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Partial amino acid sequence of porcine 1,25-dihydroxyvitamin D_3 receptor isolated by immunoaffinity chromatography

(calcium metabolism/vitamin D function/intestinal absorption/vitamin D metabolism/steroid receptors)

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ABSTRACT **Monoclonal antibodies against the porcine 1,25-dihydroxyvitamin D_3 receptor were immobilized on Sepharose CL-4B and used to obtain a highly purified 1,25-dihydroxyvitamin D_3 receptor fraction with a 45% recovery of the 1,25-dihydroxyvitamin D_3 binding capacity. The porcine receptor was purified to homogeneity by preparative electrophoresis and digested in sodium dodecyl sulfate/polyacrylamide gels with *Staphylococcus aureus* strain V8 protease. The resulting peptides were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, electrophoretically transferred to polyvinylidene difluoride membranes, and directly sequenced. The generation and isolation of peptides by this method allows sequencing of proteins present in trace amounts as well as those whose amino termini have been modified. The 1,25-dihydroxyvitamin D_3 receptor amino acid sequence corresponded to the sequence predicted from a recently cloned receptor cDNA obtained from rat kidney mRNAs.**

The high-affinity receptor protein for 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is an essential mediator of the regulation of calcium homeostasis by vitamin D_3 (1). 1,25-$(OH)_2D_3$, the hormonal form of vitamin D, is thought to act through its receptor by a mechanism analogous to that of other steroid hormones (2, 3). Observations consistent with a steroid model for vitamin D_3 action include that 1,25-$(OH)_2D_3$ is (*i*) formed from 7-dehydrocholesterol by a light-dependent reaction in the skin and sequential hydroxylations in the liver and kidney, (*ii*) absorbed by target tissues, (*iii*) rapidly localized in the nucleus of target cells, and (*iv*) responsible for the induction of specific proteins, including calcium-binding protein (2, 3). Other steroid hormone receptors, such as glucocorticoid receptor, estrogen receptor, and progesterone receptor, have been shown to bind to specific DNA sequences near target genes and to modulate the expression of these target genes (4). Although direct evidence for 1,25-$(OH)_2D_3$ receptor interaction with specific DNA sequences has not been demonstrated, the primary amino acid sequence of the 1,25-$(OH)_2D_3$ receptor putative DNA binding domain has recently been deduced from a partial avian cDNA clone; it is similar to that of other steroid hormone receptors (5). The structural similarity between the DNA-binding domains of 1,25-$(OH)_2D_3$ receptor and other steroid hormone receptors suggests a common mechanism involving the binding of receptor to DNA, which presumably alters the transcription rate of target genes and leads to the physiological response.

Considerable effort has been devoted to the isolation and characterization of the 1,25-$(OH)_2D_3$ receptor because of its central role in mediating the physiological responses to 1,25-$(OH)_2D_3$. However, due to its instability and low abundance (<0.001% in target tissues), the 1,25-$(OH)_2D_3$ receptor was not purified to apparent homogeneity until 1982 (6). Only minute amounts were obtained, insufficient for further work. Classical purification techniques were used to obtain purified chicken (7) and pig (8) receptor for the generation of monoclonal antibodies. Monoclonal antibodies have aided in the characterization of the pig 1,25-$(OH)_2D_3$ receptor as a 55-kDa protein (9), with the major form having an isoelectric point of 6.1 (10). Preliminary peptide mapping of the functional domains of the avian 1,25-$(OH)_2D_3$ receptor has also been achieved by using immunoblotting with a monoclonal antibody (11). Although immobilized monoclonal antibodies were used previously to purify avian 1,25-$(OH)_2D_3$ receptor, the isolated protein did not retain 1,25-$(OH)_2D_3$-binding activity (12).

In this paper, we report the purification and partial amino acid sequence of pig intestinal 1,25-$(OH)_2D_3$. Highly purified receptor that retained 1,25-$(OH)_2D_3$-binding activity was obtained in a one-step purification using immunoaffinity chromatography. In addition, preparative sodium dodecyl sulfate/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) followed by electroelution was used to isolate homogeneous protein. The immunoaffinity-purified 1,25-$(OH)_2D_3$ receptor was digested in $NaDodSO_4$/polyacrylamide gels with *Staphylococcus aureus* strain V8 (*Staph A*) protease according to the method of Cleveland *et al.* (13), and the resulting 1,25-$(OH)_2D_3$ receptor peptides were transferred to a polyvinylidene difluoride (PVDF) membrane. Partial amino-terminal amino acid sequences were determined by vapor-phase Edman degradation of the PVDF-bound peptides, and the amino acid sequences were compared to the sequence deduced from a rat kidney 1,25-$(OH)_2D_3$ receptor cDNA clone.

MATERIALS AND METHODS

Vitamin D Compounds. Radioactive 1,25-$(OH)_2$[26,27-^{3}H]D_3 (160 Ci/mmol; 1 Ci = 37 GBq) was produced by DuPont/NEN and prepared as previously described (14). Nonradioactive 1,25-$(OH)_2D_3$ was a gift from Hoffmann–La Roche.

Buffers. Buffers used were as follows: Phosphate-buffered saline (PBS), 1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4 (pH 8.0)/137 mM NaCl/2.7 mM KCl/0.02% NaN_3; Blotto blocking buffer, 5% (wt/vol) Carnation nonfat dry milk plus 0.05% (wt/vol) Tween 20/0.02% NaN_3 in PBS; Blotto washing buffer, 0.5% (wt/vol) Carnation nonfat dry milk/0.05% Tween 20/0.02% (wt/vol) NaN_3 in PBS; TED, 50 mM Tris·HCl (pH 7.4)/1.5 mM EDTA/5 mM dithiothreitol; TEDNa, TED/150 mM NaCl; $TEDK_{300}$, TED/300 mM KCl/10 mM $MgCl_2$; $TEDK_{400}$, TED/400 mM KCl/0.05% Tween 20.

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Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PVDF, polyvinylidene difluoride; *Staph A*, *Staphylococcus aureus* strain V8; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid.
*No reprints will be available from the authors.

Monoclonal Antibodies. Monoclonal antibodies were generated to the pig 1,25-$(OH)_2D_3$ receptor as previously described (8). Antibodies were obtained from mouse ascites fluid and purified by column chromatography on goat anti-mouse IgG-Sepharose (Hyclone, Logan, UT). Antibody XVIE10B6A5 is species specific for pig, while antibody IVG8C11 has broad species reactivity (8).

Preparation of Immobilized Monoclonal Antibody. Sepharose CL-4B (Pharmacia) was activated with CNBr by the procedure of Kohn and Wilcheck, using 9 mg of CNBr per 1 g of drained Sepharose CL-4B (15). Monoclonal antibody XVIE10B6A5 was coupled in PBS to Sepharose CL-4B by overnight incubation at 4°C of approximately 1 mg of antibody per ml of activated Sepharose. The XVIE10B6A5-Sepharose was stored at 4°C in TED with 0.025% NaN_3.

Purification Procedure. Preparative gel electrophoresis and electroelution were performed at room temperature. All other procedures were done at 0–4°C.

Intestinal Tissue Preparation. A crude nuclear extract was obtained from pig intestine as described previously (8). The small intestine was removed from eight young pigs (18–40 kg) and washed in TEDNa buffer. The mucosa was scraped from the serosa, rinsed two times with 3 vol of TEDNa buffer, and rinsed one time with 3 vol of TED buffer. One settled volume of mucosa was added to 2 vol of TED and homogenized with two 20-sec bursts from a Polytron homogenizer (Brinkmann). The homogenate was centrifuged at 3000 × *g* for 30 min to obtain a crude nuclear pellet. The nuclear pellet was washed three times with TED buffer by resuspending the pellet and centrifuging at 3000 × *g* for 10 min. The 1,25-$(OH)_2D_3$ receptor was extracted from the washed pellet by homogenization in 2 vol of $TEDK_{300}$ buffer containing 1 mM phenylmethanesulfonyl fluoride with three 20-sec bursts from a Polytron homogenizer. The homogenate was centrifuged at 27,000 × *g* for 2 hr. The supernatant fraction (nuclear extract) contained the 1,25-$(OH)_2D_3$ receptor and was used immediately or frozen in liquid nitrogen and stored at −70°C.

Immunoaffinity Chromatography. The pig nuclear extract (250 ml, 4 mg of protein per ml) was incubated overnight in 5.0 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 (2 Ci/mmol). The hormone-labeled receptor was pumped at 100 ml/hr onto a 20-ml precolumn of Sepharose CL-4B linked in series to a 1.8-ml column of XVIE10B6A5-Sepharose CL-4B. After the nuclear extract had been loaded onto the affinity column, the precolumn was removed and the antibody column was washed with the following buffers at 30 ml/hr: $TEDK_{400}$ (10 ml); TED (30 ml); $TEDK_{400}$ (30 ml); and 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (Caps), pH 9.8/5 mM dithiothreitol (15 ml). The receptor–steroid complex was then eluted at 9 ml/hr with 50 mM Caps, pH 11.2/5 mM dithiothreitol. In experiments in which hormone-binding activity was assayed the eluted receptor was added immediately to 10 vol of TED with β-lactoglobulin at 0.25 mg/ml as a carrier protein.

Preparative Electrophoresis. Discontinuous $NaDodSO_4$/polyacrylamide slab gels were prepared as described by Laemmli (16) with minor modifications. Prior to polymerization, the lower gel was overlaid with water-saturated 2-butanol to obtain a flat gel surface. The 2-butanol was replaced with lower gel buffer, and the gel was allowed to polymerize not less than 16 hr. In addition, 0.1 mM sodium thioglycolate was added to the upper buffer reservoir to prevent damage due to free radicals and oxidants trapped in the gel matrix.

Immunoaffinity-purified receptor mixed with 1 mg of β-lactoglobulin was precipitated with trichloroacetic acid in the presence of sodium deoxycholate (17). Precipitated receptor samples were subjected to electrophoresis on 9% polyacrylamide gels. After completion of electrophoresis, the gels were rinsed with water and stained 5 min with ice-cold 0.25 M KCl/1 mM dithiothreitol. The gel was rinsed and destained in ice-cold water containing 1 mM dithiothreitol until the protein band was distinctly visible in oblique lighting (approximately 15 min) (18).

Electroelution of the 1,25-$(OH)_2D_3$ receptor was performed as described by Hunkapiller *et al.* (19) except the standard Laemmli buffer system (0.025 M Tris/0.192 M glycine, pH 8.8/0.1% $NaDodSO_4$) was used and elution was completed after 4 hr at 50 V.

Quantitation of Receptor Protein on $NaDodSO_4$/PAGE. Protein on $NaDodSO_4$/PAGE was stained for 12 hr in 0.05% Coomassie brilliant blue R-250 in 95% ethanol/glacial acetic acid/water (5:1:5, vol/vol) and destained for 48 hr with 7.5% (vol/vol) glacial acetic acid. The gel was scanned with a soft laser scanning densitometer (model SL-504-XL, Biomed Instruments, Fullerton, CA). The standard curve was prepared from densitometry of 0.05–1 μg of bovine serum albumin per lane.

Peptide Mapping. Immunopurified receptor was partially digested with *Staph A* protease as described by Cleveland *et al.* (13). The purified receptor was subjected to $NaDodSO_4$/PAGE. The gel was stained for 15 min with 0.1% Coomassie blue G-250 in 50% methanol/10% acetic acid/40% water (vol/vol) and destained for 5 min in 7.5% acetic acid/5% methanol/87.5% water (vol/vol), and the receptor band was excised. The gel slices were soaked for 30 min in 0.125 M Tris·HCl, pH 6.8/0.1% $NaDodSO_4$ (buffer A). The equilibrated gel slices were placed into the sample well of a 15% polyacrylamide gel, overlaid with buffer A containing 20% (vol/vol) glycerol, and then overlaid with buffer A containing 10% glycerol and the appropriate concentration of *Staph A* protease. The Tris concentration was 0.75 M in the 15% resolving gel and 0.05 M in the reservoir buffer to allow better resolution of the low molecular weight peptides (20). Electrophoresis was carried out at 15 mA until the proteins were near the end of the stacking gel. The current was then turned off and proteolysis was allowed to proceed for 30 min at room temperature. Electrophoresis was then completed at 25 mA.

Preparation of Sample for Amino Acid Sequencing. The 1,25-$(OH)_2D_3$ receptor peptide samples for amino acid sequencing were obtained as described for the peptide mapping experiment except the $NaDodSO_4$ was recrystallized two times as described by Hunkapiller *et al.* (19) and 0.1 mM sodium thioglycolate was added to the upper reservoir buffer. For the sequencing of peptides 1 and 5, samples in three lanes containing 5 μg of immunopurified receptor per lane were digested with 1 μg *Staph A* protease per lane. For the sequencing of peptides 2, 3, 4, and 6, samples in three lanes containing 14 μg of electroeluted receptor per lane were digested with 10 μg of protease per lane. After electrophoresis, the peptides were transferred to PVDF membranes (Millipore Immobilon transfer membranes) in 10 mM Caps/10% methanol, pH 11.0, at 150 mA for 16 hr (40 V) (21). The samples were stained with Coomassie blue as described (21) and sent for sequencing.

Amino acid sequencing was done at the Biotechnology Instrumentation Facility at the University of California, Riverside, on a vapor-phase microprotein sequenator (Applied Biosystems model 470-A) with an on-line, microbore phenylthiohydantoin-amino acid analyzer (Applied Biosystems model 120-A).

Immunoblotting. Goat anti-mouse IgG was iodinated for immunoblotting by using Iodo-Beads (Pierce) essentially as described (22). One Iodo-Bead and 1 mCi of $Na^{125}I$ (DuPont/NEN) were used to iodinate 30 μg of goat anti-mouse IgG (Hyclone). The free $Na^{125}I$ was separated from the radiolabeled antibody by column chromatography on Sephadex G-25 (Pharmacia) in 0.25% (wt/vol) gelatin in PBS.

After electrophoresis, gels were equilibrated for 15 min in transfer buffer (0.025 M Tris·HCl/0.19 M glycine/20% methanol/0.1% $NaDodSO_4$, pH 8.0). The proteins were transferred to nitrocellulose (0.45-μm pore size, Schleicher & Schuell) electrophoretically at 145 mA for 12 hr in transfer buffer.

Nitrocellulose filters were rinsed 15 min in PBS, blocked in Blotto blocking buffer for 1 hr at room temperature, rinsed with Blotto washing buffer for 5 min, incubated with IVG-8C11 at 5 μg/ml in Blotto washing buffer for 2 hr, washed five times with Blotto washing buffer for 10 min each time, and incubated for 2 hr with ^{125}I-labeled goat anti-mouse IgG at 3×10^5 cpm/ml in Blotto washing buffer. Filters were washed five times for 10 min each with Blotto washing buffer, rinsed one time with PBS, air dried, and autoradiographed with preflashed Kodak XAR 5 film and a Cronex Lightning Plus intensifying screen (DuPont). The film was exposed for 6 hr at −70°C.

Receptor Binding Assay. The 1,25-$(OH)_2$[26,27-^{3}H]D_3 binding activity in the samples was determined by a modified (9) hydroxyapatite binding assay (23, 24).

Miscellaneous Methods. Silver staining was carried out according to the procedure of Oakley *et al.* (25). Protein assays were done with Bio-Rad protein stain and bovine serum albumin as a standard.

RESULTS

Purification of the 1,25-$(OH)_2D_3$ Receptor. Crude nuclear extract was labeled overnight with 1,25-$(OH)_2$[26,27-^{3}H]D_3 and loaded onto monoclonal antibody XVIE10B6A5-Sepharose CL-4B. The 1,25-$(OH)_2D_3$ receptor was monitored during the chromatography by measuring the protein-bound radioactivity by a hydroxyapatite binding assay (Fig. 1). Greater than 90% of the steroid-binding activity in nuclear extract was bound to the affinity column. The binding activity was retained on the column during successive washes with $TEDK_{400}$, TED, $TEDK_{400}$, and 50 mM Caps (pH 9.8). The bound 1,25-$(OH)_2$[26,27-^{3}H]D_3 eluted at high pH (50 mM Caps, pH 11.2) in a sharp peak. The overall yield of hydroxyapatite-precipitable binding activity was 45%. Further treatment of the column with strong denaturing reagents (2% $NaDodSO_4$ or 6 M urea) did not remove additional 1,25-$(OH)_2D_3$ receptor as determined by $NaDodSO_4$/PAGE and immunoblotting (data not shown).

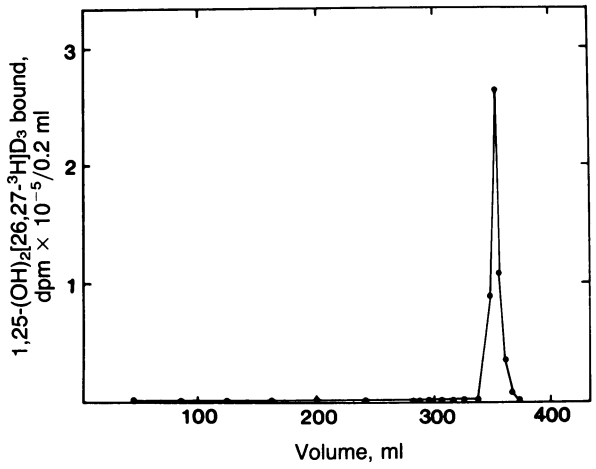

FIG. 1. Immunoaffinity chromatography of 1,25-$(OH)_2D_3$ receptor. Crude pig nuclear extract (250 ml; 4 mg of protein per ml) was labeled overnight at 4°C with 5.0 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 (2 Ci/mmol) and applied at 100 ml/hr to a 20-ml Sepharose CL-4B precolumn linked in series to a 1.8-ml XVIE10B6A5-Sepharose CL-4B column. The precolumn was removed and the antibody column was washed at 30 ml/hr with $TEDK_{400}$ (10 ml), TED (30 ml), $TEDK_{400}$ (30 ml), and 50 mM Caps (pH 9.8)/5 mM dithiothreitol (30 ml). The 1,25-$(OH)_2D_3$ receptor was eluted at 9 ml/hr with 50 mM Caps (pH 11.2)/5 mM dithiothreitol. Aliquots of fractions collected during loading, washing, and elution were assayed for bound 1,25-$(OH)_2$[26,27-^{3}H]D_3 by a hydroxyapatite binding assay.

The effectiveness of the immunoaffinity chromatography was demonstrated by using $NaDodSO_4$/PAGE with silver staining (Fig. 2*A*). Most of the proteins in the crude nuclear extract (lane 1) did not bind to the immunoaffinity column (lane 2). The peak fractions eluting at pH 11.2 (lanes 3–6) contained predominantly the 55-kDa 1,25-$(OH)_2D_3$ receptor protein with minor contaminants of 200 and 45 kDa.

Immunoaffinity fractions were also assayed by $NaDodSO_4$/PAGE with immunoblotting (Fig. 2*B*). The 55-kDa protein eluting at pH 11.2 was recognized by the monoclonal antibody IVG8C11 by immunoblotting and was concentrated considerably on the XVIE10B6A5-Sepharose column. Trace amounts of a smaller protein was also detected by immunoblotting, indicating that some proteolysis may have occurred during the chromatography. The 55-kDa receptor protein was also recognized by immunoblotting with XVIE10B6A5, whereas the 200- and 45-kDa contaminants were not recognized by immunoblotting with XVIE10B6A5 or IVG8C11 (data not shown).

Preparative $NaDodSO_4$/PAGE and electroelution were used to obtain a concentrated homogeneous preparation of the 1,25-$(OH)_2D_3$ receptor. The Coomassie blue stained gel of electroeluted receptor (Fig. 2*C*) showed no protein contaminants. The 1,25-$(OH)_2D_3$ receptor recovery during electroelution typically was 70–100% as determined by densitometry.

Peptide Mapping. Immunopurified 1,25-$(OH)_2D_3$ receptor was digested in $NaDodSO_4$/polyacrylamide gels with various concentrations of *Staph A* protease by the procedure described by Cleveland *et al.* (13) (Fig. 3). At low protease concentrations (0.0025 μg per lane in lane C and 0.025 μg per lane in lane B), some of the 55-kDa 1,25-$(OH)_2D_3$ receptor (peptide 0) was specifically cleaved to a large peptide of approximately 41 kDa (peptide 1) and a smaller peptide of approximately 11 kDa (peptide 5). As the protease concentration was increased to 0.25 μg/lane (Fig. 3, lane A), the receptor was degraded further to peptides 2, 3, 4, and 6. The *Staph A* protease was seen only as a faint series of bands at approximately 30 kDa in lane A.

Amino Acid Sequencing. The relationships between the peptides generated by proteolysis were confirmed by amino acid sequencing. The amino-terminal amino acid sequences that were determined are shown next to a sample of the Coomassie blue-stained PVDF membrane in Fig. 4. At-

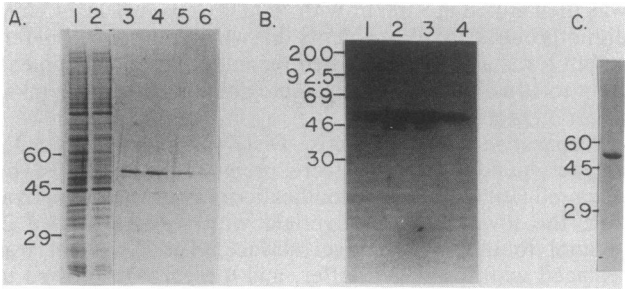

FIG. 2. $NaDodSO_4$/PAGE of 1,25-$(OH)_2D_3$ receptor. The positions of molecular mass standards (kDa) are shown on the left of the gels. (*A*) Silver staining of fractions from immunoaffinity chromatography. Lane 1, pig nuclear extract; lane 2, column wash-through; lanes 3–6, peak receptor fractions eluted at pH 11.2. (*B*) Immunoblot with monoclonal antibody IVG8C11 of receptor fractions from immunoaffinity chromatography. Lanes 1–4, fractions of receptor eluted at pH 11.2. (*C*) Electroeluted receptor visualized by Coomassie blue stain.

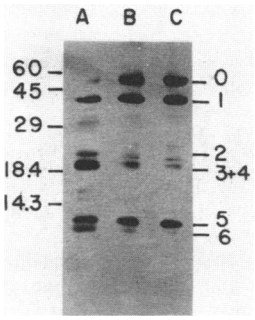

FIG. 3. Peptide maps of immunoaffinity-purified 1,25-$(OH)_2D_3$ receptor after digestion with *Staph A* protease. Immunopurified receptor was excised from $NaDodSO_4$/polyacrylamide gels and digested with *Staph A* protease as described in *Materials and Methods*. The peptides were separated in a 15% polyacrylamide gel and silver stained. Lanes A, B, and C contained 0.25 μg, 0.025 μg, and 0.0025 μg of *Staph A* protease, respectively. The positions of molecular mass standards (kDa) are shown on the left. The peptides are numbered 0–6 on the right.

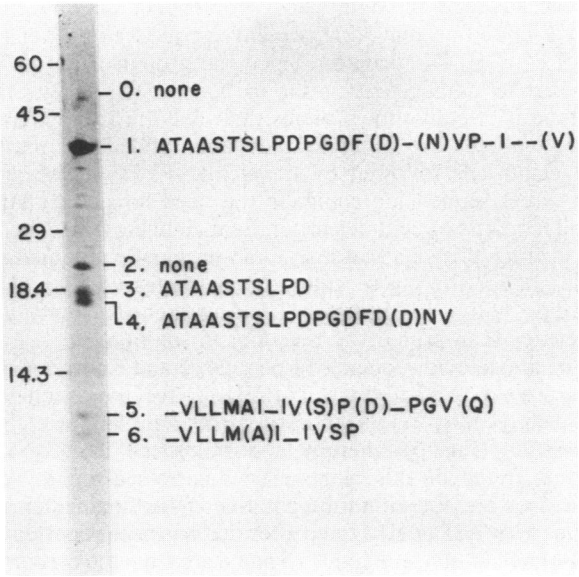

FIG. 4. Amino-terminal sequences of 1,25-$(OH)_2D_3$ receptor peptides. The 1,25-$(OH)_2D_3$ receptor (14 μg) was digested with 1 μg of *Staph A* protease by using the Cleveland procedure (13). The peptides were separated by $NaDodSO_4$/PAGE, transferred to a PVDF membrane, and stained with Coomassie blue. The amino-terminal sequences were determined from the samples described in *Materials and Methods* and are shown on the right in the standard 1-letter code next to the corresponding peptide band. Parentheses indicate uncertainty in the amino acid assignment and a dash indicates that a determination could not be made at a given position. The positions of the molecular mass standards (kDa) are shown on the left.

tempts to sequence the 55-kDa intact receptor by amino-terminal analysis were unsuccessful, presumably due to amino-terminal modification. Peptide 1 and peptide 5, generated from the cleavage of the parent 55-kDa 1,25-$(OH)_2D_3$ receptor, have distinct amino termini. Peptides 3 and 4 have amino termini identical to the terminus of peptide 1, whereas peptide 6 has an amino terminus identical to that of peptide 5. Therefore, peptides 3 and 4 are likely to have resulted from further cleavage at the carboxyl terminus of peptide 1, and peptide 6 resulted from carboxyl-terminal cleavage of peptide 5. It was not possible to determine a sequence for peptide 2 due to insufficient initial yield in the sequenator.

Sequence Comparison. The amino acid sequence determined from peptides 5 and 6 corresponds precisely with a portion of the sequence predicted from a partial 1,25-$(OH)_2D_3$ receptor cDNA clone (Fig. 5) (26). Cysteine residues could not be determined by amino acid sequencing because the peptide samples were not alkylated prior to analysis. Peptides 5 and 6 were located at the carboxyl-terminal portion of the predicted receptor sequence.

DISCUSSION

The 1,25-$(OH)_2D_3$ receptor is thought to mediate the physiological response to vitamin D_3 by directly interacting with specific genes and modulating transcription. To better understand the mechanism of the 1,25-$(OH)_2D_3$ receptor, we have developed an immunoaffinity purification of active pig intestinal receptor. This procedure was considerably more efficient and simpler than classical purification protocols (6, 8). Most importantly, unlike the previously published immunoaffinity purification method (12), the method described here maintained the 1,25-$(OH)_2D_3$ binding activity of the receptor. Although it is not possible at this time to test if the isolated receptor retains all of the biological activities necessary for regulating gene transcription, the immunopurified material is expected to be valuable in the elucidation of 1,25-$(OH)_2D_3$ receptor mechanism. It is hoped that the calcium-binding protein gene that has been recently isolated (27) will provide a suitable system with which to study 1,25-$(OH)_2D_3$ receptor binding to specific DNA sequences and the subsequent activation of transcription.

Homogeneous 1,25-$(OH)_2D_3$ receptor was isolated by immunoaffinity chromatography followed by preparative $NaDodSO_4$/PAGE. The electroeluted material, though it did not retain hormonal binding activity, will be useful for generation of polyclonal antibodies and for detailed structural studies such as peptide mapping with high-performance liquid chromatography.

By combining the Cleveland method of proteolytic cleavages within $NaDodSO_4$/polyacrylamide gels (13) with the

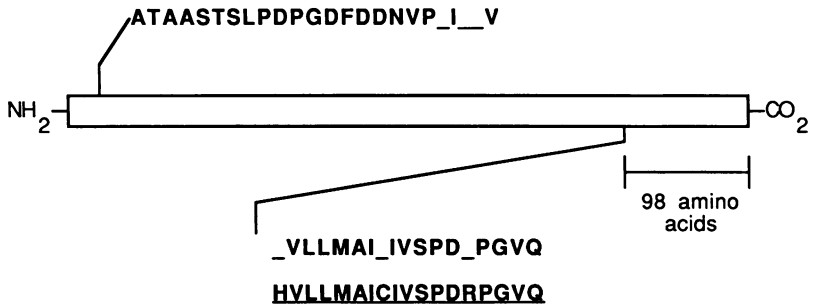

FIG. 5. Comparison of the 1,25-$(OH)_2D_3$ receptor primary sequence determined by amino acid sequencing with the sequence predicted from a receptor cDNA clone. The sequence predicted 98 amino acids upstream of the first stop codon in the rat kidney cDNA clone is underlined.

recent advance of amino acid sequencing directly from PVDF-bound proteins (21), we have sequenced portions of the 1,25-$(OH)_2D_3$ receptor. Since the protein need not be purified to homogeneity prior to $NaDodSO_4$/PAGE, this method is effective for proteins that, like the 1,25-$(OH)_2D_3$ receptor, are low in abundance and difficult to purify. Cleveland gels followed by transfer to PVDF membranes and direct sequencing eliminate the need for manipulating small amounts of peptides in solution, allowing the sequencing of trace proteins that are amino-terminally modified. In addition, for the pig receptor the cleavages with *Staph A* protease within the gel matrix had greater specificity and efficiency than proteolytic reactions in solution.

The amino acid sequence of peptides 5 and 6 corresponds precisely to part of the 1,25-$(OH)_2D_3$ receptor sequence deduced recently in this laboratory from a rat kidney cDNA clone (26) (Fig. 5), thereby confirming the identity and reading frame of this clone. The determined amino acid sequences are not within the putative DNA-binding domain of the chick 1,25-$(OH)_2D_3$ receptor that has been reported by McDonnell *et al.* (5). Peptides 5 and 6 are from the carboxyl-terminal portion of receptor, since their sequence begins 98 amino acids from the first stop codon predicted in the rat kidney cDNA. Peptides 1, 3, and 4 start near the amino terminus of mature receptor, since peptide 1 (41-kDa) and peptide 5 (11-kDa) were generated from cleavage of the 55-kDa receptor. It is not surprising that the sequence of peptides 1, 3, and 4 does not correspond to any deduced from either the rat or chick cDNA clones, as these clones do not contain the amino-terminal portion of receptor.

Peptide mapping has been widely used to show relationships between proteins (different proteins or the same protein from different sources) and to characterize structural changes in proteins (post-translational modifications such as glycosylation and phosphorylation) (28). In this report, we have generated a peptide map for the 1,25-$(OH)_2D_3$ receptor and identified the amino-terminal amino acid sequences for the peptides. The peptide map, together with the entire primary sequence, which will soon become available through DNA cloning techniques, is expected to be valuable in the characterization of receptors from different sources and in the identification and localization of modifications within the 1,25-$(OH)_2D_3$ receptor.

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