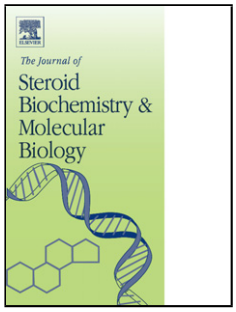


Title: Novel split luciferase-based biosensors for evaluation of vitamin D receptor ligands and their application to estimate CYP27B1 activity in living cells

Authors: Hiroki Mano, Shinichi Ikushiro, Toshiyuki Sakaki

PII: S0960-0760(18)30131-6
DOI: https://doi.org/10.1016/j.jsbmb.2018.06.017
Reference: SBMB 5175

To appear in: *Journal of Steroid Biochemistry & Molecular Biology*

Received date: 2-3-2018
Revised date: 17-6-2018
Accepted date: 30-6-2018

Please cite this article as: Mano H, Ikushiro S, Sakaki T, Novel split luciferase-based biosensors for evaluation of vitamin D receptor ligands and their application to estimate CYP27B1 activity in living cells, *Journal of Steroid Biochemistry and Molecular Biology* (2018), https://doi.org/10.1016/j.jsbmb.2018.06.017

Novel split luciferase-based biosensors for evaluation of vitamin D receptor ligands and their application to estimate CYP27B1 activity in living cells.

Hiroki Mano [a], Shinichi Ikushiro [b] and Toshiyuki Sakaki [a]*

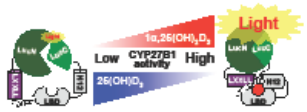
[a] Department of Pharmaceutical Engineering, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan:

[b] Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan:

*Corresponding author: Toshiyuki Sakaki
Department of Pharmaceutical Engineering,,
Faculty of Engineering, Toyama Prefectural University,
5180 Kurokawa, Imizu, Toyama 939-0398, Japan
Phone: +81-766-56-7500 Ext.601
Fax: +81-766-56-2498
E-mail: tsakaki@pu-toyama.ac.jp

Text page: 24
Table: 1
Figure: 6
Reference: 28
Abstract: 190 words
Introduction: 427 words
Discussion: 1582 words

Graphical abstract



TOC

Highlights

- Novel screening system for VDR ligands was constructed by using split luciferase.
- The novel split luciferase dramatically increased the light intensity with VDR ligands.
- This system is useful to evaluate CYP27B1 mutants from patients with rickets.

ABSTRACT

Recently, we successfully generated a novel detection system for vitamin D receptor (VDR) ligands *in vivo* and *in vitro,* using a split-luciferase technique called the LucN-LBD-LucC biosensor that is a chimeric fusion protein of firefly luciferase with the ligand binding domain (LBD) of VDR. In this system, the luciferase light intensity of the LucN-LBD-LucC biosensor was decreased by binding of VDR ligands. Although this system is quite useful for evaluation of VDR ligands in a short time, the sensitivity of the LucN-LBD-LucC biosensor is not high enough. In this study, LXXLL motif peptides involved in the interaction between LBD and coactivators, such as the steroid receptor coactivator-1 (SRC-1), transcriptional intermediary factor 2 (TIF2), and the vitamin D receptor interacting protein 205 (DRIP205) were each inserted between LucN and LBD of the LucN-LBD-LucC biosensor. Surprisingly, the resulting LucN-LXXLL-LBD-LucC biosensor increased the light intensity in response to natural VDR ligands. This high-sensitivity biosensor system may be a powerful tool for discovery of high-affinity ligands for the mutant VDR. In addition, we have successfully estimated the activity of the wild-type and mutant CYP27B1 using the LucN-LXXLL-LBD-LucC biosensor in living cells within 90 min.

Abbreviations : CYP, Cytochrome P450 ; VDR, vitamin D receptor ; LBD, ligand binding domain ; aa, amino acids; LucN, N-terminal domain of luciferase ; LucC, C-terminal domain of luciferase ; SRC-1, steroid receptor coactivating factor 1 ; RXRα,

retinoid X receptor α ; VDRE, vitamin D responsive element ; TIF2, transcriptional factor 2 ; DRIP205, vitamin D receptor interacting protein 205

Key words: vitamin D, vitamin D receptor, CYP27B1, bioluminescent sensor, split-luciferase

1. Introduction

In general, vitamin D_3 [VD_3] is converted to 25-hydroxyvitamin D_3 [$25(OH)D_3$] by two cytochrome P450 species, CYP2R1 and CYP27A1, in the liver, and $25(OH)D_3$ is converted to 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] by CYP27B1 in the kidney [1]. $1\alpha,25(OH)_2D_3$ is a high-affinity agonist of the vitamin D receptor (VDR), and plays a key role in calcium and bone homeostasis, cellular growth and differentiation, and immune responses [2-5].

An agonist-bound VDR interacts with its heterodimeric partner retinoid X receptor (RXR) to form a complex with a variety of coactivators such as the steroid receptor coactivator-1 (SRC-1), transcriptional intermediary factor 2 (TIF2) and vitamin D receptor interacting protein 205 (DRIP205) via an intramolecular specific amino acid sequence called the LXXLL motif [6-8]. Once an agonist-bound VDR-RXR-coactivator complex binds to a vitamin D response element (VDRE) on a target gene, this complex acts as the transcription factor and activates vitamin D-dependent gene expression [9,10]. Therefore, disruption of the vitamin D-dependent signaling, including inactivation of the CYP2R1, CYP27B1, and VDR leads to type I or type II rickets, respectively [11-13].

To detect the VDR ligands or evaluate the activity of CYP2R1, CYP27A1 and CYP27B1 in living cells, many researchers have widely used luciferase reporter gene assay systems that consist of a plasmid or a lentiviral vector which includes the luciferase reporter gene under the control of the VDRE-containing promoter [11,14,15,16]. However, this system needs 12-24 h to measure the activity of *de novo* synthesized luciferase proteins. Therefore, development of a simple and rapid screening system for VDR ligands is required. Recently, we have successfully generated novel detection systems for VDR ligands *in vitro* using a split-luciferase technique called the LucN-LBD-LucC biosensor that is a chimeric fusion protein of firefly luciferase with the ligand binding domain (LBD) of the VDR [17]. The detection mechanism of this system is that the luciferase light intensity of the LucN-LBD-LucC biosensor is decreased by the binding of VDR ligands such as 25(OH)D_3 and 1α,25(OH)$_2D_3$. Although this system is quite useful for evaluation of VDR ligands in a short time, the sensitivity of the LucN-LBD-LucC biosensor is not high enough for practical applications. Thus, we have tried to develop a new type of biosensor whose luciferase activity is increased by binding of VDR ligands. In this study, several peptides with the LXXLL motif were each inserted between LucN and LBD of the LucN-LBD-LucC biosensor Surprisingly, the resultant LucN-LXXLL-LBD-LucC biosensors dramatically enhanced their luciferase activity following binding of 25(OH)D_3 or 1α,25(OH)$_2D_3$. Furthermore, we applied the LucN-LXXLL-LBD-LucC biosensor to evaluate the activity of the wild-type and mutant CYP27B1 in living cells.

2. Materials and methods

2.1. Materials

Ethanol (EtOH), vitamin D_3 (VD_3) and 25(OH)D_3 were purchased from Wako Pure Chemicals (Osaka, Japan). 1α,25$(OH)_2D_3$ was purchased from Cayman Chemical. Co (Ann Arbor, MI, USA). pRL-CMV vector and *Renilla*-Glo Luciferase assay system were purchased from Promega Madison, USA. D-luciferin was purchased from Thermo Fisher Scientific Inc. USA.

2.2. cDNA cloning of human CYP27B1 and construction of expression plasmids for wild-type and CYP27B1 mutant

Total RNA was isolated from human embryonic kidney 293T (HEK293T) cell line using SV Total RNA Isolation System (Promega, Madison, USA) in accordance with the manufacturer's instruction and 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 No.1 (Table 1) to amplify the human CYP27B1 (hCYP27B1) ORF (GenBank accession number AY288916). PCR was performed with KOD-plus-neo DNA Polymerase (Toyobo, Osaka, Japan) and the resultant PCR products hCYP27B1-hexa-histidine (6×His) were cloned into pTA2 vector (Toyobo, Osaka, Japan), named pTA2-hCYP27B1-6×His vector. To obtain hCYP27B1(T321R)-6×His, PCR was performed with KOD plus-neo (Toyobo, Osaka, Japan) using pTA2-hCYP27B1-6×His vector as the template with the primer set No.2 (Table 1). cDNA encoding hCYP27B1-6×His and hCYP27B1(T321R)-6×His were each inserted between *Xho*I and *Bam*HI site of pEBMulti-Neo vector (Wako, Osaka, Japan).

The resultant plasmids were named pEBMulti-hCYP27B1-6×His and pEBMulti-hCYP27B1(T321R)-6×His, respectively.

Construction of expression plasmids for *firefly* luciferase and biosensors.

Full length of the *firefly* luciferase (FLuc) ORF (nucleotide number 1-1650) and the LucN (1-415 aa)-LBD (121-427 aa)-LucC (416-550 aa) were each cloned into pCR-BluntII-TOPO vector to yield pCR-bluntII-TOPO-FLuc and pCR-bluntII-TOPO-LucN-LBD-LucC, respectively, as described in our previous report [16]. First, to generate LucN-(GGGGS)×3-LBD-LucC, PCR was performed using pCR-BluntII-TOPO-LucN-LBD-LucC vector as a template with the primer set No.3 (Table 1). Second, to obtain LucN-NHPMLMNLLKDN-(GGGGS)×3-LBD-LucC, PCR was performed using pCR-BluntII-TOPO-LucN-(GGGGS)×3-LBD-LucC vector as a template with the primer set No.4 (Table 1). Third, to generate LucN-NHPMLMNLLKDN-LBD-LucC, LucN-NHPMLMNAAKDN-(GGGGS)×3-LBD-LucC, LucN-VDLTEMHPILTSLLQNGVDHV-(GGGGS)×3-LBD-LucC, LucN-VDLSETHPLLWTLLSSTEGDSM-(GGGGS)×3-LBD-LucC and LucN-VDLMNLLVD-(GGGGS)×3-LBD-LucC, PCR was performed using pCR-BluntII-TOPO-LucN-NHPMLMNLLKDN-(GGGGS)×3-LBD-LucC vector as a template with the primer set (No.5 to No.9), respectively (Table 1). These fragments were amplified with KOD plus mutagenesis kit (Toyobo, Osaka, Japan) in accordance with the manufacture's instruction. cDNA encoding FLuc, LucN-LBD-LucC, LucN-(GGGGS)×3-LBD-LucC, LucN-NHPMLMNLLKDN-(GGGGS)×3-LBD-LucC, LucN-NHPMLMNLLKDN-LBD-LucC, LucN-NHPMLMNAAKDN-(GGGGS)×3-LBD-

LucC, LucN-VDLTEMHPILTSLLQNGVDHV-(GGGGS)×3-LBD-LucC, LucN-VDLSETHPLLWTLLSSTEGDSM-(GGGGS)×3-LBD-LucC and LucN-VDLMNLLVD-(GGGGS)×3-LBD-LucC were each inserted between *Xho*I and *Not*I site of pIRES2-AcGFP vector (Takara, Otsu, Japan). The resultant plasmids were named pIRES2-FLuc, pIRES2-LucN-LBD-LucC, pIRES2-LucN-(GGGGS)×3-LBD-LucC, pIRES2-LucN-NHPMLMNLLKDN-(GGGGS)×3-LBD-LucC, pIRES2-LucN-NHPMLMNLLKDN-LBD-LucC, pIRES2-LucN-NHPMLMNAAKDN-(GGGGS)×3-LBD-LucC, pIRES2-LucN-VDLTEMHPILTSLLQNGVDHV-(GGGGS)×3-LBD-LucC, pIRES2-LucN-VDLSETHPLLWTLLSSTEGDSM-(GGGGS)×3-LBD-LucC, and pIRES2-LucN-VDLMNLLVD-(GGGGS)×3-LBD-LucC, respectively. To obtain VDNHPMLMNLLKDN-(GGGGS)x3-LBD-LucC, PCR was performed using pCR-bluntII-TOPO-LucN-NHPMLMNLLKDN-(GGGGS)×3-LBD-LucC vector as a template with the primer set No.10 (Table 1). PCR fragment VDNHPMLMNLLKDN-(GGGGS)×3-LBD-LucC was inserted into *Sal*I/*Not*I site of pIRES2-LucN-VDLTEMHPILTSLLQNGVDHV-(GGGGS)×3-LBD-LucC vector, and the resultant plasmid was named pIRES2-LucN-VDNHPMLMNLLKDN-(GGGGS)×3-LBD-LucC vector. To obtain LucN-VDNHPMLMNLLKDN-(GGGGS)×3-LBD(R274L)-LucC, PCR was performed with KOD plus-neo (Toyobo, Osaka, Japan) using pIRES2-LucN-VDNHPMLMNLLKDN-(GGGGS)×3-LBD-LucC vector as a template with the primer set No.11 (Table 1). All the constructs were confirmed by DNA sequencing using ABI3130 (Applied Biosystems, Tokyo, Japan).

2.3. Cell culture and transfection

The 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. The cells were harvested and transiently transfected with each of the plasmids with the pRL-CMV vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) in accordance with the manufacturer's instructions. RLuc was used as an internal control to check the transfection efficiency. Transfected cells were seeded onto 96-well plates (1.5×10^4 cells/well) or 6-well plates (3.0×10^5 cells/well). At 24 h after transfection, the culture medium was replaced with DMEM without phenol red (Nacalai Tesque, Inc., Japan) supplemented with 5 % charcoal-stripped FBS (Gibco, Rockville, MD, U.S.A.) and were incubated for an additional 24 h. At 48 h after transfection, the cells were used for luciferase complementary assay, CYP27B1 activity assay or Western blot analysis.

2.4. Luciferase complementary assay in living cells

To detect the light intensity of the *firefly* luciferase (FLuc) or biosensor, the culture medium was changed to 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.). The culture plate was incubated for 30 min at room temperature. To measure a basal level of the light intensity, the light intensity was measured before addition of EtOH, 25(OH)D_3 or 1α,25(OH)$_2$$D_3$. 25(OH)$D_3$ and 1α,25(OH)$_2$$D_3$ dissolved in EtOH was added to the wells at final concentrations of 0.01 to 1,000 nM. The final concentration of EtOH was adjusted to 0.1%. After addition of EtOH, 25(OH)D_3 or 1α,25(OH)$_2$$D_3$, the light intensity of each well was measured at several time points (0, 5, 10, 15, 30, 60, 90 and

120 min). 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 25(OH)D_3 or 1α,25$(OH)_2D_3$ was calculated in comparison with the light intensity in the absence of 25(OH)D_3 or 1α,25$(OH)_2D_3$ (0.1% EtOH) as a negative control.

2.5. Estimation of CYP27B1-dependent 1α-hydroxylation activity using biosensor system

In 96-well plates, 0.01 μg/well of pIRES2-LucN-VDNHPMLMNLLKDN-(GGGGS)×3-LBD-LucC and pRL-CMV, and 0.1 μg/well of pEBMulti-hCYP27B1(WT)-6×His or pEBMulti-hCYP27B1(T321R)-6×His were used for transfection. The COS-7 cells expressing only LucN-VDNHPMLMNLLKDN-(GGGGS)×3-LBD-LucC biosensor or co-expressing LucN-VDNHPMLMNLLKDN-(GGGGS)×3-LBD-LucC biosensor with hCYP27B1(WT)-6×His or hCYP27B1(T321R)-6×His were treated with various concentration (0, 5, 10, 25 and 50 nM) of 25(OH)D_3 for 60 min at 37°C. After additional incubation at room temperature for 30 min, the light intensity was measured. To investigate the putative conversion ratio of 25(OH)D_3 to 1α,25$(OH)_2D_3$ by hCYP27B1(WT)-6×His, the COS-7 cells expressing LucN-VDNHPMLMNLLKDN-(GGGGS)×3-LBD-LucC biosensor were treated with 5, 25 or 50 nM of 25(OH)D_3 as a conversion ratio of 0%, and co-treated with 1α,25$(OH)_2D_3$ to adjust conversion ratios from 25(OH)D_3 to 1α,25$(OH)_2D_3$ at 1, 5, and 10 %, respectively.

2.6. Western blot analysis

Cells were harvested and 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 7.4). Proteins were separated by SDS-PAGE, and electrically transferred onto a PVDF membrane (GE Healthcare, Buckinghamshire, UK). To detect the biosensor proteins, the membranes were incubated with goat anti-luciferase (1:1000, 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 room temperature for 1 h, and then reacted with secondary rabbit anti-goat IgG HRP-conjugated (1:5000, Santa Cruz, CA, U.S.A.) or goat anti-rabbit IgG HRP conjugated antibodies (1:5000, Bio Rad, CA, U.S.A.), respectively. To detect the hCYP27B1-6×His or hCYP27B1(T321R)-6×His, the membranes were incubated with anti-His-tag mAb-HRP-Direct antibody (1:5000, MBL, Nagoya, Japan) in the Can Get Signal solution (Toyobo, Osaka, Japan) for 1 h at room temperature. The immobilized proteins were visualized using an ECL prime Western blotting reagent (GE Healthcare, Buckinghamshire, UK). The chemiluminescence signals were detected using the LAS-1000 image analyzer (Fujifilm, Tokyo, Japan).

2.7. Statistical Analysis

Data are presented as mean ± standard deviation (SD). The F-test indicated that the P values for each set of two samples in Fig. 2 were not less than 0.05, suggesting the non-different variance. Thus, two-tailed Student's *t* test was used to determine statistical significance. Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Design of a biosensor based on split-luciferase for detection of VDR ligands

When natural VDR ligands bind to the LBD of the VDR, the LBD interacts with a transcription coactivator such as SRC-1, TIF-2, or DRIP-205 via the LXXLL motif [6-8] to activate vitamin D-dependent gene expression. Based on these features, we assumed that the LXXLL motif could change the enzymatic properties of luciferase-LBD biosensors. Thus, we constructed a biosensor composed of the LBD (121-427 aa) of VDR, N- and C-terminal of firefly luciferase fragments [LucN (1-415 aa) and LucC (416-550 aa)], the LXXLL peptide sequence, and the flexible linker peptide sequence [Gly-Gly-Gly-Gly-Ser (GGGGS)]×3. We named this construct the LucN-LXXLL-(GGGGS)×3-LBD-LucC WT biosensor, where WT means the wild type of LBD. A schematic diagram of the WT biosensor is shown in Fig. 1. In the absence of natural ligands, luciferase light intensity is low. When a natural VDR ligand is bound to the WT biosensor, the luciferase light intensity was immediately and dramatically increased. The remarkable activity appears to depend on reconstitution of LucN and LucC induced by positional change of helix 12 (H12) of the LBD by ligand binding, and subsequent interaction of the LXXLL-LBD [20].

3.2. Development of the WT biosensor for detection of VDR ligands

To develop a novel and high-sensitivity WT biosensor, we examined three types of LXXLL peptides (NHPMLMNLLKDN, LTEMHPILTSLLQNGVDHV, and LSETHPLLWTLLSSTEGDSM) reported by Zella et al., 2007 [21]. They reported that these peptides interact with the LBD of the VDR in response to 1α,25$(OH)_2D_3$ or other agonists. Thus, we constructed ten types of plasmids encoding biosensor proteins called

biosensors 1-10, respectively (Fig. 2A). First, we investigated the changes in light intensity and relative light intensity after treatment with 1α,25(OH)$_2$D$_3$ (Fig. 2B, C). The COS-7 cells expressing each type of biosensor were treated with 100 nM of 1α,25(OH)$_2$D$_3$ or 0.1% EtOH as a negative control, and then the luminescence was measured 90 min later. The light intensity of biosensor 1 was decreased in response to 1α,25(OH)$_2$D$_3$, and this result is consistent with our previous study [18]. However, the light intensity of biosensor 2 showed no response to 1α,25(OH)$_2$D$_3$, indicating that a (GGGGS)×3 linker abolished the negative effect of ligand binding. To our surprise, the light intensity of biosensors 3, 4, 5, 6, 7, 9, and 10, which contained the LXXLL motif, were significantly increased in response to 1α,25(OH)2D3. In contrast, the light intensity of biosensor 8, containing an LXXAA that is an alanine (A) mutant of the LXXLL motif was not increased in response to 1α,25(OH)$_2$D$_3$. These results indicate that the LXXLL motif plays an essential role in the light intensity increase induced by 1α,25(OH)$_2$D$_3$ binding to LBD. As shown in Fig. 2B, biosensor 4 was the best among 7 biosensors. The light intensity of biosensor 4 after 1α,25(OH)$_2$D$_3$ binding was approximately one-third of that of the full-length firefly luciferase (Sup Fig. 1). Therefore, biosensors 4 and 9 were used in the following experiments and referred to as the WT biosensor and the R274L biosensor, respectively.

3.3. Time- and concentration-dependent responses of the WT biosensor

To determine the light intensity detection time after addition of a ligand in this assay system, the COS-7 cells expressing WT biosensor proteins were treated with 100 nM of 1α,25(OH)$_2$D$_3$ or 0.1% EtOH (negative control) for 120 min, and the luminescence was measured at 0, 5, 10, 15, 30, 60, 90, and 120 min after addition of 1α,25(OH)$_2$D$_3$ (Fig.

3). Relative light intensity reached its maximum at 90 min, then decreased at 120 min. Thus, the optimal detection time was determined to be 90 min in the following experiments. Next, we examined the concentration-response of the WT biosensor and R274L biosensor, and effects of plasmid DNA amounts using transfection on the sensitivity of biosensors. COS-7 cells transfected with each amount of the plasmid for the WT-biosensor (0.01, 0.03, or 0,1 μg/well) were treated with various concentrations (0, 0.01, 0.1, 0.5, 1, 5, 10, 25, 50, 100, 500 or 1000 nM) of 25(OH)D_3 or 1α,25(OH)$_2D_3$ for 90 min. 1α,25(OH)$_2D_3$ increased the relative light intensity in a concentration-dependent manner in the transfected COS-7 cells (Fig. 4A). In the cases of 0.03 and 0.1 μg plasmid DNA/well, concentration-dependency was observed from 0.5 to 50 nM 1α,25(OH)$_2D_3$. In contrast, 0.01 μg plasmid DNA/well showed concentration-dependency from 0.1 to 10 nM 1α,25(OH)$_2D_3$. Therefore, we chose 0.01 μg/well as the amount of plasmid DNA using transfection in the following experiments.

As shown in Fig. 4B, 25(OH)D_3 also increased the light intensity in a concentration-dependent manner. The half maximal relative light intensity was observed at approximately 1 nM of 1α,25(OH)$_2D_3$ and at approximately 20 nM of 25(OH)D_3, respectively. These results indicate the difference in affinity for the wild type VDR between 25(OH)D_3 and 1α,25(OH)$_2D_3$. Next, we compared the affinity of 25(OH)D_3 and 1α,25(OH)$_2D_3$ towards the R274L mutant VDR using COS-7 cells expressing the R274L biosensor (Fig. 4C). Because Arg274 of the VDR forms a hydrogen bond with 1α-hydroxyl group of 1α,25(OH)$_2D_3$, the R274L mutation causes a 1000-fold decrease in affinity of 1α,25(OH)$_2D_3$ [22,23]. Expectedly, the concentration-response curve of 1α,25(OH)$_2D_3$ in the R274L biosensor was very similar to that of 25(OH)D_3. Over 500 nM of both 25(OH)D_3 and 1α,25(OH)$_2D_3$ were required to increase the maximum light

intensity of the R274L biosensor. It is noted that this biosensor system may be a powerful tool for discovery of high-affinity ligands for the mutant VDR. High affinity VDR ligands are predicted to be a therapeutic candidate for Hereditary Vitamin D Resistant Rickets (HVDRR).

3.4. Detection mechanism of the activity of CYP27B1 using the WT biosensor

When 25(OH)D_3 was added to COS7 cell culture in the concentration range used in this study, 1,25(OH)$_2D_3$ was not detected at 90 min after addition of 25(OH)D_3. Western blot analysis using anti-human CYP27B1 antibody (sc-67261, Santa Cruz Biotechnology, Dallas, USA) failed to detect monkey CYP27B1 which has 97% identity with human CYP27B1. Based on these results, monkey CYP27B1 level in COS7 cells appears to be quite low, and monkey CYP27B1-dependent 1α-hydroxylation activity could be neglected in this study.

Schema in Fig. 5 show the detection mechanism for the relative activity of CYP27B1 using the WT biosensor. The wild-type of CYP27B1 [CYP27B1(WT)] generates 1α,25(OH)$_2D_3$ from 25(OH)D_3, while in contrast, the T321R mutant of CYP27B1 [CYP27B1(T321R)], which is well-known as an inactive mutant of CYP27B1, impairs the generation of 1α,25(OH)$_2D_3$ [24,25]. Functional CYP27B1 will increase the light intensity of the WT biosensor by generation of 1α,25(OH)$_2D_3$, however, the inactive mutant of CYP27B1 will not increase the light intensity. Therefore, the CYP27B1 activity could be evaluated from the time-course of relative light intensity in the living cells expressing the WT biosensor.

3.5. Expression levels of the exogenous hCYP27B1 and WT biosensor proteins

The COS-7 cells expressing the WT biosensor, or those co-expressing the WT biosensor and hCYP27B1(WT)-6×His or hCYP27B1(T321R)-6×His proteins were used in the following experiments. To estimate the expression levels of the exogenous WT biosensor, hCYP27B1(WT)-6×His or hCYP27B1(T321R)-6×His proteins in COS-7 cells, we performed Western blot analysis using anti-luciferase and anti-His tag HRP conjugated antibody, respectively (Fig. 6A). As shown in Fig. 6A, the expression level of the WT biosensor protein was similar in the presence or absence of hCYP27B1(WT)-6×His or hCYP27B1(T321R)-6×His proteins. The specific bands of exogenous hCYP27B1 proteins were detected in hCYP27B1(WT)-6×His or hCYP27B1(T321R)-6×His transfected cells. As CYP27B1 contains N-terminal mitochondrial targeting signal peptides which are removed after translocation, the observed two bands are considered to correspond with the precursor and mature forms of hCYP27B1 proteins. In addition, there were no significant differences in the expression levels between exogenous hCYP27B1(WT)-6×His and hCYP27B1(T321R)-6×His proteins.

3.6. Estimation of hCYP27B1 activity using the WT biosensor

Next, we estimated the relative activity of CYP27B1 using the WT biosensor. The COS-7 cells expressing the WT biosensor (CTRL) or co-expressing the WT biosensor with hCYP27B1(WT) or hCYP27B1(T321R) proteins were treated with 0-50 nM of 25(OH)D_3 for 90 min (Fig. 6B). The relative light intensity in COS-7 cells expressing hCYP27B1(WT) proteins were significantly higher than that expressing hCYP27B1(T321R) proteins at each concentration of 25(OH)D_3. In contrast, the concentration response of hCYP27B1(T321R) was quite similar to that of CTRL. These

results indicate that the WT biosensor could detect *de novo* synthesized 1α,25(OH)$_2$D$_3$ by exogenous hCYP27B1 in living cells. Finally, we calculated the conversion ratio from 25(OH)D$_3$ to 1α,25(OH)$_2$D$_3$ by hCYP27B1 in this assay system (Sup. Fig. 2). The WT biosensor protein-expressing COS-7 cells were treated with 0-50 nM of 25(OH)D$_3$ containing the 1α,25(OH)$_2$D$_3$ equivalent of the 1, 5 or 10% conversion ratio, respectively. In Sup. Fig.2, "10% conversion ratio" at 50 nM means the addition of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ to the cell culture at final concentrations of 45nM and 5nM, respectively. The "5% conversion ratio" at 25 nM means the addition of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at final concentrations of 23.75 and 1.25 nM, respectively. The concentration-response curve of CYP27B1(WT) was similar to that of the 5% conversion ratio. Therefore, the conversion ratio from 25(OH)D$_3$ to 1α,25(OH)$_2$D$_3$ was estimated to be approximately 5% in our assay system. It should be noted that this assay system could detect 1α,25(OH)$_2$D$_3$ with high sensitivity, and readily evaluate the activity of CYP27B1 and its mutants within 90 min.

In this study, we have successfully modified and developed the high-sensitivity biosensor that increased the light intensity in response to the natural VDR ligands, 25(OH)D$_3$ and 1α,25(OH)$_2$D$_3$. Our biosensor could be used not only for screening of VDR ligands but also evaluation of the activity of CYP27B1 and its mutants in living cells within 90 min.

In addition, it is known that the active form of vitamin D plays important roles in developmental stages such as heart development, formation of the central nervous system and brain development [26-28]. However, tissue distribution of 25(OH)D$_3$ and 1α,25(OH)$_2$D$_3$ and its spatiotemporal action mechanism are poorly understood. In the future, we will apply our biosensor systems to the visualization and monitoring of

25(OH)D_3 and 1α,25$(OH)_2D_3$ in living animals. *In vivo* imaging of 25(OH)D_3 and 1α,25$(OH)_2D_3$ might reveal novel roles of vitamin D in developmental stages.

References

[1] T. Sakaki, N. Kagawa, K. Yamamoto, K. Inouye, Metabolism of vitamin D3 by cytochromes P450, Front. Biosci.: J. Virtual Lib. 10 (2005) 119-134.

[2] O.I. Kolek, E.R. Hines, M.D. Jones, L.K. LeSueur, M.A. Lipko, P.R. Kiela, J.F. Collins, M.R. Haussler, F.K. Ghishan, 1alpha,25-Dihydroxyvitamin D3 upregulates FGF23 gene expression in bone: the final link in a renal-gastrointestinal-skeletal axis that controls phosphate transport, Am. J. Physiol. Gastrointest. Liver Physiol. 289 (2005) G1036-1042.

[3] R.P. Heaney, The Vitamin D requirement in health and disease, J. Steroid Biochem. Mol. Biol. 97 (2005) 13-19.

[4] S. Samuel, M.D. Sitrin, Vitamin D's role in cell proliferation and differentiation, Nutr. Rev. 66 (2008) S116-124.

[5] M.T. Cantorna, Y. Zhu, M. Froicu, A. Wittke, Vitamin D status, 1,25-dihydroxyvitamin D3, and the immune system, Am. J. Clin. Nutr. 80 (2004) 1717S-1720S.

[6] C. Rachez, M. Gamble, C.P. Chang, G.B. Atkins, M.A. Lazar, L.P. Freedman, The

DRIP complex and SRC-1/p160 coactivators share similar nuclear receptor binding determinants but constitute functionally distinct complexes, Mol. Cell. Biol. 20 (2000) 2718-2726.

[7] A. Teichert, L.A. Arnold, S. Otieno, Y. Oda, I. Augustinaite, T.R. Geistlinger, R.W. Kriwacki, R.K. Guy, D.D. Bikle, Quantification of the vitamin D receptor-coregulator interaction, Biochemistry. 48 (2009) 1454-1461.

[8] B. He, E.M. Wilson, Electrostatic modulation in steroid receptor recruitment of LXXLL and FXXLF motifs, Mol. Cell. Biol. 23 (2003) 2135-2150.

[9] R. Sanchez-Martinez, A.I. Castillo, A. Steinmeyer, A. Aranda, The retinoid X receptor ligand restores defective signalling by the vitamin D receptor, EMBO Rep. 7 (2006) 1030-1034.

[10] C. Carlberg, Ligand-mediated conformational changes of the VDR are required for gene transactivation, J. Steroid Biochem. Mol. Biol. 89-90 (2004) 227-232.

[11] J.B. Cheng, M.A. Levine, N.H. Bell, D.J. Mangelsdorf, D.W. Russell, Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase, Proc. Natl. Acad. Sci. U. S.A. 101 (2004) 7711-7715.

[12] S. Kitanaka, K. Takeyama, A. Murayama, T. Sato, K. Okumura, M. Nogami, Y. Hasegawa, H. Niimi, J. Yanagisawa, T. Tanaka, S. Kato, Inactivating mutations in the 25-hydroxyvitamin D3 1alpha-hydroxylase gene in patients with pseudovitamin D-deficiency rickets, N. Engl. J. Med. 338 (1998) 653-661.

[13] P.J. Malloy, V. Tasic, D. Taha, F. Tutunculer, G.S. Ying, L.K. Yin, J. Wang, D. Feldman, Vitamin D receptor mutations in patients with hereditary 1,25-dihydroxyvitamin D-resistant rickets, Mol. Genet. Metab. 111 (2014) 33-40.

[14] K. Yamamoto, H. Masuno, M. Choi, K. Nakashima, T. Taga, H. Ooizumi, K. Umesono, W. Sicinska, J. VanHooke, H.F. DeLuca, S. Yamada, Three-dimensional modeling of and ligand docking to vitamin D receptor ligand binding domain, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 1467-1472.

[15] J.B. Cheng, D.L. Motola, D.J. Mangelsdorf, D.W. Russell, De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxilase, J. Biol. Chem. 278 (2003) 38084-38093.

[16] Staunstrup NH, Bak RO, Cai Y, Svensson L, Petersen TK, Rosada C, Stenderup K, Bolund L, Mikkelsen JG. A lentiviral vector-based genetic sensor system for comparative analysis of permeability and activity of vitamin D3 analogues in xenotransplanted human skin. Exp Dermatol. 22 (2013) 178-183

[17] H. Mano, S. Ikushiro, N. Saito, A. Kittaka, T. Sakaki, Development of a highly sensitive in vitro system to detect and discriminate between vitamin D receptor agonists and antagonists based on split-luciferase technique, J. Steroid Biochem. Mol. Biol. (2017).

[18] H. Mano, M. Nishikawa, K. Yasuda, S. Ikushiro, N. Saito, M. Takano, A. Kittaka, T. Sakaki, Development of Novel Bioluminescent Sensor to Detect and Discriminate between Vitamin D Receptor Agonists and Antagonists in Living Cells, Bioconjugate Chem. 26 (2015) 2038-2045.

[19] H. Mano, M. Nishikawa, K. Yasuda, S. Ikushiro, N. Saito, D. Sawada, S. Honzawa, M. Takano, A. Kittaka, T. Sakaki, Novel screening system for high-affinity ligand of heredity vitamin D-resistant rickets-associated vitamin D receptor mutant R274L using bioluminescent sensor, J. Steroid Biochem. Mol. Biol. 167 (2017) 61-66.

[20] Rochel, N., Wurtz, J.M., Mitschler, A., Klaholz, B., Moras, D., 2000. The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Molecular cell 5(1), 173-179.

[21] L.A. Zella, C.Y. Chang, D.P. McDonnell, J.W. Pike, The vitamin D receptor interacts preferentially with DRIP205-like LxxLL motifs, Arch. Biochem. Biophys. 460 (2007) 206-212.

[22] P.J. Malloy, J.W. Pike, D. Feldman, The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D-resistant rickets, Endocr. Rev. 20 (1999) 156-188.

[23] K. Kristjansson, A.R. Rut, M. Hewison, J.L. O'Riordan, M.R. Hughes, Two mutations in the hormone binding domain of the vitamin D receptor cause tissue resistance to 1,25 dihydroxyvitamin D3, J. Clin. Invest. 92 (1993) 12-16.

[24] S. Kitanaka, K. Takeyama, A. Murayama, S. Kato, The molecular basis of vitamin D-dependent rickets type I, Endocr. J. 48 (2001) 427-432.

[25] S. Kitanaka, A. Murayama, T. Sakaki, K. Inouye, Y. Seino, S. Fukumoto, M. Shima, S. Yukizane, M. Takayanagi, H. Niimi, K. Takeyama, S. Kato, No enzyme activity of 25-hydroxyvitamin D3 1alpha-hydroxylase gene product in pseudovitamin D deficiency rickets, including that with mild clinical manifestation, J. Clin. Endocrinol. Metab. 84 (1999) 4111-4117.

[26] H.J. Kwon, Vitamin D receptor signaling is required for heart development in zebrafish embryo, Biochem. Biophys. Res. Commun. 470 (2016) 575-578.

[27] T.D. Veenstra, K. Prufer, C. Koenigsberger, S.W. Brimijoin, J.P. Grande, R. Kumar, 1,25-Dihydroxyvitamin D3 receptors in the central nervous system of the rat embryo, Brain Res. 804 (1998) 193-205.

[28] D. Eyles, T. Burne, J. McGrath, Vitamin D in fetal brain development, Semin. Cell. Dev. Biol. 22 (2011) 629-636.

Funding

This work was supported by Grants-in-Aid from the Japan Society for the Promotion of Science (No.16H04912 and 16K14904 to T.S.).

Figure Legends

Fig. 1.

Fig. 1. Schematic diagram of the WT biosensor to detect VDR ligands. Binding of the ligands to the WT biosensor may cause a conformational change of helix12 (H12) in LBD. After conformational change of LBD, the LXXLL motif interacts with LBD in the WT biosensor. Then, this intramolecular dynamic change of the WT biosensor leads to reconstitution of the functional complex between LucN and LucC fragments of the split-luciferase. LucN:Luciferase N-terminal domain, LucC:Luciferase C-terminal domain, LBD:ligand binding domain.

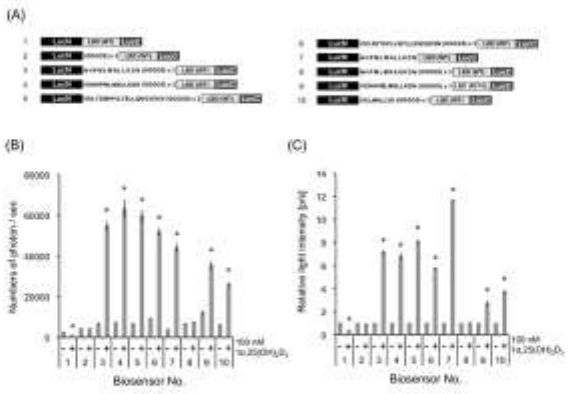


Fig. 2.

Fig. 2. Development of the split-luciferase based biosensor to detect VDR ligands in COS-7 cells. (A) Schematic structure of the biosensor proteins named 1-10, respectively. (B) Numbers of photons per second [p/s]. Data are represented as mean ± SD, $n = 4$. * $p < 0.05$. (C) relative light intensity [p/s] (fold of the control (0.1% EtOH)) of each biosensor after addition of 100 nM 1α,25$(OH)_2D_3$ were shown. The COS-7 cells expressing biosensor proteins were treated with 0.1% EtOH (-) or 100 nM 1α,25$(OH)_2D_3$ (+) and split-luciferase light intensity was measured 90 min after treatment.

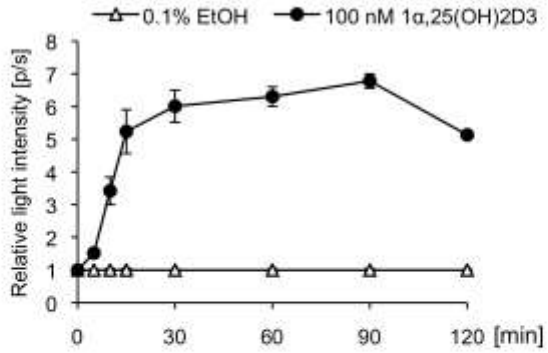


Fig. 3.

Fig. 3. Time-dependent relative light intensity of the WT biosensor. The COS-7 cells expressing WT biosensor proteins were treated with 100 nM of $1\alpha,25(OH)_2D_3$ or 0.1% EtOH for 120 min. The light intensity was measured 0, 5, 10, 15, 30, 60, 90 and 120 min after treatment. The relative light intensity [p/s] (fold of the control (0 nM=0.1% EtOH)) was shown. Data are represented as mean ± SEM, $n = 4$.

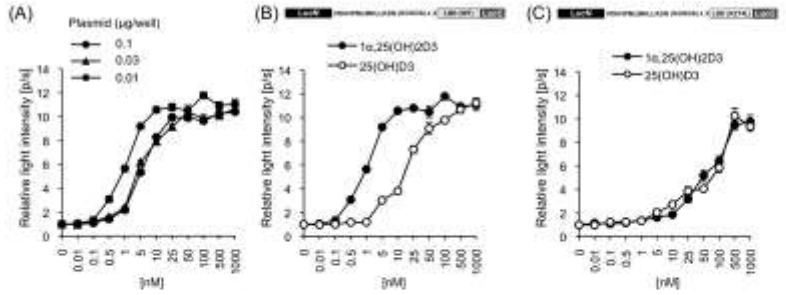


Fig. 4.

Fig. 4. Characterization of the WT biosensor and R274L biosensor in COS-7 cells. Dose-dependence of $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ on relative light intensity of the WT biosensor (A) and R274L biosensor (B). The COS-7 cells expressing WT biosensor or R274L biosensor proteins were treated with various concentrations (0-1000 nM) of $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ for 90 min. The light intensity was measured 0, 5, 10, 15, 30, 60, 90 and 120 min after treatment. The relative light intensity [p/s] (fold of the control (0 nM=0.1% EtOH)) was shown. Data are represented as mean ± SEM, $n = 4$.

It is noted that the plateau values are not reached at the measurement limit of the equipment.

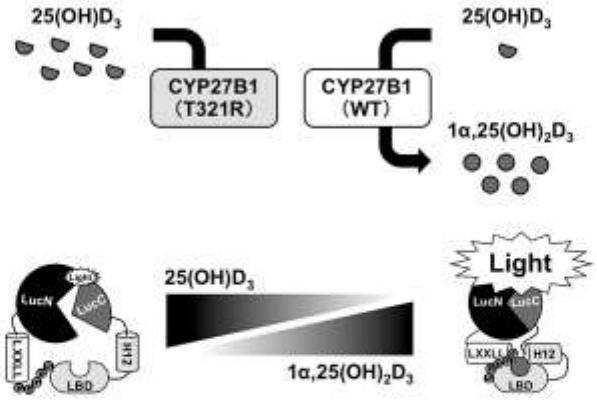


Fig. 5.

Fig. 5. Mechanism to evaluate CYP27B1 activity of the wild type (WT) or its mutants using the WT biosensor in COS-7 cells. When $25(OH)D_3$ is converted to $1\alpha,25(OH)_2D_3$ by CYP27B1 the light intensity of the WT biosensor increases. In contrast, the inactive mutant such as hCYP27B1 (T321R) cannot increase the light intensity.

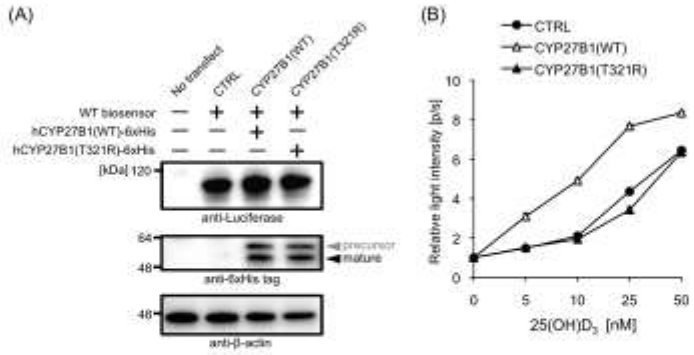


Fig. 6.

Fig. 6. Evaluation of the relative activity of hCYP27B1 in COS-7 cells using WT biosensor. (A) The amount of expressed hCYP27B1(WT)-6×His, hCYP27B1(T321R)-6×His and WT biosensor proteins was analyzed by Western blotting using anti-His tag, anti-luciferase or anti-β-actin antibody, respectively. The location of protein size markers (kDa) are shown on the left. Gray and black arrowhead indicates the precursor and mature forms of hCYP27B1, respectively. (B) Comparison of the relative activity between hCYP27B1(WT)-6×His and hCYP27B1(T321R)-6×His. The COS-7 cells co-expressing hCYP27B1(WT)-6×His or hCYP27B1(T321R)-6×His with WT biosensor were treated with various concentrations (0, 5, 10, 25 and 50 nM) of 25(OH)D_3 for 60 min at 37 °C. Subsequently, the cells were incubated for 30 min at room temperature and then total 90 min incubated cells were used for measurement of light intensity and Western blot analysis. The relative light intensity [p/s] (fold of the control (0 nM=0.1% EtOH)) was shown. Data are represented as mean ± SEM, n = 4.

Table 1 Nucleotide sequences of the PCR primer sets used to generate biosensors

Primer set No.	Primer sequence	
1	Forward	5'-AATTCTCGAGATGACCCAGACCCTCAAGTACGCC-3'
	Reverse	5'-TATTGGATCCTAGTGGTGATGGTGATGATGTCTGTCCAAAAACTGTAGGTTGATG-3'
2	Forward	5'-ATGGGACAGGGTGTCCAACACGCTC-3'
	Reverse	5'-TTGGACACCCTGTCCACTCCCGCC-3'
3	Forward	5'-CCCTCCACTACCCCCACCTCCGTCCTTGTCGATGAGAGCGTTTGTAGCC-3'
	Reverse	5'-GGAGGTAGCGGTGGCGGTGGTAGTCGGCCCAAGCTGTCTGAGGAGCAGC-3'
4	Forward	5'-CATGAGCATCGGGTGGTTGTCCTTGTCGATGAGAGCGTTTGTAGC-3'
	Reverse	5'-AACCTTCTTAAAGATAATGGAGGTGGGGGTAGTGGAGGGGGAGG-3'
5	Forward	5'-CGGCCCAAGCTGTCTGAGGAGCAG-3'
	Reverse	5'-ATTATCTTTAAGAAGGTTCATGAGCATC-3'
6	Forward	5'-AAAGATAATGGAGGTGGGGGTAGTG-3'
	Reverse	5'-AGCAGCGTTCATGAGCATCGGGTGGTTG-3'
7	Forward	5'-ACAAGCCTTCTTCAGAATGGAGTTGACCATGTGGGAGGTGGGGGTAGTGGAGGGGGAGG-3'
	Reverse	5'-GAGGATCGGGTGCATCTCTGTGAGGTCGACGTCCTTGTCGATGAGAGCGTTTGTAG-3'
8	Forward	5'-TGGACACTTCTTAGCAGCGAAGGGGACAGCATGGGAGGTGGGGGTAGTGGAGGGGGAGG-3'
	Reverse	5'-AAGAAGCGGGTGTGTCTCGCTGAGGTCGACGTCCTTGTCGATGAGAGCGTTTGTAG-3'
9	Forward	5'-GTCGACGGAGGTGGGGGTAGTGGAGGGGGAG-3'
	Reverse	5'-AAGAAGGTTCATGAGGTCGACGTCCTTGTCGATGAGAGCG-3'

10	Forwa rd	5’-ATAGTCGACAACCACCCGATGCTCATGAACCTTC-3’
	Rever se	5’-ATATGCGGCCGCTTACACGGCGATCTTGCCGCCCTTCTTG-3’
11	Forwa rd	5’-CATCATGTTGCTCTCCAATGAGTCCTTCAC-3’
	Rever se	5’-ACTCATTGGAGAGCAACATGATGACCTCAATG-3’