CHARACTERIZATION OF TWO FORMS OF GLUCOCORTICOID HORMONE-RECEPTOR COMPLEX SEPARATED BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

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SUMMARY

Two forms of [^{3}H]triamcinolone acetonide-receptor complex prepared from rat liver cytosol were successfully separated by DEAE-cellulose column chromatography. One form (Peak I) was eluted by 0.06 M, and the other form (Peak II) by 0.24 M, potassium phosphate buffer (pH 7.4). Exposure of extracts to 20° for up to 2 h progressively diminished the radioactivity found in Peak II while increasing that in Peak I, with conservation of total bound radioactivity. The radioactivity in Peak I could bind to either chromatin or DNA very efficiently. Little of the radioactivity in Peak II could bind to either. The properties of the glucocorticoid receptor therefore appear to differ from those described for the two forms of progesterone receptors.

INTRODUCTION

Purified chick oviduct progesterone receptor consists of a pair of non-identical subunits, A and B, which elute from DEAE-cellulose columns as two peaks of progesterone-binding activity (1, 2, 3, 4). Peak A, eluting at 0.10 M KCl, binds to DNA but not to chromatin, while peak B, eluting at 0.22 M KCl binds to chromatin but not to DNA (1, 2, 3, 4). Because the steroid-binding proteins for androgens, estrogens and glucocorticoids from various tissues also have been observed to separate into 2 peaks upon DEAE-cellulose chromatography (5), it has been proposed that all steroid receptors may consist of analogous non-identical subunits (5).

Our observations on the glucocorticoid receptors of rat liver, HTC cells and LA9 cells are that while these receptors do elute from DEAE-cellulose columns as two peaks, these have different DNA/chromatin binding properties than those reported for the progesterone receptor. Our data further suggest that one peak may represent a precursor for the other "activated" receptor form.

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MATERIALS AND METHODS

Preparation of cytosol. Male Fisher rats of 150-200 g were fed Purina Laboratory Chow and water ad libitum. They were decapitated and their livers perfused in situ with 40 ml of ice-cold phosphate-buffered saline (0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.4) through the portal vein. All subsequent operations were performed at 0 to 4° unless otherwise mentioned. The livers were removed and homogenized in two volumes of 5 mM KPD[1] in a Dounce homogenizer. HTC cells (6) and LA9 cells (7) were grown in a modified minimum essential medium (8) supplemented with 5% fetal calf serum. For extraction, cells were washed twice with phosphate buffered saline and similarly homogenized. After centrifuging the homogenate at 100,000 g for 1 h at 0 to 2°, the upper fatty layer was discarded and the supernatant (cytosol) was carefully removed for experiments.

Binding of [^{3}H]triamcinolone acetonide (TA)[1] to cytosolic receptor protein. Liver cytosol containing 20-30 mg protein/ml was incubated with 1-3 x 10^{-8} M [^{3}H]TA (Amersham/Searle, specific activity 36 Ci/mmole) with or without a 300-fold excess of unlabeled TA for 3 h, at which time binding was essentially complete. Unbound steroid hormone was removed by centrifugation after shaking with dextran-coated charcoal (9). (Specific binding, i.e. the difference between total [^{3}H]TA bound and that remaining after incubation with an excess of unlabeled TA, was usually about 90% at the concentration of steroid used.)

Analytical and preparative procedures. Protein was measured by the method of Lowry et al. (10), using bovine serum albumin as the standard. Chromatin was prepared from rat liver according to Huang and Huang (11) and stored frozen at -20°. DNA in nuclei and chromatin was measured by Burton's method (12) and in metrizamide fractions by the mithramycin method (13). DNA-cellulose was prepared according to Alberts and Herrick (14) using Whatman CF-11 cellulose powder and calf thymus DNA (Miles). Sucrose density gradient centrifugation was carried out by layering 0.2 ml samples on 4.6 ml linear 5-25% sucrose gradients in 5mM KPD with or without 0.3M KCl using [^{14}C]albumin as internal standard, and centrifuging in a SW 50.1 rotor at 40,000 rpm for 18 h at 2°. Fractions of 0.2 ml were collected from the top using the Beckman Fraction Recovery System. Sedimentation constants for peaks were calculated according to Martin and Ames (15). Radioactivity was estimated by mixing each fraction with 1 ml of NCS (Amersham/Searle) and counting in 10 ml of toluene containing PPO (4 g/liter) and POPOP (50 mg/liter) in a Beckman LS-255 scintillation counter. Counting efficiency was approximately 30%. Cytosols labeled with [^{3}H]TA with or without a 300-fold excess of unlabeled TA were applied to DEAE-cellulose columns (0.9 X 5 cm, bed volume 3 ml) previously equilibrated with 5 mM KPD. The columns were washed thoroughly with 5 mM KPD and eluted by a linear gradient from 5 mM to 300 mM KPD. Radioactivity of fractions from column chromatography was counted in the same way for 0.1 ml aliquots. Metrizamide gradient centrifugation (16) was carried out by layering 0.2 ml samples on 4.6 ml linear 5-20% gradient of metrizamide [2-(3-acetamido-5-N-methylacetamido-2,4,6-tri-iodobenzamido)-2-deoxy-D-glucose, Nyegaard, and Co., Oslo, Norway] in 5 mM KPD, and centrifuged at 43,000 rpm for 1.5 h at 2° in a SW 50.1 rotor in a Beckman L5-65 ultracentrifuge. Fractions of 0.2 ml were collected, 0.1 ml of each was assayed for DNA, and the remainder was counted in 10 ml of Aquasol (New England Nuclear).

1. Abbreviations: KPD, potassium phosphate buffer containing dithiothreitol at 0.5 mM, pH 7.4; TA, triamcinolone acetonide (9α-fluoro-11β, 16α, 17, 21-tetrahydroxypregna-1, 4-diene-3, 20-dione cyclic 16, 17-acetal with acetone).

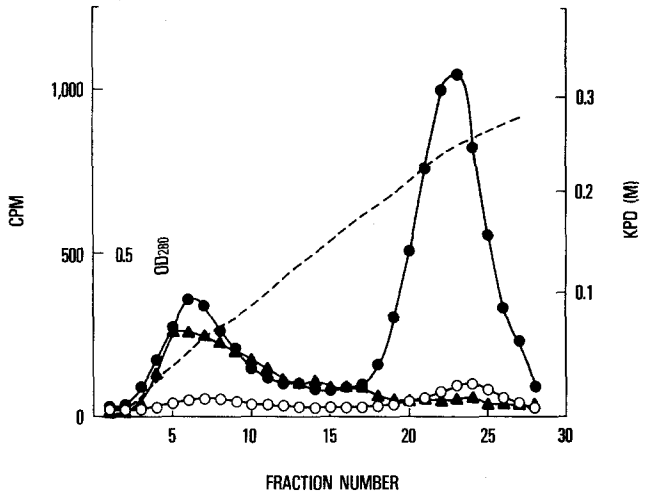

Fig. 1. DEAE-cellulose chromatography of hepatic glucocorticoid receptor. Rat liver cytosol was incubated with [^{3}H]TA as in Materials and Methods and applied on DEAE-cellulose columns. Fractions of 0.85 ml were collected and a 0.1 ml aliquot of each fraction was counted. Salt concentration (dotted line) was measured by a Radiometer electroconductivity meter and protein concentration was measured by its A_{280}. CPM without unlabeled TA (closed circles); CPM with unlabeled TA (open circles); A_{280} (closed triangles).

RESULTS AND DISCUSSION

In previous characterizations of the glucocorticoid-receptor complex, DEAE-cellulose chromatography often has been used, with KCl as the eluting salt (17, 18). By substituting a gradient of phosphate buffer, we have obtained much better resolution of the complex on DEAE-cellulose columns, with clean separation of two peaks of radioactivity (Fig. 1). Both peaks are lost when the cytosol is incubated with an excess of unlabeled steroid (Fig. 1). Of the total protein applied, about 40% is bound to the column, as is about 90% of the radioactivity, representing virtually all the "competable" steroid. The first peak of radioactivity is eluted at 0.06 M KPD, along with a major protein peak; the second peak elutes at 0.24 M KPD, and as Fig. 1 shows, is better resolved than is Peak I from other eluted proteins. Virtually identical results were obtained with extracts from HTC and LA9 cells, rat thymus and rat brain.

To check chromatographic homogeneity, fractions corresponding to Peak I and II were prepared by stepwise elution as specified in the legend to Fig. 2

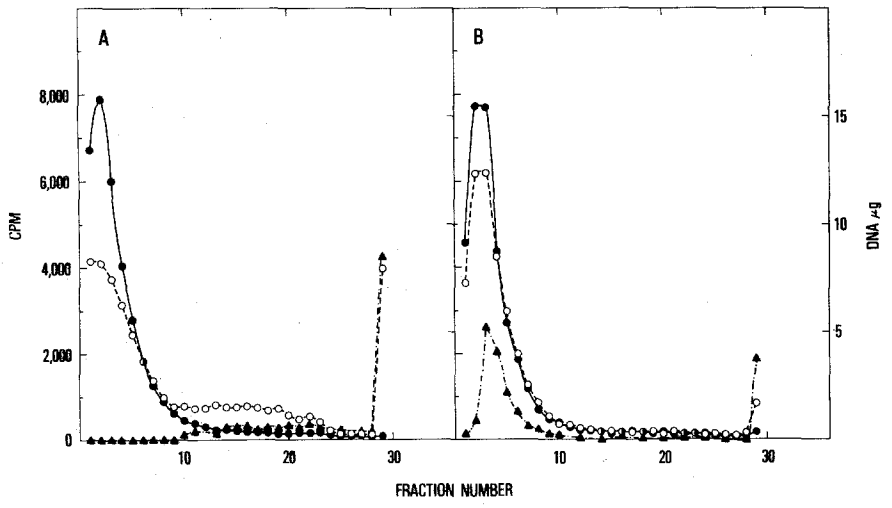

Fig. 2. Rat liver cytosol labeled with [^{3}H]TA as in Materials and Methods was first incubated at 20° for 30 min and then applied to a DEAE-cellulose column. Fractions corresponding to Peak I and Peak II were obtained by the following stepwise elution: 1) thorough 5 mM KPD wash; 2) 0.1 M KPD elution (Peak I); 3) 0.15 M KPD wash; and 4) 0.3 M KPD elution (Peak II). Both the 0.1 M KPD and the 0.3 M KPD eluates were passed through a Sephadex G-25 column which had been equilibrated with 5 mM KPD. Aliquots of 0.2 ml of Peak I or Peak II were preincubated together with or without 40 μg DNA at 0° for 2 h and then analyzed by metrizamide gradient centrifugation. Panel A shows the results of experiments with Peak I, and Panel B those with Peak II. For both, radioactivity of a control sample which did not contain DNA is shown by closed circles, and of the experimental by open circles. Amount of DNA in the latter samples is shown by closed triangles. The last fraction in each (no. 29) shows the DNA and radioactivity found at the bottom of the gradient.

and rechromatographed. Peak I rechromatographed in its original position, but Peak II separated into a small peak in the Peak I position, with the remainder in the Peak II position. Therefore Peak II is either contaminated with Peak I or is partially converted to Peak I during chromatography. Clear separation of two peaks by DEAE-cellulose chromatography is consistent with the suggestion that two forms of glucocorticoid receptor exist (5, 18, 19).

Progesterone receptors from chick oviduct have been purified to homogeneity and found to consist of a pair of non-identical, non-interconvertible subunits (1, 2, 3, 4). These subunits were apparent on DEAE-cellulose chromatography of cytosols as two equal peaks (1, 3). Because of the apparently similar fractionation on DEAE-cellulose of glucocorticoid receptor activity,

Table 1. Binding Properties of Two Forms of Receptor

		NUCLEI			CHROMATIN			DNA-CELLULOSE		
		cpm applied	cpm bound	% bound	cpm applied	cpm bound	% bound	cpm applied	cpm bound	% bound
EXPERIMENT 1	Peak I	3,018	2,681	89	3,018	2,383	79	3,018	2,745	91
	Peak II	3,724	541	15	3,724	-35	0	3,724	1,502	40
EXPERIMENT 2	Peak I	3,260	2,473	76	3,260	2,571	79	3,260	2,255	69
	Peak II	682	-97	0	682	104	15	682	89	13
EXPERIMENT 3	Peak I	4,010	3,295	82	4,010	3,451	86	4,010	2,557	64*
	Peak II	4,488	760	17	4,488	822	18	4,488	1,025	23*

Binding of Peaks I and II to nuclei, chromatin and DNA-cellulose. Peaks I and II samples were prepared as described in the legend to Fig. 2. Nuclei, chromatin, and DNA-cellulose were used in pelleted form in plastic tubes. Samples in 0.2 ml were added to the pellets of nuclei, chromatin and DNA-cellulose and mixed thoroughly by a Vortex mixer. During 1 h incubation at 0-2°, the tubes containing the mixtures were agitated every 2-3 min to ensure the binding. The mixtures were centrifuged at 12,000 g for 10 min and a 0.1 ml aliquot of each supernatant was counted. Radioactivity bound to nuclei, chromatin and DNA-cellulose was calculated by subtracting the radioactivity remaining in the supernatant from the total radioactivity applied to each tube. Nuclei, chromatin and DNA-cellulose were calibrated for these experiments so that their binding activity was not exceeded (data not shown). When nuclei, chromatin and DNA-cellulose were washed once with 2 ml of 5 mM KPD and then counted, the results were essentially the same as are shown here except for small decrease in binding (not shown). In experiment 3, the data obtained by subtracting the radioactivity bound to plain cellulose are also shown.

	bound	% bound
Peak I	1,658	41
Peak II	599	13

* Corrected for binding to cellulose alone

we examined the binding properties of our two crude fractions. Peak I bound effectively to nuclei, chromatin and DNA-cellulose, while Peak II bound slightly to nuclei and DNA but little or not at all to chromatin (Table 1). In other experiments (not shown) unfractionated cytosol preincubated with

[^{3}H]TA was heated for 30 min at 20° and mixed with an excess of rat liver chromatin for 1.5 h at 0°. Then the chromatin was removed by centrifugation, and the supernatant chromatographed on DEAE-cellulose. The cytosol was found to be depleted for Peak I by about 90%, while Peak II remained essentially unchanged. Although Peak I consistently showed greater binding to DNA-cellulose than did Peak II, considerably less than 100% bound. Consequently, DNA binding of Peak I and Peak II was further explored using metrizamide gradient centrifugation (Fig. 2). Peak I, Peak II or DNA alone (not shown) barely entered the gradient. When Peak I and DNA were preincubated together, about 40% of the radioactivity and all of the DNA moved into the gradient without a defined peak, and a large fraction of both was found at the bottom of the gradient. On the other hand, when Peak II was preincubated with DNA, both remained at the top except for a slight precipitate. These data also suggest that Peak I interacts with DNA to a much greater extent than Peak II does.

Taken together, these preliminary experiments show that the two DEAE fractions of crude glucocorticoid-receptor complex do not have the properties found for the equivalent fractions of progesterone receptor. Species and organ differences (chick oviduct vs rat liver) could be invoked to explain the differences. Other possibilities doubtless exist as well: for instance, Peak I might contain both subunits in an unresolved form. Nevertheless these data, while they do not rule out the existence of nonidentical subunits for the glucocorticoid receptor, do not support that model.

Relation between Peak I and Peak II receptors

Glucocorticoid receptor activity in nearly every system described to date has been found to be a single class of binding sites when analyzed by Scatchard analysis (17, 20, 21, 22). Multiple peaks have been reported by Litwack et al., but only one of them was thought to be the true glucocorticoid receptor (23). It is also well established that a heat-dependent "activation" is necessary for receptor to transfer to nuclei *in vivo* or bind to nuclei *in vitro* (24). The differential binding properties in the two crude fractions we have sepa-

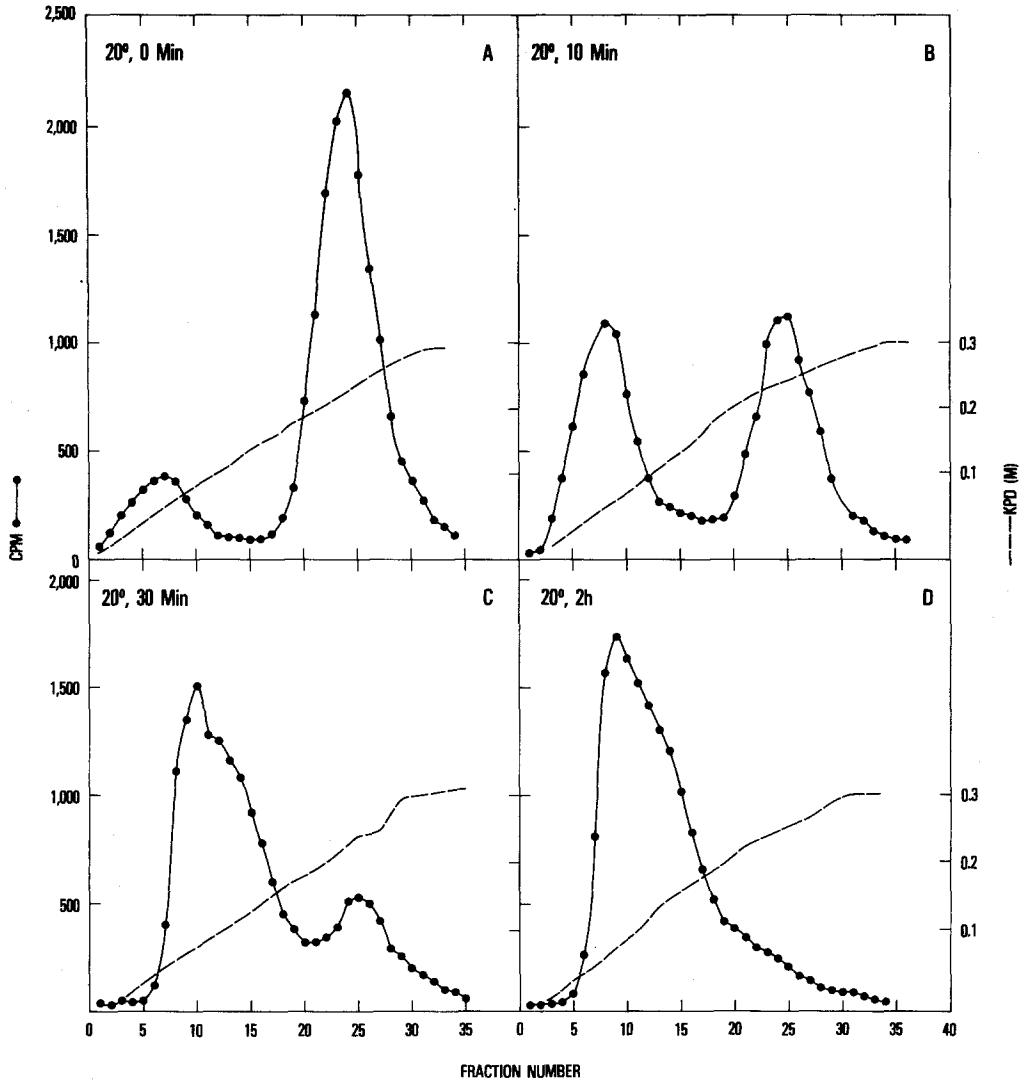

Fig. 3. DEAE-cellulose chromatography of heated liver cytosol. Rat liver cytosol labeled with 1.9×10^{-8} M [^{3}H]TA and charcoal-treated was divided in four equal portions and heated at 20° for 0 min (A), 10 min (B), 30 min (C), and 2 h (D), respectively and then chromatographed as described in the legend to Fig. 1. After 2 h at 20°, a small fraction of the 0 time material no longer bound to the column.

rated, with only Peak I binding well to nuclei, chromatin, and DNA, raise the possibility that Peak II represents an unactivated and Peak I an activated form of the steroid-receptor complex.

Several experiments support this idea. First, if a steroid-cytosol

preparation is held at 20° for increasing periods of time, more and more of the label is found in Peak I (Fig. 3). The time course of this process is consistent with that known to be required for heat activation of the complex (24, 25). Second, it is known that exposure to high salt concentrations activates the complex, and in experiments analogous to that of Fig. 1 (not shown) we found that shifting the salt concentration to 0.2-0.5 M KCl for 30-60 min resulted in the complexed radioactivity appearing as Peak I. Also, a shift of Peak II to Peak I material could be observed when Peak II, after initial fractionation on DEAE-cellulose, was treated with either heat or salt and then rechromatographed (data not shown). Finally, the shift of Peak II to Peak I could be inhibited by exposure to a crude fraction containing the recently described heat-stable inhibitor of activation (26). Cytosols from liver were heated at 100° for 30 min, centrifuged at 2500 `g for 10 min and the supernatant solution reserved. When this supernatant was mixed with Peak II, heating at 20° for 1 h no longer resulted in the shift of counts to the Peak I position as was seen in Fig. 3. Thus, these preliminary data are consistent with the view that Peak II represents an unactivated precursor to an activated steroid-receptor complex found in Peak I.

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