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BACTERIAL SYNTHESIS OF TRUNCATED FORMS OF THE HUMAN VITAMIN D RECEPTOR AND CHARACTERIZATION OF ANTI-RECEPTOR MONOCLONAL ANTIBODIES

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We biosynthesized full-length (amino acids 1-427) and truncated human 1,25-dihydroxyvitamin D_3 receptor proteins that encompassed only the putative DNA binding domain (amino acids 1-112) or the DNA binding domain and parts of the sterol binding domain (amino acids 1-193 and 1-328) in a bacterial expression system. We also prepared monoclonal antibodies against the full-length vitamin D receptor. The binding properties of the monoclonal antibodies were characterized by their ability to bind to full-length and truncated vitamin D receptor protein constructs. Seven of twelve monoclonal antibodies recognized the full-length receptor protein. These antibodies bound to truncated hVDR proteins with decreasing affinities as successive truncations were made from the carboxy-terminal end of the receptor protein. The five remaining monoclonal antibodies recognized the full-length and truncated receptor proteins with equally low affinities. Truncated forms of the vitamin D receptor and region-specific antibodies will be useful in assessing the properties of the receptor.

1,25-Dihydroxyvitamin D_3, the hormonal form of vitamin D_3, acts in various calcium-transporting and other tissues by associating with an intracellular receptor, the 1,25-dihydroxyvitamin D receptor (VDR), that acts in association with 1,25-dihydroxyvitamin D_3 as a transcription factor to increase, and sometimes decrease, the expression of genes (1-8). Examples of genes whose activity is influenced by 1,25-dihydroxyvitamin D_3 include the vitamin D-dependent calcium-binding proteins, osteocalcin, the 25-hydroxyvitamin D_3 24-hydroxylase, the plasma membrane calcium pump, the vitamin D receptor itself, and parathyroid hormone among others (1,5-14).

The VDR is related to thyroid hormone and retinoic acid receptors, and like these two receptors, it has domains that bind to hormone or deoxyribonucleic acid sequences present in responsive genes (1,2). The VDR binds to DNA response elements either as a homodimer or as a heterodimer in association with the retinoid X receptor (11-22). Based on the analysis of mutant receptors from patients with hereditary vitamin D dependency rickets, type II, the DNA binding

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domain of VDR is thought to be located in the amino-terminal portion of the protein, while the sterol binding domain of the receptor is in the carboxy-terminal portion of the molecule (1,2,31,32). The DNA binding domain has nine cysteine residues, eight of which are presumably part of "zinc fingers" that might associate with the DNA of the response elements (1-4).

Until recently, when we reported on a method for the expression of milligram amounts of VDR protein in a bacterial expression system, the expression of receptor protein in amounts sufficient to use in biophysical experiments was frustrated by relatively low yields of the receptor in various expression systems and the need for extensive purification procedures after expression (33-36). Furthermore, although analysis of receptor mutations has yielded information about domains of the receptor that are involved in sterol and DNA binding, direct experiments testing the ability of defined segments of the receptor protein to bind to sterol or DNA have not been carried out. To learn more about the structure of these domains and to develop reagents that recognize different domains of the receptor, we expressed truncated forms of the receptor that contained successive deletions of the molecule from the carboxy-terminal region.

MATERIALS AND METHODS

General. Ultraviolet spectra of oligonucleotides and nucleic acids were obtained on a Beckman DU-70 spectrophotometer (Beckman Instruments, Fullerton, CA). Oligonucleotides were synthesized on an Applied Biosystems Oligonucleotide synthesizer (Applied Biosystems, Foster City, CA) using phosphoramidate chemistry (37). Initial subcloning of PCR DNA fragments was carried out using an Invitrogen TA Cloning kit (Invitrogen Corporation, San Diego, CA). DNA sequencing was performed using Sanger dideoxy sequencing methods (38) on an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, CA). Protein determinations were carried out with a Bio-Rad Protein assay (Bio-Rad, Richmond, CA). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using precast mini-gels and a Pharmacia Phast Gel System (Pharmacia Instruments, Piscataway, NJ). Amino acid composition analysis and sequencing were carried out as described (39,40). Enzyme linked immunosorbent assay (ELISA) procedures were carried out as described (41). The original human vitamin D receptor clone was obtained from Dr. J. W. Pike (Ligand Pharmaceuticals, San Diego, CA).

Synthesis of Oligonucleotides: The following oligonucleotides were synthesized and used in polymerase chain reactions to generate the appropriate constructs for insertion into the *pET 3a* expression vector (42). These chimeric plasmids were used in subsequent experiments to be described below. The sequence of the 5' oligonucleotide incorporated an *Nde I* restriction site for cloning into the *pET 3a* expression vector. The same 5' oligonucleotide was used to generate all constructs. The *Nde I* site is underlined. The sequence of this oligonucleotide is:

5' ATGCCATATGGAGGCAATGGCGGC 3'

The sequence of the 3' oligonucleotides incorporated a *Bam HI* restriction site for cloning into the *pET 3a* expression vector. The *Bam HI* site is underlined. The sequences of the 3' oligonucleotides are as follows:

Full-length hVDR construct 1-427: **5' CGTGGATCCTCAGGAGATCTCATTGCCAAA 3';**
hVDR construct 1-328: **5' ATGCATCTAGAGGATCCTCACTCCTCATGCAAGTTCAGCTTC 3';**
hVDR construct 1-193: **5' ATGCATCTAGAGGATCCTCAAGAGGTGATACAGTGATCTGAGCA 3';**
hVDR construct 1-112: **5' ATGCATCTAGAGGATCCTCACTCCTTCCGCTTCAGGATCAT 3'.**

Synthesis of Constructs by PCR: The hVDR-*pGEM* chimeric plasmid was linearized as described previously and used with the appropriate primers in a polymerase chain reaction to

construct the truncated hVDR DNA fragments (42). The PCR products were each ligated into the *pCR II* vector, amplified and excised by digestion with *Nde 1* and *Bam H1*. The inserts were then ligated into *Nde 1* and *Bam H1* digested *pET 3a* plasmid.

Expression of Full-Length and Truncated Proteins in *BL 21 (DE3) pLysS* Cells: Expression was carried out by transforming *E. coli BL 21 (DE3) pLysS* cells with the appropriate hVDR-*pET 3a* plasmid constructs and inducing protein expression as described by Studier et al and earlier by us in detail for the full-length protein (33,43). Briefly, individual colonies of appropriately transformed *E. coli BL 21 (DE3) pLysS* cells grown on LB medium containing ampicillin and chloramphenicol were grown overnight at 37°C in M9ZB medium containing 0.02 M glucose, 1 mM magnesium sulfate and 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. A 5 ml aliquot of this culture was added to 200 ml of M9ZB medium, prepared as noted above. The cells were grown at 37°C to an OD_{600} of 0.55 and protein synthesis was induced under the direction of bacterial T_7 polymerase by adding isopropylthiogalactoside to a final concentration of 0.4 mM. Incubation of the culture was continued for another 4 hours. Protein expression was monitored by SDS-PAGE.

Purification of Expressed Proteins: The expressed proteins were treated in a manner identical to that described earlier (33). All of the expressed hVDR proteins were present in bacterial inclusion bodies. Homogenization in buffer, Triton X-100 treatment in the presence of reducing agents and solubilization in SDS were carried out exactly as described earlier (33). The individual hVDR proteins were dialyzed extensively against water at 4°C. At this stage the expressed proteins were sufficiently pure to be used for further analysis.

Generation of Monoclonal Antibodies: Monoclonal antibodies were produced as described by Katzman et al using established procedures (41).

ELISA was performed according to a standard protocol (41). Briefly, the monoclonal antibodies were detected by coating the wells of microtiter plates with the full-length, 1-328, 1-193, or 1-112 hVDR proteins, incubating the coated plates with solutions containing specific antibodies and washing away unbound antibodies. A solution containing an alkaline phosphatase conjugated secondary antibody was then added to the plate. After incubation, unbound conjugate was washed away and substrate solution was added. After further incubation, the amount of substrate hydrolyzed was measured with a spectrophotometer.

RESULTS AND DISCUSSION

By using the same 5' oligonucleotide and varying the 3' oligonucleotide, we generated DNA fragments by PCR methods that represent distinct portions of the hVDR (Figure 1, Lanes B-E). The products were of the appropriate sizes. Following purification, the PCR products were ligated into the *pCR II* vector for amplification. Sequencing of the insert DNAs revealed that they had the appropriate nucleotide sequences. The *Nde 1 - Bam H1* DNA fragments were then subcloned into a *pET 3a* vector.

Full-length and truncated hVDR-*pET 3a* chimeric plasmids were used to transform *E. coli BL 21 (DE3) pLysS* cells. Transformed cells were initially grown without isopropylthiogalactoside in the culture medium. Following the addition of isopropylthiogalactoside to induce bacterial T_7 polymerase, full-length and truncated hVDR proteins of the appropriate length were synthesized. In the induced bacteria, protein bands of appropriate sizes were noted for the full-length, 1-328, 1-193 and 1-112 hVDRs respectively (Figure 2, Lanes B-E). Prior to induction of protein synthesis no bands corresponding to the expressed proteins were visible (Figure 2, Lane F). Following purification procedure, hVDRs of the appropriate M_r were noted on SDS-PAGE. These were virtually pure and were used as such for further analysis by the monoclonal antibodies. Each of the truncated hVDR proteins had the appropriate amino acid composition and the appropriate amino-terminal amino acid

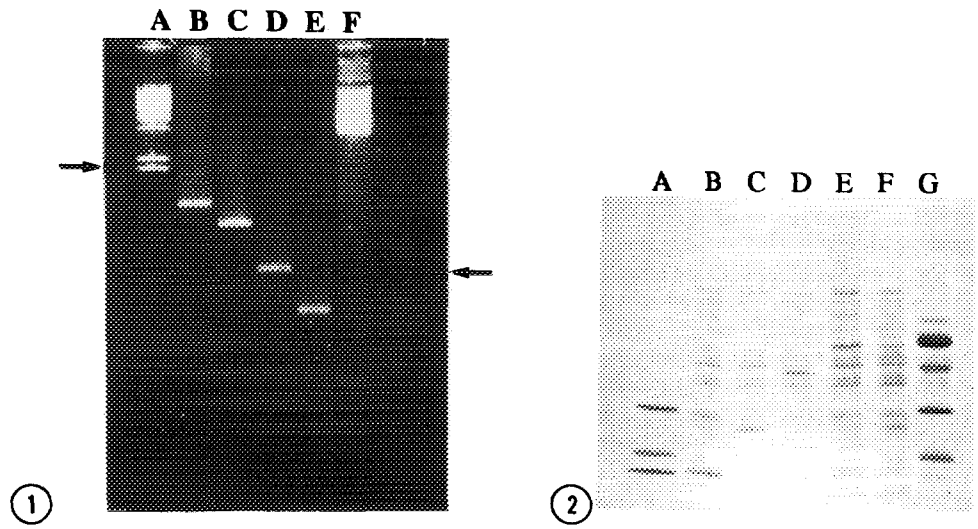

Figure 1. PCR products of the full-length and truncated receptors prior to insertion into the *pET 3a* expression vector. Lane A: Lambda Hind III markers (arrow indicates the 2.0 kb fragment). B: Full-length hVDR PCR product (1299 bp including the extra nucleotides present in the oligonucleotide primers). C: 1-328 aa hVDR PCR product (1020 bp). D: 1-193 aa hVDR PCR product (616 bp). E: 1-112 aa hVDR PCR product (373 bp). F: 123 bp DNA ladder (arrow indicates the 615 bp fragment).

Figure 2. SDS-PAGE of the bacterial proteins prior to and following induction with isopropylthiogalactoside. Lane A: Molecular weight markers: Carbonic Anhydrase, M_r 29,000, Trypsin Inhibitor, M_r 20,100, Lysozyme, M_r 14,300. Lane B: Induced 1-112 hVDR protein (predicted MW 12,931). Lane C: Induced 1-193 hVDR protein (predicted MW 21,911). Lane D: Induced 1-328 hVDR protein (predicted MW 37,073). Lane E: Induced full-length 1-427 hVDR protein (predicted MW 48,285). Lane F: Uninduced bacterial expression culture. Lane G: Molecular weight markers: Bovine Serum Albumin, M_r 66,000, Ovalbumin, M_r 45,000, Carbonic Anhydrase, M_r 29,000, Trypsin Inhibitor, M_r 20,100.

sequence (data not shown). Yields of each of the expressed hVDR proteins ranged between 5-10 mg per 200 ml of bacterial culture.

The full-length hVDR was used to generate monoclonal antibodies according to established procedures. Twelve hybridomas that were positive on initial screening were used to generate antibodies. The monoclonal antibodies produced were used to perform enzyme-linked immunosorbent assays with the full-length and truncated hVDR proteins serving as antigen. Seven of the monoclonals (24.8, 24.45, 42.11, 46.40, 68.30, 80.6 and 80.12) bound to the full-length receptor with high affinity but showed a reduced signal when assayed against truncated receptors. The signal was not abolished even with the shortest protein construct (hVDR 1-112). This indicates that the monoclonals bind optimally to the entire VDR molecule. Specific epitopes in the truncated receptors, however, still allow binding of antibody, although with a reduced affinity. The five remaining monoclonals recognized the full-length and truncated receptors at the same low affinity levels.

In summary, we have shown that a bacterial expression system can be used to successfully generate truncated human VDR proteins in amounts sufficient for further biophysical analysis. We have also shown that one can generate monoclonal antibodies that bind to the full-length and truncated vitamin D receptor. These reagents should prove useful in the analysis of the function of different domains of the receptor.

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