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Cloning the human vitamin D receptor into the pTwin-1 expression vector☆

Elaine D. Collins*, Aileen Espinoza, Lily Thao-Nhi Le, Mallory Kato

Department of Chemistry, San Jose State University, One Washington Square, San Jose, CA 95192-0101, USA

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ABSTRACT

Many of the actions of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] are mediated by binding to the nuclear vitamin D receptor (VDR). VDR is a member of a superfamily of nuclear receptors that are ligand-dependent transcription factors. Ligand binding induces conformational changes in the VDR that enable the receptor to interact with other coactivators to modulate gene transcription. In order to better characterize the binding of the VDR to 1,25$(OH)_2D_3$ and to analogs of 1,25$(OH)_2D_3$, we have cloned the cDNA for the human VDR into the pTwin1 expression system. The expression system results in the cDNA for a chitin-binding peptide and a yeast intein fused in frame with the N-terminal end of the cDNA for VDR. The intein cDNA codes for a self-cleaving peptide that can release VDR, without any additional amino acids, from a chitin column by changing the pH of the buffer. Western blot analysis of the VDR-fusion protein indicates that a protein of approximately 75 kDA was obtained as expected.

1. Introduction

The hormonal form of vitamin D is a key regulator of calcium and phosphorus homeostasis in the human body. It promotes their absorption in the intestines, and retains calcium at the kidney for bone growth, mineralization, and remodeling. Vitamin D is converted into the hormonal form of vitamin D, 1,25$(OH)_2$ vitamin D_3 by two hydroxylation reactions, first in the liver and finally in the kidneys [1]. Hormonal vitamin D mediates most of its actions by binding to the nuclear vitamin D receptor (VDR) in target tissues. VDR is a member of the nuclear receptor superfamily. The proteins of the superfamily are ligand-dependent transcription factors that contain two highly conserved domains: a DNA binding domain and a ligand binding domain [2]. When the cognate ligand binds to the receptor, the receptor undergoes a conformational change that allows it to interact with a number of other proteins to form an active transcriptional complex [3]. This complex can then modulate gene expression of sensitive genes [4].

The human VDR has a polymorphism, FokI, within its coding sequence that can change the amino acid sequence of the receptor. The polymorphism is in the ATG start codon and is a "T" to "C" conversion. The result is that the protein is initiated from the fourth codon, the next ATG in the sequence [1]. The final VDR protein is then three amino acids smaller (Fig. 1). The polymorphism also results in the ablation of a FokI restriction site within the coding sequence since the recognition sequence for FokI is GGATG that has been changed to GGACG. The FokI polymorphism produces two VDR forms, a 427 form designated "f" or a 424 form designated "F". The polymorphism is related to bone mineral density in human populations. The FF genotype is related to higher bone mineral density, and thus stronger bones, than the ff genotype. The smaller VDR has been shown to interact more strongly with transcription factor TFIIB than the 427 VDR [5].

We have designed primers to amplify the cDNA for three different VDR constructs: the full-length VDR (1-427), the FokI (F) polymorphism VDR (4-427) and the ligand binding domain VDR (118-427). We have cloned these constructs into the expression vector, pTwin-1 (New England Biolabs). The pTwin expression system will result in the expression of an N-terminal chitin-binding peptide–intein fusion protein with the VDR construct. The expressed protein is purified with a chitin affinity column, and the VDR is released from the column by self-cleavage of the intein at the peptide bond between the intein and VDR. The purified VDR can then be used for biophysical techniques that require purified protein.

2. Materials and methods

2.1. Polymerase chain reaction to amplify the three VDR constructs

PCR primers were designed so that the forward primer contained a SapI recognition site directly upstream from the initiating ATG, and the reverse primer contained a BamHI site adjacent to the stop codon. The primers were obtained from Eurofins MWG Operon. The template DNA was the cDNA for human VDR cloned

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* Corresponding author. Tel.: +1 408 924 4969; fax: +1 408 924 4945.
E-mail address: ecollins@science.sjsu.edu (E.D. Collins).

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MEAMAASTSLPDPGDFDRNVPRICGVCGDRATGFHFNAMT - 40
CEGCKGFFRRSMKRKALFTCPFNGDCRITKDNRRHCQACR - 80
LKRCVDIGMMKEFILTDEEVQRKREMILKRKEEEALKDSL -120
RPKLSEEQQRIIAILLDAHHKTYDPTYSDFCQFRPPVRVN -160
DGGGSHPSRPNSRHTPSFSGDSSSSCSDHCITSSDMMDSS -200
SFSNLDLSEEDSDDPSVTLELSQLSMLPHLADLVSYSIQK -240
VIGFAKMIPGFRDLTSEDQIVLLKSSAIEVIMLRSNESFT -280
MDDMSWTCGNQDYKYRVSDVTKAGHSLELIEPLIKFQVGL -320
KKLNLHEEEHVLLMAICIVSPDRPGVQDAALIEAIQDRLS -360
NTLQTYIRCRHPPPGSHLLYAKMIQKLADLRSLNEEHSKQ -400
YRCLSFQPECSMKLTPLVLEVFGNEIS               -427
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Fig. 1. Amino acid sequence of human VDR. Bold residues indicate the first amino acids of the three constructs.

into the EcoRI and NsiI sites of pcDNA 1.1-Amp (Invitrogen) to produce plasmid pcVDR. PCR was performed using HotStart Taq polymerase (Fermentas) according to manufacturer's instructions in a 50 μL reaction volume with 1 ng of template DNA, 0.2 μM of forward and reverse primers, and 1.5 mM $MgCl_2$. The samples were incubated in a Robocycler thermalcycler (Stratagene) programmed for the following cycles: 94 °C for 2 min; 40 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min; 72 °C for 10 min; hold at 6 °C. PCR products were purified using PCR cleanup (Bay Gene, Inc, San Francisco, CA) according to manufacturer's instructions. Size and concentration of PCR products were determined by agarose gel electrophoresis.

2.2. Cloning the VDR construct into pTwin-1

The purified PCR products or plasmid pTwin1 (New England Biolabs) were digested with SapI and BamHI using FastDigest enzymes (Fermentas) at 37 °C. The pTwin1 was also treated with thermal sensitive alkaline phosphatase, TSAP (Promega), for an additional 15 min at 37 °C. Enzymes were removed from the reaction using PCR cleanup (Bay Gene, Inc). The VDR fragments were ligated into 50 ng of pTwin1 at a ratio of 1:3 vector:insert using Ligafast Rapid DNA Ligation System (Promega). The ligation was incubated for 1 h at room temperature. XL2-blue ultracompetent cells (Stratagene) were transformed with 2 μL of the ligation reaction and plated onto LB-agar plates containing 100 μg/mL ampicillin and incubated overnight at 37 °C. Positive clones were selected and screened for plasmids with the expected size insert. Positive clones were verified by sequencing (Alpha Biolaboratory Inc).

2.3. Expression of VDR (1-427) in Escherichia coli ER2566 cells

Plasmids with VDR inserts were transformed into *E. coli* strain ER2566 cells (New England Biolabs) with TransformAid Bacterial Transformation Kit (Fermentas). Expression of cultures in LB broth at 12 °C was induced with 0.4 mM IPTG. Cells were collected after overnight growth by centrifugation at 5000 × *g* for 10 min at 4 °C. The pellet was resuspended in 20 mL column buffer (20 mM Tris–Cl, pH 8.0, 0.5 M NaCl, 1 mM EDTA), and the cells were lysed by sonication on ice. Cell extracts were prepared by centrifugation at 20,000 × *g* for 30 min at 4 °C. The cell extract was resolved by electrophoresis on 4–12% NuPage Bis–Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Western blot analysis was performed using rabbit anti-chitin-binding protein antibody (New England Biolabs) and horseradish peroxidase-linked goat anti-rabbit antibody (Santa Cruz Biotechnology) using the Super-Signal West Pico Rabbit IgG kit (Thermo Scientific) according to manufacturer's protocol. Chemiluminescence was detected using FluorChem Imaging System (AlphaInnotech).

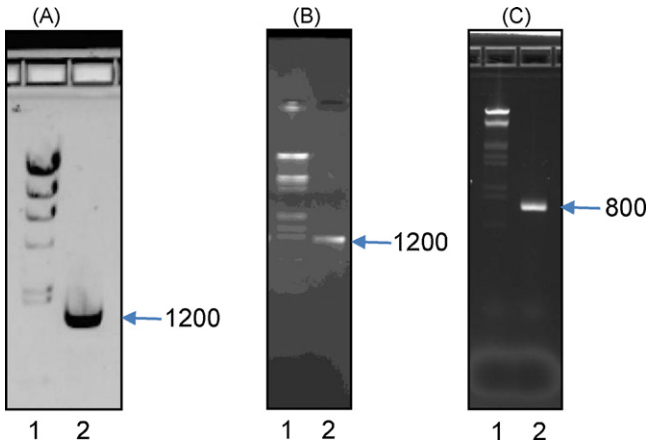

Fig. 2. PCR of the three VDR constructs. PCR was performed as described in Section 2 to produce the three VDR constructs: 1-427, 4-427 and 118-427. The PCR products were resolved on 1% agarose gels with ethidium bromide (5 μg/mL). In Panel A, Lane 1 is Lambda Hind III cut DNA standard (0.5 μg) and in Lane 2 is 5 μL of PCR reaction for VDR 1-427. A single fragment of ≈1200 bp was obtained, the expected size for the construct. In Panel B, Lane 1 is Lambda HindIII/EcoRI cut DNA standard (0.5 μg) and in Lane 2 is 5 μL of PCR reaction for VDR 4-427. A single fragment of ≈1200 bp was obtained, the expected size for the construct. In Panel C, Lane 1 is Lambda HindIII/EcoRI cut DNA standard (0.5 μg) and in Lane 2 is 5 μL of PCR reaction for VDR 118-427. A single fragment of ≈800 bp was obtained, the expected size for the construct.

3. Results

3.1. Polymerase chain reaction to amplify the three VDR constructs

Forward and reverse PCR primers were designed with a SapI site in the forward primer and a BamHI site in the reverse primer. The primers were used in PCR reactions to amplify the cDNA for each of the three VDR constructs. The results of the PCR reactions are shown in Fig. 2. All three reactions yielded a fragment of the

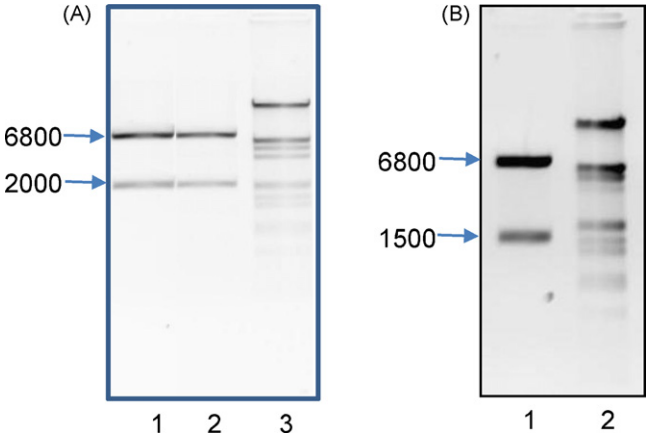

Fig. 3. Purification and digestion of pTwin with VDR construct insert. The VDR PCR fragments were digested with SapI and BamHI and ligated into pTwin. The ligated plasmids were transformed into XL2-blue ultracompetent cells as described in Section 2 and positive clones were selected. Plasmids were purified from the bacterial colonies and digested with NdeI and BamHI. Digests were resolved on 1% agarose gels with ethidium bromide (5 μg/mL). In Panel A, Lane 1 is 1 μL of digested plasmid pTwin-VDR 1-427, Lane 2 is 1 μL of digested plasmid pTwin-VDR 4-427, and in Lane 3 is Lambda HindIII/EcoRI cut DNA standard (0.5 μg). Two bands were obtained for each digestion. The top band at 6800 bp is the expected size of the pTwin vector; the lower band at 2000 bp is the VDR insert. Both VDR 1-427 and VDR 4-427 have expected sizes of ≈2000 bp. In Panel B, Lane 1 is 1 μL of digested plasmid pTwin-VDR 118-427, and in Lane 2 is Lambda HindIII/EcoRI cut DNA standard (0.5 μg). The top band at 6800 bp is the expected size of the pTwin vector; the lower band at 1500 bp is the expected size for the VDR 118-427 insert.

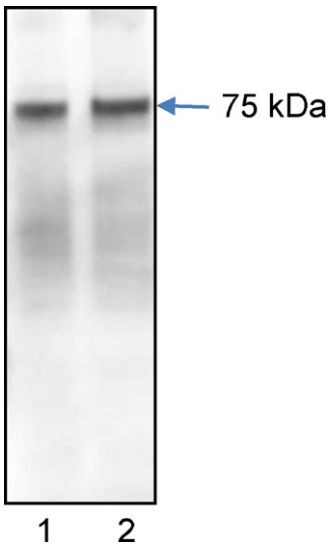

Fig. 4. Western blot analysis of expressed VDR 1-427. VDR 1-427 was expressed in *E. coli* ER2466 cells as described in Section 2. The cells were lysed by sonication and cell extract was electrophoresed on 4–12% Tris–Bis gels and blotted to nitrocellulose filters. The primary antibody used was rabbit anti-chitin-binding peptide, and the secondary antibody was HRP-linked goat anti-rabbit. The substrate used was Supersignal west pico chemiluminescent substrate as described in Section 2. Chemiluminescence was detected with FluorChem 6400. Both Lanes 1 and 2 contain 7.5 μL of cell extract from VDR 1-427 expression. A single band of 75 kDa that interacts with the chitin-binding protein was detected for both samples. This is the expected size for the VDR-chitin-binding protein–intein fusion protein.

expected size, ≈1200 bp for (1-427) and (4-427) and ≈800 bp for (118-427).

3.2. Cloning the VDR construct into pTwin-1

The three PCR fragments were purified to remove the Taq polymerase and unincorporated primers and nucleotides. The fragments were then digested with SapI and BamHI restriction endonucleases. The pTwin vector was digested with the same restriction enzymes and also with thermal sensitive alkaline phosphatase to prevent religation of the vector with itself. After digestion, the VDR fragments were ligated into pTwin and used to transform competent bacteria. Positive clones were selected and plasmids were purified from the selected clones. The plasmids were double digested with NdeI and BamHI to verify they contained the correct size insert. The results are shown in Fig. 3. The digestions of all three plasmids resulted in two bands, a band of 6800 bp corresponding to the pTwin plasmid and a smaller band corresponding to the VDR insert. For the VDR (1-427) and (4-427), the band was the expected size of ≈2000 bp. For the VDR (118-427) the band was the expected size of ≈1500 bp. The identities of the inserts were verified by sequencing. All of the inserts had the correct sequence, and the coding frame was determined to be in frame with the chitin-binding peptide and intein.

3.3. Expression of VDR (1-427) in E. coli ER2566 cells

The pTwin plasmid containing the VDR (1-427) insert was transformed into expression cells, *E. coli* ER 2566. The cells were grown in LB broth and induced with IPTG. The cells were harvested and lysed by sonication. The cell extract was then analyzed for the presence of the VDR-chitin-binding peptide–intein fusion protein by Western blotting. The results are seen in Fig. 4. A single band was detected by the anti-chitin-binding peptide antibody at 75 kDA. This is the expected size for the VDR-fusion protein.

4. Discussion

Three constructs of the VDR have been successfully cloned into the pTwin expression plasmid. Preliminary expression of the VDR (1-427) construct indicates that a protein corresponding to the expected size of the VDR-fusion protein was obtained. The VDR (1-427) will be purified on a chitin column, and the other two constructs will also be expressed and purified. The purified proteins will allow further studies of the biophysical properties of the human nuclear VDR.

Acknowledgements

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References

[1] R. Bouillon, G. Carmeliet, L. Verlinden, E. van Etten, A. Verstuyf, H.F. Luderer, L. Lieben, C. Mathieu, M. Demay, Vitamin D and human health: lessons from vitamin D receptor null mice, Endocr. Rev. 29 (6) (2008) 726–776.
[2] E.D. Collins, Molecular biology of the vitamin D endocrine system, Recent Res. Dev. Steroid Biochem. Mol. Biol. 1 (1) (2000) 81–106.
[3] S. Peleg, M. Shastry, E.D. Collins, J.E. Bishop, A.W. Norman, Distinct conformational changes induced by 20-epi analogues of 1α,25-dihydroxyvitamin D_3 are associated with enhanced activation of the vitamin D receptor, J. Biol. Chem. 270 (18) (1995) 10551–10558.
[4] M.R. Haussler, C.A. Haussler, L. Bartik, G.K. Whitfield, J.C. Hsieh, S. Slater, P.W. Jurutka, Vitamin D receptor: molecular signaling and actions of nutritional ligands in disease prevention, Nutr. Rev. 66 (10 Suppl. 2) (2008) S98–S112.
[5] P.W. Jurutka, L.S. Remus, G.K. Whitfield, P.D. Thompson, J.C. Hsieh, H. Zitzer, P. Tavakkoli, M.A. Galligan, H.T. Dang, C.A. Haussler, M.R. Haussler, The polymorphic N terminus in human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIB, Mol. Endocrinol. 14 (3) (2000) 401–420.