Purification of chicken intestinal receptor for 1,25-dihydroxyvitamin D

cytosol hormone receptor/Polymy P/DNA-cellulose/pseudo-affinity chromatography/gene expression

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ABSTRACT The 3.3S chicken intestinal receptor for 1,25-dihydroxyvitamin D [1,25-(OH)\textsubscript{2}D] has been purified approximately 86,000-fold from the cytosolic fraction. The receptor was selectively precipitated with Polymy P from high-speed supernatants derived from 800 g of intestinal mucosa and then sequentially chromatographed on DNA-cellulose, Sephacryl, blue dextran-Sepharose, DNA-cellulose, and heparin-Sepharose. Polyacrylamide gel electrophoresis of 8-10 \mu g of the purified receptor in sodium dodecyl sulfate indicated the presence of one major and three minor protein bands of molecular weights 50,000-85,000. Sucrose gradient analysis of the purified material in 0.3 M KCl suggested that a fraction of the receptor remained complexed to the 1,25-(OH)\textsubscript{2}D and that its sedimentation properties of 3.3S remained unchanged. These results represent a major purification of the chick intestinal receptor for 1,25-(OH)\textsubscript{2}D, an extremely rare and labile protein whose isolation is estimated to require a 200,000-fold purification. Of primary importance is the observation that affinity ligands such as DNA and blue dextran can effect major purification of this protein, lending credence to the hypothesis that the 1,25-(OH)\textsubscript{2}D receptor functions within the cell nucleus by altering the expression of specific genes.

In the course of the molecular action of 1,25-dihydroxyvitamin D [1,25-(OH)\textsubscript{2}D] on the chicken intestinal mucosal cell, the hormone first undergoes an obligatory coupling within the cytoplasm to a specific high-affinity receptor molecule (1, 2), and then rapidly accumulates within the nucleus as a functionally active complex bound to chromatin. This model is strongly substantiated by both biochemical observations (3, 4) and recent autoradiographic localization studies (5, 6). Although details of the events that follow remain obscure, most evidence suggests that the complex specifically induces the biosynthesis of nucleic acids that are ultimately modified, transported, and then translated in the cytoplasm into biologically active components of mineral transport (7-10). Thus, vitamin D, through its hormonal metabolite 1,25-(OH)\textsubscript{2}D, promotes the enhanced absorption of calcium and phosphate across the intestinal epithelium, thereby contributing to the maintenance of mineral homeostasis.

A specific receptor for 1,25-(OH)\textsubscript{2}D was first observed in the intestines of vitamin D-deficient (rachitic) chickens. This receptor remains the most extensively studied, despite its identification recently in a number of other tissues including parathyroid gland (11), bone (12), pancreas (13, 14), and kidney (13, 14). The intestinal receptor sediments in high-salt sucrose gradiants as a 3-3.7S macromolecule, has a molecular weight (estimated by agarose gel filtration) of 47,000, and displays all the characteristics typical of true steroid hormone receptors, including specificity, high affinity, and low capacity (2). Moderate purifications of this protein have been achieved recently by using ion exchange and blue dextran-Sepharose chromatography (15). However, efforts have been generally hampered by its lack of abundance within the cell and also by its extreme lability as purification proceeds. Nevertheless, we report here a major purification of the 1,25-(OH)\textsubscript{2}D receptor from rachitic chicken intestine achieved by utilizing selective precipitation, group selective (pseudo) affinity chromatography, and gel filtration.

MATERIALS AND METHODS

Animals and Materials. White Leghorn chickens were raised on a vitamin D-deficient diet (16) for 4 weeks before sacrifice. \textsuperscript{3}H-Labeled 25-hydroxyvitamin D\textsubscript{3} (110 Ci/mmol; 1 Ci = 3.7 \times 10\textsuperscript{10} becquerels) was obtained from Amersham, converted biologically into 1,25-(OH)\textsubscript{2}D\textsubscript{3} (110 Ci/mmol) as described (17), and then used directly or adjusted to 7 Ci/mmol by the addition of nonradioactive 1,25-(OH)\textsubscript{2}D\textsubscript{3} (M. Uskoković, Hoffman-LaRoche). All chemicals were reagent grade or better.

Intestinal Tissue Preparation. Treatment of intestinal tissue and preparation of cytosol were as described (15). Ammonium sulfate precipitation of the receptor from cytosol was carried out as described (15). Polymy P (Bethesda Research Laboratories, diluted to 10% with distilled water and adjusted to pH 7.9 with HCl) precipitation was performed as described in Results. After centrifugation of precipitates from either ammonium sulfate or Polymy P treatment, the pellets were frozen in liquid nitrogen and stored at -80°C.

Assay for 1,25-(OH)\textsubscript{2}D-Receptor Activity. A modification of the DEAE-Filler binding assay of Santi et al. (18) was used basically as described (17). Receptor-containing samples were incubated with a saturating concentration of 1,25-(OH)\textsubscript{2}D\textsubscript{3} (7 Ci/\textsuperscript{10}mol) or with a 100-fold excess of nonradioactive hormone and then filtered on Whatman DE-81 filters.

Column Chromatography. General methods for the chromatography of analytical amounts of receptor, using resolubilized ammonium sulfate precipitates of cytosol, have been described (15). DNA-cellulose was prepared by a modification of the procedure of Alberts and Herrick (19) using calf thymus DNA (Worthington) and cellulose (Bio-Rad, Cellex N-1). Blue dextran-Sepharose was prepared (20) with blue dextran (Pharmacia) and CNBr-activated Sepharose (Pharmacia). Heparin-Sepharose was prepared (21) using heparin (Sigma, grade II) and CNBr-activated Sepharose (Pharmacia). All resins were washed in KETT-0.05. Sephacryl S-200 (Pharmacia) was equilibrated in KETT-0.3; then, 1-ml samples were applied and resolved in KETT-0.3. Purification of the receptor on a pre-

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Results

Receptor Labeling. Receptor pellets were redissolved in KETT-0 and then incubated with saturating concentrations of 1,25-(OH)$_2$D$_3$ [added in cold ethanol to 5–6% (vol/vol)] for 1 hr or 12 hr at 0–4°C.

Sodium Dodecyl Sulfate (NaDodSO$_4$)/Polyacrylamide Gel Electrophoresis. This was performed essentially as described by Laemmli (22) on 11% polyacrylamide/0.3% N,N-methylenebisacrylamide slab gels. Protein samples were precipitated with 6% trichloroacetic acid, washed with ether, and dissolved in standard denaturing buffer. Electrophoresis was at 24°C. Gels were stained according to the Fairbanks technique (23).

General Techniques. Protein was determined by a modification (24) of the method of Lowry et al. (25) using sodium deoxycholate/trichloroacetic acid precipitation or by absorbance at 280 nm. Salt gradients were measured by conductivity. Tritium was assayed, in aliquots taken from column fractions, by liquid scintillation techniques (33% efficiency). Sucrose gradient analysis was carried out as described by Hughes and Haussler (11).

RESULTS

Analysis of 1,25-(OH)$_2$D-Receptor Binding to Resins. The intestinal cytosol receptor sediments in a 5–20% sucrose gradient (in KETT-0.3) as a 3–3.7S macromolecule (2, 15). When the sediment was precipitated from cytosol with ammonium sulfate at 40% saturation and redissolved and labeled with hormone, there was no apparent change in the sedimentation value (data not shown). This treatment concentrates the receptor and also is capable of minor purification of 2- to 3-fold. Utilizing this material as receptor source, we then determined whether the receptor could be purified on resins such as DNA-cellulose, blue dextran-Sepharose, and heparin-Sepharose, as has been demonstrated for other nuclear proteins and receptors.

Chromatography of analytical amounts of receptor (4–8 g equivalents of mucosa) revealed that a macromolecular hormone complex bound to DNA-cellulose (Fig. 1A), blue dextran-Sepharose (Fig. 1B), and heparin-Sepharose (Fig. 1C) and that during a linear KCl gradient, the complex could be eluted from the resin as a single peak at 0.28, 0.40, and 0.22 M, respectively. The extent of nonspecifically bound fall-through $^3$H (compare Fig. 1A and C) was related to the extent of hormone saturation of the receptor preparation. Subsequent sucrose gradient analysis of an aliquot of the eluted $^3$H peak in each case demonstrated the presence of a 3.3S macromolecule bound to 1,25-(OH)$_2$D. Because most other intestinal proteins present in the receptor preparation either did not bind or bound weakly to these resins (see protein profiles, Fig. 1A and B), the resulting purification of the receptor over salt-precipitated starting source was 200-fold (DNA-cellulose), 100-fold (blue dextran-Sepharose), and 30-fold (heparin-Sepharose). These purifications are much greater than those observed for a number of ion exchange resins (15) and thus were considered of potential importance in a receptor isolation scheme.

Gel Filtration. When salt-precipitated receptor material was subjected to gel filtration on Sephacryl S-200 (Fig. 2), there was a minor purification (10- to 12-fold), with the major protein eluting in or near the void volume. Resolution was sacrificed for high flow rates to minimize the extent of receptor–hormone dissociation. The receptor comigrated with bovine serum albumin (Mr 68,000); this is slightly larger than what is seen with agarose (2).

Selective Precipitation with Polymin P. For large-scale receptor purification, a more selective precipitation procedure than that with ammonium sulfate was developed with Polymin P, basically as described (26). Concentrations of Polymin P greater than 0.04% (Fig. 3A) began to precipitate the receptor effectively; at 0.08%, all was sedimented in pellet form (18,000 g, 10 min) with only minor coprecipitation of other proteins. It could be eluted from the polymer in the pellet at 0.5 M KCl (Fig. 3B), when subsequent salt precipitation with ammonium sulfate was carried out, the receptor could be collected free of residual Polymin P and stored at -80°C. Sucrose
gradient analysis of redissolved material demonstrated an unchanged 3.3S macromolecule (data not shown). In practice, a selective cut of cytosol was taken between 0.04 and 0.08% Polymin P, which led to a 10- to 15-fold purification of the receptor over cytosol, in yields of approximately 50-70%.

**Purification of the 1,25-(OH)2D Receptor.** A major purification of the 1,25-(OH)2D receptor complex entailed selective precipitation of cytosol with Polymin P and then sequential column chromatography. The results of such an experiment are summarized in Table 1. Cytosol was prepared from the intestinal mucosa of 350 rachitic chickens (800 g of tissue), and the receptor was precipitated by Polymin P. Upon dissolving and labeling with 35 nM 1,25-(OH)2D3 (7 Ci/mmol), protein had decreased from 22 g (cytosol) to 1.17 g, and the specific activity of the receptor was enhanced 13-fold. This material was applied to DNA-cellulose and, after extensive washing with KETT-0.1, was eluted during a KCl gradient. A major reduction in protein was achieved at this step, to 20 mg, with an attendant 780-fold overall purification of the 1,25-(OH)2D-receptor complex. This single 0.28 M KCl receptor peak was pooled.

**Table 1. Purification of the chicken intestinal receptor for 1,25-(OH)2D**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein, mg</th>
<th>Receptor, cpm x 10^-6</th>
<th>Specific activity, cpm x 10^-9/mg</th>
<th>Yield, %</th>
<th>Purification, fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>22,400</td>
<td>--</td>
<td>0.58</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>Polymin P (precipitate)</td>
<td>1,170</td>
<td>9.10</td>
<td>7.69</td>
<td>100</td>
<td>13</td>
</tr>
<tr>
<td>DNA-cellulose (0.28 M)</td>
<td>19.8</td>
<td>9.00</td>
<td>454.5</td>
<td>99</td>
<td>780</td>
</tr>
<tr>
<td>Sephacryl</td>
<td>--</td>
<td>6.35</td>
<td>--</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Blue dextran-Sephacryl (0.4 M)</td>
<td>0.85</td>
<td>4.50</td>
<td>5,290</td>
<td>49</td>
<td>9,100</td>
</tr>
<tr>
<td>DNA-cellulose (0.28 M)</td>
<td>0.216</td>
<td>2.75</td>
<td>12,700</td>
<td>30</td>
<td>22,000</td>
</tr>
<tr>
<td>Heparin-Sepharose (0.22 M)</td>
<td>0.026</td>
<td>1.30</td>
<td>50,000</td>
<td>14</td>
<td>86,000</td>
</tr>
</tbody>
</table>

The specific activity of the 1,25-(OH)2D receptor in cytosol was first determined on an aliquot, and then the remaining cytosol, prepared from 800 g of intestinal mucosa, was treated with Polymin P as described in Results. The receptor-containing pellets were redissolved in 100 ml of KETT-0 and labeled with 35 nM 1,25-(OH)2D3 (7 Ci/mmol, added in 5 ml of cold ethanol) for 12 hr at 0-4°C. The sample was then applied to and chromatographed successively on DNA-cellulose (5 x 13 cm), Sephacryl (1.6 x 60 cm), blue dextran-Sephacryl (2.5 x 5 cm), DNA-cellulose (2 x 4 cm), and heparin-Sepharose (2 x 3 cm). Conditions: initial DNA-cellulose, 180 ml/hr, 600-ml linear gradient of 0.1-0.8 M KCl, 15-ml fractions; blue dextran-Sepharose, 60 ml/hr, 240-ml linear gradient of 0.2-0.6 M KCl, 5-ml fractions; DNA-cellulose, 60 ml/hr, 120-ml linear gradient of 0.1-0.6 M KCl, 3-ml fractions; heparin-Sepharose, 50 ml/hr, 100-ml linear gradient of 0.05-0.4 M KCl, 3-ml fractions. Gel filtration on Sephacryl was achieved in KETT-0.3 at an ascending flow rate of 60 ml/hr (3-ml fractions). Ammonium sulfate precipitation was used to concentrate the receptor peaks at early stages, and chromatography on an hydroxylapatite column (0.8 x 0.5 cm) was used to concentrate the final heparin-Sepharose-purified receptor peak. One percent of each fraction was assayed for 3H.
Concentrated by precipitation, dissolved in 1 ml of KETT-0.3, and chromatographed on Sephacryl. Although protein was not measured at this step, other experiments have demonstrated a 2- to 3-fold enhancement of receptor specific activity. The harvested peak fractions from this step were in turn concentrated and then chromatographed on blue dextran-Sepharose. The receptor peak that eluted at 0.40 M KCl was pooled, diluted to 0.1 M KCl with KETT-0, and then chromatographed sequentially on the remaining resins listed in Table 1 until it was eluted at 0.22 M KCl from heparin-Sepharose. Protein content of the pooled receptor peak was 26 μg with a resultant total enhancement of specific activity over cytosol of 86,000-fold in 14% yield. Because homogeneity has been estimated to require a 200,000-fold purification, these results would suggest that the 1,25-(OH)₂D receptor is approximately 50% pure.

**NaDodsSO₄/Polyacrylamide Gel Electrophoresis.** Purification of protein was also followed by electrophoresis of samples derived from the last three chromatographic steps. Aliquots containing 8-10 μg of protein were taken from the receptor preparation after purification by blue dextran-Sepharose (precipitated by trichloroacetic acid), the second DNA-cellulose (precipitated by trichloroacetic acid), and heparin-Sepharose [concentrated by hydroxylapatite (15) and precipitated by trichloroacetic acid]. These aliquots represent 1, 5, and 40%, respectively, of the entire protein from that step. The results (Fig. 4) show that significant purification was achieved with each successive step. One major and three minor protein bands, with molecular weights between 50,000 and 65,000, represented 95% of the 8-10 protein species seen after heparin-Sepharose chromatography, indicating the power of this purification procedure. On the basis of total receptor-bound hormone present just prior to denaturation for electrophoresis, it is likely that one of these bands represents the receptor for 1,25-(OH)₂D, although it cannot be identified at this time.

**Sucrose Gradient Analysis of Purified 1,25-(OH)₂D Receptor.** The presence of a 3.3S macromolecule was monitored throughout purification on 5-20% sucrose gradients in 0.3 M KCl. The 3.3S 1,25-(OH)₂D receptor could be demonstrated at each step during purification, particularly after DNA-cellulose and heparin-Sepharose chromatography (Fig. 5). The receptor continued to cosegregate with unpurified receptor from cytosol (Fig. 5B).

**DISCUSSION**

On the basis of specific activity measurements, an 86,000-fold purification of the chicken intestinal receptor for 1,25-(OH)₂D has been achieved, and theoretical considerations (15) would suggest that the receptor has been purified to approximately 50% of homogeneity. The principle steps involved in this procedure are Polymac P precipitation of cytosol and the sequential use of DNA-cellulose, blue dextran-Sepharose, and heparin-Sepharose. NaDodsSO₄/polyacrylamide gel electrophoresis of an aliquot of the purified sample indicates that the procedure has isolated approximately four proteins, all near 55,000 in molecular weight; and sucrose gradient analysis supports the presence of a 3.3S macromolecule that is complexed with 1,25-(OH)₂D. Thus, the results suggest that the present technique are capable of purifying the receptor at least 100-fold over what has been achieved by using ion exchange and blue dextran-Sepharose chromatography (15).

The receptor for 1,25-(OH)₂D has been difficult to purify, primarily because of its rarity as a cytosolic protein and because of its extreme lability, particularly as purification progresses. Because the receptor exists as 0.001% of the total cytosolic protein (approximately 200 μg/kg equivalent of intestinal mucosal cytosol), major problems have been encountered in developing procedures to cope efficiently with large amounts...
of protein. Additional difficulties have arisen in dealing with the small amounts of protein resulting from high purification. With regard to lability, this characteristic appears to be expressed in the apparent tendency for the receptor to dissociate its hormonal ligand and subsequently denature. Relabeling of the dissociated receptor has not been possible, and, therefore, all efforts to stabilize the complex have been unsuccessful. Nevertheless, the results reported here are a significant advance in our ability to purify this interesting protein.

The binding of the 1,25-(OH)_{2}D receptor to blue dextran-Sepharose, DNA-cellulose, and heparin-Sepharose may imply that the receptor functions to interact with the intestinal cell genome. It has been suggested that blue dextran has a biospecific affinity for nucleotide-requiring enzymes, because of the structural similarity between nucleotide cofactors and the blue chromophore. Thus, blue dextran has been useful in purifying a number of dehydrogenases (20, 27). The resin also appears to bind proteins that interact with DNA, including RNA polymerase (28) and DNA polymerase (29), suggesting that it also has affinity for those proteins that bind nucleic acids. This resin has been used to study properties of an estrogen receptor (30). DNA-cellulose has been used to study and purify an extensive number of nuclear proteins and steroid hormone receptors such as the progesterone A subunit (31), the estrogen receptor (32, 33), the glucocorticoid receptor (34), and the androgen receptor (35). In addition, heparin-Sepharose has been used to purify the "native" form of the estrogen receptor (21). Thus, the observation that the 1,25-(OH)_{2}D receptor can be extensively purified by these techniques suggests that this receptor belongs to a class of proteins which bind to, and possibly modify, DNA.

The 1,25-(OH)_{2}D receptor remains to be purified to homogeneity and unequivocally identified, and its physical and functional properties remain to be investigated in detail. Preliminary results with semipurified material (10- to 15-fold) suggest that the receptor–hormone complex can be resolved on a nondenaturing electrophoretic gel, thus providing a means of identifying and then characterizing the protein once it is pure. Furthermore, because kinetic and sterol-specificity studies have been carried out with the crude cytosol receptor, similar experiments must now be accomplished on the purified material. Once this is achieved, however, interesting functional studies of this receptor's action on the expression of specific genes may be initiated.

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