PARTIAL PURIFICATION OF THE CHICK INTESTINAL RECEPTOR FOR 1,25-DIHYDROXY VITAMIN D BY ION EXCHANGE AND BLUE DEXTRAN–SEPHAROSE CHROMATOGRAPHY

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1. Introduction

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is now considered to function analogously to classic steroid hormones. The ultimate site of action of 1,25-$(OH)_2D_3$ is the intestinal mucosa cell nucleus [1,2], where it apparently induces the formation of new mRNAs which code for proteins functional in calcium and phosphate absorption [3–6]. The 1,25-$(OH)_2D_3$ sterol is transported to the intestinal chromatin via a specific, high affinity cytoplasmic receptor protein which migrates at 3.7 S in high salt-sucrose gradients [7–11]. Other than agarose gel filtration of analytical amounts [9], no chromatographic purification of the 1,25-$(OH)_2D_3$ receptor has been reported to date. Other steroid hormone receptors, such as those for progesterone, have been purified to homogeneity, either by ion-exchange [12] or affinity [13] chromatography. However, this has proven to be difficult because of the extremely low percentage of cytosolic proteins representing receptor molecules and the high lability of these receptors, especially in more purified states.

In the present study, we report that the intestinal cytosol receptor for 1,25-$(OH)_2D_3$ from the chick can be purified approx. 800-fold by ammonium sulfate precipitation, ion-exchange chromatography on phosphocellulose, hydroxylapatite, and DEAE-cellulose, and by blue dextran–Sepharose chromatography.

Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; KETT-buffer, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4, 12 mM thioglycerol (KETT-0, no KCl; KETT-0.1, 0.1 M KCl); HAP (hydroxylapatite) buffer, 0.05 M Tris–HCl, pH 7.4, 1 mM EDTA, 12 mM thioglycerol, 0.1 M KCl (HAP-0, no KH_2PO_4; HAP-0.1, 0.1 M KH_2PO_4)

2. Materials and methods

Four-week rachitic chicks were raised as in [14]. 1,25-$(OH)_2[^3H]D_3$ (6.7 Ci/mmol) was generated and purified as in [7].

2.1. *Assay for specific 1,25-$(OH)_2D_3$ receptor binding*

A modification of the DEAE filter binding assay [15] was employed to monitor receptor activity. An aliquot of crude soluble protein ($\leq$ 4 mg) was diluted to 1.3 ml in ice-cold KETT-0 buffer (1 mM EDTA, 10 mM Tris–HCl, pH 7.4, 12 mM thioglycerol) and 500 μl portions were then incubated 1 h at 0°C with 50 μl ethanol containing either 30 000 dpm of 1,25-$(OH)_2[^3H]D_3$ (2 pmol), or the same amount of radioactive hormone plus a 100-fold excess of nonradioactive 1,25-$(OH)_2D_3$ (M. Uskoković, Hoffmann-LaRoche). After addition of 1 ml 1% Triton X-100 in 0.01 M Tris–HCl, pH 7.5, each sample was filtered slowly through 2 presoaked DE-81 (Whatman) filters. The filters were washed with 2 ml 1% Triton X-100 in 0.01 M Tris under a light vacuum, removed and placed in scintillation vials. After addition of 5 ml acetone, the filters were dried in the vials and counted for tritium (50% efficiency) via standard liquid scintillation techniques. In a particular sample, the difference in dpm bound to the filters between the incubation containing 1,25-$(OH)_2[^3H]D_3$ alone and that containing a 100-fold excess of unlabeled hormone represents specific binding.

2.2. *Preparation of intestinal mucosa cytosol*

Small intestines of rachitic chicks were rinsed extensively with ice-cold 0.25 M sucrose, 0.05 M Tris–HCl, pH 7.4, 0.025 M KCl, and 0.005 M $MgCl_2$ before preparing 30% mucosa homogenates in the same buffer. Cytosol was isolated by centrifugation [8] and extreme care was taken to avoid warming above 4°C during these and all subsequent procedures.

2.3. *Ammonium sulfate precipitation of receptor*

Ammonium sulfate (enzyme grade, Schwarz/Mann) was preground to a powder and added slowly to stirring cytosol at 0°C, to achieve 40% saturation. After 30 min, the solution was centrifuged at 15 000 × *g* for 15 min to pellet the receptor fraction.

2.4. *Column chromatography*

Columns were formed in 5–50 ml disposable plastic syringes. After application of receptor preparations labeled with 1,25-$(OH)_2$[^{3}H]D_3, linear gradients of increasing salt concentration were run; gradients were analyzed via conductivity measurement. Aliquots of the fractions were measured for protein by 10% trichloroacetic acid precipitation, solubilization in 1 N NaOH and utilization of the method [16]. Separate aliquots were counted for tritium using a scintillation mixture for aqueous samples (3% Liquifluor in toluene–Triton X-100, 3:1; efficiency ≈ 35%).

Whatman DE-52 cellulose resin and Pharmacia DEAE-Sephadex A-50 were washed and extensively equilibrated in KETT-0 buffer prior to eluting 10–30 ml columns first with KETT-0 buffer and then with a KETT-0 to KETT-0.5 (KETT buffer with 0.5 M KCl) gradient. Bio-Rad hydroxylapatite (Bio-Gel HTP) was washed initially with 10 vol. degassed buffer containing 0.05 M Tris–HCl, pH 7.4, 1 mM EDTA, 12 mM thioglycerol, 0.1 M KCl, 0.1 M KH_2PO_4 (HAP-0.1 buffer); followed by 3 washes with the same buffer minus KH_2PO_4. Columns (5 ml) were eluted with either linear gradients from 0–0.3 M KH_2PO_4 or by batch technique with HAP-0.3 buffer. Whatman P-11 phosphocellulose was precycled by washing successively with water, 0.5 M NaOH, water, 0.5 M HCl and water and then adjusted to pH 7.4 with Tris. After a wash with 1 M KCl, the resin was equilibrated in KETT-0.05 buffer and 20 ml columns were run with gradients from KETT-0 to KETT-0.5. Blue dextran–Sepharose resin was prepared from blue dextran (Sigma) and CNBr-activated Sepharose (Pharmacia) as in [17]. Receptor solutions were applied to 10–45 ml columns.

3. Results and discussion

From preliminary experiments, it was determined that the 1,25-$(OH)_2D_3$ receptor in chick intestinal cytosol precipitated between 25% and 35% saturation of ammonium sulfate at 0°C. Consequently, a 0–40%-saturated ammonium sulfate precipitate of cytosol was routinely used as a crude receptor preparation in all subsequent experiments. Ammonium sulfate precipitation 'activates' the cytosol receptor for 1,25-$(OH)_2D_3$ (T.A. McC, M.R. H, M. R.H, unpublished observation) and, analogous to the estrogen receptor [18], renders it capable of rapid binding to chromatin at 0°C. The stability of this receptor fraction, after redissolving in KETT-0 buffer, was investigated first. Solubilized receptor fraction at 0°C was either labeled

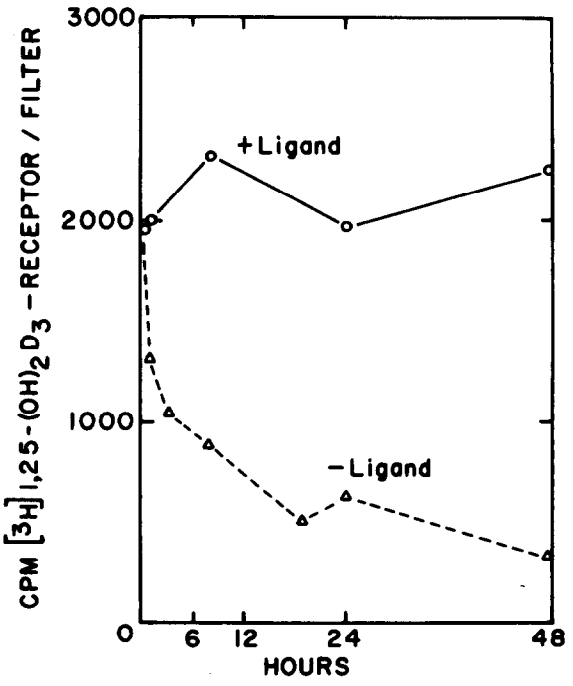

Fig.1. Lability of the ammonium sulfate-precipitated cytosol receptor at 0°C in the absence of 1,25-$(OH)_2D_3$.

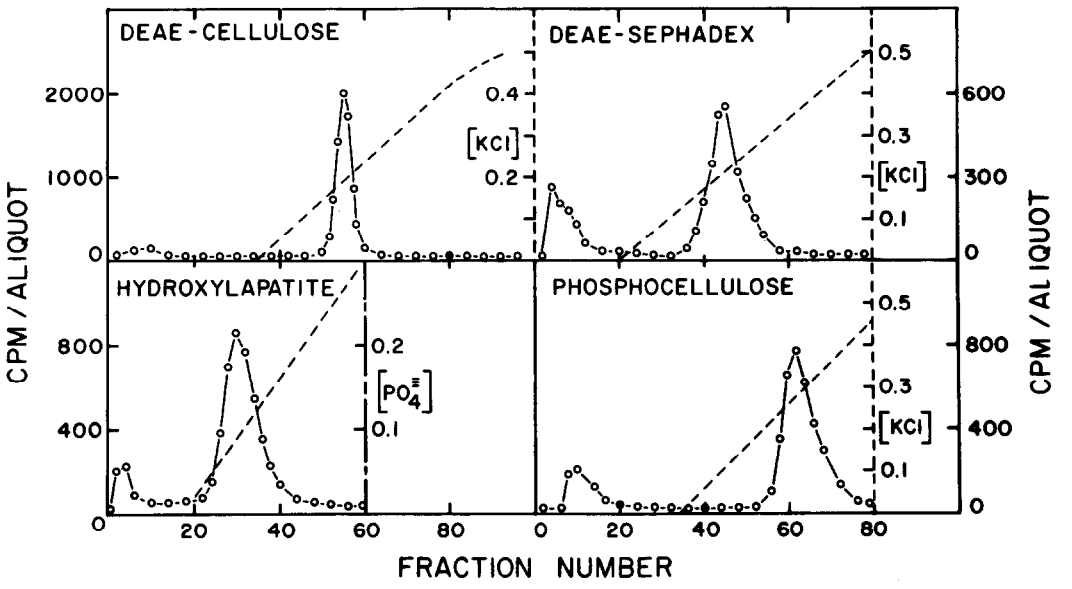

Fig.2. Ion-exchange chromatography of ammonium sulfate-precipitated cytosol receptor for 1,25-$(OH)_2D_3$ from chick intestine. Columns were run as described in section 2. Fractions (2–4 ml) were collected and 25–50% aliquots were counted (-o-). Yields of 1,25-$(OH)_2[^3H]D_3$ from original incubations which appeared in receptor peaks were 40–75%. Salt gradients are denoted with a dashed line.

initially with radioactive 1,25-$(OH)_2D_3$ (4 nM) or kept in the unliganded state, and then DEAE filter assays were performed as a function of time to determine rate of receptor degradation. Fig.1 shows that at 0°C the receptor is stable for at least 48 h in the liganded form, but is rapidly inactivated in the absence of the ligand; temperatures above 4°C also destroy the receptor even when hormone is bound (data not shown). A similar protective effect of ligand on the glucocorticoid and mineralocorticoid receptors of rat kidney at 0°C has been noted [19], and this fact dictated that any further purification of the 1,25-$(OH)_2D_3$ receptor be performed on preparations saturated with hormone.

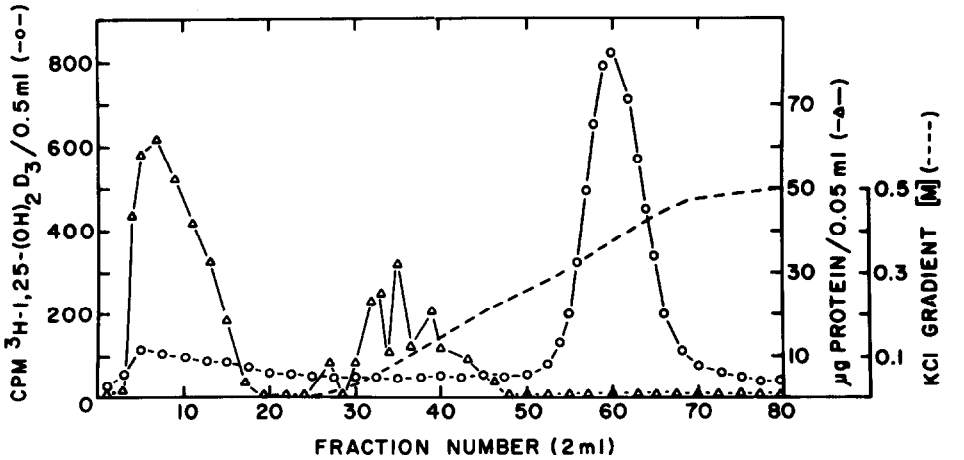

Fig.3. Blue dextran–Sepharose chromatography of ammonium sulfate-precipitated 1,25-$(OH)_2D_3$ receptor.

We next determined if ion-exchange chromatography [12,20,21] could be employed to purify the ammonium sulfate-precipitated receptor. Receptor fractions from 3–6 g intestinal mucosa were labeled with 1,25-$(OH)_2[^3H]D_3$ (2 nM) for 1 h in KETT-0 buffer and applied to small (5–20 ml) columns of DEAE-cellulose, DEAE-Sephadex, hydroxylapatite and phosphocellulose. As can be seen in fig.2, the 1,25-$(OH)_2D_3$ receptor binds to these anion-exchange resins quite effectively and elutes from either resin at 0.2 M KCl, from phosphocellulose at 0.28 M KCl and from hydroxylapatite at 0.07 M phosphate. A significant fraction of the total protein 'falls through' these columns (data not shown) and 4-, 6-, 3- and 30-fold purifications are obtained on DEAE-cellulose, DEAE–Sephadex, hydroxylapatite and phosphocellulose, respectively. In all cases, the 1,25-$(OH)_2D_3$ receptor emerges as a single peak, indicating that unlike the progesterone receptor which contains two separable subunits [23], the vitamin D hormone apparently has a single binding species in the intestine.

A second type of resin utilized in this study is blue dextran–Sepharose. This material has apparent biospecific affinity for nucleotide-requiring enzymes, probably due to the structural similarity between its blue chromophore and nucleotide cofactors. Numerous kinases and dehydrogenases have been purified with blue dextran–Sepharose [23,24], although Wilson [25] has recently suggested that a 'dinucleotide fold' may not be required for certain enzymes to interact with the resin. Fig.3 illustrates the striking purification of the 1,25-$(OH)_2D_3$ receptor which can be obtained using blue dextran–Sepharose. Many contaminating proteins in the 0–40%-saturated ammonium sulfate precipitate pass through the column in KETT-0 buffer and most other proteins elute at a KCl concentration lower than 0.45 M, where the receptor is removed from the column. Neither the cytosol protein which selectively binds 25-hydroxyvitamin D_3 [26,27], nor the serum-binding protein for 1,25-$(OH)_2D_3$ and 25-hydroxyvitamin D_3 [28] will adsorb to this resin in KETT-0 buffer (data not shown). At least a 30-fold purification of the intestinal 1,25-$(OH)_2D_3$ receptor can be accomplished via this method. The tight association of the 1,25-$(OH)_2D_3$ receptor with blue dextran–Sepharose intimates that the protein–hormone complex may bind nucleotides during its interaction with the intestinal cell genome to alter mRNA biosynthesis [29]. Perhaps of broader interest is the possibility that all steroid hormone receptors associate with this resin, thereby providing both insight into

Table 1
Purification of intestinal 1,25-$(OH)_2D_3$ receptor

Step	Total protein (mg)	1,25-$(OH)_2D_3$ bound (dpm × 10^{-6})	Liganded receptor yield (%)	Spec. act. (dpm/mg, protein × 10^{-3})	Fold purification
Cytosol	10 200	–	–[a]	1.50	–
Ammonium sulfate (0–40% sat.)	1712	8.00	100	4.67	3
Phosphocellulose batch (0.1–0.6 M KCl)[b]	146	6.08	76	41.6	28
Blue dextran–Sepharose pool (0.45 M KCl)	11.7	3.55	44	303	202
DEAE-cellulose pool (0.2 M KCl)	4.0	1.80	23	450	300
Phosphocellulose pool (0.28 M KCl)	2.3	1.27	16	552	368
Hydroxylapatite pool (0.3 M KH_2PO_4)	2.0	0.952	12	476	317
Blue dextran–Sepharose peak (0.45 M KCl)	0.168	0.207	2.6	1235	823

[a] Cytosol was not labeled with hormone because of the large volume, but specific activity was determined on an aliquot; about 50% of receptor activity (unliganded) is lost due to degradation prior to and during the ammonium sulfate precipitation. Pellets were redissolved in total 100 ml KETT-0 buffer and labeled with 36 × 10^6 dpm (24 nM) 1,25-$(OH)_2D_3$ for 90 min

[b] After dilution to 400 ml to give a final KCl concentration of 0.1 M, the receptor was adsorbed to 400 ml slurry of phosphocellulose. Following extensive washes of the resin with KETT-0.1 buffer, the receptor was batch eluted with KETT-0.6 M KCl

their molecular nature and a facile means of isolation.

To test the combined power of the above procedures for isolating the 1,25-$(OH)_2D_3$ receptor, we started with 140 g intestinal mucosa to prepare cytosol. Table 1 summarizes the sequence of separations and purification results. A key step is the phosphocellulose batch elution which effects a 10-fold purification over the crude ammonium sulfate precipitate. After this operation, the receptor was concentrated by ammonium sulfate precipitation and split in half for chromatography on two 45 ml blue dextran–Sepharose columns. The combined peaks from these columns (approx. 7-fold purified over previous step) were ammonium sulfate precipitated and chromatographed on columns of DEAE-cellulose (30 ml) and phosphocellulose (20 ml), although these steps accomplished only minor purifications over the blue dextran–Sepharose. Next, the receptor fraction was concentrated to 6 ml with a 5 ml hydroxylapatite column eluted with HAP-0.3 buffer. 100 μl of material was centrifuged on a 0.4 M KCl, 5–20% sucrose gradient [9] yielding a macromolecule sedimenting at 3.7 S (data not shown); the balance was placed on a 20 ml blue dextran–Sepharose column and eluted with an extremely gentle gradient of 0.007 M KCl increment per fraction (100 of 2 ml fractions total). The center 7 fractions of the peak were harvested and found to contain receptor which was 823-fold purified over the original cytosol.

Assuming mol. wt 50 000 for the receptor protein as approximated by agarose gel filtration [9], it can be calculated that an approx. 200 000-fold purification will be required to isolate the cytosol receptor to homogeneity. Therefore, the 823-fold purification attained so far is still approx. 250-fold short of this ultimate goal and vitamin D-specific affinity chromatography will most likely be required to obtain absolute purity. Nevertheless, the partially purified receptor, which is free of other vitamin D-binding proteins, will be useful in further defining the properties of the protein and screening 1,25-$(OH)_2D_3$ analogs. This preparation should also have value as a basic tool in elucidating the nuclear mechanism of 1,25-$(OH)_2D_3$ action and a simplified version of the present isolation procedure could provide a source of binding protein for diagnostically-important radioreceptor assays for the hormone [30–32].

Acknowledgement

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