Dimerization with Retinoid X Receptors Promotes Nuclear Localization and Subnuclear Targeting of Vitamin D Receptors*

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The vitamin D receptor (VDR) acts as heterodimer with the retinoid X receptor α (RXR) to control transcriptional activity of target genes. To explore the influence of heterodimerization on the subcellular distribution of these receptors in living cells, we developed a series of fluorescent-protein chimeras. The steady-state distribution of the yellow fluorescent protein-RXR was more nuclear than the unliganded green fluorescent protein (GFP)-VDR. Coexpression of RXR-blue fluorescent protein (BFP) promoted nuclear accumulation of GFP-VDR by influencing both nuclear import and retention. Fluorescence resonance energy transfer microscopy (FRET) demonstrated that the unliganded GFP-VDR and RXR-BFP form heterodimers. The increase in nuclear heterodimer content correlated with an increase in basal transcriptional activity. FRET also revealed that calcitriol induces formation of multiple nuclear foci of heterodimers. Mutational analysis showed a correlation between hormone-dependent nuclear VDR foci formation and DNA binding. RXR-BFP also promoted hormone-dependent nuclear accumulation and intranuclear foci formation of a nuclear localization signal mutant receptor (nlsGFP-VDR) and rescued its transcriptional activity. Heterodimerization mutant RXR failed to alter GFP-VDR and nlsGFP-VDR distribution or activity. These experiments suggest that RXR has a profound effect on VDR distribution. This effect of RXR to promote nuclear accumulation and intranuclear targeting contributes to the regulation of VDR activity and probably the activity of other heterodimerization partners.

Proteins of the nuclear receptor superfamily mediate response to hormones or intracellular signals into transcriptional responses and regulate an array of important cellular functions. A member of the nuclear receptor superfamily, the vitamin D receptor (VDR)1, mediates effects of calcitriol on bone development and maintenance, calcium homeostasis, immune functions, endocrine functions, vitamin D metabolism, and cellular proliferation and differentiation. Like other class II nuclear receptors, such as the thyroid hormone receptor, the retinoic acid receptor, and many orphan receptors, VDR requires heterodimerization with the retinoid X receptor (RXR) for high affinity binding to target genes (1, 2). VDR and RXR can heterodimerize in the absence of calcitriol, and these heterodimers regulate basal transcriptional activity of target genes and exert transcriptional silencing functions (3). The addition of calcitriol stabilizes the heterodimers and promotes their binding to the vitamin D response elements (4). The importance of heterodimerization in VDR functions led us to investigate the spatial and temporal relationships between these receptors in living cells.

Recently we and others have used green fluorescent protein chimeras of VDR to study the receptor distribution in living cells (5–7). Unlike the glucocorticoid receptor (GR), which stays in the cytoplasm without the ligand, the unliganded VDR distributes evenly between the cytoplasm and the nucleus. This indicates that the regulation of VDR distribution is more complex than the regulation of GR distribution. The fact that VDR distribution is similar to thyroid hormone receptor distribution (8) raised the possibility that heterodimerization with RXR could account for the partial nuclear localization of both VDR and thyroid hormone receptor.

Most of what we know about the subcellular distribution of RXR comes from immunohistological and cell fractionation studies. In these studies, RXR was found in the nuclei of normal human skin cells (9), the rat kidney (10), and the human pituitary (11). RXR was also detected in the cytoplasm by cell fractionation experiments (12). Although a nuclear localization of RXR is to be expected, looking in live cells sometimes gives surprises, as has been the partial cytoplasmic localization of the thyroid hormone receptor (8). In a recent paper, focusing on the localization of an orphan nuclear receptor, the transiently expressed mouse GFP-RXRβ was found in the nuclei of phaeochromocytoma cells (36). Because previous studies indicated that VDR preferentially associates with RXRα to stimulate transcription (13), the subcellular localization of the RXRα had to be explored in detail. Even less is known about the subcellular location of the VDR/RXR heterodimers. Receptors that heterodimerize with RXR are not only synthesized in the cytoplasm but reside in the cytoplasm to some degree before ligand binding. Thus, heterodimerization may influence subcellular localization of RXR or its partner receptors. Using transcriptionally active and stably expressed yellow, green, and blue fluorescent protein chimeras of RXR and VDR and their mutants, we visualized the differences in the distribution of VDR and RXR in living cells by confocal laser-scanning microscopy.

Current developments of multicolor fluorescent protein variants also allow visualization of protein interactions in living cells by fluorescence resonance energy transfer microscopy.

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1 The abbreviations used are: VDR, vitamin D receptor; RXR, human retinoid X receptor α; GFP, green fluorescent protein; YFP, yellow fluorescent protein; BFP, blue fluorescent protein; hd, heterodimerization; NLS, nuclear localization signal; 8c-RA, 9-cis-retinoic acid; ROS, 17β-28 rat osteosarcoma cells; CYR, CV-1-derived cell line stably expressing YFP-RXR; GL48, 283-derived cell line stably expressing GFP-VDR; 240H/Luc, luciferase gene that is under control of the 25-hydroxyvitamin D3 24-hydroxylase promoter; FRET, fluorescence resonance energy transfer; GR, glucocorticoid receptor.

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G418-resistant cell clones were then selected for 3–5 wk by culturing reagents (Life Technologies) according to manufacturer’s instructions. pQBI25 plasmid (Quantum Biotechnologies). 293 cells were transfected the human VDR cDNA (a gift from Dr. J. W. Pike, University of Lines—

were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone), 2 mM glutamine, 0.1 

promotes DNA binding of VDR in living cells. We used FRET microscopy to visualize the effect of RXR to connection between DNA binding and nuclear foci formation

Ligand-induced intranuclear pattern changes, including foci formation, have been reported for the GR (19), the androgen receptor (22, 27), and the VDR (5). Here, we report similar receptors and other signaling proteins could be regulated by the control of translocation across the nuclear membrane (17). We generated a cell line stably expressing GFP-VDR that allowed us to correlate nuclear receptor content with basal transcriptional activity. Our experiments revealed that RXR promotes nuclear accumulation of VDR. To study the mechanisms of this RXR effect we explored both nuclear import and export of the unliganded VDR by mutational analysis and by temperature changes.

During hormone-induced receptor activation, a redistribution from the cytoplasm into the nucleus has been shown in living cells for GFP chimeras of the mineralocorticoid receptor (18), the GR (19), the thyroid hormone receptor (8), the proges-terone receptor (20), and the androgen receptor (21). We found earlier that the cytoplasmic portion of VDR-GFP also translocates into the nucleus in a ligand-dependent fashion (5, 6). The mechanisms controlling VDR translocation, however, re-

mean and S.D. of brightness values were calculated for each receptor, and cytoplasmic brightness values were calculated after background subtraction from 50 cells. The experiments were repeated at least four times.

Detection of Heterodimer Distribution in Living Cells—Protein–protein interactions of GFP-VDR and RXR-BFP were studied using FRET experiments. CV-1 cells were grown on 24.5-mm diameter coverslips and used for experiments. CV-1 cells were stably expressing GFP-VDR that was selected by culturing with G418 and used for experiments.

Point mutations were introduced into the coding sequences of the GFP-VDR, the YFP-RXR, and the RXR-BFP using the QuickChange mutagenesis kit (Stratagene). Up- and downstream oligonucleotides were designed according to manufacturer’s instructions. To create a GFP-VDR mutant impaired in nuclear localization (nlsGFP-VDR), we introduced point mutations in the NLS1 of VDR at amino acids 53–55 (K53Q, lysine to glutamate; R54G, arginine to glycine; K55E, lysine to glutamic acid) (7). To create the DNA binding mutant of GFP-VDR (dnGFP-VDR), we introduced a point mutation at amino acid 77 (R77Q). This mutation was found in a patient with hereditary resistance to calcitriol, and in vitro studies demonstrated that the mutation weakened DNA binding defect without altering hormone binding (29) or nuclear import (30). To create heterodimerization mutants of RXR-BFP (hdRXR-BFP), we mutated the amino acids 419 and 420 of the RXR from leucine to arginine, because these amino acids are crucial for the heterodimerization function of RXR (31). In all experiments, including the mutants, the coding sequences for the fusion proteins were confirmed using the ABI Prism sequencing kit (PerkinElmer).

Determinations of Receptor Distribution—Cells were transiently transfected with one or two plasmids using LipofectAMINE Plus reagents with 0.125 μg of DNA/chamber for each plasmid. Transfected cells were used for microscopy after 48 h. GL48 and CYR cells were subcultured into coverglass chamber slides (Nunc) 24 h before micro-

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I restriction sites of the 10C plasmid (a gift from J.

A krypton-argon laser with a band-pass 510–525-nm emission filter was used for GFP and YFP detection, and the 364-nm line of an UV

laser with a 397-nm long-pass emission filter was used for BFP detection.

For morphometric analysis, mean brightness values were obtained from images of cytoplasm, nucleus, and background. The ratios of nuclear and cytoplasmic brightness values were calculated after background subtraction from 50 cells. The experiments were repeated at least four times.

Construction of Expression Vectors and Generation of Stable Cell Lines—A transcriptionally active GFP-VDR was generated by cloning the human VDR cDNA (a gift from Dr. J. W. Pike, University of Cincinnati College of Medicine, OH) C-terminal to the GFP of the pQBI25 plasmid (Quantum Biotechnologies). 293 cells were transfected with this GFP-VDR expression plasmid using LipofectAMINE Plus reagents (Life Technologies) according to manufacturer’s instructions. G41S-resistant cell clones were then selected for 3–5 wk by culturing cells in media with 1 μg G418 (Life Technologies). One of the high-expressing clones, GL48, was characterized and used for subsequent experiments (49). The RXR-BFP was generated by cloning the human RXRα cDNA (RXR) (a gift from P. Chambon, CNRS/INSERM, Stras-

Determination of Receptor Distribution in Living Cells—Protein–protein interactions of GFP-VDR and RXR-BFP were studied using FRET experiments. CV-1 cells were grown on 24.5-mm diameter coverslips and used for experiments. Cells were subcultured into glass coverslip chambers (NUNC) 24 h before micros-

1.4 NA objective. The 488-nm line of an argon laser was used for GFP and YFP detection, and the 364-nm line of an UV laser with a 397-nm long-pass emission filter was used for BFP detection. For morphometric analysis, mean brightness values were obtained from images of cytoplasm, nucleus, and background. The ratios of nuclear and cytoplasmic brightness values were calculated after background subtraction from 50 cells. The experiments were repeated at least four times.

Detection of Heterodimer Distribution in Living Cells—Protein–protein interactions of GFP-VDR and RXR-BFP were studied using FRET experiments. CV-1 cells were grown on 24.5-mm diameter coverslips and used for experiments. Cells were subcultured into coverglass chamber slides (Nunc) 24 h before micros-

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VDR and the same plasmids in combinations with either RXR-BFP or hdRXR-BFP (0.3 μg/well each). These plasmids were cotransfected with the reporter plasmid p240H/Luc-23 (0.07 μg/well; a gift from H. F. DeLuca, University of Wisconsin, Madison, WI) and the β-galactosidase standardization plasmid pGL3 (0.07 μg/well; Promega). The p240H/Luc-23 encodes a luciferase gene that is under the control of the 25-hydroxyvitamin D3 24-hydroxylase promoter (32). DNA contents were equalized by the addition of herring sperm DNA (Roche Molecular Biochemicals). Transfected cells were incubated for 24 h with either calcitriol (Solvay Duphar) or vehicle in the media. Transcriptional activities of wild type and mutant RXR chimeras (0.3 μg/well) were also tested by a luciferase reporter assay using the retinoid X response element-Luc (0.07 μg/well; a gift from Dr. Karathanasis, Wyeth-Ayerst Research, NY). This retinoid X response element-Luc encodes a luciferase gene that is under the control of a minimal ApoAI enhancer (33).

RESULTS

RXR Expression and Subcellular Localization—To determine subcellular localization of RXR in living cells, we developed expression constructs encoding functional GFP chimeras of RXR. Both N-terminal and C-terminal chimeras were generated with the RXR and the yellow or blue fluorescing variants of GFP. Also, point mutations were introduced into the wild-type RXR-BFP to generate a heterodimerization mutant (hdRXR-BFP). To characterize these fusion proteins, we evaluated protein expression and transcriptional activities. We confirmed the expression of intact fusion proteins by Western blot analysis (not shown). In addition, fluorescence microscopy showed similar expression levels for the wild-type RXR-BFP and the hdRXR-BFP. We evaluated the functionality of the fusion proteins by luciferase reporter assays. The ligand 9c-RA (100 nM) caused an 8-fold induction of retinoid X response element-luciferase reporter activity by YFP-RXR, a 7-fold induction by RXR-BFP, and a 5-fold induction by the untagged RXR. As expected from a previous report (31), the mutant hdRXR-BFP displayed impaired transcriptional activity (2-fold induction). These results show that the tagged RXR proteins retained the ability of the untagged RXR to induce transactivation.

To evaluate subcellular distribution of YFP-RXR and RXR-BFP, chimeric proteins were transiently expressed in CV-1, COS-7, 293, and ROS cells. Microscopy showed the unliganded YFP-RXR and RXR-BFP predominantly in the nucleus of each cell type tested. The unliganded RXR distributed evenly within the nucleus excluding the nucleoli, as shown in ROS cells (Fig. 1a). Treatment with 9c-RA induced development of prominent intranuclear foci (Fig. 1b). In addition to the nuclear YFP-RXR, a sub-population of cells contained variable amounts of cytoplasmic YFP-RXR (Fig. 1c). Time-lapse recordings revealed that this cytoplasmic YFP-RXR does not translocate from the cytoplasm into the nucleus after hormone treatment (not shown). During our previous studies with transiently expressed GFP-GR and GFP-VDR, we noticed that overexpression of receptors can cause predominantly nuclear mislocalization. To confirm that nuclear localization of RXR is not due to protein overexpression, we generated a subclone of CV-1 cells that stably expresses the intact YFP-RXR (CYR) cells. Fig. 1d shows that the YFP-RXR signal is detectable in every CYR cell, predominantly within the nucleus. The addition of 9c-RA to CYR cells also induced intranuclear YFP-RXR redistribution (not shown), suggesting that overexpression does not contribute to foci formation. Taken together, our studies with Western blot analysis, microscopy, and transactivation assays demonstrate that YFP-RXR and RXR-BFP encode intact, functional, and fluorescing receptors. These receptors appear to reside in the cell nucleus and respond to hormone treatment by forming intranuclear foci.

VDR Expression and Subcellular Localization—We previously reported that VDR partitions between the cytoplasm and the nucleus without calcitriol and translocates into the nucleus after calcitriol treatment in experiments using a transiently expressed N-terminal GFP chimera of VDR (5). Here we generated a C-terminal fusion of VDR with GFP (GFP-VDR) and stably expressed it in 293 cells. One moderately expressing clone was selected for characterization (GL48). Western blot analysis of GL48 cell extracts confirmed the expression of intact GFP-VDR. The expression level of GFP-VDR was approximately twice the expression level of the endogenous VDR in ROS 17/2.8 cells (data not shown). Transactivation assay in transiently transfected COS-7 cells showed that the potency of calcitriol to induce transcription of the 24OH/Luc-23 reporter is the same in GFP-VDR and in VDR-expressing cells. The half-maximal effect was detected at 0.5 nM calcitriol, and the maximal induction was at 10 nM calcitriol (5-fold for GFP-VDR and 4-fold for VDR over vehicle-treated controls).

Microscopy showed that without hormone, GFP-VDR partitions between the cytoplasm and the nucleus of GL48 cells (Fig. 2a) and that after the addition of hormone (0.01–100 nM calcitriol), GFP-VDR localizes predominantly in the nucleus (Fig. 2b). The subcellular distribution of dnaGFP-VDR was indistinguishable from the distribution of wild-type GFP-VDR (Fig. 2, c–d), and calcitriol induced dnaGFP-VDR translocation from the cytoplasm into the nucleus. This translocation is probably not related to homodimerization and a “piggyback” mechanism (34), since CV-1 cells contain negligible amounts of endogenous VDR. We also generated a nuclear localization sequence mutant of the GFP-VDR (nlsGFP-VDR). The functional signifi-
The fluorescence intensity over cells expressing GFP-VDR alone were only approximately 20% of the intensities generated from FRET. Thus, we did not calculate ratios unless the cells expressed a blue protein. In cells expressing BFP or RXR-BFP, the blue signal intensities were high, whereas the green intensities remained low; the green intensities after background subtraction were only 40% of the intensities from FRET (Fig. 3c). As a result, ratio intensities from the bleed-through of BFP were 0.9 ± 0.02, and intensities from the bleed-through of RXR-BFP were 0.9 ± 0.1. To detect FRET signals from heterodimers, we generated ratio images from cells coexpressing GFP-VDR and RXR-BFP (Fig. 3a). Control ratio images were generated from cells coexpressing the GFP-VDR and the mutant hdRXR-BFP (Fig. 3b). The ratio values of the GFP-VDR/RXR-BFP-expressing cells were 1.7 ± 0.5 over the nuclei and 1.2 ± 0.2 over the cytoplasm. However, control ratio values of the GFP-VDR/hdRXR-BFP-expressing cells were significantly lower: 1.0 ± 0.2 over the nuclei (p < 0.025) and 0.9 ± 0.1 over the cytoplasm (p < 0.05). After a 30-min incubation with 50 nM calcitriol, heterodimers were no longer detectable in the cytoplasm. This finding suggests that the heterodimers translocate from the cytoplasm into the nucleus, which is similar to calcitriol-induced GFP-VDR translocation (Fig. 3d). These FRET experiments demonstrated that the tagged receptors heterodimerize in intact living cells and indicated that heterodimers are present in both the cytoplasm and nucleus with significantly more heterodimers present in the nucleus than in the cytoplasm. Most importantly, FRET revealed that calcitriol promotes nuclear redistribution of heterodimers.

**Heterodimerization between VDR and RXR Shifts VDR into the Nucleus**—The results obtained in living cells indicated that RXR and VDR/RXR heterodimers localize predominantly in the nucleus. There was no apparent change in RXR-BFP distribution when it was coexpressed with GFP-VDR in CV-1 cells. However, GFP-VDR was more nuclear when coexpressed with RXR-BFP. To explore the possibility that heterodimerization with RXR modifies VDR localization in more detail, we studied the effect of RXR-BFP expression on GFP-VDR localization in GL48 cells. Multi-channel confocal microscopy of living GL48 cells showed steady-state GFP-VDR distribution in the green channel and RXR-BFP distribution in the blue channel. Without RXR, the unliganded GFP-VDR is unevenly distributed between cytoplasm and nucleus (Fig. 2). When these cells were transiently transfected with RXR-BFP, 30% of cells expressed the blue-tagged RXR (Fig. 4b). In those cells expressing RXR-BFP (arrows), 69 ± 7% GFP-VDR accumulated in the nucleus, whereas in the nontransfected cells 46 ± 7% of total GFP-VDR signal was in the nucleus (p < 0.0001) (Fig. 4a). This effect of RXR-BFP to induce a nuclear shift of GFP-VDR could have been the result of heterodimerization, but several other nonspecific effects had to be considered. To test the possibility that nonspecific protein overexpression caused mislocalization of VDR, we transfected GL48 cells with large amounts of BFP. This experiment showed that overexpression of the BFP protein does not influence VDR distribution (Fig. 4, c-d) and clarified that the more intense nuclear GFP-VDR signal does not derive from a bleed-through of the BFP signal. Another possi-
CV-1 cells were cotransfected with GFP-VDR and RXR-BFP (a and d), GFP-VDR and hdRXR-BFP (b), or with BFP alone (c). Representative ratio images are shown from FRET experiments, with a color table that was applied to show intensity differences. Ratio images were generated from the images taken with a 370–390-nm excitation filter and with either a 515–555-nm or a 435–485-nm emission filter as indicated. Ratio image of cells expressing both wild type receptors show energy transfer between the RXR-BFP and the GFP-VDR. The ratios in the green range indicate heterodimers; most of them are in the nuclei, and a few of them are in the cytoplasm (a). The ratio image from cells expressing the GFP-VDR and the hdRXR-BFP shows ratio values in the blue range because the BFP emission is stronger than the GFP emission (b). The ratio image from cells expressing only the BFP shows similar ratio values (c). After treating the GFP-VDR/RXR-BFP-expressing cells with 10 nM calcitriol, ratio images indicate the strongest green signal for heterodimers within intranuclear foci (d). Bar, 10 μm.

Fig. 3. FRET shows VDR and RXR heterodimers in the nucleus and in the cytoplasm. CV-1 cells were cotransfected with GFP-VDR and RXR-BFP (a and d), GFP-VDR and hdRXR-BFP (b), or with BFP alone (c). Representative ratio images are shown from FRET experiments, with a color table that was applied to show intensity differences. Ratio images were generated from the images taken with a 370–390-nm excitation filter and with either a 515–555-nm or a 435–485-nm emission filter as indicated. Ratio image of cells expressing both wild type receptors show energy transfer between the RXR-BFP and the GFP-VDR. The ratios in the green range indicate heterodimers; most of them are in the nuclei, and a few of them are in the cytoplasm (a). The ratio image from cells expressing the GFP-VDR and the hdRXR-BFP shows ratio values in the blue range because the BFP emission is stronger than the GFP emission (b). The ratio image from cells expressing only the BFP shows similar ratio values (c). After treating the GFP-VDR/RXR-BFP-expressing cells with 10 nM calcitriol, ratio images indicate the strongest green signal for heterodimers within intranuclear foci (d). Bar, 10 μm.

### RXR Inhibits VDR Export

We noticed during microscopy that after keeping GL48 cells at room temperature, GFP-VDR moves out of the nucleus into the cytoplasm (Fig. 5a–b). This temperature sensitivity was present in other cell types but to a lesser extent. We took advantage of this temperature-dependent export to evaluate the ability of RXR to promote nuclear retention of VDR. We incubated GL48 cells for 2 h at either 37 °C or at 22 °C before microscopy and maintained these conditions during microscopy. As expected, GFP-VDR moved out from the nucleus and accumulated in the cytoplasm when GL48 cells were incubated at 22 °C for 2 h (Fig. 5a–b). This redistribution was specific for VDR, as YFP-RXR was not exported at 22 °C (Fig. 5c–d). GFP-VDR remained functional at this temperature, as the addition of 10 nM calcitriol induced a complete translocation of cytoplasmic GFP-VDR within 2 h at 22 °C (not shown). It was also reversible; increasing the temperature to 37 °C restored nuclear localization. RXR-BFP and GFP distribution did not change when the temperature was lowered (not shown). Then we transfected GL48 cells with RXR-BFP and compared temperature sensitivity of GFP-VDR distribution in RXR-BFP-expressing and nontransfected cells. GFP-VDR remained in the nucleus after lowering the temperature in cells expressing both proteins, but GFP-VDR moved into the cytoplasm in the nontransfected cells (Fig. 5c–f). Morphometric analysis confirmed that RXR-BFP inhibits temperature-dependent GFP-VDR export. Without RXR-BFP, the nuclear/cytoplasmic ratios of GFP-VDR signal intensities in GL48 cells were 1.28 ± 0.03% at 37 °C and 0.86 ± 0.01 at 22 °C (p < 0.01). Nuclear/cytoplasmic ratios of the YFP-RXR in the CYR cells did not decrease after lowering the temperature (6.3 ± 1.1 at 37 °C and 8.1 ± 0.36 at 22 °C). When GL48 cells express RXR-BFP, VDR ratios were 5.1 ± 0.49 at 37 °C and 3.8 ± 0.49 at 22 °C (p = 0.167). This change is smaller than the change without RXR-BFP and suggests that nuclear retention of VDR by RXR could contribute to the nuclear accumulation.

### RXR Promotes VDR Import

To test the ability of RXR to influence nuclear import, we coexpressed the nlsGFP-VDR and the RXR-BFP in CV-1 cells. Multi-channel confocal microscopy showed that the nlsGFP-VDR remained mostly in the cytoplasm of cells, which did not express RXR-BFP but became nuclear in cells that expressed the RXR-BFP (Fig. 6a–b). The ability of RXR-BFP to shift the nlsGFP-VDR into the nucleus was also reproducible in COS-7 and 293 cells (not shown). To test the role of heterodimerization, we expressed the nlsGFP-VDR together with the heterodimerization mutant hdRXR-BFP. Microscopy showed that the hdRXR-BFP has no effect on the nlsGFP-VDR distribution, as the nlsGFP-VDR remained in the cytoplasm in both the hdRXR-BFP-expressing and nonex-
pressing cells (Fig. 6, c–d). Thus, the effect of RXR on VDR localization requires an intact heterodimerization function of RXR.

Next, we studied the effect of RXR on the calcitriol-induced translocation of GFP-VDR. Because in RXR-BFP-expressing cells most of the GFP-VDR is already in the nucleus without the hormone, the effect of calcitriol was not easily noticeable. However, RXR-BFP caused only a partial translocation of the nlsGFP-VDR without the hormone (Fig. 2, e–f), thus allowing for the detection of calcitriol-induced translocation. Microscopy showed that RXR-BFP restored the ability of calcitriol to induce translocation of the nlsGFP-VDR into the nucleus (Fig. 6). The mutant hdRXR-BFP was ineffective in rescuing the calcitriol-induced translocation of the nlsGFP-VDR. Morphometric analysis of these images showed that without the presence of RXR, calcitriol caused a minimal nuclear shift of nlsGFP-VDR ($p = 0.06$) (Fig. 7). In RXR-coexpressing cells, calcitriol caused a significant shift of the nlsGFP-VDR into the nucleus ($p < 0.0001$) (Fig. 7). Taken together, these data suggest that dimerization of RXR with VDR facilitates VDR nuclear import.

**RXR Influences Calcitriol-dependent Intranuclear Redistribution of VDR**—The addition of calcitriol to GL48 cells induced formation of multiple small intranuclear foci (Fig. 8). We have detected similar intranuclear VDR-GFP foci formation after hormone treatment (5). However, the addition of calcitriol did not cause such focal nuclear accumulation of the dnaGFP-VDR and the nlsGFP-VDR (Fig. 8, b and c). Coexpression of RXR-BFP restored the ability of calcitriol to induce foci formation of nlsGFP-VDR (Fig. 8d). In contrast, coexpression of RXR-BFP with the dnaGFP-VDR did not allow calcitriol-dependent intranuclear redistribution (not shown). These results indicate an association between the lack of foci formation and the lack of DNA binding. The effect of RXR on foci formation could be interpreted as an increase of DNA binding.

FRET experiments showed that calcitriol also induces intranuclear redistribution of VDR/RXR heterodimers. The ratio image (Fig. 3d) showed that FRET is strongest in the foci. There were no focal intranuclear ratio increases in the hormone-treated cells expressing GFP-VDR/dRXR-BFP (data not shown). Because previous studies showed that RXR and VDR bind to DNA as heterodimers, the hormone-induced heterodimer accumulation within discrete nuclear foci could signify receptor binding to DNA.

**RXR Effect on VDR Distribution Is Functionally Significant**—To explore the physiological importance of RXR-BFP effect on GFP-VDR localization, we performed transactivation
assays using a suitable vitamin D response element-luciferase reporter system (24OH/Luc). This 24-hydroxylase promoter contains vitamin D response element sequences separated by three nucleotides (DR3), which typically bind VDR/RXR heterodimers. Moreover, previous studies indicated that coexpression of RXR stimulates basal transcription of this reporter by VDR (13). First, we compared basal transcriptional activities in GL48 cells transfected with increasing concentrations of RXR-BFP. Transfection with 0.003 µg/well RXR-BFP plasmid resulted in a 2.7-fold increase and, with 0.02 µg/well RXR-BFP, in a 3.6-fold increase in basal transcriptional activity compared with activities from GL48 cells transfected with the reporter plasmid and herring sperm DNA. We then compared the luciferase activities of extracts from CV-1 cells that were transfected with 24OH/Luc, GFP-VDR, and RXR-BFP with activities

![Image](http://example.com/image1.png)

**FIG. 6.** RXR-BFP brings nlsGFP-VDR into the nucleus. Representative images are shown from dual channel confocal microscopy experiments, with the GFP channel shown in green and the BFP channel shown in blue. CV-1 cells were transfected with nlsGFP-VDR together with either RXR-BFP (a–b) or hdRXR-BFP (c–d). The nlsGFP-VDR accumulated in the nucleus in every cell that also expressed the RXR-BFP (arrow) (a). In the nontransfected cells the nlsGFP-VDR remained in the cytoplasm (such as the cell that is not marked by an arrow in the lower left corner of panel a). The hdRXR-BFP did not influence the nlsGFP-VDR distribution (c).

![Image](http://example.com/image2.png)

**FIG. 7.** Morphometric analysis demonstrates that RXR-BFP promotes calcitriol-dependent translocation of nlsGFP-VDR. CV-1 cells were transiently transfected with expression plasmids and exposed to calcitriol for 1 h before microscopy. Images were taken from vehicle-treated (open bars) and calcitriol-treated (shaded bars) living cells. The ratios of nuclear and cytoplasmic brightness values were calculated as described under “Experimental Procedures.” RXR-BFP increased the nuclear GFP-VDR levels both in the presence and absence of calcitriol and restored the ability of calcitriol to shift the nlsGFP-VDR into the nucleus. Data represent mean ± 1 S.E.

![Image](http://example.com/image3.png)

**FIG. 8.** RXR-BFP promotes calcitriol-dependent intranuclear foci formation of nlsGFP-VDR. CV-1 cells were transfected with GFP-VDR (a), dnaGFP-VDR (b), nlsGFP-VDR (c) or cotransfected with nlsGFP-VDR and RXR-BFP (d). Cells were treated with calcitriol for 1 h before microscopy. Confocal images were taken from living cells. Hormone treatment resulted in a nuclear accumulation of the wild-type GFP-VDR and the formation of small bright foci within the nucleus (a). After calcitriol, the dnaGFP-VDR and the nlsGFP-VDR did not form intranuclear foci (b–c). Cotransfection of RXR-BFP restored the ability of calcitriol to induce intranuclear nlsGFP-VDR foci formation (d). Bar, 10 µm.
RXR Effect on VDR Distribution

**Fig. 9.** RXR-BFP changes basal and calcitriol-induced transcriptional activities of wild type, nlsGFP-VDR, and dnaGFP-VDR. CV-1 cells were cotransfected with the 24OH/Luc-23 reporter and the expression plasmids as described under “Experimental Procedures.” Luciferase activities of cells vehicle treated are shown as open bars, and those treated with calcitriol are shown as shaded bars. Activities of the wild type (first group), nlsGFP-VDR (second group) and dnaGFP-VDR (third group) are shown without coexpression of RXR (A) and with coexpression of RXR (B). Luminescence values were normalized with β-galactosidase activities, and data are expressed as mean ± S.E. Basal transcriptional activity of the GFP-VDR increased 2.5-fold by RXR-BFP coexpression. RXR-BFP expression caused a similar increase in the basal activity for the nlsGFP-VDR and the dnaGFP-VDR. RXR-BFP rescued ligand-dependent transcriptional activity of the nlsGFP-VDR. The calcitriol-induced transcriptional activity of the dnaGFP-VDR was only slightly increased.

from cells that were transfected with 24OH/Luc, GFP-VDR, and hdRXR-BFP. The coexpression of RXR-BFP with GFP-VDR increased basal transcription by 2.5-fold (Fig. 9, A–B, open bars). In contrast, the coexpression of the hdRXR-BFP had a moderate inhibitory effect (not shown), possibly due to a dominant negative effect on the endogenous RXR (35). Similar experiments were carried out with the nlsGFP-VDR. RXR-BFP coexpression increased reporter activity 4.2-fold (Fig. 9, A–B nlsGFP-VDR, open bars), whereas the coexpression of the hdRXR-BFP had no effect (not shown). This effect of RXR to increase basal transcription correlated with our observation that RXR increases nuclear import of VDR. The connection is further supported by the correlation between the cytoplasmic localization and the 3-fold lower basal transcriptional activity of the nlsGFP-VDR compared with GFP-VDR. These results all suggest that heterodimerization with RXR increases the hormone-independent transcriptional activity of VDR by facilitating its hormone-independent nuclear import.

We used similar cotransfection assays to test if this shift in VDR localization by RXR influences hormone-dependent functions of VDR. Calcitriol (10 nm) addition for 24 h induced a 15-fold increase in reporter activity compared with vehicle-treated controls in GFP-VDR-expressing cells (Fig. 9A, GFP-VDR, shaded bar). Coexpression of RXR did not influence calcitriol-induced maximal luciferase activity by GFP-VDR (Fig. 9B, GFP-VDR, shaded bar). Calcitriol did not increase luciferase activity by the nlsGFP-VDR (Fig. 9A, nlsGFP-VDR, shaded bar). Coexpression of RXR-BFP, however, completely restored the calcitriol-dependent transcriptional activity of the nlsGFP-VDR (Fig. 9B, nlsGFP-VDR, shaded bar). Coexpression of the hdRXR-BFP with the nlsGFP-VDR failed to increase calcitriol-dependent transcriptional activity (not shown). Because mutations of the nlsGFP-VDR also impair DNA binding, we tested the effect of RXR-BFP coexpression on the transcriptional activity of another DNA binding mutant of GFP-VDR (dnaGFP-VDR). Calcitriol had no effect on transcriptional activity of dnaGFP-VDR (Fig. 9A, dnaGFP-VDR), and RXR-BFP coexpression caused only a small increase in the calcitriol-dependent transcriptional activity by the dnaGFP-VDR (Fig. 9B, dnaGFP-VDR).

Collectively, these assays reveal that the effects of RXR on nuclear VDR accumulation and intranuclear reorganization correlate with the effects of RXR on VDR transcriptional activity. By bringing the unliganded GFP-VDR into the nucleus, RXR increases basal activity, and by bringing the nlsGFP-VDR into the nucleus, RXR rescues its hormone-dependent transcriptional activity.

**DISCUSSION**

Our experiments with green and blue fluorescent chimeras of the receptors and FRET microscopy provided the initial evidence for the distribution of VDR/RXR heterodimers in living cells. Confocal microscopy also allowed us to recognize that dimerizing RXR facilitates nuclear accumulation of VDR. In addition, we observed that VDR, RXR, and their respective heterodimers all responded to hormone by forming intranuclear bright foci. Mutational analysis showed a correlation between receptor distribution and transcriptional activity and between nuclear foci formation and DNA binding.

At first, we generated N-terminal-tagged GFP-VDR and YFP-RXR and a C-terminal-tagged RXR-BFP expression plasmids. The transcriptional activities of the tagged receptors were confirmed by luciferase reporter assays, and the expression of the correct size proteins were confirmed by Western blot analysis. We also cloned cell lines to stably express the GFP-VDR (GL48) and the YFP-RXR (CYR) to establish reproducible systems for the investigation of hormone-independent and hormone-dependent receptor trafficking. Previously, we and others used alternate GFP chimeras of VDR to show hormone-dependent translocation from the cytoplasm into the nucleus (5, 6). Confocal microscopy on GL48 cells confirmed our previous findings on the steady-state subcellular distribution of VDR by
showing that unliganded GFP-VDR remained evenly distributed between the cytoplasm and nucleus (Fig. 2).

Our finding on the steady-state nuclear localization of YFP-RXRA in living cells was expected from previous immunolocalization studies, and it is similar to the nuclear localization of the transiently expressed GFP-RXRβ in PC12 cells (36). In contrast, the presence of YFP-RXR in the cytoplasm is new and suggests that heterodimers of VDR and RXR could also form in the cytoplasm (Fig. 1). Furthermore, the difference between the steady-state localization of RXR and VDR raised the possibility that heterodimerization could influence the distribution of either VDR or RXR.

FRET microscopy has not been used extensively in the nuclear receptor field. Here, utilizing a heterodimerization mutant of RXR as a control, we located dimers of RXR-BFP and GFP-VDR for the first time in intact living cells. These FRET studies showed that the tagged receptors are competent to form heterodimers; therefore, they were suitable for studies on the impact of dimerization on subcellular trafficking. FRET results demonstrated that most of the VDR formed dimers with the RXR without the ligand and that these heterodimers were predominantly in the nucleus in a diffuse pattern excluding the nucleoli.

Our experiments showed that the dimerization-competent RXR can shift the VDR from the cytoplasm into the nucleus, but the dimerization-deficient RXR is ineffective. This effect of RXR on VDR distribution could involve changes in either VDR export or import across the nuclear envelope. We tested the effect of RXR on VDR export by taking advantage of the differences in their temperature sensitivity. Lowering the temperature of GL48 cells induced export of GFP-VDR into the cytoplasm, but GFP-VDR/RXR-BFP heterodimers were less sensitive to temperature change and remained in the nucleus (Fig. 5). This finding suggests that the export mechanisms for the heterodimer differ from that of VDR. It is possible that export receptors are involved because both VDR and RXR have leucine-rich sequences, which could serve as nuclear export signal (37). Mutational analysis of these GFP-tagged receptors opens several potential avenues for the investigations of these export mechanisms.

We also found that RXR influences nuclear import of VDR. This is most apparent in our experiments with the nlsGFP-VDR, which harbors a mutation in one of the nuclear localization signals of the VDR (7). This mutation severely compromised nuclear import of VDR but did not completely abolish it. The remaining import is likely to be mediated by other domains of the VDR, such as the one suggested within the hinge region (24). However, we did not find any defect in nuclear import when we deleted this region of GFP-VDR.2 The ability of RXR to bring the NLS mutant GFP-VDR into the nucleus can be explained by a piggyback mechanism. Such a mechanism was reported to bring the NLS mutant of progesterone receptor into the nucleus by homodimerizing wild-type receptors (38). The effect of RXR could also be indirect; RXR could weaken the VDR interactions with cytoplasmic docking sites, as suggested by a recent report showing that RXR increases the solubility and perhaps assists the folding of the retinoic acid receptor in Escherichia coli (39). Finally, RXR could also act by complexing with Hsp70 and Hsp90 chaperones (40). Interestingly, RXR not only influenced the steady-state distribution of the nlsGFP-VDR but also restored the effect of calcitriol to induce a rapid nuclear accumulation of the mutant receptors (Figs. 6 and 7). This effect is unexpected and implies a role for RXR in the regulation of VDR nuclear import.

We found a strong correlation between the ability of RXR to shift VDR into the nucleus and to regulate its transcriptional activity. First, the increase in the nuclear VDR content correlated with an increase in basal transcriptional activity by VDR both in GL48 and CV-1 cells. This hormone-like effect of RXR is consistent with the phantom ligand effect of RXR described earlier (41). This effect on basal activity may be physiologically important, since previous studies indicate that the unliganded VDR can activate several target genes (42, 43). Second, RXR facilitated hormone-independent nuclear localization of the nlsGFP-VDR and increased basal transcriptional activity by the nlsGFP-VDR. Third, RXR restored the ability of the nlsGFP-VDR to accumulate in the nucleus after calcitriol addition and to activate the reporter gene after calcitriol addition. This indicates that the regulation of VDR nuclear import by RXR may play a role in hormone-dependent transcriptional regulation. Such regulation could explain how, in some target tissues, nuclear accumulation of VDR is a general mechanism that controls the activity of many transcriptional regulators.

Regulation of subnuclear trafficking can be one of the ways RXR influences the speed and specificity of transcriptional activities. Here we found that hormone induces YFP-RXR and GFP-VDR to accumulate in foci (Figs. 1 and 2). Our earlier finding that a DNA binding mutant of GFP-GR failed to form foci (46) and our present finding that the DNA binding mutant of GFP-VDR also fails to form nuclear foci suggest that these foci could signify receptor binding to DNA target sites. This notion is further supported by the FRET experiments, which show that calcitriol treatment causes focal accumulation of heterodimers in the nucleus (Fig. 3). The use of the heterodimerization mutant of RXR-BFP for control makes this argument even more powerful. Moreover, coexpression of RXR-BFP restored the ability of the nlsGFP-VDR to form foci and activate transcription after calcitriol treatment (Figs. 7 and 9). The dimerization-deficient form of RXR-BFP had no effect. This observation is in agreement with earlier findings on the effect of calcitriol to promote binding of VDR/RXR heterodimers to vitamin D response elements (47, 48) and suggests that RXR could promote DNA binding by influencing intranuclear targeting of VDR. A local change in receptor dynamics could account for the calcitriol-induced accumulation of heterodimers in discrete subnuclear regions and warrant further investigations by photobleaching techniques.

Multicolor fluorescent protein tagging and advanced microscopy methods allowed us to visualize subcellular distribution of VDR, RXR, and their respective heterodimers in living cells. These studies revealed that RXR heterodimerization plays a key role in the translocation and nuclear targeting of VDR, which could explain tissue-specific differences in VDR distribution observed previously by immunocytochemistry (25). We also visualized the ligand-dependent interaction of heterodimers with possible DNA binding sites by FRET imaging and found a correlation between intranuclear foci formation and transcriptional activity by mutational analysis. Mutational analysis also indicated that nuclear targeting is independent of DNA binding. As the RXR forms heterodimers with other nuclear receptors and associates with coactivators and corepressors, RXR could also regulate the activity of other partners by regulating subcellular trafficking. The techniques described here open the possibility of exploring the location and kinetics for a multitude of regulatory protein interactions and exploring the mechanisms of transcriptional regulation in living cells.

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