Mutant K246A in the predicted helix 3 of the ligand-binding domain, as well as mutants L417S and E420Q in helix 12, which contains the core ligand-dependent transcriptional activation domain (AF-2), were generated to examine AF-2 activity of the vitamin D receptor (VDR). These mutations abolished vitamin D-dependent transactivation. In addition, VDR mediates a ligand-dependent repression of the response of the retinoic acid receptor β2 promoter to retinoic acid, and the helix 3 and helix 12 mutants were unable to mediate transrepression. Furthermore, the VDR mutants, but not the native receptor, enhanced phorbol ester induction of the activator protein-1-containing collagenase promoter. The helix 3 and helix 12 mutations strikingly reduced the ability of VDR to interact with the coactivators steroid receptor coactivator-1, ACTR, and the CREB-binding protein. As a consequence, overexpression of steroid receptor coactivator-1 increased vitamin D-dependent transactivation by VDR but not by the K246A mutant. These results indicate that the lysine 246 participates, together with residues in helix 12, in the recruitment of coactivators and that AF-2 activity is involved both in ligand-dependent transactivation and in transrepression by VDR.

The vitamin D receptor (VDR) is a member of the superfamily of nuclear receptors that act as ligand-inducible transcription factors (1, 2). VDR binds, as a heterodimer with the retinoid X receptor (RXR), to hormone response elements (VDREs) in target genes (3–7). Transcriptional actions of nuclear receptors can result not only from direct modulation of target gene expression but also from cross-talk with other nuclear receptors and signal transduction pathways (e.g. AP-1 activity) (8).

Nuclear receptors display a modular structure with an N-terminal region A/B, followed by region C (the DNA-binding domain), a hinge region D, and the C-terminal E/F region containing the ligand-binding domain (LBD) and the dimerization domain (1, 2). An autonomous ligand-dependent transcriptional activation function (AF-2) is located in the LBD (9). The core AF-2 domain has been characterized in the C terminus of different nuclear receptors (10–15) and consists of a well-conserved amphipathic α-helix motif. The crystal structure of five different nuclear receptor LBDs has been solved (16–20). Although some variability exists, the ligand-binding domains contain a similar helical fold with twelve α-helices (numbered helix 1 to helix 12) (21). The most striking difference between the unoccupied (apo) and ligand-bound (holo-) receptors is the position of the C-terminal helix 12, which contains the core AF-2 domain. This helix projects away from the LBD in the apo-receptors but is tightly packed against helix 3 of the LBD upon ligand binding. In addition, the LBDs of the holo-receptors are more compact that those of the unliganded receptors, indicating that the ligands may reconfigure other surface features of the LBD. The conformational change in helix 12, together with other changes that occur after ligand binding such as the bending of helix 3, are believed to create a surface that allows binding of coactivator proteins required to transduce the signal to the basal transcriptional apparatus (9).

Several of these proteins have histone acetylase activity, which can disrupt nucleosomes (26, 27, 31, 32). Therefore, the current model for transcriptional regulation by nuclear receptors suggests that the AF-2 of nuclear receptors serves to recruit a multicomponent coactivator complex that can open up chromatin (33, 34).

By analyzing the transcriptional activity of mutant receptors, it has been confirmed that residues located not only in helix 12 but also in helix 3 are required for ligand-dependent transcriptional activity. In particular, mutation of a highly conserved lysine residue at the C terminus of helix 3 strongly impairs transactivation by estrogen receptor (35), thyroid hormone receptor (36–38), and VDR (39). This mutation, which could disrupt interaction between helices helix 12 and helix 3, has been very recently shown to reduce binding of the coactivators SRC-1 and TIF-2 to estrogen receptor (35). In this study we have analyzed the role of mutations in the conserved VDR residues Leu147 and Glu428 (in helix 12) and Lys246 (in helix 3) on transcriptional responses to vitamin D. Our data confirm that these residues are required for ligand-dependent activa-
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**Plasmids**—In the plasmid Spp1-tk-CAT, the oligonucleotide 5′-AGCTTGGACACAAAGTTCAGGTTCTCCTCT-3′ conferring the VDRE of the osteopontin-1 (Spp-1) promoter was cloned into the HindIII and XbaI sites of pBluescript in + in the thymidine kinase promoter. The R140T CAT construct containing the fragment −124 to +14 of the human RARα 2 promoter was obtained from the previously described plasmid (40). The promoter fragment was obtained by PCR with the oligonucleotides 5′-GGGAGGCTTGGATCTGGGAGTTGTTG-3′ and 5′-GCTCTTAGAGCTACTTCTACTAC-3′ and subcloned in HindIII and XbaI sites of pBluescript replacing the thymidine kinase promoter. The construct −73CdLuc, which contains the collagenase promoter fused to luciferase, has been described previously (41). Expression vectors for human VDR, RARα, RARβ, and SRC-1 cloned in pSG5 (35, 42, 43) and RSV-Jun (44) have been also described. The expression vectors for the VDR point mutants K246A, K417A, and E420Q were obtained by PCR using established protocols (45). In the first PCR reaction the sense oligonucleotides 5′-CGGCTCCTCTGGCTCCTGGA-3′ (for L417S), 5′-CCCTTGGTCTGCCAATGGTG-3′ (for E420Q), and 5′-GCTTGGTGGATCTGGGAGTTGTTG-3′ (for K246A) were used to generate mutated fragments. After an elongation phase, a second PCR reaction was performed. In the case of L417S and E420Q the resulting products were cloned with the oligonucleotides 5′-GCGGTCCTCCAGGATTCTGTCG-3′ with a BamHI site were used to generate mutated fragments. After an elongation phase, a second PCR reaction was performed. In the case of L417S and E420Q the sense oligonucleotide for this reaction was 5′-GCTTGCACCTACGACGAGCAT-3′ containing the unique NcoI site of the VDR cDNA. For the K246A mutant the sense oligonucleotide, containing a BstXI site was 5′-GGAGGGACCCGACGCCATCATT-3′. The resulting products were cloned into the pGEX expression vector, and the mutations were confirmed by sequencing (pGST-VDR, pGST-VDR(K246A), and pGST-VDR(E420Q)). The GST fusion proteins were expressed and purified by standard techniques following the recommendations of Pharmacia Biotech Inc. The expression of correctly sized proteins was monitored by SDS-PAGE.

**Limited Proteolytic Digestion**—In vitro translated 35S-labeled wild type or K246A VDR were incubated in glass tubes with 100 nm vitamin D3 or ethanol for 20 min at room temperature. Aliquots of receptors were then incubated with increasing amounts of trypsin (between 0 and 50 μg/ml) for 10 min. Proteolysis was stopped by adding SDS sample buffer and boiling for 10 min. The proteolytic fragments were separated by SDS-PAGE in a 12% polyacrylamide gel and identified by autoradiography.

**Mobility Shift Assays**— Gel retardation assays were performed with the in vitro translated receptors and the oligonucleotide corresponding to the VDRE of the osteopontin-1 (Spp-1) promoter: 5′-agcttccatcagagctacactc-3′. For the binding reaction, the proteins were incubated on ice for 15 min in a buffer (200 mmoles Tris-HCl, pH 7.5, 75 mM KCl, 1 mM dithiothreitol, 5 μg/ml bovine serum albumin, 13% glycerol) containing 3 μl of poly(dI-dC) and then for 15–20 min at room temperature with approximately 50,000 cpm of labeled double-stranded oligonucleotide end-labeled with 32PIdCTP, using Klenow fragment as kinase. DNA-protein complexes were resolved on 6% polyacrylamide gels in 0.5 x TBE buffer. The gels were then dried and autoradiographed at 70°C.

**Protein-Protein Interactions**—GST pull-down assays were performed with 5 μl of the in vitro translated 32P-labeled proteins. These proteins were incubated with 1 μg of the GST fusion protein or with the same amount of GST as a control and immobilized in glutathione-Sepharose beads. The proteins were first incubated in the presence of 100 μl vitamin D or ethanol for 20 min at room temperature in glass tubes. The reaction with the beads was performed for 1 h at 4°C in a binding buffer containing 25 mM Hepes KOH, pH 7.9, 1% glycerol, 5 mM MgCl2, 1 mM dithiothreitol, 0.05% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. Free proteins were washed from the beads with a buffer containing increasing concentrations (50, 100, and 200 mM) of KCl, and the bound proteins were analyzed by SDS-PAGE and autoradiography.

**RESULTS**

**Transcriptional Activation by Mutant Vitamin D Receptors**—As shown in Fig. 1, the amphipathic α-helix that contains the core AF-2 domain as well as sequences contained in helix 3 and helix 4 are well conserved among different nuclear receptors. A glutamic acid residue at position 420 in the predicted helix 12 of VDR is also present in thyroid, steroid, and retinoid receptors. We investigated the importance of the glutamic acid residue in the transactivational actions of VDR by replacing it with the unreacted and acetylated [14C]chloramphenicol were separated by thin layer chromatography and quantified with an InstantImager. The data are expressed as the percentages of acetylated forms after each treatment. Each treatment with the ligands was performed at least in duplicate cultures that normally exhibited less than 10% variation in CAT activity, and the experiments were repeated at least three times. The results are expressed as the means ± S.D. of the CAT or luciferase values obtained.
with a glutamine to generate E420Q. Additionally, the leucine residue 417 conserved in most receptors was changed by a serine (L417S). These mutations in VDR have been previously shown to impair transactivation and to be essential for interaction with coactivators (14). On the other hand, lysine residue 246 at the C terminus of the predicted helix 3 in VDR was mutated to an alanine to render K246A. This lysine residue is also extremely well conserved in the different nuclear receptors and also appears to play an important role in transactivation (35–39).

We compared K246A, L417S, and E420Q with the native VDR for the ability to stimulate transcription from a CAT reporter gene containing the VDRE of the osteopontin (Spp-1) gene fused to the thymidine kinase promoter in transiently transfected COS-7 cells. These cells contain low levels of endogenous VDR. Fig. 2A shows that upon transfection with wild type VDR, treatment with vitamin D activated the Spp-1-tk-CAT construct in COS-7 cells, whereas the helix 12 and helix 3 mutants were transcriptionally inactive. Fig. 2B shows that, as assessed by Western blot analysis, K246A, L417S, and E420Q, and the wild type receptor were expressed in COS-7 cells at similar levels. The DNA binding properties of the mutant receptors were determined in gel retardation assays with the Spp-1-tk-CAT construct. As shown in Fig. 2C, the mutant VDRs bound DNA as strongly as the native receptor.

The transcriptional activity of the helix 3 and helix 12 mutants was also tested in HeLa cells in which vitamin D causes a 6–7-fold activation of the Spp-1-tk-CAT construct. Whereas overexpression of wild type VDR increased further the response of the reporter plasmid to vitamin D, the mutant receptors had a dominant negative activity and blocked the response to the vitamin mediated by endogenous VDR (data not shown).

VDR and VDR K246A Present Identical Ligand-dependent Protease Sensitivity—Ligand-induced conformational changes within the receptors appear to result in an increased resistance to limited proteolytic digestion. To analyze whether the inability of the helix 3 mutant to activate transcription could result from an inadequate conformation upon ligand binding, we used a trypsin digestion assay. Fig. 3 shows that both VDR and the K246A mutant are digested to small peptides at a trypsin concentration of 50 μg/ml within 10 min at room temperature. At lower trypsin concentrations several proteolytic fragments with molecular masses between 38 and 25 kDa were also observed. Incubation with vitamin D strongly inhibited the proteolysis of these fragments. The size of the main resistant fragment was found to be approximately 33 kDa. Trypsin digestion of the helix 3 mutant in the presence of vitamin D generated a proteolytic pattern indistinguishable from that obtained with the native VDR.

The Core AF-2 Domain as Well as Lys-246 Are Required for Transrepression of the RARb2 Promoter—We have observed that incubation with vitamin D reduces retinoic acid-mediated transactivation of the RARb2 promoter in pituitary GH4C1 cells (47). VDR interferes with the activation of this promoter, which contains a strong retinoic acid response element (40). We then examined whether mutations in helix 3 and helix 12 could also affect transrepression mediated by VDR. Fig. 4 compares the influence of VDR and the mutants K246A, L417S, and E420Q on the response of the RARb2 promoter in GH4C1 cells (panel A) and in HeLa cells (panel B). In GH4C1 cells incubation with 1 μM retinoic acid increased by about 7-fold the activity of the RARb2 promoter, and this response was reduced to approximately 5-fold in cells incubated in the presence of 1 nM vitamin D. Transfection with native VDR enhanced this inhibitory response, whereas the helix 12 and helix 3 mutants were unable to elicit a further reduction in the response to retinoic acid. Similar results were observed in HeLa cells. In this cell type vitamin D repressed the retinoic acid response in cells transfected with VDR, and the different mutants were again inactive.

The AF-2 Mutants, but Not Wild Type VDR, Affect Collagenase Promoter Activity—12-O-Tetradecanoyl-phorbol-13-acetate (TPA) leads to the induction of genes through activation of the AP-1 complex, and different nuclear receptors have been shown to functionally interact with TPA-inducible gene expression (8). The collagenase promoter has been widely used as a model for the study of AP-1-dependent gene activation. In Fig. 5A we examined the effect of wild type VDR and the mutants K246A, L417S, and E420Q on the activity of the collagenase promoter in HeLa cells. Activation by TPA was not modified by the different VDRs in the absence of ligand. In the presence of vitamin D the collagenase response to TPA was minimally increased in cells transfected with the wild type receptor. However, both the helix 12 mutants and the helix 3 mutant significantly enhanced the response to TPA. The data shown in Fig. 6B confirm that these mutants are able to mediate a ligand-dependent increase in AP-1-dependent collagenase activation. In this case, the effect of the receptors was assessed in cells transfected with c-jun, a component of the AP-1 complex. Whereas vitamin D did not activate the collagenase promoter in HeLa cells expressing the native VDR, the K246A, L417S, and E420Q mutants mediated a significant activation.

Lys-246 Is Required for Vitamin D-dependent Binding of Coactivators—Transcriptional stimulation by nuclear receptors correlates with their ability to interact with coactivator
proteins in a ligand-dependent manner. Therefore, we tested the effect of K246A mutation on in vitro interaction between VDR and SRC-1 in GST pull-down assays. As shown in Fig. 6A in the presence of vitamin D a significant portion of the input of 35S-labeled SRC-1 was specifically retained by wild type GST-VDR fusion protein immobilized in glutathione-agarose beads, whereas no significant binding was observed either in the absence of vitamin D or by GST alone. The helix 3 mutation dramatically reduced binding to the coactivator, and the 35S-labeled SRC-1 was not retained by GST-VDR(K246A) in the...
presence of vitamin D. That this reduction is specific for the coactivator is shown by the finding that association with 35S-labeled RXR was unaffected by mutation K246A. In addition, no interaction of the receptors with 35S-labeled luciferase used as a negative control was detected.

The ability of wild type and mutant K246A, L417S, and E420Q receptors to interact with the coactivators ACTR and CBP was also analyzed. In these assays the coactivators fused to GST, and in vitro translated 35S-labeled receptors were used. As illustrated in Fig. 6B, wild type VDR was significantly retained by GST-ACMR in the presence of vitamin D, whereas mutation K246A abolished this association. In addition, the helix 12 mutants were unable to interact with GST-ACMR in a vitamin D-dependent manner. Similar results were obtained with a fragment of GST-CBP (amino acids 1–1099) that contains the receptor interaction domain. In this case, whereas 35S-labeled luciferase was not retained by the CBP fragment, some interaction with the different VDR mutants in the absence of vitamin D was observed. However, incubation with vitamin D increased binding of GST-CBP to the wild type receptor, whereas binding to the helix 3 or helix 12 mutants was unaffected.

**SRC-1 Does Not Enhance Transactivation by the K246A Mutant VDR**—In view of the differences in the ability of wild type and mutant K246A VDR to interact with coactivators in vitro, we analyzed the effect of overexpression of SRC-1 on the activity of these receptors in COS-7 cells transfected with Spp1-tk-CAT. As shown in Fig. 7, the vitamin D-stimulated activity of the wild type receptor was enhanced with increasing amounts of SRC-1. In contrast, the K246A mutant was a very poor transcriptional activator, and in agreement with the lack of binding to SRC-1 in vitro, its activity was unaffected by expression of SRC-1.

**DISCUSSION**

Previous work has shown the importance of the C-terminal helix 12 for AF-2 activity of nuclear receptors. Helix 12 contains residues with significant negative charges and forms an amphipathic a-helix that functions as an interaction surface for coactivators (17, 18). In this study we confirmed the require-
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Fig. 6. Mutation of lysine 246 disrupts the vitamin D-dependent interaction between VDR and coactivators. A, GST alone and the GST fusion proteins wild type VDR (wt) and VDR(K246A) were immobilized in glutathione-Sepharose beads. In vitro translated 35S-labeled SRC-1, RXR, and Luciferase were incubated with the beads in the absence (−) or presence (+) of 100 nM vitamin D (Vit.D). B, in vitro translated 35S-labeled VDR wild type (wt), VDR(L417S), VDR(E420Q), VDR(K246A), and luciferase were incubated with immobilized GST or the fusion proteins GST-ACTR or GST-CBP in the presence and absence of vitamin D. In both panels the bound proteins were analyzed by SDS-PAGE and visualized by autoradiography. The first lane in each case shows a 20% of the input of the corresponding labeled protein.

Fig. 7. Influence of SRC-1 on the transcriptional activity of the wild type and K246A VDR. COS-7 cells were transfected with 5 μg of Sp1-tk-CAT and expression vectors for VDR or VDR(K246A) (20 ng) in the presence of the amounts indicated of SRC-1 expression vector. After transfection the cells were treated with medium containing no additions or 100 nM vitamin D (vit.D) for 24 h, and CAT activity was determined.

ment of an intact AF-2 core domain for vitamin D-activated transcription. Point mutants E420Q and L417S were transcriptionally silent, although they have been described to bind vitamin D with equilibrium constants similar to wild type VDR (14). These amino acids in helix 12 are required for the interaction with SRC-1, SUG-1, or RIP140 in the yeast two-hybrid system (14), and our in vitro interaction assays indicate that in the presence of vitamin D the coactivators ACTR (the human homologue of mouse p/CIP) and CBP also fail to associate in a ligand-dependent manner with VDRs containing these inactivating mutations in helix 12. This defect can explain the lack of transcriptional activation.

Although the minimal AF-2 domain of VDR (residues 408–427) appears to be sufficient to mediate ligand-dependent transactivation as well as interaction with coactivators, the activity of this region is only partial (14). This suggested that additional elements of the LBD outside the core AF-2 domain are important for generating an efficient AF-2. These elements could include helix 3 because this helix is in close proximity to helix 12 in the holo-receptors (16–21). Our studies demonstrate that indeed the conserved lysine 246 at the predicted C terminus of helix 3 plays an important role in the transcriptional actions of VDR. The replacement of this lysine with alanine dramatically reduced AF-2 activity without affecting heterodimerization with RXR or DNA binding activity.

It was possible that the inactivity of the lysine 246 mutant could be a consequence of an altered receptor conformation upon ligand binding. It has been postulated that the ligand-induced conformational changes within the receptor result in a more compact folding of the LBD and increased resistance to proteolysis (48). Our results show that vitamin D treatment of wild type and K246A receptors generates indistinguishable peptide maps with a 33-kDa fragment becoming resistant to tryptic digestion. Given that the helix 3 mutation does not affect this property, it can be concluded that the overall structure of the mutant VDR resembles that of the wild type receptor and that both undergo similar conformational changes after binding of vitamin D. On the other hand, the changes elicited by vitamin D demonstrate that the loss of transcriptional activation by the K246A mutant was not due to a defect in ligand binding. It had been previously shown that replacing this lysine with a glycine also impaired transcriptional activity of VDR and that this mutant receptor was normal with respect to ligand binding (39).

Our studies also reveal that not only the C-terminal domain of the receptor but also the conserved lysine 246 residue are required for interaction with SRC-1. The transcriptionally inactive mutant receptor K246A was found to be defective for in vitro binding to the coactivators SRC-1, ACTR, and CBP. Functional studies also show that overexpression of SRC-1 in COS-7 cells enhanced ligand-dependent transcriptional activation by VDR and that lysine 246 is essential for this function. These results suggest that the transcriptional defect of this mutant results from its inability to recruit coactivators upon vitamin D binding. The fact that this mutant does not interact with the coactivators suggests subtle differences in conformation between the native and mutant VDR that were not detected at the level of resolution afforded by the protease digestion assay. The transcriptional activities of the thyroid hormone and the estrogen receptors were reported to be also strongly reduced when the conserved lysine was replaced with another amino acid (35–38). Furthermore, the mutant estrogen receptor also fails to interact with SRC-1 upon estrogen binding (35). These results as well as our results with the VDR indicate that residues in helix 12 as well as the lysine residue in helix 3 are required to form the surface by which the nuclear receptors interact with different coactivators. Mutation K246A could destabilize the helix 3-helix 12 interaction that appears to be
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importantly for generating an efficient AF-2. Because both the charged residues in helix 12 and the lysine residue in helix 3 are exposed on the surface of the ligand-binding domain (9), they may generate the hydrophilic surface of interaction with the coactivators required to mediate AF-2 activity (35).

In addition to their inability to stimulate transcription of a reporter gene containing a positive VDRE, our results also reveal that the VDR mutants are unable to inhibit retinoic acid-mediated transactivation of the RARp2 promoter. We have previously observed that the ability of a mutant VDR lacking the last 12 C-terminal amino acids to mediate transrepression by vitamin D was strongly decreased (47). Our present results indicate that the integrity of not only helix 12 but also helix 3 is essential for the dominant negative activity of VDR. The requirement of the residues responsible for AF-2 activity suggests that titration of coactivators or common associated proteins may be involved in the inhibitory effect of vitamin D, and in agreement with this hypothesis over-expression of E1A, which can act as a RARp2 promoter-specific coactivator, significantly reversed repression by vitamin D (47). These results also indicate that vitamin D-dependent transactivation and transrepression might depend on similar or the same interaction surfaces, although we cannot dismiss the possibility that the proteins interacting might be different.

Our results have also demonstrated an unanticipated property of the VDR mutants. They can enhance the response of the AP-1 containing collagenase promoter to TPA, a characteristic not shared by the native receptor. Although several receptors have been shown to antagonize the effect of TPA or c-Jun on the collagenase promoter, the functional interaction of nuclear receptors with AP-1 is complex, and Fos and Jun can have cell-specific inhibitory and stimulatory effects on transcription activation by nuclear receptors (8). Interestingly, some unliganded mutants of the thyroid hormone receptors have an effect similar to that of the liganded VDR mutants shown here (49). The fact that the VDR mutants have the common characteristic of being AF-2-defective suggests the involvement of coactivators in this property. A good candidate for being implicated in this function is CBP/p300, which also plays an essential coactivator role for several classes of transcription factors, including the AP-1 complex (26–29, 33, 34).

Overexpression of CBP has been shown to relieve AP-1 antagonism by the retinoic acid and glucocorticoid receptors, consistent with the hypothesis that competition between AP-1 and nuclear receptors for limiting amounts of CBP/p300 accounts for transrepression (26). We have shown that vitamin D does not enhance binding of CBP to the helix 3 and helix 12 VDR mutants, which could allow a greater availability of this common coactivator for the TPA-inducible signal transduction pathway. On the other hand, we cannot dismiss the possibility that the mutations create a new surface for interaction with other coactivator proteins or that they abolished interaction with putative inhibitory factors. Therefore, the mechanism by which the VDR mutants enhance c-Jun-mediated transactivation is unclear, and further work will be required to explain this effect.

In summary, our results have established that AF-2 activity of the VDR depends on the conserved lysine in helix 3 and support the view that recruitment of coactivators is essential in vitamin D-dependent transactivation as well as in vitamin D-dependent transrepression. Moreover, the facts that mutation of this lysine also abolishes ligand-independent transactivation by the thyroid hormone receptor (37, 38) and that this residue is conserved in most orphan receptors, which are believed to be constitutively active, suggest the existence of common mechanisms for ligand-dependent and ligand-independent transactivation. Future studies will hopefully clarify the role of different coactivator complexes in the diverse transcriptional actions of the nuclear receptors.

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