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Development of Novel Bioluminescent Sensor to Detect and Discriminate between Vitamin D Receptor Agonists and Antagonists in Living Cells

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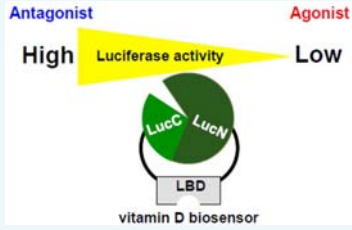
ABSTRACT: Active forms of vitamin D regulate the expression of multiple genes that play essential roles in calcium and phosphate homeostasis, cell differentiation, and the immune system via the vitamin D receptor (VDR). Many vitamin D analogs have been synthesized for clinical use in the treatment of type I rickets, osteoporosis, renal osteodystrophy, psoriasis, leukemia, and breast cancer. We have constructed two fusion proteins containing split-luciferase and the ligand binding domain (LBD) of the VDR designated as LucN-LBD-LucC and LucC-LBD-LucN. Remarkably, the LucC-LBD-LucN, which has the C-terminal domain of luciferase at the N-terminus of the fusion protein, was a significantly better biosensor than LucN-LBD-LucC. Addition of the VDR agonists to COS-7 cells expressing LucC-LBD-LucN dramatically reduced luciferase activity. In contrast, the VDR antagonist significantly increased the chimeric luciferase activity in a dose- and time-dependent manner. Our results on chimeric luciferases containing the LBDs of mutant VDRs derived from patients with vitamin D-dependent type II rickets indicated that our system could detect a conformational change of the LBD of the VDR likely based on a positional change of the helix 12, which occurs upon ligand binding. This novel system to detect and discriminate between VDR agonists and antagonists could be useful for the screening and identification of chemical compounds that bind to normal or mutant VDRs with high affinity.



INTRODUCTION

Vitamin D_3 is initially converted into 25(OH)D_3 in the liver, and then 25(OH)D_3 is further converted into a functionally active form, 1α,25(OH)$_2D_3$, in the kidney.[1] The resultant 1α,25(OH)$_2D_3$ regulates expression of multiple genes which play essential roles in bone formation, calcium and phosphate homeostasis, cell differentiation, and the immune system via binding to the vitamin D receptor (VDR).[2−5] The human VDR is a member of a family of 48 nuclear receptors that function as classical endocrine receptors. The most biologically active vitamin D metabolite, 1α,25(OH)$_2D_3$ binds tightly to the VDR.

VDR contains a DNA-binding domain of 66 amino acids at its amino terminus, and a ligand-binding domain (LBD) of approximately 300 amino acids at its carboxy terminus. The LBD consists of 12 α-helices, and the most carboxy-terminal α helix (helix 12) localizes closely to the ligand-binding pocket and plays an essential role in ligand binding.[6,7] In addition, the position and conformation of the helix 12 is involved in the interaction of the LBD with a variety of coactivator proteins such as SRC-1, TIF2, and RAC3, and corepressor proteins such as NCoR and SMRT.[8,9]

More than 1000 vitamin D analogs have been synthesized and characterized for potential clinical use in the treatment of diseases such as type I rickets, osteoporosis, psoriasis, leukemia, and breast cancer.[10] Many useful analogs with high potency or selective activity have also been developed. For example, eldecalcitol (ED-71), a 2β-hydroxylpropoxylated analog of the active form of vitamin D_3 (1α,25(OH)$_2D_3$) was approved as a new drug for the treatment of osteoporosis in Japan in 2011.[11−13] 22-Oxa-1α,25(OH)$_2D_3$ (OCT), which shows more potent activity in cell differentiation than in calcemic effect, has been clinically used as a drug for secondary hyperparathyroidism and psoriasis.[14]

Several different systems evaluating the affinity of ligands for the VDR have been constructed. In vitro assay systems using VDR itself are useful to directly determine the affinity of a compound for the VDR.[15] However, this system might select poorly cell-permeable and/or cytotoxic compounds as good ligands for the VDR. A commercially available VDR luciferase reporter assay system consists of a plasmid containing the luciferase reporter gene under the control of a potent promoter containing a vitamin D responsive element (VDRE).[16−18] Thus, this construct can monitor both increases and decreases in the transcriptional activity of the VDR. However, this system might select compounds that affect interactions between VDR and retinoid X receptor (RXRα) or coactivators or

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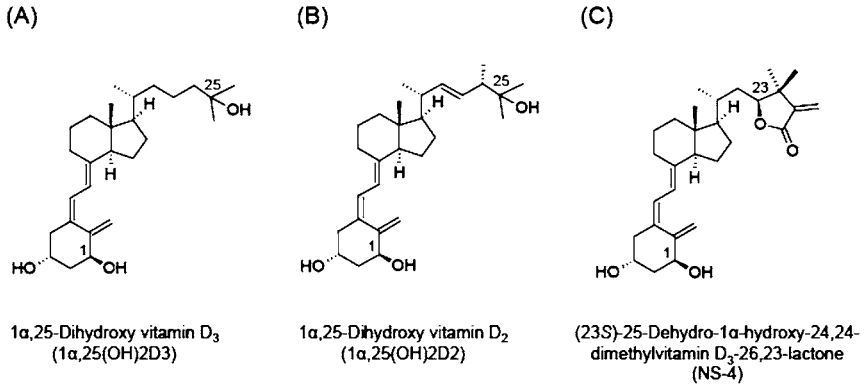

Figure 1. Chemical structures of VDR agonists $1\alpha,25(OH)_2D_3$ (A) and $1\alpha,25(OH)_2D_2$ (B), and a VDR antagonist NS-4 (C).

corepressors. In this study, we have constructed a highly sensitive and quantitative system to screen compounds that bind to VDR with high affinity in tissue culture cells, without cytotoxicity. In addition, our system can monitor the conversion of vitamin D into its active form, as well as inactivation of the active form in living cells.

Split-luciferase complementation assay systems have been developed to study protein−protein interactions and protein−nucleic acid interactions.[19,20] These systems can also be used as intramolecular folding sensors. Ataei et al. generated a sensor to detect inositol 1,4,5-trisphosphate (IP_3) using split luciferase fragments on either side of IP_3-binding core (IBC) domain of the IP_3-receptor.[21] Paulmurugan and Gambhir constructed a novel sensor system to distinguish estrogen receptor (ER)-agonists, antagonists, and partial agonist/antagonists using a fusion protein consisting of the N-terminal domain of luciferase (LucN), the ER-ligand binding domain (LBD), and the C-terminal domain of luciferase (LucC).[22] This system is based on a distinct conformational change of helix 12 of the ER when each of agonist, antagonist, or partial agonisit/antagonist is bound. Thus, we have constructed the fusion proteins LucN-VDR(LBD)-LucC and LucC-VDR(LBD)-LucN in an attempt to generate a highly sensitive and quantitative system to screen VDR ligands including agonists and antagonists.

RESULTS

Design of a Split Luciferase Biosensor to Detect VDR Ligands. The LBD of hVDR is composed of 12 α-helices (aa 121−427). When the ligand binds to the LBD, a conformational change of the helix-12 is believed to occur, triggering interaction with RXRα and cofactors. Based on this assumption, we designed split luciferase biosensor proteins to detect VDR ligands such as $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_2$ (Figure 1A,B).

The LucN (aa 1−415) and LucC (aa 416−550) domains of firefly luciferase were fused to the N and C terminus of the LBD, respectively. The split site of luciferase was determined as described in the previous report.[23] Since the reverse construct having LucC at the N-terminus and LucN at the C-terminus might function better,[24] we constructed both types of biosensors named LucN-LBD-LucC (normal type) or LucC-LBD-LucN (reverse type) (Figure 2A,B).

Expression of LucN-LBD-LucC or LucC-LBD-LucN in COS-7 Cells and Comparison of Their Function. LucN-LBD-LucC or LucC-LBD-LucN was expressed in COS-7 cells, and the transfected cells were treated with either 10 or 100 nM of $1\alpha,25(OH)_2D_3$, or with ethanol (EtOH) as a negative

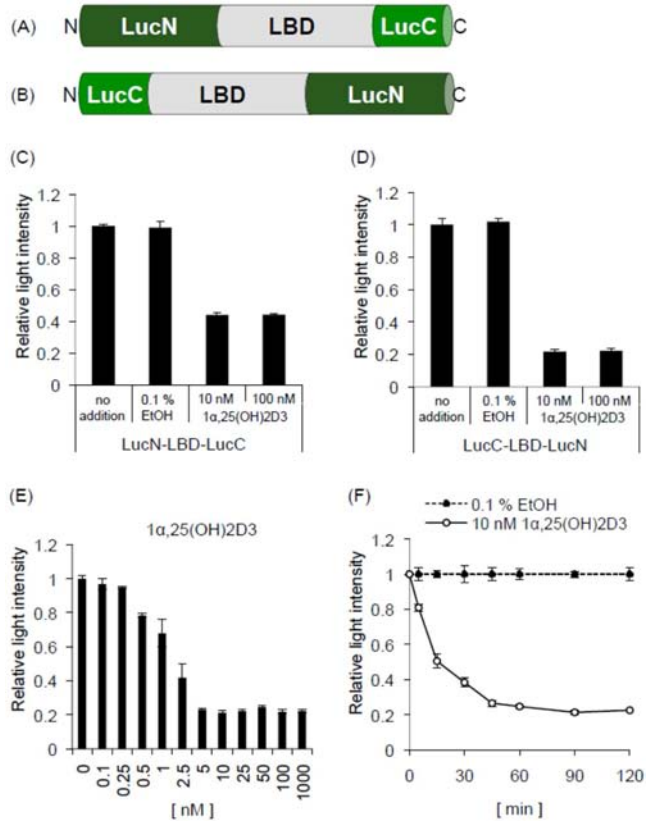

Figure 2. Schematic structures and characteristics of vitamin D biosensors. (A,B) LucN (aa 1−415) or LucC (aa 416−550) domain of the luciferase was fused to the N-terminal or C-terminal of ligand binding domain (LBD) (aa 121−427) of VDR. Each protein was expressed in COS-7 cells under the control of the CAG promoter. (C,D) COS-7 cells were transfected with the plasmid encoding LucN-LBD-LucC or LucC-LBD-LucN, and treated with 10 nM or 100 nM of $1\alpha,25(OH)_2D_3$ for 90 min. The relative light intensity compared to the control (designated as no addition) was shown. Data are represented as mean ± SEM, n = 4. (E) The dose- and (F) time-dependent relative light intensity of LucC-LBD-LucN in COS-7 cells were shown. The COS-7 cells expressing LucC-LBD-LucN were treated with various concentrations (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, or 1000 nM) of $1\alpha,25(OH)_2D_3$ for 90 min (E). The COS-7 cells expressing LucC-LBD-LucN were treated with 10 nM $1\alpha,25(OH)_2D_3$, and the luminescence was measured at 0, 5, 15, 30, 45, 60, 90, and 120 min after addition of $1\alpha,25(OH)_2D_3$ (F). Data are represented as mean ± SEM, n = 4.

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control. At 90 min after addition of 0.1% EtOH, no significant change in light intensity was observed for LucN-LBD-LucC or LucC-LBD-LucN. However, addition of $1\alpha,25(OH)_2D_3$ decreased the light intensity of LucN-LBD-LucC and LucC-LBD-LucN to approximately 40% and 20%, respectively (Figure 2C,D). These results suggest that these chimeric luciferases lose activity with the addition of $1\alpha,25(OH)_2D_3$, probably due to its binding to the LBD. In addition, the luciferase activity of LucC-LBD-LucN deceased more upon binding ligand than did LucN-LBD-LucC, indicating that the former is more useful for detecting VDR ligands. Therefore, the LucC-LBD-LucN construct was used in subsequent experiments. The light intensity in the COS-7 cells expressing LucC-LBD-LucN was approximately 5% of that in the COS-7 cells expressing wild type luciferase.

Dose- and Time-Dependent Responses of LucC-LBD-LucN. Dose- and time-dependent responses of LucC-LBD-LucN were examined in tissue culture cells. The COS-7 cells expressing LucC-LBD-LucN were treated with various concentrations (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, or 1000 nM) of $1\alpha,25(OH)_2D_3$ for 90 min. As shown in Figure 2E, $1\alpha,25(OH)_2D_3$ induced a dose-dependent decrease in the relative light intensity. Minimal light intensity was observed at 5 nM, and no further decrease was observed upon treatment with higher concentrations of $1\alpha,25(OH)_2D_3$ (Figure 2E). To examine a time course of the relative light intensity, COS-7 cells expressing LucC-LBD-LucN were treated with 10 nM $1\alpha,25(OH)_2D_3$, and the luminescence was measured at 0, 5, 15, 30, 45, 60, 90, and 120 min after addition of $1\alpha,25(OH)_2D_3$ (Figure 2F). The relative light intensity was decreased linearly for 30 min, reached to almost the lowest point the bottom at 60 min, with no further change after 90 min. Thus, we chose a 90 min time point in the following experiments.

Effects of Vitamin D-Related Compounds on the Activity of LucC-LBD-LucN. As $1\alpha,25(OH)_2D_2$ is known to be another active form of vitamin D, effects of various types of natural vitamin D on the activity of LucC-LBD-LucN were examined. The COS-7 cells expressing LucC-LBD-LucN were treated with various concentration of VD_3, $25(OH)D_3$, VD_2, $25(OH)D_2$, or $1\alpha,25(OH)_2D_2$ for 90 min. Although VD_3 and VD_2 did not change the light intensity in each concentration (Figure 3A,B), $25(OH)D_3$ and $25(OH)D_2$ decreased the light intensity in a dose-dependent manner (Figure 3C,D). The relative light intensity was decreased to approximately 20% with the addition of high concentration (500 to 10000 nM) of $25(OH)D_2$ or $25(OH)D_3$. Moreover, $1\alpha,25(OH)_2D_2$ decreased the relative light intensity to approximately 20% in a dose-dependent manner as did $1\alpha,25(OH)_2D_3$ (Figure 3E). Because $25(OH)D_2$ and $25(OH)D_3$ are known to be low affinity VDR ligands,[25,26] LucC-LBD-LucN should be useful for screening low affinity VDR ligands.

Effects of $1\alpha,25(OH)_2D_3$ on the Luciferase Activity and the Expression Level of LucC-LBD-LucN. It might be possible that the activity loss of LucC-LBD-LucN originates from $1\alpha,25(OH)_2D_3$-induced degradation of LucC-LBD-LucN. To examine the effect of the VDR ligand on luciferase enzyme activity and protein expression levels, COS-7 cells expressing wild-type luciferase (Luciferase) proteins were treated with 0.1% EtOH or 10 nM $1\alpha,25(OH)_2D_3$ for 90 min. Figure 4 shows light intensity in the absence or presence of $1\alpha,25(OH)_2D_3$ in the COS-7 cells expressing luciferase itself, LucC-LBD-LucN, LucC-LBD(R274L)-LucN, or LucC-LBD(R391L)-LucN.

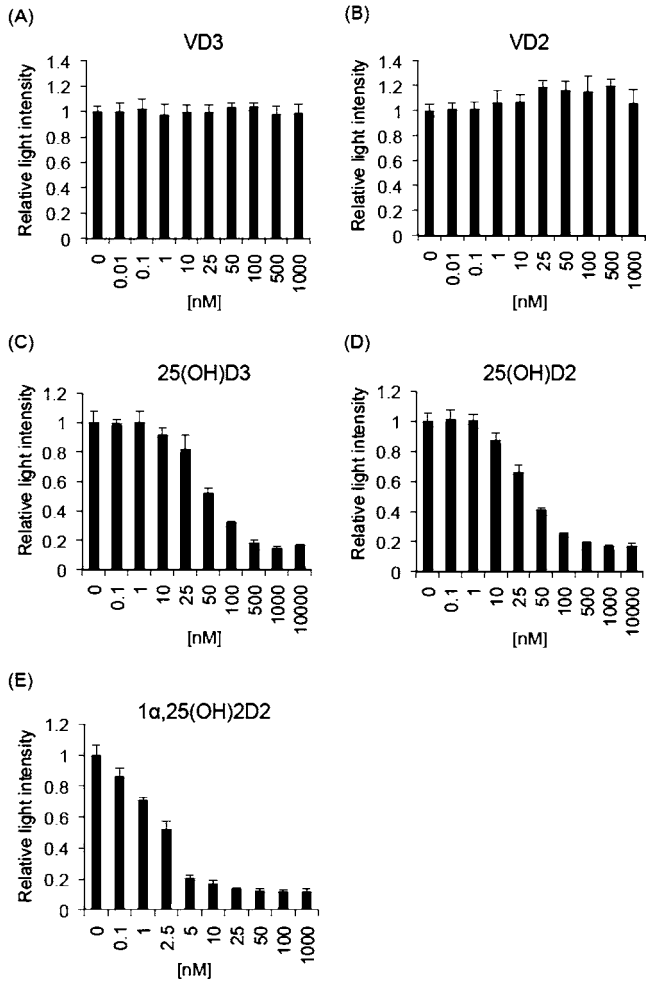

Figure 3. Effects of vitamin D-related compounds on the luciferase activity of LucC-LBD-LucN in COS-7 cells. The COS-7 cells expressing LucC-LBD-LucN were treated with various concentration of VD_3 (A), VD_2 (B), $25(OH)D_3$ (C), $25(OH)D_2$ (D), and $1\alpha,25(OH)_2D_2$ (E) for 90 min. The relative light intensity compared to the control (0.1% EtOH) was shown. Data are represented as mean ± SEM, $n = 4$.

Although a significant decrease in luminescence was observed for LucC-LBD-LucN and LucC-LBD(R391L)-LucN, no change was observed for the other luciferases. It was noted that the mutant VDR(R274L) corresponding to a mutation in a patient with vitamin D-dependent type II rickets cannot bind $1\alpha,25(OH)_2D_3$.[16,27−29] In contrast, the mutant VDR(R391C) derived from a patient having vitamin D-dependent type II rickets can bind a ligand as well as the wild type, but cannot interact with RXRα.[27,28] These results suggest that the change in luminescence intensity fully depends on binding of $1\alpha,25(OH)_2D_3$ to the LBD.

Next, we performed Western blot analysis of LucC-LBD-LucN using anti-luciferase antibody to investigate effects of $1\alpha,25(OH)_2D_3$ on LucC-LBD-LucN expression level and degradation in COS-7 cells. A distinct band with an apparent molecular weight of 90 kDa which is consistent with its calculated MW of 95 kDa was observed in the cells, and no degradation of this protein was observed in the presence of $1\alpha,25(OH)_2D_3$ (Figure 5). These results suggest that the decrease of the relative light intensity upon $1\alpha,25(OH)_2D_3$

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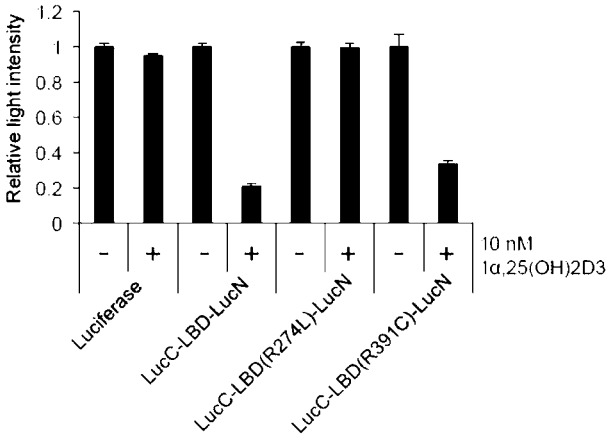

Figure 4. Effect of 10 nM $1\alpha,25(OH)_2D_3$ on the activity of luciferase itself, wild-type or mutant-type of vitamin D biosensors. The luminescence in the COS-7 cells expressing luciferase itself, LucC-LBD-LucN, LucC-LBD(R274L)-LucN, or LucC-LBD(R391L)-LucN was measured in the absence or presence of $1\alpha,25(OH)_2D_3$. Cells were treated with 0.1% EtOH (−) or 10 nM $1\alpha,25(OH)_2D_3$ (+) for 90 min. The relative light intensity compared to the control (0.1% EtOH) was shown. Data are represented as mean ± SEM, n = 4.

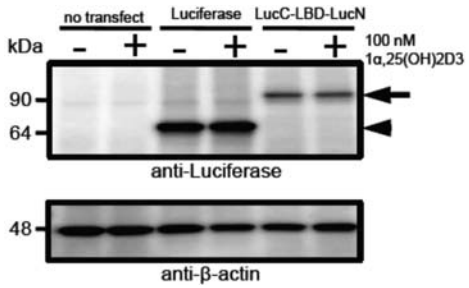

Figure 5. Western blot analysis of luciferase and LucC-LBD-LucN expressed in COS-7 cells. The transfected COS-7 cells were treated with 0.1% EtOH (−) or 100 nM $1\alpha,25(OH)_2D_3$ (+) for 120 min. The expressed proteins and β-actin were analyzed using anti-luciferase or anti β-actin antibody. The bands of luciferase and LucC-LBD-LucN are indicated by arrowhead and arrow, respectively. The designated numbers 90, 64, and 48 (kDa) mean sizes of marker proteins, bovine serum albumin, glutamine dehydrogenase, and ovalbumin, respectively.

addition is not caused by degradation of LucC-LBD-LucN, but by loss of luciferase activity.

Effects of the VDR Antagonist on the Activity of LucC-LBD-LucN. (23*S*)-25-Dehydro-1α-hydroxy-24,24-dimethylvitamin D_3-26,23-lactone named NS-4 is known to be a potent VDR antagonist.[30,31] NS-4 binds to the LBD of the VDR to inhibit vitamin D-induced genomic actions.

Finally, we investigated the effect of the VDR antagonist on the light intensity of the LucC-LBD-LucN biosensor. The COS-7 cells expressing LucC-LBD-LucN were treated with various concentration (0, 0.1, 0.5, 1, 5, 10, 50, 100, 500, or 1000 nM) of NS-4 for 90 min. Surprisingly, NS-4 induced a dose-dependent increase in the relative light intensity. In the presence of over 50 nM of NS-4, the light intensity was increased to approximately 3.5-fold (Figure 6A). To examine a time course of the relative light intensity, COS-7 cells expressing LucC-LBD-LucN were treated with 50 nM NS-4, and the luminescence was measured at 0, 5, 10, 15, 30, 60, 90, and 120 min after addition of NS-4 (Figure 6B). The relative

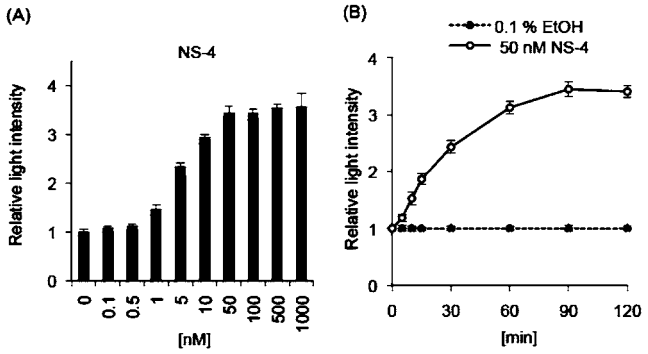

Figure 6. Effects of VDR antagonist on the luciferase activity of LucC-LBD-LucN in COS-7 cells. The COS-7 cells expressing LucC-LBD-LucN were treated with various concentration of NS-4 for 120 min. The dose-(A) and time-(B) dependent relative light intensity of LucC-LBD-LucN in COS-7 cells were shown. The COS-7 cells expressing LucC-LBD-LucN were treated with various concentrations (0, 0.1, 0.5, 1, 5, 10, 50, 100, 500, or 1000 nM) of NS-4 for 90 min (A). The COS-7 cells expressing LucC-LBD-LucN were treated with 50 nM NS-4, and the luminescence was measured at 0, 5, 10, 15, 30, 60, 90, and 120 min after addition of NS-4 (B). The relative light intensity compared to the control (0.1% EtOH) was shown. Data are represented as mean ± SEM, n = 4.

light intensity was rapidly increased until 60 min, and then gently increased after 60 min. It is noted that we can screen VDR antagonists using the LucC-LBD-LucN biosensor with the increase of the light intensity.

DISCUSSION

In this study, we have successfully developed a novel biosensor to detect the VDR ligands in living cells. Remarkably, our biosensor can discriminate between agonists and antagonists based on the decrease or increase of split-luciferase activity. Detection limits for $1\alpha,25(OH)_2D$ and $25(OH)D$ are 0.1−0.5 nM (Figures 2E and 3E) and 25−50 nM (Figure 3C,D), respectively, while that for NS-4 is 1−5 nM (Figure 6A). Based on our previous studies showing the biological effects of $1\alpha,25(OH)_2D$ and $25(OH)D$, this system appears significantly sensitive and quantitative enough to allow the concentrations of these compounds in cells to be estimated.[26] Three unexpected results were obtained in this study. First, the relative light intensity was decreased to approximately 20% by binding of VDR agonists, suggesting that LucN and LucC could form a functional complex exhibiting luciferase activity in the absence of a ligand, and luciferase activity could be reduced by binding VDR agonists, as shown in Figure 7.

Second, LucC-LBD-LucN was a significantly better sensor than LucN-LBD-LucC as shown in Figure 2C and D. The luciferase activity of the fusion proteins was markedly decreased by VDR agonists in a concentration-dependent manner. Although the luciferase activity of the LucC-LBD-LucN is much lower than that of luciferase itself, the sensitivity of our system might be sufficient enough for its practical use. We examined each of three types of modified LucN-LBD-LucC, and LucC-LBD-LucN with a flexible linker GGGGS × 3. The flexible linker was inserted between LucN (LucC) and LBD, and/or LBD and LucC (LucN), However, they showed no better properties than the original ones. Based on the previous studies,[32] optimization of the fusion construct including screening of better split sites and flexible linkers might improve our sensor ability.

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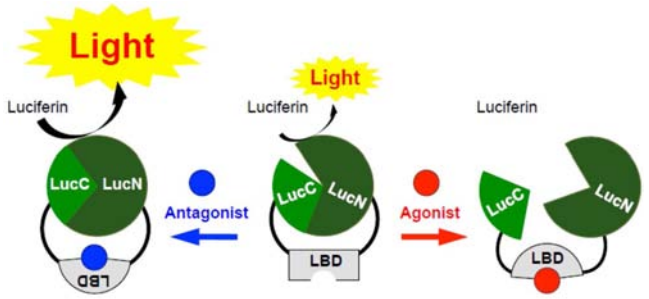

Figure 7. Putative mechanism of LucC-LBD-LucN for detecting VDR ligands. Binding of the VDR agonists to the LBD may cause a conformational change of the LBD that leads to disruption of the functional complex between N-terminal and C-terminal domains of the luciferase. In contrast, binding of the antagonist leads to the reassembly of N-terminal and C-terminal domains of the luciferase to increase the activity.

Based on the time course of the luminescence decrease, the luminescence was measured at 90 min after addition of the chemical compounds (Figure 2F). Because widely used VDRE-luciferase reporter gene assay systems require at least 12 h incubation, our system appears to be more rapid and convenient. Western blot analysis indicated that the decrease of the relative light intensity upon 1α,25$(OH)_2D_3$ addition is caused not by degradation of LucC-LBD-LucN, but by loss of luciferase activity (Figure 5). Third, the relative light intensity of LucC-LBD-LucN biosensor was increased in response to the addition of the antagonist NS-4. Since NS-4 did not change native luciferase activity (data not shown), the increase of LucC-LBD-LucN activity appears to be based on the conformational change of the LucC-LucN complex by binding of NS-4 to LBD as shown in Figure 7. As suggested in previous reports, the positioning of helix 12 is different between agonist-binding LBD and antagonist-binding LBD.[33] Thus, the luminescence decrease by agonists and increase by antagonist observed in our biosensor may depend on positional changes of helix12 of LBD to result in the conformational change of LucC-LucN complex (Figure 7). VDR antagonists could be useful to treat some diseases caused by hypersensitivity to 1α,25-$(OH)_2D_3$, such as Paget's bone disease that is a second common bone disease after osteoporosis.[34]

To determine whether VDR/RXRα heterodimer formation contributes to the behavior of the biosensor, we examined a mutant VDR (R391C) derived from a patient with vitamin D-dependent type II rickets.[27,28] This mutant can bind a ligand as well as the wild-type receptor, but cannot interact with RXRα. The luciferase activity of LucC-LBD (R391C)-LucN was decreased significantly by binding of 1α,25$(OH)_2D_3$, suggesting that the change in luciferase activity likely does not depends on RXRα. However, the decrease of the relative light intensity of LucC-LBD (R391C)-LucN is somewhat less than that of LucC-LBD (wild type)-LucN (Figure 4). Thus, we cannot exclude a small contribution of RXRα at the present time. On the other hand, 10 nM 1α,25$(OH)_2D_3$ did not change the luciferase activity of the mutant LucC-LBD (R274L)-LucN as shown in Figure 4. This mutant VDR (R274L) loses a hydrogen bond between Arg274 and the 1α-hydroxyl group of 1α,25-$(OH)_2D_3$,[16,29] resulting in the loss of binding to 1α,25-$(OH)_2D_3$. Based on these results, our system could detect a conformational change of the LBD of VDR, likely based on a positional change of the helix 12 upon ligand binding, resulting in a loss of functional complex formation between the N-terminal and C-terminal domains of the luciferase (Figure 7). Although 1α,25$(OH)_2D_3$ cannot bind to VDR(R274L), some vitamin D analogs may tightly bind to this mutant VDR.[29] Thus, our system might also be useful for developing a mutant VDR-specific drug.

Table 1. List of Template DNA, Primer Sequence, and PCR Product No

Template DNA	Sets of primers and sequence			PCR product No.
cDNA from THP-1 cells	(i)	For	5'- AATTCTCGAGATGGAGGCAATGGCGGCCAGCACTTC -3'	1
		Rev	5'- ATATGCGGCCGCTCAGGAGATCTCATTGCCAAACAC -3'	
pGL4.31	(ii)	For	5'- AATTCTCGAGATGGAAGATGCCAAAAACATTAAG -3'	2
		Rev	5'- ATATGCGGCCGCTTACACGGCGATCTTGCCGCCCTTCTTG -3'	
pGL4.31	(iii)	For	5'- AATTCTCGAGATGGAAGATGCCAAAAACATTAAG -3'	3
		Rev	5'- CTTCTTAATGTTTTTGGCATCTTCGGAGATCTCATTGCCAAACACTTCGA -3'	
pCR-bluntII-TOPO -hVDR	(iv)	For	5'- TACAAACGCTCTCATCGACAAGGACCGGCCCAAGCTGTCTGAGGAGCAGC -3'	4
		Rev	5'- GCTGCTCCTCAGACAGCTTGGGCCGCACGGCGATCTTGCCGCCCTTCTTG -3'	
pGL4.31	(v)	For	5'- CGAAGTGTTTGGCAATGAGATCTCCGGCTGGCTGCACAGCGGCGACATCG -3'	5
		Rev	5'- ATATGCGGCCGCTTACACGGCGATCTTGCCGCCCTTCTTG -3'	
PCR product No.3, 4, 5	(vi)	For	5'- AATTCTCGAGATGGAAGATGCCAAAAACATTAAG -3'	6
		Rev	5'- ATATGCGGCCGCTTACACGGCGATCTTGCCGCCCTTCTTG -3'	
pGL4.31	(vii)	For	5'- AATCTCGAGATGGGCTGGCTGCACAGCGGC -3'	7
		Rev	5'- CGATGTCGCCGCTGTGCAGCCAGCCGGAGATCTCATTGCCAAACACTTCG -3'	
pCR-bluntII-TOPO -hVDR	(viii)	For	5'- CCAAGAAGGGCGGCAAGATCGCCGTGCGGCCCAAGCTGTCTGAGGAGCAG-3'	8
		Rev	5'- GCTGCTCCTCAGACAGCTTGGGCCGGTCCTTGTCGATGAGAGCGTTTGTA -3'	
pGL4.31	(ix)	For	5'- TCGAAGTGTTTGGCAATGAGATCTCCGAAGATGCCAAAAACATTAAGAAG -3'	9
		Rev	5'- TAAGCGGCCGCTTAGTCCTTGTCGATGAGAGCGTTTGTAG -3'	
PCR product No.7, 8, 9	(x)	For	5'- AATCTCGAGATGGGCTGGCTGCACAGCGGC -3'	10
		Rev	5'- TAAGCGGCCGCTTAGTCCTTGTCGATGAGAGCGTTTGTAG -3'	
pCR-bluntII-TOPO -LucC-LBD-LucN	(xi)	For	5'- CATCATGTTGCTCTCCAATGAGTCCTTCAC -3'	11
		Rev	5'- ACTCATTGGAGAGCAACATGATGACCTCAATG -3'	
pCR-bluntII-TOPO -LucC-LBD-LucN	(xii)	For	5'- CCGACCTGTGCAGCCTCAATGAGGAGC -3'	12
		Rev	5'- TGAGGCTGCACAGGTCGGCTAGCTTCTG -3'	

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In this study, we used COS-7 cells (derived from African Green Monkey kidney) to express LucN-LBD-LucC and LucC-LBD-LucN proteins. Because kidney is a vitamin D target tissue, it is reasonable to use COS-7 cells as host cells to screen and evaluate the candidate therapeutic compounds for drugs against osteoporosis or cancer, and others. Additionally, we must carefully examine the effects of CYP24A1-dependent metabolism of the compound. When a VDR agonist binds to the VDR, transcriptional induction of CYP24A1 may occur in the COS-7 cells, causing metabolism of the VDR agonist. However, no metabolites were observed within 90 min after addition of the VDR agonists such as 25(OH)D_2, 25(OH)D_3, 1α,25(OH)$_2D_2$, or 1α,25(OH)$_2D_3$ (data not shown). Thus, effects of metabolism could be neglected in all the compounds tested in this study. However, the remarkable advantage of a luminescent biosensor in the living cells is the direct monitoring of the conversion of vitamin D in the living cells. As shown in Figure 3, the concentration-dependence in the luciferase activity of LucC-LBD-LucN is quite different for VD, 25(OH)D, and 1α,25(OH)$_2$D. Thus, we could monitor conversion of vitamin D into 1α,25(OH)$_2$D via 25(OH)D, and/or degradation of 1α,25(OH)$_2$D by CYP24A1 in the living cells. Then, we could analyze a relationship between the cellular level of the active form of vitamin D and VDR-mediated responses. We found that CYP24A1 is induced more than 90 min later after addition of the active forms of vitamin D in COS-7 cells. Our preliminary results suggest that our system could be used as a surrogate for measuring the activity of CYP27B1 and/or CYP24A1 in the cells (data not shown). Therefore, the COS-7 cells expressing LucC-LBD-LucN will be useful in analyzing VDR-mediated cellular responses.

EXPERIMENTAL PROCEDURES

Materials. Ethanol (EtOH), vitamin D_2 (VD_2), vitamin D_3 (VD_3) and 25(OH)D_3 were purchased from Wako Pure Chemicals (Osaka, Japan). 25(OH)D_2 was purchased from LKT Laboratories. 1α,25(OH)$_2D_2$ and 1α,25(OH)$_2D_3$ were purchased from Cayman Chemical. Co (Ann Arbor, MI, USA). (23*S*)-25-Dehydro-1α-hydroxy-24,24-dimethylvitamin D_3-26,23-lactone (NS-4) was synthesized as described previously (Figure 1C).[30,31]

cDNA Cloning of Human VDR. Total RNA was isolated from human monocytic cell line THP-1 cells using SV Total RNA Isolation System (Promega, Madison, USA) in accordance with the manufacturer's instruction. cDNAs were prepared using Prime Script RT reagent kit (Takara, Otsu, Japan) from 1 μg of total RNA. PCR was carried out using cDNA product as a template with the primer set (i) in Table 1 to amplify the human VDR (hVDR) ORF (GenBank accession number AB002168). PCR was performed with KOD-plus-neo DNA Polymerase (Toyobo, Osaka, Japan) and the resultant PCR products (PCR product No. 1 in Table 1) were cloned into pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.).

Construction of Expression Plasmids for Bioluminescent Sensor Proteins. To obtain the firefly luciferase ORF (nucleotide numbers 1−1650), PCR was carried out using pGL4.31 vector (Promega, Madison, USA) as a template with the primer set (ii) (Table 1). The PCR products (designated as No. 2 in Table 1) were cloned into pCR-BluntII-TOPO vector to obtain pCR-bluntII-TOPO-Luciferase. Overlap PCR method is frequently used to ligate the multiple fragments on several times of PCR. At the first PCR, the PCR products (PCR products No. 3, 4, 5, 7, 8, and 9) were amplified. To obtain the LucN (nucleotide number 1−1245 of luciferase ORF)-LBD (nucleotide number 361−1284 of hVDR ORF)-LucC (nucleotide number 1246−1650) or LucC-LBD-LucN, secondary PCR was carried out using the PCR fragments (PCR products No. 3, 4, and 5) or (PCR products No. 7, 8, and 9) as the templates with two sets of primers (Table 1 (vi) and (x)). These PCR products (PCR product No. 6 or 10) were cloned into pCR-BluntII-TOPO vector and named pCR-bluntII-TOPO-LucN-LBD-LucC or pCR-bluntII-TOPO-LucC-LBD-LucN, respectively. To generate the R274L or R391C mutant type of the LucC-LBD-LucN biosensor, PCR was performed with KOD-plus-neo DNA Polymerase using the set of primers (Table 1 (xi) or (xii)), and the resultant plasmids (PCR products No. 11 and 12) were named pCR-BluntII-TOPO-LucN-LBD-R274L-LucC and pCR-BluntII-TOPO-LucN-LBD-R391C-LucC, respectively. All the constructs were confirmed by DNA sequencing using ABI3100 (Applied Biosystems, Tokyo, Japan). Finally, cDNAs encoding luciferase, LucN-LBD-LucC, LucC-LBD-LucN, LucC-LBD-R274L-LucN, and LucC-LBD-R391C-LucN were each inserted between *Xho*I and *Not*I site of pEBMulti-Neo vector (Wako, Osaka, Japan).

Cell Culture and Transfection. COS-7 cells derived from African Green Monkey kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Inc., Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing phenol red (Nacalai Tesque, Inc., Japan) at 37 °C in an incubator with 5% CO_2. Then, cells were plated in 10 cm dish for 24 h before transfection. The cells were transfected with each of the plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.). After 24 h, the transfected cells were harvested and seeded onto 6-well plates (1.5×10^5 cells/well) for Western blot analysis or 96-well plates (1.5×10^4 cells/well) for luciferase complementary assay. Then the seeded cells were incubated in DMEM without phenol red, supplemented with 5% charcoal-stripped FBS #Lot No. 1581400 (Gibco, Rockville, MD, U.S.A.) for an additional 24 h. At 48 h after transfection, the cells were used for luciferase complementary assay or Western blot analysis.

Luciferase Complementary Assay in Living Cells. The culture medium was replaced with phenol red free Leibovitz's L-15 medium (Gibco, Rockville, MD, U.S.A.) containing 0.5 mM D-luciferin (Thermo Scientific, CA, U.S.A.) and incubated for 30 min at room temperature. Then, the light intensity was measured before addition of vitamin D-related compound to know a basal level of the light intensity. Each of vitamin D-related compounds (VD_2, VD_3, 25(OH)D_2, 25(OH)D_3, 1α,25(OH)$_2D_2$, 1α,25(OH)$_2D_3$, or NS-4) dissolved in EtOH was added to the wells at final concentrations of 0.01 to 10 000 nM. The final concentration of EtOH was adjusted to 0.1%. After addition of the vitamin D-related compound or EtOH, the light intensity of each well was measured at several time points. The luminescence was measured using a luminometer (Infinite 200 Pro 96-microplate luminometer, Tecan). In this study, the relative light intensity in the presence of a VDR ligand was calculated in comparison with the light intensity in the absence of VDR ligands as a control.

Western Blot Analysis. Cells were lysed using a lysis buffer containing 150 mM NaCl, 0.1% SDS, 1% sodium cholate, and 1% Triton-X in 50 mM Tris-HCl (pH 7.0). Proteins were separated by SDS-PAGE, and electrically transferred onto a PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membranes were incubated with goat anti-luciferase (1:1000,

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Promega, Madison, USA) or rabbit anti-β-actin (1:1000, Santa Cruz, CA, U.S.A.) antibody in the Can Get Signal solution (Toyobo, Osaka, Japan) at 4 °C for 12 h, and then reacted reacted with secondary rabbit anti-goat IgG HRP-conjugated (1:2000, Santa Cruz, CA, U.S.A.) or goat anti-rabbit IgG HRP conjugated antibodies (1:2000, Bio Rad, CA, U.S.A.). The immobilized proteins were visualized using an ECL prime Western blotting reagent (GE Healthcare, Buckinghamshire, UK). Finally, the chemiluminescence signals were detected using the LAS-1000 image analyzer (Fujifilm, Tokyo, Japan).

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

VDR, vitamin D receptor; RXRα, retinoid X receptor α; LBD, ligand binding domain; SRC-1, steroid receptor coactivator-1; TIF2, transcriptional intermediary factor 2; RAC3, receptor-associated coactivator 3; NCoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoic acid receptors and thyroid hormone receptors; VDRE, vitamin D responsive element; IP_3, inositol 1,4,5-trisphosphate; IBC, IP_3-binding core; ER, estrogen receptor; LucN, N-terminal domain of luciferase; LucC, C-terminal domain of luciferase

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