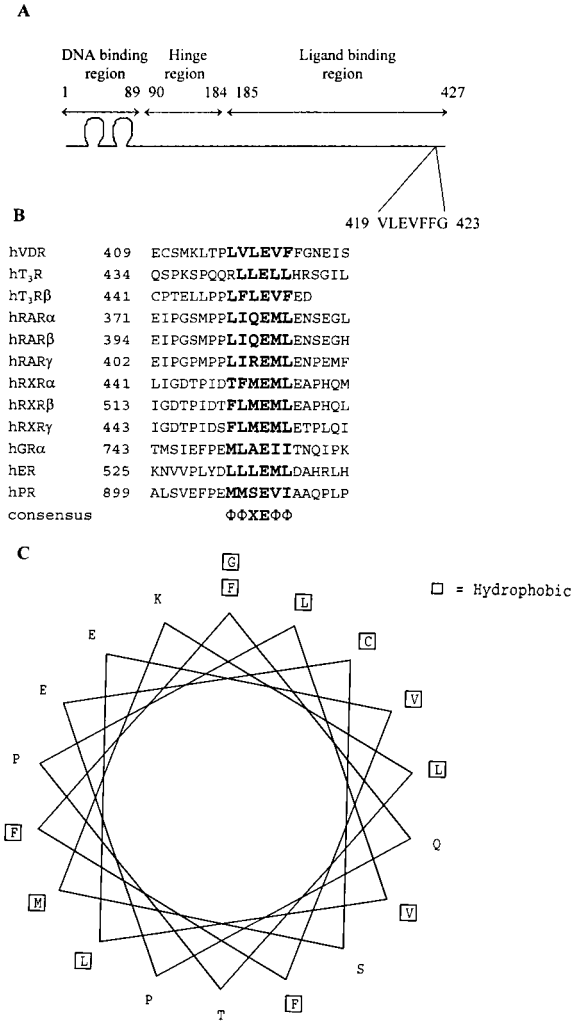

Fig. 4. Demonstration That the AF-2 Domain of the VDR Interacts with the SRC-1

A, Diagrammatic representation of the amino acids essential for interaction in the two regions. B, Alignment of the AF-2 region of various nuclear receptors. The consensus sequence is ΦΦXEΦΦ and is represented as *bold letters*. Φ represents conserved hydrophobic amino acids, X represents nonconserved amino acids, and E is conserved glutamic acid residue. Nuclear receptors with their accession numbers are human VDR (J03258), human $T_3R\alpha$ (M24899), human $T_3R\beta$ (m26747), human RARα (X06538), RARβ (X07282 or Y00291), RARγ (M57707), human RXRα (X52773), RXRβ (X63522), mouse RXRγ (M84819), human glucocorticoid receptor (X03225), human ER (M11457), and human PR (M15716). C, Helical wheel representation of amphipathic helix of the AF-2 region of the VDR showing charged and polar amino acids on one side of the helix.

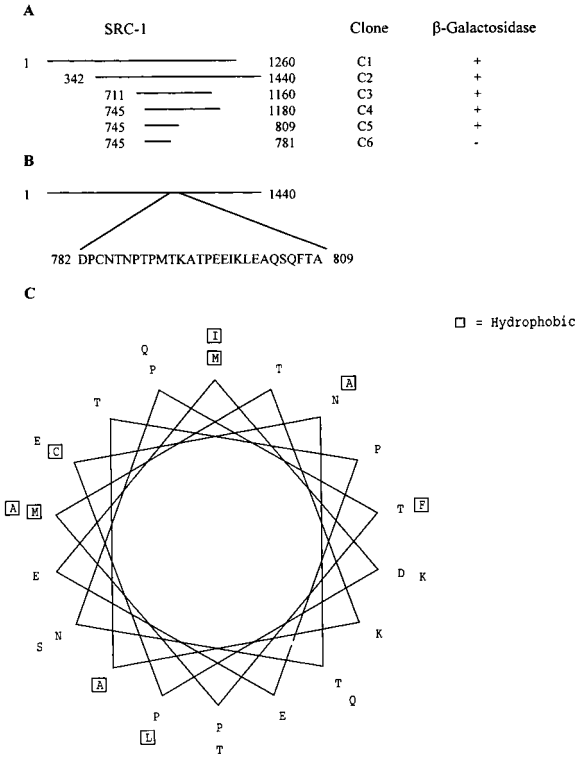

Fig. 5. Identification of the Region of the SRC-1 That Interacts with the VDR

A, Deletion mutant analysis of the SRC-1. SRC-1 deletion mutation constructs were cotransformed with Gal^4DB-VDR, and transformants were analyzed for β-galactosidase activity. B, Diagrammatic representation of SRC-1 required for interaction with the VDR. C, Helical wheel representation of amphipathic helix of SRC-1 amino acids 781–809.

demonstrated. RIP140 (36) and GRIP (glucocorticoid receptor interacting protein) (37) have recently been identified as coactivators, and N-CoR (nuclear receptor corepressor) (38, 39) as corepressor.

Our studies reveal that an AF-2 core domain plays a central role in SRC-1-mediated signaling. Deletion mutation analysis of the VDR (Fig. 3) demonstrated that the C-terminal domain of the VDR is required for interaction with the SRC-1. As SRC-1 binds to the 1,25-$(OH)_2D_3$-VDR complex, we analyzed deletion mutants for ligand binding. We found that in a yeast system, deletion of 34 amino acids at the C-terminus of the VDR (Δ393–427) resulted in failure to bind ligand, and deletion of amino acids 1–116, the DNA-binding domain, had no effect on ligand binding or on the interaction with SRC-1 (Fig. 3A). These results support previous studies in mammalian cells in which deletion of the DNA-binding domain (amino acids 1–116) had no effect on ligand binding, and removal of amino acids 383–427 resulted in failure to bind ligand (40, 41). In addition, a deletion mutant lacking amino acids 418–427 of the VDR can bind ligand. These results are in agreement with those of previous studies in which ΔVDR-(1–409) was shown to bind ligand (40, 41). The C-terminus amino acids 419–423 of the VDR are required for the interaction with SRC-1 (Figs. 3A and 4A). This region contains consensus AF-2 motif (amino acids 416–422) that is conserved among all nuclear receptors (Fig. 4B). It has been established that the integrity of AF-2 is required for ligand-dependent receptor-mediated activity (42–44). Mutagenesis

of this conserved region abrogates AF2 activity without significantly altering DNA binding, heterodimerization, and ligand binding (42–44). Interestingly, the corresponding motif is absent in v-*erb*A, which has no AF2 activity (45, 46), and a point mutation in this region has little or no effect on steroid or DNA binding (40–44). In the present study, we established that deletion of this AF-2 region abrogates the binding to SRC-1 without affecting ligand binding (Fig. 3A). The conserved AF-2 region contains amino acids with significant negative charges and forms an amphipathic α-helix. Amphipathic helixes have been implicated in the function of a variety of transcription factors (for review, see Ref. 47). Thus, deletion of amino acids encompassing this putative amphipathic helix in the VDR resulted in failure to interact with the SRC-1. These results demonstrate that the SRC-1 activates the transcription by interacting with the AF-2 activation domain of the steroid hormone receptor. Interestingly, these amino acids are found adjacent to a heptad repeat 9 that has been shown to function as a dimerization interface (37). Recent crystal structural analysis of the ligand-binding domains of TR, RXR, and RAR suggests that the AF-2 core domain, which forms an amphipathic helix also known as helix 12, undergoes striking conformational changes upon hormone binding (48–50). In the unoccupied receptor, helix 12 projects into the solvent (48). In the hormone-occupied receptor, the helix folds back toward the receptor to form part of the ligand binding cavity (49, 50). The helix is packed loosely with hydrophobic residues facing inward toward the ligand-binding pocket, and charged residues extend into the solvent (49, 50). It is possible that in this conformation, the helix presents itself for interaction with SRC-1. Recently, Henttu *et al.* (51) established that AF-2 activity and lysine 366 of the ER are essential for interaction with the SRC-1.

Both the RXR, as demonstrated previously (25, 26), and the VDR, as shown in the present study, were shown to interact with the SRC-1. As the VDR-RXR heterodimer mediates 1,25-$(OH)_2D_3$-regulated gene transcription, it is not clear whether one SRC-1 molecule binds both heterodimer partners or each partner binds to a different SRC-1 molecule. However, in studies using RXR mutants in which the AF-2 domain of RXR was deleted, a significantly reduced *trans*-activation was observed by RXR-RAR, -TR, and -VDR heterodimers in the presence of hormones specific for the RXR heterodimeric partner (52), demonstrating that the AF-2 domain of the RXR is necessary for hormone-dependent *trans*-activation. It is possible that the SRC-1 integrates the AF-2 functions of both partners of the VDR-RXR heterodimer in the same manner, as previously demonstrated for AF-1 and AF-2 functions of the ER (53).

The 27 amino acids of SRC-1 that are required for interaction with the VDR also form an amphipathic α-helix. As shown in Fig. 5C, helical wheel analysis of these amino acids of SRC-1 demonstrates that there are clusters of charged and polar amino acids on one side of the amphipathic helix of this region of SRC-1 that may be involved in interaction. These charged amino acids probably extend into the solvent from amphipathic α-helix formed by the AF-2 region of the VDR.

In summary, the present study demonstrate that the AF-2 domain of the VDR interacts with 27 amino acids of the SRC-1. Six amino acids in the ligand-binding domain of the VDR, which represent AF-2, are involved in this interaction (Fig. 4). The results provide further insight into the molecular events associated with ligand-dependent gene activation by 1,25-$(OH)_2D_3$.

MATERIALS AND METHODS

Bacterial and Yeast Strains

Yeast strains SFY526 [genotype MATa, Ura^{3-52}, His^{3-200}, Ade^{2-101}, Lys^{2-801}, Trp^{1-901}, Leu^{2-3112}, Can^r, Gal^{4-542}, Gal^{80-538}, Ura3::Gal1-*lacZ*] and DY150 [genotype MATa, Ura^{3-52}, Leu^{2-3112}, Trp^{1-1}, Ade^{2-1}, His^{3-11}, and Can^{1-100}) were purchased from Clontech (Palo Alto, CA). Yeast strain YPB6 [genotype MATa, Ura^{3-52}, His^{3-200}, Ade^{2-101}, Lys^{2-801}, Trp^{1-90}, Leu^{2-3112}, Can^R, Gal^{4-542}, Gal^{80-538}, Gal-*lacZ*::Ura^3,Gal^1-His^3(pBM1499)::Lys^2] was a gift from Dr. Paul Bartel (Department of Microbiology, State University of New York, Stony Brook, NY). The *Escherichia coli* strain of DH5α (Life Technologies, Grand Island, NY) was used for rescue of plasmids from yeast cells.

Construction of Plasmids

Gal^4DB-VDR Fusion Protein and Gal^4DB-VDR Deletion Mutant Fusion Protein Plasmids Yeast shuttle vector pGBT9 (Gal^4-DB) was a gift from Dr. Paul Bartel (Department of Microbiology, State University of New York, Stony Brook, NY). The pGBT9 was described in detail previously (54). The pGBT9 contains the DNA-binding domain of the yeast transcription factor Gal^4. A cDNA clone coding for VDR (55) was purchased from American Type Culture Collection (Rockville, MD). To express VDR as a Gal^4 fusion protein, a full-length coding region (1–427 amino acids) of the VDR was PCR amplified with primer containing *Eco*RI and *Bam*HI linkers at 5′ and 3′, respectively. After digestion with *Eco*RI and *Bam*HI, the PCR-amplified VDR cDNA was subcloned into *Eco*RI and *Bam*HI of pGBT9, and this plasmid was designated pGVDR-(1–427). The pGVDR-(1–427) was sequenced to confirm in-frame fusion of the VDR with the Gal^4-DB. Similarly, the deletion mutants of VDR were PCR amplified and cloned into pGBT9, and these plasmids were designated pGVDR followed by the amino acid position in the VDR protein.

GST-VDR Fusion Protein Plasmid To express VDR as a GST fusion protein, the cDNA insert was removed from pGVDR-(1–427) by digestion with *Eco*RI and *Sal*I restriction enzymes and ligated into *Eco*RI- and *Sal*I-digested pYEX 4T1 yeast vector. This recombinant vector was pGST-VDR. The resulting plasmid produced an in-frame fusion of GST and VDR from Met^1-Ser^{427}.

***In vitro* Transcription and Translation Vector** A clone containing 3.5 kb cDNA corresponding to the SRC-1 was subcloned into pBluescript, and the recombinant clone was digested with *Xba*I before *in vitro* transcription and translation.

Two-Hybrid Library Screening We used the two-hybrid system developed by Field and Song (56). Briefly, one hybrid is a fusion protein between the Gal^4 DNA-binding domain and the VDR, whereas the other hybrid is a fusion protein between

the activation domain of Gal4 and a second protein. *Trans*-activation of His3 and the *lacZ* reporter gene occurs only if both proteins of interest interact with each other when coexpressed in an appropriate yeast strain. The interaction can, therefore, be monitored by β-galactosidase activity and/or prototrophy for histidine. The YPB6 yeast reporter strain was used for the library screening. Yeast was grown in SD containing 2% sucrose (wt/vol) and 0.67% nitrogen base without amino acids (wt/vol; Difco, Detroit, MI) supplemented with the appropriate amino acids. To identify proteins that interact with the VDR in the presence of 1,25-$(OH)_2D_3$, the *Saccaromyces cerevisiae* YPB6 reporter strain containing pGVDR-(1–427) was transformed with a human kidney cDNA library constructed in pGAD10 vector. Approximately 2.5×10^7 transformants were plated onto Trp^-,Leu^-,His^- SD containing 30 mM 3-aminotrizole and 10^{-7} M 1,25-$(OH)_2D_3$. His^+ colonies that appeared from 4–8 days after plating were tested for β-galactosidase by the filter assay with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as previously described (57). The library plasmids (Gal4 activation domain plasmid) were rescued by growing the yeast clone in Leu^- SD medium for 40 h and then selecting for growth on Leu^- SD medium and not Leu^-,Trp^- SD medium. This plasmid was transformed into either the YPB6 or SFY526 strain alone or cotransformed with either pGVDR or unrelated plasmid (Gal4DB-lamin) to confirm interaction and to test for false positive clones.

Subcloning and Sequencing of the cDNA The partial sequence of each clone was determined by double-stranded sequence with the Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland OH) according to the manufacturer's instructions. The partial sequence of each clone was compared to determine the number of times each clone was independently isolated and to determine the reading frame of the fusion protein. The cDNA insert from the rescued plasmid was excised with restriction enzymes and subcloned into pBluescript for sequencing. A search for homology with known sequences from GenBank was carried out with the Fasta A software of Wisconsin package (Genetic Computer Group, Madison, WI).

Quantitation of β-Galactosidase Activity by Liquid Assay The liquid β-galactosidase assay was performed by following the published protocol (57). Briefly, the YPB6 cells transformed with the indicated plasmid pair were cultured into Leu^-,Trp^- SD in the absence or presence of 1,25-$(OH)_2D_3$ to midlog phase (OD_{600} = 0.3–0.7), and a 100-μl aliquot of culture was used for the liquid β-galactosidase assay. After the reaction, absorbance was measured at OD_{420}. The specific β-galactosidase activity was calculated with the following formula and expressed as Miller units (mean of at least four determinations ± SEM): β-galactosidase assay = $1000 \times [OD_{420}/(t \times V \times OD_{600})]$, where t is time of incubation in minutes, V is the volume of culture added in milliliters, OD_{600} is optical density of yeast culture at λ600, OD_{420} is absorbance at λ420.

Protein-Protein Interactions The yeast DY150 strain was transformed with pGST-VDR. The GST-VDR fusion protein was expressed according to the published protocol (58). Yeast whole cell extract was prepared in KTEDM buffer [300 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM EDTA, and 10 mM sodium molybdate] containing soybean trypsin inhibitor (10 μg/ml), leupeptin (2 μg/ml), pepstatin (2 μg/ml), and aprotinin (2 μg/ml). The GST-VDR was activated *in vivo* by the addition of 10^{-8} M 1,25-$(OH)_2D_3$ during induction of the protein. Yeast cell extracts were treated for an additional 15 min at room temperature with 10^{-6} M hormone before purification. Approximately 400 μg total protein extract were incubated with 20 μl GST-Sepharose beads in suspension for 2 h at 4 C. Resins were then washed twice with NENT buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 0.5% milk powder] and washed twice more with transcription buffer [20 mM HEPES (pH 7.9), 60 mM NaCl, 1 mM dithiothreitol, 6 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol]. Subsequently, the beads were mixed with 20 μl *in vitro* transcribed and translated [^{35}S]methionine-labeled SRC-1, and the interaction was allowed to occur for 4 C for 1 h. The bound protein was eluted with SDS sample buffer, fractionated on SDS-PAGE, and analyzed by fluorography for ^{35}S.

Ligand Binding Assay Whole cell extract of YPB6 cells expressing Gal4DB-VDR-(1–427) and various deletion mutations was prepared in KTEDM buffer as described above. The cell suspension was centrifuged at 210,000 × *g* for 35 min at 4 C. The protein concentration of the supernatant was determined by the method of Bradford (59). The ligand binding assay was performed according to a published protocol (60).

Analysis of Gal4-VDR Fusion Protein Expression

Whole cell yeast extracts of YPB6 cells and YPB6 cells transformed with the Gal4DB-VDR and Gal4DB-VDR deletion mutants were prepared in KTEDM. The proteins were applied to Immobilon-P (polyvinyldifluoridine) with a slot blotter, and then the blot was dried for 20 min at 37 C as described previously (61). The membranes were blocked in 1% BSA in Tris-buffered saline and then incubated with anti-Gal4DB monoclonal antibody (Clontech, Palo Alto, CA). The membrane was incubated with secondary antimouse antibodies conjugated to peroxidase for 1 h at room temperature, followed by development of color.

Acknowledgments

We thank Drs. Paul Bartel and Stanley Fields for yeast matchmaker plasmids and yeast strain YPB6.

Received July 25, 1997. Re-revision received and accepted October 16, 1997.

Address requests for reprints to: Rajbir K. Gill, Ph.D., Research Service/Veterans Administration Medical Center, 109 Bee Street, Charleston, South Carolina 29401-5799. E-mail: gillrk@musc.edu.

This work was supported in part by an institutional grant from the Medical University of South Carolina (to R.G.).

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