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Identification of an Autonomous Transactivation Domain in Helix H3 of the Vitamin D Receptor

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Abstract The vitamin D receptor (VDR) contains an α-helical, ligand-inducible activation function (AF-2) at the COOH-terminus of the ligand-binding domain (LBD). In this study, a second distinct activation domain was identified in the VDR LBD. Using a yeast-based system to screen a random mutant library of GAL4-VDR (93–427), a mutant GAL4-VDR fusion protein with constitutive transcriptional activity was isolated. Sequence analysis identified a C to T transition that introduced a stop codon at glutamine 239 eliminating a large portion of the LBD, including the AF-2 domain. The GAL4-VDR (93–238) mutant exhibited ligand-independent transactivation activity both in yeast and in mammalian cells. Deletion analysis defined a minimal activation domain within helix H3 between D195 and I 238 in the VDR. An aspartic acid residue (D232) within helix H3 was essential for the autonomous transactivation activity since altering this residue to an alanine or an asparagine dramatically reduced its transactivation potential. Expression of the minimal helix H3 activation domain interfered with ligand-activated transcription by full-length VDR suggesting that helix H3 interacts with limiting cellular factors important for VDR-activated transcription. Consequently, we have identified a novel activation domain in helix H3 of the VDR that apparently plays an important role in 1,25-$(OH)_2D_3$-activated transcription. J. Cell. Biochem. 75:82–92, 1999.

Key words: VDR; 1,25-$(OH)_2D_3$; helix H3; AF-2 domain; transactivation; transcription

The vitamin D receptor (VDR) is a member of the superfamily of nuclear receptors that functions as a ligand-activated transcription factor in response to 1α,25-dihydroxyvitamin D3 [1,25-$(OH)_2D_3$] [Haussler et al., 1997; Haussler et al., 1998; Kraichely and MacDonald, 1998; MacDonald et al., 1994; Strugnell and Deluca, 1997]. The binding of 1,25-$(OH)_2D_3$ to the VDR induces VDR/RXR heterodimerization and the heterodimer specifically binds vitamin D response elements (VDREs) in the promoter regions of vitamin D-responsive genes. In addition to contacting other nuclear receptors and DNA, the VDR also interacts with general transcription factors, including transcription factor IIB [Blanco et al., 1995; MacDonald et al., 1995], transcription factor IIA [Lemon et al., 1997], and several TATA binding protein-associated factors [May et al., 1996; Mengus et al., 1997]. VDR also interacts with comodulator proteins including several coactivator and corepressor proteins [Baudino et al., 1998; Hong et al., 1996; Masuyama et al., 1997a; Li et al., 1997; Schulman et al., 1996]. Many of the coactivators described thus far selectively interact in a ligand-dependent manner with the AF-2 transactivation domain of nuclear receptors [Henttu et al., 1997; Hong et al., 1997; Jeyakumar et al., 1997; Masuyama et al., 1997a], clearly implicating the AF-2 domain as a central protein-protein interface mediating ligand-activated transcription by the nuclear receptors.

Abbreviations used: AF-1, activation function 1; AF-2, activation function 2; AF-2a, activation function 2a; AF-3, activation function 3; ER, estrogen receptor; GRIP 1, glucocorticoid receptor interacting protein 1; LBD, ligand binding domain; NCoA-62, nuclear receptor coactivator, 62,000 Da; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PCR, polymerase chain reaction; PR, progesterone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator-1; TR, thyroid hormone receptor; TRAM-1, thyroid receptor activator molecule-1; VDR, vitamin D receptor; VDRE, vitamin D response element; WT, Wild-type.

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Previous studies have demonstrated that the AF-2 domain of the VDR is required for 1,25-$(OH)_2D_3$-dependent transactivation and for co-activator interaction [Jurutka et al., 1997; Masuyama et al., 1997a]. While the VDR expresses classical AF-2 activity, there is no strong evidence for the existence of additional, distinct activation domains in the VDR as has been described for other nuclear receptors. These additional activation functions include the AF-1 [Giguere et al., 1986; Hadzic et al., 1995; Kumar et al., 1987; Nagpal et al., 1993; Pham et al., 1992] AF-3 [Sartorius et al., 1994], and AF-2a domains [Pierrat et al., 1994; Norris et al., 1997]. Both the AF-1 and the AF-2 domains of the nuclear receptors express autonomous trans-activation activity when assayed outside of the context of the receptor [Berry et al., 1990; Masuyama et al., 1997a; Tora et al., 1989]. However, in the context of the full-length receptor, the activity of the AF-1 domain appears to be considerably weaker and is ligand-independent, while the stronger activity of the AF-2 domain is absolutely dependent upon hormone [Lees et al., 1989; Nagpal et al., 1993; Pham et al., 1992; Tora et al., 1989]. Recent evidence has suggested that the AF-1 and AF-2 domains may, in fact, function together to synergistically increase the transcriptional activity of nuclear receptors that possess both domains [Webb et al., 1998]. Moreover, the ligand-independent activity of the AF-1 domain of nuclear receptors may be due, at least in part, to the binding of p160 coactivator proteins to this amino-terminal domain [Webb et al., 1998]. Since the VDR lacks a classical NH_2-terminal AF-1 activity, it is possible that other activation domains exist in the VDR that serve to complement the activity of the VDR AF-2 domain to elicit full 1,25-$(OH)_2D_3$-activated transcription.

In this paper, we report the isolation of a mutant GAL4-VDR fusion protein that exhibits potent autonomous transactivation activity in yeast and in mammalian cells. Additional studies in mammalian cells determined that this ligand-independent transactivation activity is contained in a minimal region of the VDR between residues D195 and I 238 that includes the NH_2-terminal part of helix H3. This minimal domain squelched transactivation mediated by full-length VDR suggesting a functional role for the helix H3 transactivation domain in VDR-mediated transcription.

MATERIALS AND METHODS

Generation of a Random Mutant VDR Library

The generation of random point mutations within a defined region of the human VDR cDNA was accomplished by error-prone polymerase chain reaction (PCR) amplification [Leung et al., 1989; Uppaluri and Towle, 1995] of a segment between residues 93–427. The reaction mixture contained 1 ng of linear pSG5 GAL4-VDR (93–427, 16 mM $(NH)_4SO_4$, 67 mM Tris-HCl (pH 8.8), 6.1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 6.7 μM EDTA, 10 mM β-mercaptoethanol, 1 μM each dGTP, dTTP, and dCTP, 0.2 μM dATP, 1 μM of each primer and 5 units of Taq DNA polymerase (FisherBiotech, Pittsburgh, PA). The sequences of the primers used were previously described [MacDonald et al., 1995]. The amplified product was digested with EcoRI and BamHI then inserted into the pAS1 GAL4 DBD fusion plasmid [Durfee et al., 1993]. The ligation products were transformed into MAX Efficiency DH5α Competent Cells (GIBCO BRL, Gaithersburg, MD). The combined colonies from the entire transformation were then pooled and amplified to generate a random mutant library of GAL4-VDR (~20,000 clones).

Yeast Two-Hybrid Expression Vectors, Transformation, and β-Galactosidase Assays

All two-hybrid plasmid constructs used the pAS1 [Durfee et al., 1993] and pAD-GAL4 (Stratagene, La Jolla, CA) yeast expression vectors. The pAS1-VDR (93–427) construct, containing the GAL4 DNA-binding domain (amino acids 1–147) and the carboxyl-terminal region of VDR (amino acids 93–427), was previously described [MacDonald et al., 1995]. The pAD-mSRC-1 (1169–1465; steroid receptor coactivator-1) construct was previously isolated as a ligand-dependent VDR interacting protein [Masuyama et al., 1997a]. Competent yeast were transformed with 10 μg of the random mutant library of GAL4-VDR (93–427) and 10 μg pAD-mSRC-1 (1169–1465). The transformations were plated on solid media deficient in leucine, tryptophan, and histidine [SC (-leu, -trp, -his)] and containing 5 mM 3-amino-1,2,4-triazole (an inhibitor of histidine biosynthesis). Histidine-positive colonies were assayed for *LacZ* expression in liquid cultures [Fagan et al., 1994; Masuyama et al., 1997a].

Oligonucleotide-Directed Mutagenesis

Single-stranded DNA for the mammalian expression vector pSG5 GAL4-VDR was isolated using phage M13K07 in LB media containing 100 μg/mL ampicillin and 50 μg/mL kanamycin. Mutations were introduced into the VDR using the GeneEditor in vitro oligonucleotide-directed mutagenesis system (Promega, Madison, WI). All mutations were confirmed by DNA sequencing.

Plasmids and Transient Transfection Studies

The $(VDRE)^4$-TATA-GH reporter plasmid contains four copies of the rat osteocalcin VDRE adjacent to the rat osteocalcin promoter (-40 to +32) driving a human GH reporter sequence [Masuyama et al., 1997b]. The $(GAL4)^5$-TATA-GH plasmid contains five copies of the GAL4-responsive element, the Elb TATA element, and the human GH reporter sequence. The pSG5 GAL4 construct was previously described [Masuyama et al., 1997a]. pSG5 GAL4-VDR (93–427) was constructed by fusing the LBD of VDR (residues 93–427) to the GAL4 DBD (residues 1–147). The pSG5-VDR (4–427) expression plasmid was described previously [Hsieh et al., 1991; MacDonald et al., 1993].

COS-7 cells were transfected by standard calcium phosphate precipitation procedures as previously described [MacDonald et al., 1993]. In all transfections, the amount of total DNA was kept constant at 15 μg by adding pBluescript II KS^+ (Stratagene) as a carrier plasmid. Transfected cells were treated with 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle for 24 h and an immunoassay was used to determine the amount of secreted GH (Nichols Institute, San Juan Capistrano, CA).

RESULTS

Identification of a GAL4-VDR Fusion Protein With Constitutive Transcriptional Activity

A random mutant library of GAL4-VDR (93–427) was created by error-prone PCR amplification of the DNA segment that encodes the VDR LBD. A yeast-based, GAL4-responsive reporter gene system was used to screen a GAL4-VDR mutant library to identify mutant VDRs that interact with the SRC-1 coactivator in the absence of ligand. Colonies which grew out of the histidine selection indicated either a ligand-independent interaction of VDR with SRC-1 or that the mutant VDR clones possessed autonomous transactivation activity. The clones were colony purified, the VDR plasmids were rescued from the yeast, and then amplified in DH5α cells as previously described. Each mutant plasmid was re-introduced into the Hf7c strain of yeast along with either pAD-mSRC-1 (1169–1465) or pAD-GAL4 (the GAL4 activation domain parent plasmid) to retest for growth on histidine-deficient media and to determine the β-galactosidase activity in the absence of ligand. The VDR mutant described here (mutant 5.1) strongly expressed both the *His3* and *LacZ* reporter genes in a ligand-independent manner with either the pAD-mSRC-1 (1169–1465) or the parent AD-GAL4 plasmid (Fig. 1A). These data indicated that VDR mutant 5.1 was functioning as a constitutive transcriptional activator in yeast.

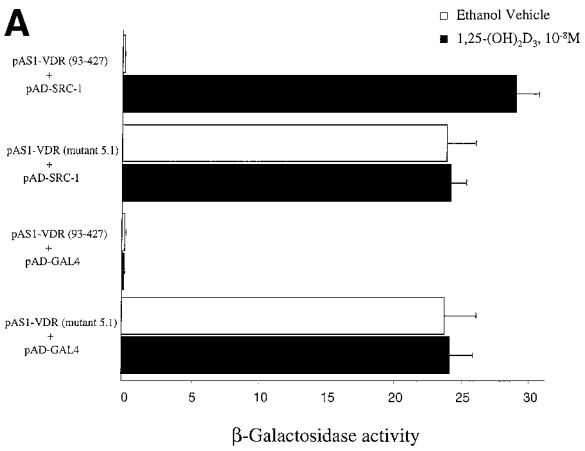

Fig. 1. Autonomous transactivation by a deletion mutant of hVDR in yeast. **A**: A GAL4-VDR mutant expressing autonomous transcriptional activity. Yeast expressing the AS1-VDR (93–427) or AS1-VDR (mutant 5.1) and pAD-SRC-1 or pAD-GAL4 were grown for 16 h at 30°C in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$. Induction of *LacZ* expression from a GAL4-responsive *LacZ* reporter gene was determined by assaying for β-galactosidase activity. Results are presented as the mean ± SD of triplicate independent cultures. **B**: DNA sequence and amino acid sequence of Wild-type hVDR and mutant 5.1. Sequence analysis identified a C to T transition that introduced a TAA stop codon at Q239 in the AS1-VDR (mutant 5.1) sequence.

Western blot analysis using the 9A7 antibody against VDR revealed that GAL4-VDR (mutant 5.1) was truncated (34-kDa) compared to the WT (Wild-type) GAL4-VDR (93–427) (55-kDa) (data not shown). Direct DNA sequencing of the mutant 5.1 receptor expression plasmid identified a C to T transition which produced a TAA termination codon at glutamine 239 (Fig. 1B). Thus, mutant 5.1 was designated GAL4-VDR (93–238). From this screening strategy, the GAL4-VDR (93–238) mutant was isolated seven times as well as a number of other clones that will be described elsewhere (Kraichely DM, Nakai YD, MacDonald PN, unpublished data).

Autonomous Transcriptional Activity by GAL4-VDR (93–238) in Mammalian Cells

The ligand-independent transcriptional activity by GAL4-VDR (93–238) observed in yeast was also observed in mammalian COS-7 cells using a $(GAL4)^5$-TATA-GH reporter gene construct. Expressing the full-length, Wild-type VDR LBD fused to the GAL4 DBD [pSG5 GAL4-VDR (93–427)], demonstrated no transcriptional activity in the absence of hormone and a dramatic increase in reporter gene expression when COS-7 cells were treated with 10^{-8} M 1,25-$(OH)_2D_3$. COS-7 cells transfected with the GAL4-VDR (93–238) mutant exhibited a potent ligand-independent increase in GH reporter expression compared to unliganded Wild-type VDR LBD. In fact, the level of reporter gene expression by GAL4-VDR (93–238) in the absence of 1,25-$(OH)_2D_3$ was nearly 70% of the activity of the liganded Wild-type GAL4-VDR (93–427; Fig. 2A).

Introducing this stop codon into the native hVDR cDNA [pSG5 hVDR (4–238)] produced a truncated receptor that activated the expression of a $(VDRE)^4$-TATA-GH reporter gene construct in the absence of 1,25-$(OH)_2D_3$ (Fig. 2B). A similar constitutively active mutant of VDR [Δ190–427 or VDR (4–189)] was previously reported [McDonnell et al., 1989]. Importantly, hVDR (4–238) did not affect the expression of a reporter gene vector driven by estrogen response elements $[(ERE)^2$-TATA-GH] or a vector that lacks response elements (TATA-GH) indicating that this was a VDRE-mediated transcriptional effect. The activity of the pSG5 hVDR (4–238) is weak compared to the liganded full-length VDR (four-fold vs. 20–40 fold activation, respectively). This is likely the result of weak monomer binding by VDR (4–238) to the VDRE since this deletion construct lacks the ability to heterodimerize with RXR (data not shown). Regardless, the Q239 STOP truncation confers constitutive transactivation to the VDR in the context of a GAL4-VDR fusion and in the context of native VDR on a natural VDRE reporter gene construct.

Delineation of a Minimal Autonomous Transactivation Domain in the hVDR LBD

To identify a minimal autonomous transactivation domain within GAL4-VDR, a series of

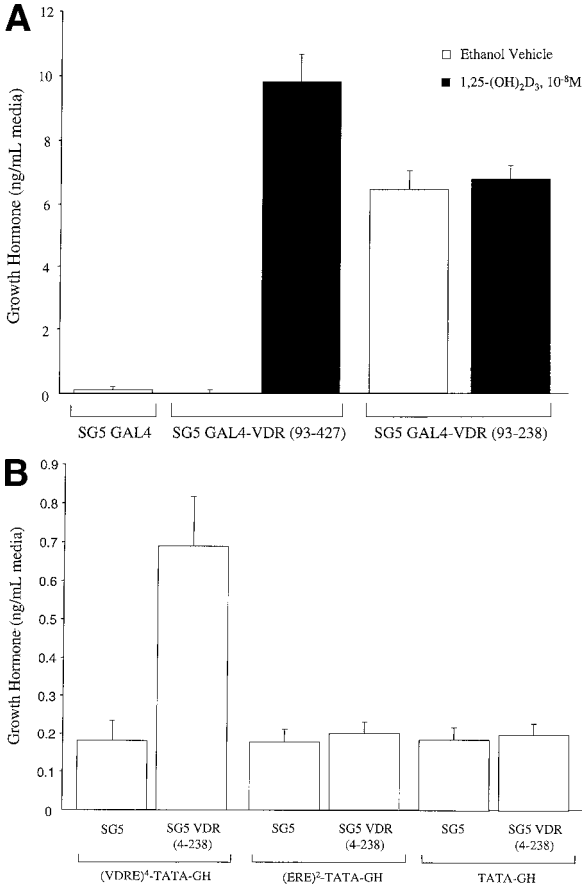

Fig. 2. Ligand-independent transactivation mediated by GAL4-VDR (93–238) in mammalian cells. **A**: GAL4-VDR (93–238) exhibits strong, ligand-independent transactivation of a GAL4-responsive GH reporter construct in mammalian cells. COS-7 cells were transfected with 5 μg of a reporter construct $(GAL4)^5$-TATA-GH and 0.5 μg of pSG5 GAL4, pSG5 GAL4 VDR (93–427), or pSG5 GAL4 VDR (93–238). Cells were treated in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$ for 24 h and GH secretion was determined. **B**: Constitutive transactivation of a vitamin D-responsive reporter gene construct by VDR (4–238). COS-7 cells were transfected with 3 μg of the $(VDRE)^4$-TATA-GH or the (TATA-GH) reporter constructs and 1 μg of pSG5 or pSG5 VDR (4–238). All constructs were assayed in the absence of 1,25-$(OH)_2D_3$. GH secreted into the media was quantitated 24 h post-transfection.

carboxyl-terminal truncations and amino-terminal deletions were generated. Premature termination codons were introduced into the VDR in the context of the mammalian expression vector pSG5 GAL4-VDR (93–427) at amino acids Ser 199 and Glu 220 by oligonucleotide-directed mutagenesis. The plasmid encoding pSG5 GAL4-VDR (93–198), pSG5 GAL4-VDR (93–219), or pSG5 GAL4-VDR (93–238) were individually transfected into COS-7 cells together with the $(GAL4)^5$-TATA-GH reporter gene construct. As illustrated in Figure 3 the GAL4-VDR (93–198) and GAL4-VDR (93–219) deletions were inactive compared to the GAL4-VDR (93–238) construct suggesting that key residues for the transactivation activity reside between residues 220 and 238. Several NH_2-terminal deletions of the pSG5 GAL4-VDR (93–238) expression vector were also examined. These deletions were produced as in-frame GAL4-DBD fusions using unique restriction endonuclease sites. Transient transfection provided additional evidence that a transactivation domain was located between amino acid residues 195 and 238. This conclusion is supported by the finding that GAL4-VDR (195–238) showed a strong ligand-independent increase (greater than 27-fold) in transactivation activity compared to the GAL4 control. Paradoxically, two other NH_2-terminal deletions [GAL4-VDR (116–238) and GAL4-VDR (166–238)] showed only minimal transactivation activity on the $(GAL4)^5$-TATA-GH reporter construct (data not shown) suggesting that the secondary structure of these fusions may be altered, thus disrupting the activity of the minimal domain. Western immunoblot analysis revealed that the expression levels were similar for all the mutants examined (data not shown). From the combined transfection data for both the COOH-terminal truncations and the NH_2-terminal VDR deletion mutants, the minimal domain which is sufficient for autonomous transactivation resides in the ligand-binding domain of the vitamin D receptor in a region between amino acid residues D195 and I 238 (Fig 3).

A more refined mapping of this minimal domain further indicated that residues between D232 and I 238 were important for the autonomous transactivation activity (Fig. 4). Several additional COOH-terminal truncations were generated by introducing stop codons at amino acids Leu 233, Tyr 236, and Lys 240 in pSG5 GAL4-VDR (93–427). As illustrated in Figure 4, each additional GAL4-VDR LBD truncation mutant tested showed some level of autonomous transactivation activity (two- to six-fold) when compared with Wild-type GAL4-VDR (93–427) in the absence of hormone. However, a dra-

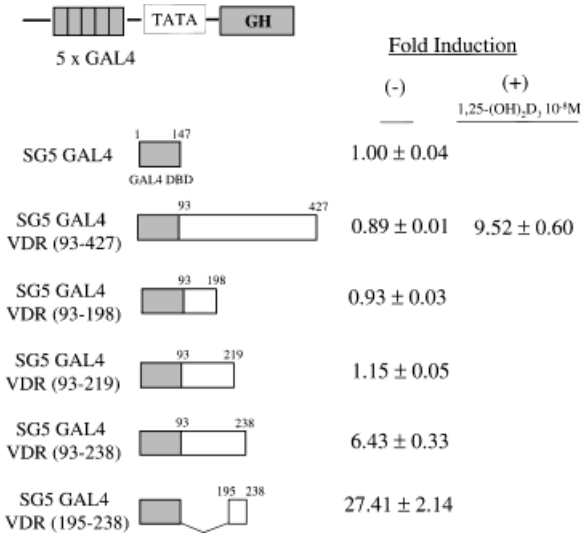

Fig. 3. A minimal autonomous transactivation domain resides between amino acids D195 and I 238 in the hVDR. Carboxyl-terminal and amino-terminal deletions in GAL4-VDR (93–427) were tested in COS-7 cells. Each transfection included 5 µg of $(GAL4)^5$-TATA-GH and 0.5 µg of the GAL4 VDR derivative. GH secretion was quantitated after 24 h.

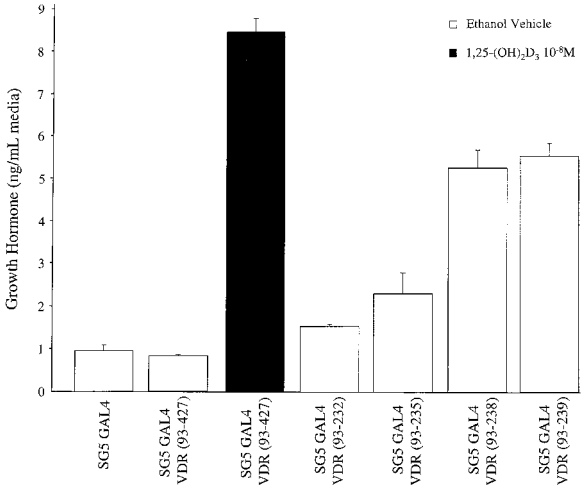

Fig. 4. Refined mapping of the activation domain. Termination codons were generated in pSG5 GAL4-VDR (93–427) vector by oligonucelotide-directed mutagenesis. COS-7 cells were transfected with 5 µg of a reporter construct $(GAL4)^5$-TATA-GH and 0.5 µg of pSG5 GAL4, pSG5 GAL4-VDR (93–427), (93–232), (93–235), (93–238), or (93–239). The cells were treated in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$ as indicated for 24 h and GH secretion was determined by an immunoassay.

matic decrease in activity was observed with the 93–235 and the 93–232 constructs suggesting that important residues for this activity reside between L233 and I 238.

The Minimal Autonomous Transactivation Domain GAL4-VDR (195–238) Contains the NH_2-Terminal Part of Helix H3

Interestingly, the minimal transactivation domain (D195-I 238) delineated above comprises a major portion of α-helix H3 (a.a. 224–246) in the VDR LBD. A helical wheel plot of helix H3 demonstrates the amphipathic nature of this helix with the distribution of hydrophobic and hydrophilic residues on opposite faces of the helix (Fig. 5).

To identify specific residues in this helix that participate in the transactivation activity of the minimal domain, we introduced point mutations into the minimal transactivation domain GAL4-VDR (195–238) and examined the ability of these mutants (S225A, H229A, D232A, D232N, S235A, and Y236A) to activate transcription of the $(GAL4)^5$-TATA-GH reporter gene in COS-7 cells. Mutations generated in the minimal transactivation domain were compared to the Wild-type minimal domain of GAL4-VDR (195–238) to identify those residue(s) which abrogated or attenuated the constitutive transcriptional activity (Fig. 6). While the mutations of Ser 225 and Ser 235 modestly enhanced the autonomous transactivation activity of the minimal domain, the mutations of His 229 and Tyr 236 produced significant decreases. Strikingly, mutation of Asp 232 (D232A and D232N) ablated this constitutive transcriptional activity. These findings indicate that these helix H3 residues, in particular D232, play an important role in the transactivation potential of this minimal domain.

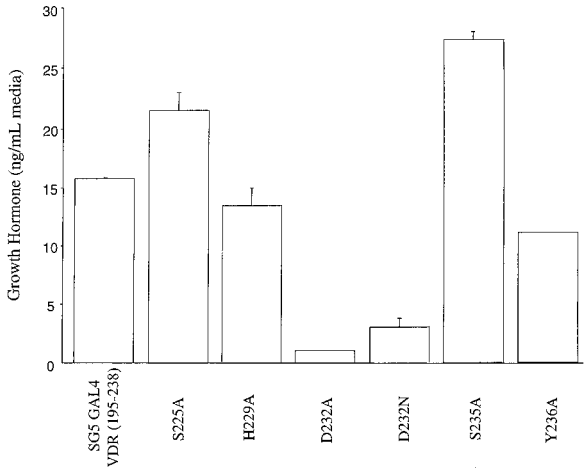

Fig. 6. Delineation of specific residues in helix H3 that are important for autonomous transactivation. A series of point mutations were generated in the minimal transactivation domain of GAL4-VDR (195–238) and these mutants were tested in a GAL4-responsive reporter gene assay in mammalian cells. COS-7 cells were transfected with 5 μg of a $(GAL4)^5$-TATA-GH reporter construct and 0.5 μg of pSG5 GAL4-VDR (195–238) Wild-type or S225A, H229A, D232A, D232N, S235A, or Y236A mutants. All constructs were assayed in the absence of 1,25-$(OH)_2D_3$. GH secreted into the media was quantitated 24 h post-transfection.

The functional significance of this transactivation domain is supported by findings in which the minimal activation domain GAL4-VDR (195–238) squelched ligand-activated transcription mediated by full-length VDR. As illus-

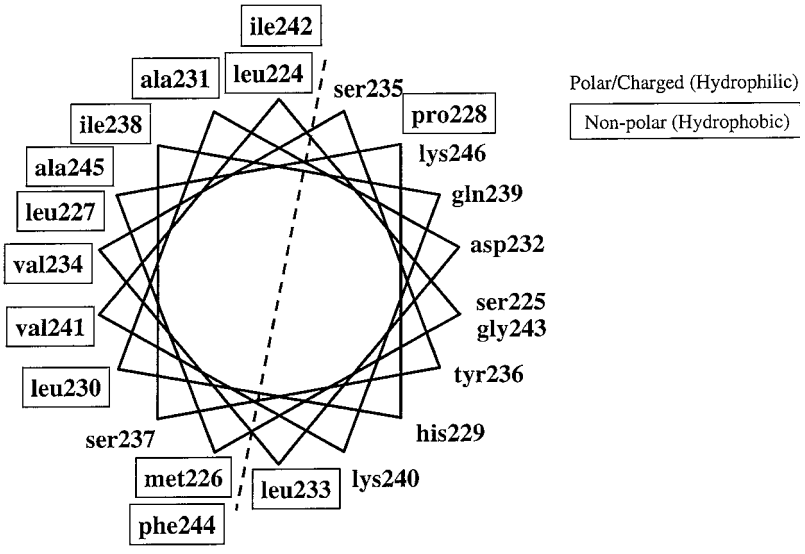

Fig. 5. A helical wheel plot of amphipathic α-helix H3 in the hVDR LBD. A computer-generated plot demonstrates the amphipathic nature of helix H3 in the ligand-binding domain of the human vitamin D receptor. Note that the non-polar residues (*boxed*) and the polar/charged residues reside on opposite faces of the helix.

trated in Figure 7, cotransfection of the minimal domain construct [pSG5 GAL4-VDR (195–238)] and pSG5 hVDR (4–427) into COS-7 cells resulted in a significant (nearly 35%) reduction in the 1,25-$(OH)_2D_3$-activated transcription of a $(VDRE)^4$-TATA-GH reporter construct. Similarly, under these conditions the liganded GAL4-VDR (93–427) inhibited VDR-activated transcription by approximately 55%. Thus, pSG5 GAL4-VDR (195–238) exhibited a modestly weaker, but significant dominant negative effect on VDR-activated transcription. Importantly, the inactive GAL4-VDR (195–238) D232A mutation did not interfere with ligand-activated transcription. The expression of these GAL4 constructs had no effect on basal transcription of the receptor (open bars in Fig. 7) or on the transcription of a (TATA-GH) reporter construct (data not shown). For the GAL4-VDR (195–238) construct, the observed squelch is not due to a simple titration of RXR away from the Wild-type VDR since no interaction was detected between GAL4-VDR (195–238) and GST-RXRα in an in vitro interaction assay (data not shown). These results suggest that VDR (195–238) contains a domain involved in binding limiting cofactors that are important for activated transcription (coactivator proteins).

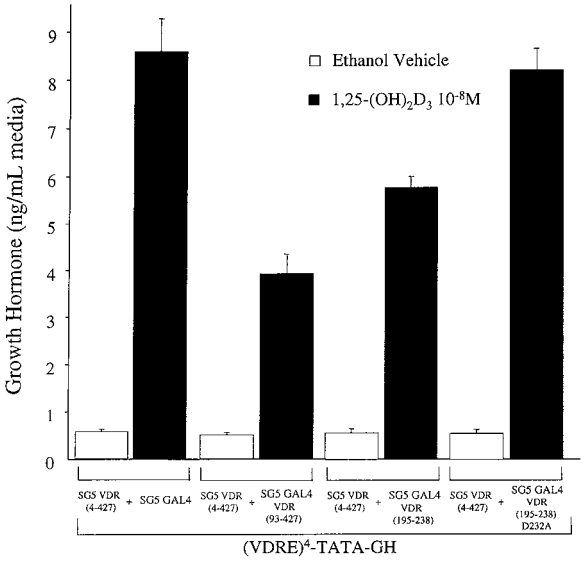

Fig. 7. Expression of the minimal transactivation domain interferes with activated transcription by full-length VDR. COS-7 cells were transfected with 3 μg of $(VDRE)^4$-TATA-GH and 100 ng pSG5 VDR (4–427). In addition, each experimental group was transfected with either 1 μg pSG5 GAL4, 1 μg pSG5 GAL4-VDR (93–427), 1 μg pSG5 GAL4-VDR (195–238), or 1 μg pSG5 GAL4-VDR (195–238) D232A. The cells were treated in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$ for 24 h and GH secretion was determined.

DISCUSSION

Ligand-induced activation of transcription by the nuclear receptors is a complex process that requires multiple, discrete transactivation domains within the receptor. These domains are known to serve as protein-protein interaction sites for a variety of important transcription factors. The AF-2 α-helix in the COOH-terminus of the receptors is of central importance in this mechanism serving as a ligand-dependent interaction domain for the class of nuclear receptor coactivator proteins that include SRC-1 and glucocorticoid receptor interacting protein 1 (GRIP 1) [Onate et al., 1995; Hong et al., 1997]. In addition, a ligand-independent activation function 1 (AF-1) resides in the NH_2-terminal, A/B domain of the receptors and this domain is necessary for full-transcriptional activity of the nuclear receptors [Giguere et al., 1986; Gronenmeyer et al., 1987; Hadzic et al., 1995; Kumar et al., 1987]. Both the AF-1 [Hollenberg and Evans, 1988; Thompson and Evans, 1989; Webster et al., 1988] and AF-2 [Masuyama et al., 1997a] domains function autonomously when the minimal domains are fused to a heterologous DNA-binding domain such as GAL4 (1–147). The VDR expresses AF-2 activity that is required for 1,25-$(OH)_2D_3$-dependent transactivation and coactivator interaction [Jurutka et al., 1997; Masuyama et al., 1997a]. However, the VDR lacks an AF-1 transactivation function in the NH_2-terminus. In fact, the A/B domain of the VDR consists of only 20 amino acids and the deletion of these residues has no impact on VDR-mediated transcription [Sone et al., 1991]. The lack of a classical AF-1 activity in the VDR suggests the possibility that other activation domains exist within the VDR that might participate in 1,25-$(OH)_2D_3$-dependent transcription. Recently, a unique activation function 2a (AF-2a) domain was identified in the mid-region of the ER LBD [Norris et al., 1997; Pierrat et al., 1994]. The identification of other autonomous transactivation domains in the VDR has not been reported. In the present manuscript, we have identified one such domain that is distinct from AF-1, AF-2, and AF-2a activities and that is located in the central portion of the VDR LBD, specifically in helix H3.

The classification of helix H3 as a transactivation domain is based on the following data. First, a VDR mutant was identified in a yeast-based genetic screen that introduced a TAA stop codon at Q239 in the hVDR cDNA. This truncated GAL4-VDR fusion protein lacks the AF-2 domain and a large portion of the LBD yet it expressed strong, ligand-independent transactivation potential both in yeast and in mammalian cells. Additional experiments in mammalian cells delineated a minimal domain between residues D195 and I 238, which contains the NH_2-terminal part of helix H3. Selected residues within helix H3 were important for this activity since D232A or D232N mutations ablated the transactivation potential and the H229A and Y236A mutations reduced the transcriptional activity. Thus, helix H3 of the VDR, when fused to a heterologous DNA binding domain such as GAL4 (1–147) is sufficient to activate transcription a GAL4-reponsive reporter gene construct in yeast and in mammalian cells. Moreover, this domain is also sufficient to mediate transactivation of a VDRE-driven reporter gene construct when tethered to the VDR DNA-binding domain as observed with the VDR (4–238) construct. However, the transcriptional activity of the pSG5-VDR (4–238) is considerably weaker than that observed for Wild-type VDR. This is most likely due to weak monomer binding of VDR (4–238) to the VDRE, since this mutant does not heterodimerize with RXR and it does not bind ligand (data not shown), both of which are essential for high affinity interaction of VDR with VDREs [Cheskis and Freedman, 1994; Kliewer et al., 1992; MacDonald et al., 1993; Sone et al., 1991].

In addition to the amphipathic α-helical nature of the helix H3 (Fig. 5), an interesting feature of the minimal activation domain identified in this study, GAL4-VDR (195–238), is the relative abundance of acidic residues (10 out of 44 residues or 23%). This suggests the possibility that the helix H3 domain may fall into the class of acidic activation domains reminiscent of GAL4 and VP16 activators. However, most of this charge distribution is clustered in the 20 most NH_2-terminal residues (195–213) and rather dramatic losses in activity occur when mutating the 20 most COOH-terminal residues in this region. For example, the deletion of six uncharged residues at the COOH-terminus of the minimal domain results in a loss of transactivation activity [compare GAL4-VDR (93–238) to GAL4 (93–232) in Fig. 4]. With all 10 acidic residues intact, the GAL4-VDR (93–232) construct is essentially inactive. Thus, the acidic nature of this particular domain probably cannot account for its observed activity.

The functional significance of the helix H3 transactivation domain is supported by the observation that the minimal transactivation domain interfered with, or squelched, 1,25-$(OH)_2D_3$-activated transcription by the intact VDR when co-expressed in COS-7 cells (Fig. 7). The observed squelch was not a general effect since basal transcription was unaffected and the inactive D232A mutation had no ability to squelch in this system. These data indicate that the minimal domain containing helix H3 may compete with the full-length, liganded receptor for common transcription factors (i.e., coactivator proteins) and the D232A mutation of the minimal domain disrupts that interaction. Interestingly, expression of the minimal domain interfered with the full-length receptor to a maximal extent of only 35% inhibition, while the liganded GAL4-VDR (93–427) inhibited VDR activated-transcription by approximately 55%. This weaker dominant negative effect of GAL4-VDR (195–238) is likely due to the lack of other important domains and protein interaction surfaces (i.e., the AF-2 domain) in this construct. Thus, while helix H3 is transcriptionally active on its own, it is not able to fully compete with the intact VDR in the transactivation process. This suggests a more efficient interaction of these transcription factor(s) to liganded, intact VDR compared to the minimal helix H3 domain. Consequently, other regions of the VDR may be required for interaction and interference in this assay (see discussion below). Regardless, these findings suggest that helix H3 is a critical region of VDR for ligand-activated transcription and may be essential for interaction with cofactors that are required for activated transcription.

The importance of the helix H3 region of the VDR and other nuclear receptors in ligand-activated transcription is supported in previous studies. Whitfield et al. demonstrated that K246 at the COOH-terminus of helix H3 is a critical residue for transactivation [Whitfield et al., 1995b]. Mutation of this lysine residue to a glycine (K246G) did not affect 1,25-$(OH)_2D_3$ binding, RXR heterodimerization, VDRE interaction, or nuclear localization, but the mutant was transcriptionally silent. Similarly, the con-

served lysine residue K366 in the mouse estrogen receptor (ER) [corresponding to K246 within helix H3 in the hVDR] has been shown to be important for estradiol-dependent transcription and ER-coactivator interaction [Henttu et al., 1997]. This conserved lysine is not present in the VDR deletion mutant characterized here, thus highlighting the concept that multiple residues in this crucial domain are involved in the transcriptional response. This is supported by the studies of O'Donnell and Koenig and their identification of three point mutants within helix H3 in the thyroid hormone receptor ($TR\beta_1$) that reduced transcriptional activity without altering binding of T_3 or interaction with T_3 response elements [O'Donnell and Koenig, 1990]. Thus, these data support the concept that helix H3 is a key domain for modulating ligand-activated transcription by nuclear receptors.

The precise role of helix H3 of the VDR in ligand-activated transcription remains to be elucidated. It is possible that helix H3 of the VDR forms a distinct transactivation surface that functions independent of the AF-2 domain. In this scenario, one might expect that helix H3 would contact coactivator proteins that are distinct from those of the SRC-1/GRIP 1 family which function via the AF-2 domain. Recent findings by Takeshita and Chin suggest that thyroid receptor activator molecule-1 (TRAM-1) may preferentially bind to helix H3 in the LBD of TR [Takeshita et al., 1997]. NCoA-62 is a nuclear receptor coactivator that augments VDR-mediated transcription and its interaction with the LBD of VDR is also independent of the AF-2 domain [Baudino et al., 1998]. Thus, helix H3 may play a role in mediating interactions between nuclear receptors and AF-2-independent coactivators such as TRAM-1 and NCoA-62.

Alternatively, it is possible that helix H3 and helix H12 (the AF-2 domain) function in concert to form a common transactivation surface that requires both helices for efficient coactivator interaction and transactivation. Recent data from our laboratory clearly indicate that this helix H3 transactivation domain is critical for ligand-activated transcription in the full-length VDR [Kraichely et al., J. Biol. Chem., in press]. Mutations within helix H3 disrupt both transactivation by VDR and abolish p160 coactivator interaction with VDR. Thus, mutations in two separate distinct helices (i.e., helices H12 and H3) disrupt 1,25-$(OH)_2D_3$-induced interaction with p160 coactivators. These data support the formation of a common p160 interaction surface on VDR comprised, in part, of helices H12 (the AF-2 domain) and H3. A similar p160 coactivator binding motif was recently described on the thyroid hormone receptor [Feng et al. 1998].

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