The Autonomous Transactivation Domain in Helix H3 of the Vitamin D Receptor Is Required For Transactivation and Coactivator Interaction*

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A ligand-inducible transactivation function (AF-2) exists in the extreme carboxyl terminus of the vitamin D receptor (VDR) that is essential for 1α,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃)-activated transcription and p160 coactivator interaction. Crystallographic data of related nuclear receptors suggest that binding of 1,25-(OH)₂D₃ by VDR induces conformational changes in the ligand-binding domain (LBD), the most striking of which is a packing of the AF-2 helix onto the LBD adjacent to helices H3 and H4. In this study, a panel of VDR helix H3 mutants was generated, and residues in helix H3 that are important for ligand-activated transcription by the full-length VDR were identified. In particular, one mutant (VDR (Y236A)) exhibited normal ligand binding and heterodimerization with the retinoid X receptor (RXR), VDR-RXR binding to vitamin D-responsive nuclear receptor coactivators.

The vitamin D receptor is a ligand-activated transcription factor that mediates the biological effects of 1α,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃). The mechanism involves a 1,25-(OH)₂D₃-induced heterodimerization of VDR with the retinoid X receptor (RXR). VDR-RXR binding to vitamin D-responsive elements (VDREs) in the promoter regions of specific genes, and the subsequent impact of the VDR-RXR heterodimer on transcriptional control of vitamin D-responsive genes (1, 2).

Discrete domains of the VDR that are important to this mechanism are the 90-residue zinc finger DNA-binding module in the NH₂-terminal portion of the VDR, the large COOH-terminal ligand-binding domain (LBD), an RXR heterodimerization interface located within the COOH-terminal portion of the LBD, and the activation function 2 (AF-2) domain. The AF-2 domain is a small α-helical region located at the extreme COOH terminus of the VDR. The precise role of the AF-2 domain in VDR-activated transcription is not fully understood.

Recent studies show that it plays a central role in mediating ligand-dependent protein-protein interactions with the p160 family of nuclear receptor coactivator proteins such as SRC-1 and GRIP-1 (3–6). Coactivator interaction with nuclear receptors, including the VDR, is thought to be necessary for the ligand-activated transcriptional process (6–11). Indeed, the deletion of the AF-2 domain or mutations within the AF-2 motif of the VDR eliminate coactivator interaction and ligand-activated transcription (6, 12).

Important aspects of this mechanism are the ligand-dependent nature of the nuclear receptor-coactivator interaction and the effect of ligand on the AF-2 motif. Structural analyses of related nuclear receptors indicate that the AF-2 domain undergoes a dramatic movement upon binding ligand (13–18). In the unliganded RXR, helix H12 (which contains the AF-2 motif) projects out from the globular LBD core (13). In the liganded receptor (e.g., the estrogen receptor, the retinoic acid receptor, or the thyroid hormone receptor), the AF-2 helix is folded down onto the surface of the LBD where it is thought to close off the hydrophobic ligand binding pocket (14–16). Interestingly, p160 coactivator proteins do not interact appreciably with unliganded receptors in which the AF-2 domain may, in fact, be surface exposed (8, 10). High affinity coactivator interaction with nuclear receptors apparently requires a ligand-induced repositioning of the AF-2 helix against the core of the LBD. This suggests that the AF-2 helix alone is required but is not completely sufficient to mediate coactivator interaction and that an interaction surface comprised of the AF-2 domain and other surface-exposed domains is needed for efficient coactivator interaction and transactivation. Candidate domains surrounding the AF-2 core that may form a part of a coactivator interaction surface include exposed residues on the surfaces of helices H3 and H4, the loop between H11 and H12, and the region between H1 and H3 comprising the Ω loop (15–18).

Recently, we identified a novel autonomous activation function in helix H3 of the VDR that is distinct from the AF-2 activation domain. Here, we show that select residues within the helix H3 activation domain are also required for ligand-dependent transcription and interaction of VDR with the p160...

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1 The abbreviations used are: 1,25-(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; AF-2, activation function 2; LBD, ligand-binding domain; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator 1; GRIP-1, glucocorticoid receptor-interacting protein 1; GST, glutathione S-transferase; VDR, vitamin D response element; AF-1 activation function 1, AF-2a, activation function 2a; TR, thyroid hormone receptor; GH, growth hormone.
2 Kraichely, D. M., Nakai, Y. D., and MacDonald, P. N., (1999) J. Cell. Biochem., in press.

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family of nuclear receptor coactivators including SRC-1 and GRIP-1. Specifically, a Y236A helix H3 mutation in the VDR selectively disrupted 1,25-(OH)$_2$D$_3$-induced interactions between VDR and SRC-1 or GRIP-1. These data support a model for VDR in which ligand binding creates a transactivation surface or platform for the binding of p160 coactivator proteins. Important components of this transactivation platform for the VDR are helices H3 and H12.

EXPERIMENTAL PROCEDURES

Oligonucleotide-directed Mutagenesis—Single-stranded DNA for the mammalian expression vector pSG5-VDR (4–427) was isolated as described. Mutations were introduced using the GeneEditor oligonucleotide-directed mutagenesis system (Promega, Madison, WI). All mutations were confirmed by DNA sequencing.

Plasmids and Transient Transfection Studies—The reporter constructs, (VDR)$_5$-TATA-GH and (GAL4)$_5$-TATA-GH were described previously (6, 19). The expression plasmids for pSG5 GAL4-VDR-(195–238) wild-type, and the H229A, D232A, or Y236A mutants. GH secreted into the media was quantitated 24 h post-transfection.

Ligand-binding Assay—Ligand binding experiments were performed in the DH5a strain of E. coli and purified by glutathione-Sepharose affinity chromatography as described previously (23, 24). 3H-Labeled VDR-(4–427) wild-type or helix H3 point mutants (S225A, H229A, D232A, S235A, Y236A, and K240A) and SRC-1 or GRIP-1 were each generated using the TNT-coupled transcription-translation system according to the manufacturer (Promega, Madison, WI). Interactions between the purified GST fusion proteins and $^{35}$S-labeled proteins were assessed as described (23). Autoradiographic images were scanned and densitometrically quantitated using ImageQuant, Version 3.0, software from Molecular Dynamics.

RESULTS

Specific Point Mutations within Helix H3 Reduce Both the Autonomous Activity of a Minimal Transactivation Domain and the Ligand-dependent Activity of Full-length VDR—We have identified a distinct transactivation function within helix H3 of the human vitamin D receptor that resides between residues Asp$^{195}$ and Ile$^{238}$.

VDR Helix H3 in Transactivation and Coactivator Interaction

![Figure 1: Point mutations within helix H3 impair the ligand-independent activity of a minimal transactivation domain in the hVDR LBD.](image)

To investigate the functional importance of amino acid residues within helix H3 for 1,25-(OH)$_2$D$_3$-activated transcription by full-length VDR, point mutations were introduced into the full-length pSG5-VDR-(4–427) plasmid, and the ability of these mutants (S225A, H229A, D232A, S235A, Y236A, and K240A) and SRC-1 or GRIP-1 to interact with the purified GST fusion proteins and [35S]methionine-labeled proteins were assessed as described (23). Autoradiographic images were scanned and densitometrically quantitated using ImageQuant, Version 3.0, software from Molecular Dynamics.

In Vitro Protein Interaction Assay—GST-VDR-(116–427) wild-type or Y236A mutant and GST-RXRalpha(235–493) were each individually expressed in the DH5a strain of E. coli and purified by glutathione-Sepharose affinity chromatography as described previously (23, 24). $^{35}$S-Labeled VDR-(4–427) wild-type or helix H3 point mutants (S225A, H229A, D232A, S235A, Y236A, and K240A) and SRC-1 or GRIP-1 were each generated using the TNT-coupled transcription-translation system according to the manufacturer (Promega, Madison, WI). Interactions between the purified GST fusion proteins and [35S]methionine-labeled proteins were assessed as described (23). Autoradiographic images were scanned and densitometrically quantitated using ImageQuant, Version 3.0, software from Molecular Dynamics.

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Fig. 2. Point mutations within helix H3 in the full-length VDR disrupt ligand-activated transcription. A series of point mutations were generated within helix H3 in the full-length pSG5-VDR-(4–427) by site-directed mutagenesis, and these mutants were tested in COS-7 cells. Each transfection included 3 μg of a (VDRE)4-TATA-GH reporter construct and either 10 ng of pSG5-VDR-(4–427) wild-type or a mutant version (S225A, H229A, D232A, S235A, Y236A, or K240A). The cells were treated in the absence or presence of 10−8 M 1,25-(OH)2D3 as indicated for 24 h, and GH secretion was determined by an immunoassay. A western immunoblot analysis of pSG5-VDR-(4–427) wild-type and mutants using the anti-VDR-9A7 antibody is shown in the bottom panel. Extracts were prepared from duplicate plates of COS-7 cells transfected with the wild-type pSG5-VDR-(4–427) and the helix H3 mutants (S225A, H229A, D232A, S235A, Y236A, and K240A) in pSG5-VDR-(4–427).

K240A) to activate transcription of a (VDRE)4-TATA-GH reporter gene in COS-7 cells was examined. As illustrated in Fig. 2, the S225A and K240A mutations showed only modest effects (20–30% decrease compared with wild-type VDR), whereas the mutation of Ser229 led to a greater than 55% reduction in the ligand-dependent transcription of a vitamin D-responsive reporter. However, most striking were the mutations of His229, Asp232, and Tyr236 which individually eliminated VDR-activated transcription. As shown in the lower panel of Fig. 2, Western blot analysis of whole cell lysates from duplicate transfection plates revealed nearly equivalent expression of VDR for the mutants examined in this assay, with the exception being the D232A mutant which showed somewhat reduced levels of expression or stability. Consequently, His229, Asp232, and Tyr236 are three residues within helix H3 that are determined to be important for both the autonomous activity of the minimal domain (Fig. 1) and for ligand-activated transcription of full-length VDR (Fig. 2).

Characterization of the Helix H3 Mutants of VDR—To determine the precise molecular basis for the loss of ligand-dependent transactivation in these mutant VDRs, a number of important functional parameters were analyzed including RXR heterodimerization, ligand-binding, and coactivator interaction. To determine whether these mutations altered VDR heterodimerization with RXR, in vitro protein-protein interaction assays were performed (Fig. 3A), and a quantitation of these interactions is presented in Fig. 3B. A 5-fold increase in the amount of 35S-labeled VDR (wild-type) binding to GST-RXR was observed in the presence of 10−8 M 1,25-(OH)2D3 (compare lanes 3–4). Similarly, 35S-labeled full-length VDR mutants (S225A, S235A, Y236A, and K240A) showed ligand-dependent interaction with RXR that was comparable with wild-type (lanes 7–8, 19–20, 23–24, and 27–28). However, 35S-labeled full-length VDR mutants H229A and D232A were impaired in their ability to interact with RXR in a ligand-dependent manner (lanes 11–12 and 15–16). Neither wild-type nor any mutant showed interaction with GST alone (lanes 2, 6, 10, 14, 18, 22, and 26). These data suggest that the H229A and D232A mutations in full-length VDR compromised the ability of the receptor to bind ligand or to heterodimerize with RXR.

Several helix H3 VDR mutants were then examined in an in vitro ligand-binding assay. Specific, saturable binding curves were generated for the binding of 1,25-(OH)2-1H1D3 by purified wild-type VDR and mutants. As suggested from the previous experiment, the VDR mutants H229A and D232A exhibited impaired abilities to bind the 1,25-(OH)2D3 ligand (Fig. 4). The Y236A mutation appeared to bind ligand with an affinity comparable with wild-type VDR. Dissociation constants for the binding of 1,25-(OH)2-1H1D3 by the wild-type VDR and mutants were determined from Scatchard plots of the saturable binding curves (Fig. 4, inset). The Y236A mutant had a Kd similar to wild-type VDR (0.2–0.3 nM), whereas the H229A and D232A mutants had 4.5- and 8-fold lower affinities, respectively. Cumulatively, these data indicate that the H229A and D232A mutations were inactive in hormone-mediated VDR transcription, presumably because of impaired ligand binding and subsequent inability to heterodimerize with RXR. In contrast, the loss of 1,25-(OH)2D3-mediated transactivation by the Y236A mutation could not be explained by this defect because it bound ligand with an affinity similar to that of wild-type VDR, and it interacted in a ligand-dependent manner with RXR.

Interactions between the Wild-Type VDR or Helix H3 Mutant Y236A and Coactivator Proteins—These data suggested that the Y236A mutant must be defective in some other aspect of receptor action that did not include hormone binding or RXR heterodimerization. Therefore, the yeast two-hybrid system was used to assess the ability of wild-type VDR and mutant Y236A to interact with nuclear receptor coactivator proteins (Fig. 5). In this system, ligand-dependent interactions were observed between the wild-type VDR (pAS1-VDR-(116–427)) and fusion proteins of SRC-1 and RIP-140 (pAD-mSRC-1-(1169–1465) and pAD-mRIP-140-(867–1158), respectively). These 1,25-(OH)2D3-dependent interactions between wild-type VDR and SRC-1 or RIP-140 were observed previously (6). Interestingly, the helix H3 point mutant Y236A (pAS1-VDR-(116–427) Y236A) showed markedly reduced interaction with RIP-140 and essentially no interaction with SRC-1. Both wild-type and mutant Y236A retained strong interaction with RXRα in this system (data not shown). No interaction was observed with the AD-GAL4 parent plasmid for either wild-type VDR or...
mutant Y236A. Thus, 1,25-(OH)2D3-dependent interactions between VDR (Y236A) and AF-2 coactivator proteins were dramatically impaired compared with wild-type VDR as determined by the yeast two-hybrid system.

To confirm these in vivo yeast two-hybrid data, interactions between VDR (Y236A) and p160 coactivator proteins were examined in vitro by GST pull-down analysis. As shown in Fig. 6A, 35S-labeled full-length wild-type VDR and helix H3 mutants were each incubated with 5 μg of GST (lanes 2, 6, 10, 14, 18, 22, and 26) or with 5 μg of GST-RXRα in the absence (lanes 3, 7, 11, 15, 19, 23, and 27) or presence (lanes 4, 8, 12, 16, 20, 24, and 28) of 10−8 M 1,25-(OH)2D3. Protein-protein complexes were washed, analyzed by SDS-PAGE, and visualized by autoradiography. The input lanes represent 10% of the protein in the binding assay. B, the data were plotted as relative densitometric units from a representative experiment.

Findings indicate that mutant Y236A, despite retaining the ability to bind hormone and to heterodimerize with RXR, is selectively impaired in interaction with coactivator proteins both in yeast and in in vitro binding assays. Bacterial-expressed and -purified GST-VDR-(116–427) wild-type () and mutants (C), Y236A, H229A, D232A, or GST (A) (0.5 μg of protein) were combined with 4.5 μg of nuclear extract obtained from HeLa cells and incubated with increasing concentrations of 1,25-(OH)2D3. Following the removal of free ligand with dextran-coated charcoal, specific counts in the bound fraction were determined by scintillation counting. Nonspecific binding was determined by competition with 400-fold molar excess of unlabeled 1,25-(OH)2D3 and was generally less than 10% of specific binding values. As shown in the inset, dissociation constants for the binding of 1,25-(OH)2D3 by the wild-type and mutant VDRs were determined from Scatchard plots of the saturable binding curves.
The AF-2 domain is a small α-helix at the COOH terminus of the nuclear receptors that is required for ligand-activated transcription. The AF-2 core sequence is highly conserved throughout the nuclear receptor superfamily consisting of a centrally conserved glutamic acid residue flanked by two pairs of hydrophobic residues (6, 29). Recently, the AF-2 domain has been shown to be the central motif through which coactivator proteins such as SRC-1 and GRIP-1 interact in a ligand-dependent manner with the nuclear receptors (3–6). The ligand-dependent interaction between coactivators and the AF-2 helix is thought to be critical for transcriptional regulation mediated by the nuclear receptor family members. For example, in the VDR, the deletion of the AF-2 helix or subtle point mutations within its conserved residues selectively abrogates coactivator interaction (6) as well as ligand-activated transcription (6, 12). Consistent with the properties of other activation domains identified in unrelated transactivating proteins, the AF-2 helix is active when assayed outside of the context of the nuclear receptor, meaning that it alone confers transactivation capacity to a heterologous DNA binding domain such as GAL4 (1–147). Specifically, for the VDR, our laboratory demonstrated that a fusion protein consisting of the VDR AF-2 helix and GAL4 (1–147) was sufficient for both transactivation and for interaction with coactivator proteins including SRC-1 (6). Importantly, in this heterologous context, both transactivation and coactivator interaction mediated through the minimal AF-2 motif were substantially weaker compared with the liganded, intact VDR. These findings suggested that other residues outside the AF-2 core domain may be required for optimal coactivator interaction and for the full transcriptional activity of the VDR.

Helix H3 is an intriguing candidate region outside the AF-2 motif of the VDR that may be important for coactivator contact and transactivation. Based on the structural analyses of related nuclear receptors, helix H3 of the VDR is a putative α-helix that resides between Leu224 and Lys246 (18). Recently, we identified a distinct transactivation domain in the VDR that encompasses a major portion of helix H3. This domain alone was sufficient for transactivation, suggesting that it may serve an important role in VDR function, perhaps forming a part of a binding interface for other transcriptional regulatory proteins.
Indeed, expression of the minimal helix H3 activation domain in COS-7 cells interfered with or squelched VDR-mediated transactivation, indicating that this domain interacted with limiting factors in the cell which are essential for VDR-activated transcription.\(^7\) It is possible that the helix H3 interacting proteins are distinct from the AF-2 coactivators SRC-1 and GRIP-1 of the p160 family and are selective for the helix H3 transactivation domain of the VDR. Alternatively, helices H3 and H12 in the VDR may fold in a manner that creates a single transactivation surface in which both helices are required for optimal interaction with the SRC-1/GRIP-1 family of coactivator proteins. Such a model was proposed by Barettino et al.\(^{(30)}\) who suggested that the activity of the AF-2 domain of nuclear receptors depends on the cooperation of a number of activation domains that are dispersed throughout the hormone binding domain and that are brought together upon ligand binding.

This hypothesis is strongly supported by crystallographic data that examined the ligand-binding domain of related nuclear receptors (13–17). These structural data indicate that ligand induces a dramatic structural change that involves a folding or packing of the helix H12 (AF-2 domain) onto the surface of the globular LBD core with a juxtapositioning of helix H12 and helices H3/H4. Moreover, scanning surface mutagenesis of the thyroid hormone receptor (hTR\(^\beta\)) recently identified a hydrophobic cleft formed by helices H3, H5, H6, and H12 that is crucial for thyroid hormone-dependent binding of the coactivator proteins SRC-1 and GRIP-1 and for ligand-activated transactivation (31). Cumulatively, these data suggest that ligand binding induces a repositioning of helix H12 to create a coactivator interaction surface composed of helix H12 and surrounding residues within helices H3, H5, and H6. Our current data strongly support a similar paradigm for the VDR. This was most strikingly apparent in the Y236A mutation within the VDR helix H3 activation domain. While the Y236A mutation did not affect the ability of VDR to bind ligand or to heterodimerize with RXR, alteration of this single tyrosine residue selectively impaired both 1,25-(OH)\(_2\)D\(_3\)-dependent interaction with the AF-2 coactivators SRC-1 and GRIP-1 as well as 1,25-(OH)\(_2\)D\(_3\)-activated transactivation. The essential nature of this helix H3 residue in coactivator function is apparent in Fig. 7, wherein overexpression of SRC-1 or GRIP-1 dramatically augments 1,25-(OH)\(_2\)D\(_3\)-activated transactivation mediated by wild-type VDR, but each exhibited absolutely no effect on transactivation mediated by the VDR (Y236A) mutant. Thus, in addition to the AF-2 domain, Y236 located within the helix H3 activation domain of the VDR is required for efficient interaction with coactivators of the SRC-1/GRIP-1 family and the subsequent transcriptional regulatory effects of the VDR.

Asp\(^{232}\) of hVDR is a critical residue for transactivation mediated by the minimal helix H3 domain (Fig. 1).\(^2\) However, its role in mediating the transcriptional activity of the full-length VDR is unknown. The role of Asp\(^{232}\) in the intact VDR could not be tested directly because mutation of Asp\(^{232}\) in the full-length VDR disrupts the ligand-dependent interaction of VDR with RXR (Fig. 3) and binding of 1,25-(OH)\(_2\)D\(_3\) (Fig. 4). Whether Asp\(^{232}\) directly contacts the ligand or if it is responsible for maintaining an important structural role required for high order binding or RXR heterodimerization remains to be determined. However, some insight may be gained from modeling studies of the ligand-binding domain of the VDR.\(^{(32)}\) Based on the crystal structure of holo-RAR\(\gamma\), this study strongly suggests that Asp\(^{232}\) of hVDR (corresponding to Glu\(^{232}\) in RAR\(\gamma\)) is spatially distant from the ligand (> 5 Å) and does not directly contact the ligand (32). In fact, Glu\(^{232}\) of RAR\(\gamma\) (corresponding to Asp\(^{232}\) of hVDR) is solvent-exposed and projects out away from the ligand, residing on the outer face of helix H3 (data not shown). These structural modeling data suggest that Asp\(^{232}\) of the VDR may not be directly involved in ligand binding, and its mutation in the full-length VDR may affect the positioning of other nearby residues that do directly contact ligand. Regardless, its putative surface localization and its key role in transactivation by the minimal domain strongly suggest that it also may play a role in the transactivation surface of the full-length receptor.

An intriguing paradox arises from our studies of the VDR activation domains as well as previous work of others on related nuclear receptors. The AF-2 domain of most nuclear receptors is autonomously active when assayed outside the context of the native receptor. The same is true for the AF-1 (33–37) and AF-2a (38, 39) motifs of other nuclear receptors and the helix H3 activation domain that we identified in the VDR.\(^2\) Yet mutations that inactivate the VDR AF-2 core in the intact receptor completely abolish 1,25-(OH)\(_2\)D\(_3\)-activated transactivation, indicating that, in the context of the full-length
VDR with an inactive AF-2 domain, other resident activation functions such as the helix H3 domain are also nonfunctional. However, our data also demonstrate that inactivation of the helix H3 domain through the Y236A mutation abolishes 1,25-(OH)$_2$D$_3$-mediated transactivation, indicating that the AF-2 domain is also nonfunctional in this context. Thus, additional structural complexities likely exist in the liganded intact receptors that preclude observation of the resident activities of the AF-2 or helix H3 activation domains, both of which are readily apparent when assayed outside the context of the full-length VDR. Moreover, the co-dependence of the AF-2 and helix H3 domains is not consistent with a mechanism that involves distinct coactivator proteins functioning exclusively through either the AF-2 or the helix H3 domains.

In conclusion, although it is difficult to predict the three-dimensional positioning of specific amino acids in the VDR in the absence of any structural data, our studies suggest that Tyr$^{236}$ is an important residue that may comprise a portion of the interaction surface through which p160 coactivator proteins such as SRC-1 or GRIP-1 contact the VDR. Taken together, the findings described in this manuscript and structural data of related nuclear receptors suggest that helix H3 of the VDR is a transactivation domain that functions in concert with the AF-2 domain to form a transactivation surface that mediates 1,25-(OH)$_2$D$_3$-VDR-mediated transcription.

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