Partial purification of a glucocorticoid receptor

(hormones/steroids/proteins/affinity chromatography)

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ABSTRACT A simple method for purification of the glucocorticoid receptor from hepatoma tissue culture cells has been developed. The procedure, which requires only about 24 hr, involves biospecific adsorption of the receptor to deoxycorticosterone derivatized agarose, elution with a glucocorticoid, and gel filtration. The receptor-steroid complex is obtained in 35–40% yield and is about 2000-fold purified. It possesses properties similar to those reported in crude extracts, including sedimentation coefficient in glycerol gradients and activation-dependent binding to nuclei.

Extensive investigations of the mechanism of steroid action clearly indicate that the biological effects of these agents are mediated via protein receptors found in the cytoplasm of target cells (for a review see ref. 1). From studies with crude extracts, a general mechanism of action for glucocorticoids, which is similar in most respects to those of other steroid hormones, has been proposed (2, 3).

[1] The receptor (R) binds tightly to the corticosteroid (S) to form a receptor-steroid complex (RS). [2] The RS complex undergoes a temperature- and/or salt-dependent conversion to an "activated" form (R*S). [3] The activated complex, having high affinity for a nuclear component (N), presumably chromatin, enters the nucleus and forms RSN complexes. This association precedes accumulation of specific mRNAs, presumably resulting from modulation of gene transcription, which ultimately produces characteristic biological responses.

Although this model is likely to be correct in its general features, it has been derived from studies in very crude preparations; an understanding of the detailed mechanisms of action of these hormones awaits studies with systems reconstituted with purified components. We have been attempting to develop a method of purifying the glucocorticoid receptor from hepatoma tissue culture (HTC) cells. A number of difficulties have precluded successful purification of the glucocorticoid receptor in the past. In crude cytosol, the receptor has a half-life of less than 24 hr at 0–4° and, because it is present in low concentrations, requires substantial purification. Additional problems result from source limitations, the lability of the receptor to conditions classically used to purify proteins, and the tendency of receptor-steroid

complexes to undergo conformational changes and/or disaggregation (1). We report here a preliminary description of a facile method of purifying the glucocorticoid receptor-steroid complex to a stage that should advance investigations of biochemical mechanisms of steroid action.

MATERIALS AND METHODS

[1,2,4-³H]Dexamethasone (21 Ci/mmol) and [1,2-³H]tetrahydrocortisol (42 Ci/mmol) were from New England Nuclear. [1,2,4-³H]Triamcinolone acetonide ([³H]TA, 10.7 Ci/mmol) was from Schwarz/Mann. Aminoalkylagarose derivatives (1.2 μmol of amine per ml) were prepared from Bio-Gel A-50 m, 50–100 mesh Bio-Rad by reported procedures (4, 5). Cytosol from frozen HTC cells was prepared as described (6), using a homogenization buffer of 50 mM 2-(N-morpholino)ethanesulfonic acid (Mes, pH 6.5) 50 mM KF, 1 mM EDTA, and 1 mM dithiothreitol. Glassware used in purification experiments was pretreated with Siliclad. Protein concentrations were determined by the fluorescamine method (7) with bovine serum albumin as standard, and pH measurements were made at about 20°.

Measurements of Macromolecular Steroid Binding. Cytosol was incubated with 0.1 μM [³H]dexamethasone or [³H]TA at 0–4° for 1–24 hr. Macromolecular steroid binding (300 μl) in crude and purified samples was determined by gel filtration on Sephadex G-25 (5 ml) using a buffer consisting of 10 mM Mes (pH 6.5) containing 50 mM KF, 0.5 mM EDTA, and 0.5 mM dithiothreitol (buffer A). The macromolecular fraction was dissolved in xylene/Triton X-114 (3:1) containing 0.3% Omnifluor (New England Nuclear) and radioactivity was measured by liquid scintillation spectrophotometry at 45% counting efficiency. DEAE-cellulose filter assays of receptor-steroid complexes were performed by the method of Santi *et al.* (8). Nonspecific steroid binding in cytosol was estimated by similar analysis of samples labeled with 0.1 μM [³H]steroid in the presence of 1000-fold excess of the same nonradioactive steroid (9). In contrast to the specific, high affinity receptor-steroid binding, the nonspecific binding is heat stable and can also be estimated in purified samples by heat denaturation (37°, 4 hr) prior to analysis for bound steroid.

Glycerol gradient centrifugations (10–30%) were performed in buffer A or buffer A containing 0.4 M KCl. Receptor-steroid complexes (about 10,000 dpm) were applied to the gradients and centrifuged in a SW-50.1 rotor at 45,000 rpm for 18 hr at 0°. [¹⁴C]Acetyl-bovine serum albumin was used as marker.

Preparation of Affinity Adsorbents. A solution of 0.1 M 21-chloroprogesterone (10) and 0.1 M of the sodium salt of methyl *p*-hydroxybenzoate in *N,N*-dimethylformamide was stirred at room temperature for 8 hr. After the mixture was poured onto ice water, the product was collected by filtration and recrystallized from methanol to give 21-(4-carbom-

Abbreviations: DOC-BAP-A and DOC-BAH-A, deoxycorticosterone-benzamidopentyl and hexyl agaroses, respectively; buffer A, 10 mM 2-(N-morpholino)ethanesulfonic acid (Mes), 50 mM KF, 0.5 mM EDTA, 0.5 mM dithiothreitol; HTC cells, hepatoma tissue culture cells; [³H]TA, [1,2,4-³H]triamcinolone acetonide.

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ethoxyphenoxy)progesterone, mp 170–172° (d), in 82% yield. Hydrolysis of the ester with 1.2 mol equivalents of potassium carbonate by refluxing 24 hr in methanol/water (4:1) gave, after neutralization and recrystallization from CHCl_3 /hexane, the corresponding acid in 84% yield; mp 220–222° (d). The ester and acid had the expected infrared spectra and C,H combustion data. The steroid acid was converted to its *N*-hydroxysuccinimide ester (11) and then reacted in 10-fold molar excess with aminoalkyl agarose to give the affinity adsorbents deoxycorticosterone-benzamido-pentyl and hexyl agaroses (DOC-BAP-A and DOC-BAH-A) (Fig. 1). Steroid concentrations of the adsorbents were determined to be about 1 $\mu\text{mol/ml}$ of packed gel by quantitation of the gel-bound amine (12) prior and subsequent to treatment with the steroid active ester. Residual gel-bound amines were acetylated by treatment with *N*-acetoxy succinimide (13) (2 mmol/100 ml of gel) at room temperature for 12 hr. Gels were then extensively washed with dioxane and 90% acetone to remove noncovalently bound steroids (14) and stored in 95% EtOH at 4°. Treatment of DOC-BAP-A with excess sodium borohydride gave the reduced gel formulated as $\text{H}_2\text{DOC-BAP-A}$; similar treatment of 3-keto-4-ene steroids results in complete reduction of the 3-keto group and partial reduction of the 4,5-double bond (15).

Receptor Purification with Affinity Gel. All operations were performed at 0–4°. The adsorbent (2 ml) was successively washed with 40 ml of 95% ethanol, 40 ml of water, and 40 ml of buffer A. Freshly prepared cytosol (6 ml, about 200 mg of protein) was added and the mixture was gently agitated for 3 hr. After centrifugation, the supernatant was assayed for specific binding of [^3H]glucocorticoid and protein content. The gel was washed batchwise with six portions of buffer A (10 ml each) over 30 min, and then 2 ml of buffer A containing 1 μM [^3H]TA was added. After 18 hr the mixture was transferred to a 3-ml plastic syringe fitted with a glass wool plug and the supernatant removed by centrifugal (1000 $\times g$) filtration. The gel was washed with 1 ml of buffer A by the same technique. The combined supernatant and wash was assayed for protein content and receptor–steroid complex.

Filtration of Affinity Gel Product Through Bio-Gel A-0.5 m. A 500- μl portion of the material eluted from the affinity gel was filtered through a column (0.7 $\times$ 13 cm) containing 5 ml of Bio-Gel A-0.5 m, 100–200 mesh, equilibrated in buffer A at 0–4°. The column was eluted with buffer A and the void volume (1.8 ml) collected. Nonspecific binding was determined by treatment of the receptor–steroid complex at 37° for 4 hr prior to gel filtration. DEAE-cellulose filter assays were also performed on the purified receptor–steroid complex immediately after gel filtration; in this case nonspecific binding was determined by 37° treatment of the gel-filtered material.

RESULTS AND DISCUSSION

Because of the difficulties anticipated in purification of the glucocorticoid receptor which were mentioned in the introduction, it was apparent that a rapid and effective initial purification step was necessary. In addition, since we wished to study the activation of receptor–steroid complexes, the procedures used for purification could not involve exposure to high salt concentrations. Affinity chromatography appeared to be a most attractive method despite difficulties (16) encountered by other workers in purification of steroid–receptor proteins using this technique. In choosing a bio-adsorbent, we desired a ligand that could be easily modified in a

FIG. 1. Structure of the affinity adsorbents DOC-BAP-A and DOC-BAH-A.

tolerated position such that it could be attached to a solid support; moreover, we felt it necessary that all linkages connecting the ligand with the solid matrix be as stable as possible. From previous studies (6, 17), it appeared that the 21-carbon of glucocorticoids was the most suitable position for substitution. Deoxycorticosterone, a steroid with high affinity for the HTC cell glucocorticoid receptor ($K_d = 4.0$ nM at 0°), could be easily converted to 21-(4-carboxyphenoxy)progesterone; the latter had $K_d = 0.25$ μM (0–4°) for the glucocorticoid receptor and at 1 μM inhibited tyrosine aminotransferase induction by 40% of that observed with 0.1 μM dexamethasone alone. This ligand can easily be connected to agarose derivatives by linkages not susceptible to esterases expected to be present in crude cytosol, a problem encountered in previous attempts at affinity chromatography using ester derivatives of similar ligands (14). The ligand was attached by conventional means to aminopentyl and amino-hexyl agarose to give the adsorbents DOC-BAP-A and DOC-BAH-A, respectively (Fig. 1). Residual amines on the affinity adsorbent were masked by acetyl groups to preclude ion exchange effects.

When HTC cytosol was treated with DOC-BAP-A or DOC-BAH-A, 76 and 87%, respectively, of the specific glucocorticoid binding proteins were removed from solution without removing significant amounts of protein (Table 1). Leakage of the ligand from the matrix over the period required for adsorption could not be detected (16). With agarose, there was no adsorption of glucocorticoid binding proteins, and using a matrix in which the 3-keto group of the ligand had been reduced with NaBH_4 ($\text{H}_2\text{DOC-BAP-A}$), less than 20% was removed from solution. The 3-keto group is essential for binding to the glucocorticoid receptor (17) and the small amount of binding to $\text{H}_2\text{DOC-BAP-A}$ was proba-

Table 1. Adsorption of the glucocorticoid receptor to agarose and steroid–agarose*

Adsorbent	Specifically bound steroid (nM)		
	Cytosol	Treated cytosol	% Adsorbed
Agarose	6.70	6.7	0
DOC-BAP-A	6.70	1.61	76
$\text{H}_2\text{DOC-BAP-A}$	6.70	5.40	19
DOC-BAH-A	9.40	1.22	87

* Cytosol (0.6 ml) was treated with 0.2 ml of the gel as described in *Materials and Methods*. After 2 hr, the tubes were centrifuged and supernatants assayed for specific and nonspecific binding to [^3H]TA; the control contained buffer A instead of agarose or derivatized agarose.

Table 2. Purification of the glucocorticoid receptor*

Fraction	Protein (mg/ml)	Vol. (ml)	Receptor-[³ H]TA	
			Total dpm × 10 ⁻⁴ (%) yield)	dpm × 10 ³ /mg of protein (purif.)
Cytosol	29	4.5	125	9.49 (1)
DOC-BAH-A				
cytosol	22	6	16.5	1.25
DOC-BAH-A eluate†	0.085	2.6	55.6 (44)	2516 (265)
BioGel-Gel A-0.5 m	0.003	9.4	45.9 (37)	16380 (1726)

* The procedure used is described in *Materials and Methods*. Assays were performed using 0.1 μM [³H]TA and values presented are corrected for nonspecific binding.

† Nonspecific binding was estimated by heat denaturation of the receptor-[³H]TA complex.

bly due to incomplete reduction or nonspecific adsorption. The DOC-BAH-A bound receptor could be freed of most contaminating proteins by batchwise washing with KF-Mes buffer (μ = 0.055) for 30 min. The buffer composition and time period used were derived from the results of numerous experiments designed to achieve a balance of maximal yield and purification of the receptor upon subsequent elution.

The receptor was competitively eluted from the bioadsorbent DOC-BAH-A by an 18-hr equilibration with a glucocorticoid for which it has a high affinity (Table 2). The recovery of the receptor-[³H]steroid complex from the matrix was 51% (44% overall), and the purification was 250- to 300-fold over that in crude cytosol. For reasons unknown at this time, other proteins bind to the matrix which are not removed by the initial washing, but slowly elute over the period required for dissociation of the receptor. Attempts at removal of these proteins by modification of washing and eluting conditions have thus far been unfruitful.

A number of proteins bind glucocorticoids and it is necessary, although often neglected, to utilize criteria in addition

Table 3. Binding of receptor-[³H]TA to HTC cell nuclei*

Source	Receptor-[³ H]TA dpm added	dpm specifically bound to nuclei†	
		Unactivated	Activated‡
Cytosol	44,000	9,030	23,600
Aff-C§	64,000	13,300	36,400
Aff-C + Bio-Gel¶	29,400	2,940	7,560

* Nuclei were prepared as described (23) and suspended in 5 mM piperazinoethanesulfonate (pH 7.4), 50 mM KF, 0.5 mM dithiothreitol, and 0.25 M sucrose. The receptor-[³H]TA complex and nuclei from about 2.5 × 10⁸ cells in a total volume of 1.3 ml were kept at 4° for 90 min and analyzed for specific binding by the method of Higgins *et al.* (3).

† Specific nuclear bound radioactivity was corrected for background binding by parallel experiments using receptor-[³H]TA that had been treated at 37° for 24 hr.

‡ Activation was performed by maintaining the receptor-[³H]TA preparations at 20° for 1/2 hr in the presence of 0.1 M added KF (final μ = 0.15).

§ Aff-C refers to receptor-[³H]TA complex purified by affinity chromatography.

¶ The buffer was 10 mM morpholinopropanesulfonate (pH 7.4).

to steroid binding to establish the authenticity of steroid receptors. Proteins that bind a variety of glucocorticoids with low affinity are present in sufficient concentration in cytosol that binding is linear with steroid concentrations up to at least 1 μM; furthermore, unlike the glucocorticoid receptor-steroid complexes, nonspecific complexes are stable or increase upon treatment at 37° (ref. 14; unpublished results). That the [³H]TA-protein eluted from the DOC-agarose matrix is not a nonspecific steroid binding protein was initially indicated by the observation that the complex was unstable at 37° for 4 hr. In addition, when the DOC-BAH-A adsorbed receptor was treated with [³H]tetrahydrocortisol, a steroid that does not bind to the glucocorticoid receptor (17) but does complex with nonspecific steroid binding proteins, heat-labile macromolecular bound radioactivity was not found in the supernatant.

Proteins that specifically bind glucocorticoids show a high affinity and structural specificity for these steroids, and are present in saturable amounts. The glucocorticoid receptor belongs to this class of proteins, as do other corticosteroid binding proteins of liver (18, 19) and the corticosteroid binding protein (CBG) of serum (20). It appears unlikely that the protein we obtain is related to the latter since the CBG does not bind dexamethasone (19, 21), and this steroid may be used to elute the receptor described here from the DOC-agarose matrix.

Direct evidence that the eluted protein we obtain is the receptor previously characterized in crude HTC cytosol is as follows: (i) The purified and crude receptor-[³H]TA complexes have identical sedimentation properties in glycerol gradients (see below). (ii) Unlike other glucocorticoid binding proteins, the glucocorticoid receptor-steroid complex undergoes a temperature- and/or salt-dependent activation and subsequent nuclear binding (3, 21). Table 3 compares binding of crude and purified glucocorticoid receptor-[³H]TA complexes to isolated nuclei before and after such activation. The fact that the purified protein we obtain undergoes this reaction substantiates that it is indeed the glucocorticoid receptor. At this stage of purification the receptor-[³H]TA complex can be stored at 0-4° for at least 1 week without loss in binding activity providing an excess of [³H]TA was present.

To achieve additional purification, we attempted various ion-exchange chromatographic methods. Although these were successful to varying degrees, elution of the receptor required high salt and concomitant conversion to the activated (R*S) form. Since one of our objectives is to study the RS to R*S conversion, purification methods requiring salt treatment were not pursued in detail. An obvious purification method adaptable to conditions of low salt was gel filtration. Initial attempts using agarose in which the receptor-steroid complex was included in the gel indicated that the prolonged treatment required resulted in destruction of the complex. We reasoned that a gel with an effective molecular weight cutoff just below that of the receptor-[³H]TA complex would retain low-molecular-weight proteins and provide the receptor-[³H]TA complex in the void volume, thus minimizing its exposure to the gel and time required for the purification. Bio-Gel A-0.5 m, 100-200 mesh was found to be well suited for this application. Filtration of the receptor-[³H]TA complex, purified by DOC-BAH-A, through 5 ml of this gel provides an additional 6- to 8-fold purification (1600-2000 overall) and 83% recovery. This purified complex sediments in glycerol gradients as a single 9S peak, as does the receptor-[³H]TA complex in crude cytosol, and re-

tains the ability to undergo activation-dependent nuclear binding (Table 3).

It is difficult to assess the degree of purification of the receptor obtained here because of ambiguities in assignment of molecular weight. The complex sediments at 8–9 S in low salt glycerol gradients and, together with gel filtration, an apparent molecular weight of about 450,000 may be calculated. At salt concentrations sufficient for activation, the receptor–steroid complex sediments at 4 S, and an apparent molecular weight of about 10^5 is calculated. Although a number of explanations can be forwarded for this behavior, the molecular nature of the 8–9S to 4S conversion, and thus the molecular weight, remains uncertain. The purified receptor–steroid complex we obtain contains about 1 nmol of bound steroid per mg of protein. With the assumptions that the molecular weight of the 9S form isolated is in the range of 1 to 4.5×10^5 and that 1 mol of steroid is bound per mol of receptor, the receptor should be between 10 and 45% pure. Since we are currently obtaining amounts of protein that are close to the lower limit of detection, definitive assignment of molecular weight and purity await our obtaining sufficient quantities so that the necessary physical measurements may be made. Nevertheless, the preparation described here is sufficiently pure to pursue definitive biochemical studies on the nature of activation and nuclear binding.

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