INVOLVEMENT OF A LOW MOLECULAR WEIGHT COMPONENT(S) IN THE MECHANISM OF ACTION OF THE GLUCOCORTICOID RECEPTOR*

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SUMMARY: [^{3}H]Dexamethasone-receptor complexes from rat liver cytosol preincubated at 0° bind poorly to DNA-cellulose. However, if the steroid-receptor complex is subjected to gel filtration at 0-4° separating it from the low molecular weight components of cytosol, the steroid-receptor complex becomes "activated" enabling its binding to DNA-cellulose. This activation can be prevented if the gel filtration column is first equilibrated with the low molecular weight components of cytosol. In addition, if adrenalectomized rat liver cytosol, in the absence of exogeneous steroid, is subjected to gel filtration the macromolecular fractions separated from the "small molecules" of that cytosol have much reduced binding activity towards [^{3}H]dexamethasone. These results suggest that rat liver cytosol contains a low molecular weight component(s) which maintains the glucocorticoid receptor in a conformational state that allows the binding of dexamethasone. Furthermore, this component must be removed from the steroid-receptor complex before binding to DNA can occur.

INTRODUCTION

The glucocorticoid receptor-steroid complex present in cytosol needs to undergo an "activation" to enable it to bind to nuclei (1-5), chromatin (6) or purified DNA (1,3,7). In vitro, this activation can be brought about by raising the temperature either prior to or during the nuclear incubation (1-4). In addition, it has been shown that the steroid-receptor complex can be activated at low temperature by increasing the ionic strength (2,3,7), by the addition of calcium ions (7) or by the addition of theophylline (8).

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The chemical nature of the activation of the steroid-receptor which enables it to bind to DNA has remained obscure. Kalimi et al. (7) were unable to detect any change in the sedimentation behavior of the glucocorticoid-receptor complex as it underwent activation. They demonstrated that heat activation leads to a decrease in the isoelectric point, but no such change was observed if the receptor was activated by increasing the ionic strength. What is evident is that upon activation the steroid-receptor complex undergoes a transformation such that positively charged regions become exposed at the surface of the complex enabling it to bind to various polyanions (3,7). These include homologous and heterologous DNA (3,6,7,9), carboxymethyl- and sulfopropyl-Sephadex (3) and phosphocellulose (7).

Recent evidence suggests that cytosol contains a factor(s) which inhibits either the activation or the binding of receptor to DNA (3,6). We have shown (unpublished observations) that activation can be brought about at low temperature by removal of this component(s) by gel filtration or dilution. In this report we demonstrate that this component(s) has a low molecular weight. Furthermore, we show that if a low molecular weight component(s) is removed from adrenalectomized rat liver cytosol in the absence of exogenous steroid, the glucocorticoid receptor present in that cytosol is either rendered inactive to bind steroid or becomes unstable.

METHODS

Preparation of cytosol - Adrenalectomized male rats (110 to 140 g; CD Sprague-Dawley strain from Charles River Breeding Labs) were used 7 to 14 days following surgery. Animals were killed by decapitation and the livers perfused in situ with cold 0.145 M NaCl through the portal vein. The livers were removed and homogenized in an equal volume of ice-cold homogenization buffer (50 mM tris-HCl, pH 7.5, 0.25 M sucrose, 3 mM $MgCl_2$) and cytosol prepared as previously described (8).

Specific cytosol binding of dexamethasone - The cytosol was incubated with [^{3}H]dexamethasone (New England Nuclear; 22.6 Ci/mmole) at a final concentration of 3 x 10^{-8} M in the presence or absence of a 1000-fold excess of non-radioactive steroid. After a 2 hour incubation at 0°, when steroid binding was essentially complete, the specific macromolecular bound fraction was determined using the dextran-coated charcoal technique (10).

Assay of receptor binding to DNA-cellulose - The binding of the glucocorticoid receptor to DNA-cellulose was determined by the procedure of Kalimi et al. (7).

A 100 µl aliquot of cytosol, preincubated with [^{3}H]dexamethasone, was mixed with 50 µl of packed DNA-cellulose (P-L Biochemical Co.; 1.0 mg native DNA/ml cellulose) and incubated at 0° for 45 min with gentle shaking. At the end of the incubation, 2 ml of ice cold assay buffer (10 mM tris-HCl, pH 7.5, 1 mM EDTA) was added, mixed, and centrifuged for 5 min at 600 x g. The DNA-cellulose pellet obtained after 3 washes with the same buffer was suspended and an aliquot assayed for radioactivity as previously described (11). Under these conditions the DNA is in excess and 90% of the receptor in the activated state binds to the DNA (unpublished observations). Results are expressed as the percentage of specifically bound [^{3}H]dexamethasone which binds to the DNA-cellulose.

Chromatographic procedures - Bio-Gel P-4 - buffer equilibrated: Chromatography was carried out on minicolumns (1.5 x 6.0 cm) of Bio-Gel P-4 (Bio Rad Laboratories; 50-100 mesh) equilibrated with homogenization buffer. Cytosol (15 ml) was applied and eluted with the same buffer, and 1.0 ml fractions were collected. By this means it was possible, in the early stages of elution, to obtain fractions which were undiluted with respect to macromolecules but separated from small molecules. With continued elution the small molecules began to elute and eventually fractions were obtained which contained both macromolecules and small molecules at a concentration equivalent to the applied cytosol. The columns used in these studies were characterized using a solution of blue dextran and KCl.

Bio-Gel P-4 - "Small molecule" equilibrated : When a Bio-Gel P-4 minicolumn, previously equilibrated with 15 ml cytosol, is eluted with buffer the fractions collected immediately after the macromolecules have been completely eluted contain small molecules at a concentration equivalent to the applied cytosol. These fractions were applied to another Bio-Gel P-4 minicolumn already pre-equilibrated with 15 ml cytosol. This procedure provided a column which was equilibrated with small molecules but devoid of macromolecules. To this column 15 ml of [^{3}H]dexamethasone-labeled cytosol was added and 1.0 ml fractions collected. Each fraction was analyzed for specific dexamethasone binding and ability to bind to DNA-cellulose.

Sephadex chromatography: Minicolumns (1.5 x 6.0 cm) of Sephadex G-15, G-25 and G-50 were prepared and equilibrated with homogenization buffer. To these columns 500 µl of [^{3}H]dexamethasone labeled cytosol was applied and eluted with the same buffer. After discarding the first 3.0 ml eluted, the macromolecular fraction was obtained by collecting the next 2.5 ml. Each of these fractions was assayed for specific dexamethasone binding and ability to bind to DNA-cellulose.

RESULTS AND DISCUSSION

When a relatively large volume (15-20 ml) of rat liver cytosol previously incubated at low temperature with 30 nM [^{3}H]dexamethasone was applied to a minicolumn of Bio-Gel P-4 (10 ml bed volume), the receptor-bound steroid was found to elute immediately after the void volume (Fig. 1). This is consistent with the macromolecular nature of the hepatic glucocorticoid receptor (10,12,13). These early fractions containing macromolecules separated from the low molecular weight components of cytosol were "activated" and thus able to bind to DNA-cellulose (Fig. 1). With continued elution the degree of activation of the [^{3}H]dexamethasone-receptor complexes progressively declined until they were

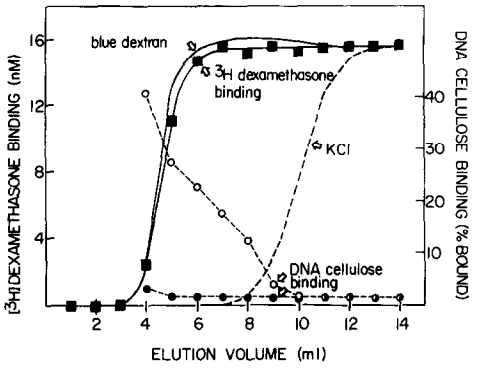

Figure 1. Effect of gel filtration on glucocorticoid receptor activation

[^{3}H]Dexamethasone-labeled cytosol was applied to a Bio-Gel P-4 minicolumn equilibrated with either buffer or "small molecules" and 1.0 ml fractions collected. Each fraction was assayed for [^{3}H]dexamethasone binding (●) and ability to bind to DNA-cellulose (buffer-equilibrated, ○; "small molecule"-equilibrated, ●). The columns were characterized using a solution of blue dextran and KCl. These fractions were assayed for blue dextran by absorption at 625 nm and for salt by conductivity measurements. Each point represents the mean of two separate experiments.

virtually inactive with respect to DNA-cellulose binding (only 1.3% of the complexes were capable of binding to DNA). However, [^{3}H]dexamethasone-labeled cytosol does not undergo activation when subjected to gel filtration if the Bio-Gel P-4 minicolumn is first equilibrated with the low molecular components of rat liver cytosol (Fig. 1). This implies that the activation of the steroid-receptor complexes on the buffer-equilibrated column is the result of a separation of the complex from a low molecular weight inhibitory component(s). Simons et al. (6) have reported the existence of an inhibitory material in HTC cell cytosol but on the basis of it being excluded by Sephadex G-25 they conclude it to be macromolecular.

If the gel filtration procedure does not completely remove the low molecular weight component from the steroid-receptor complex, dissociation of this component from the complex would be facilitated by dilution of the macromolecules. The degree of activation in samples diluted with respect to both macromolecules and "small molecules" (fractions 4 and 5) would then be expected to be greater than the degree of activation in samples diluted with respect to "small molecules" only (fraction 6). This, in fact, is the situation observed on buffer-equilibrated columns and suggests that the low molecular weight component is, indeed, not completely removed from the steroid-receptor complex by gel filtration. Further, the concentration of the inhibitory component remaining associated with the complex, and hence the degree of activation of the complex, is dependent on the amount of dilution the complex undergoes. Indeed, Milgrom et al. (3) have shown that when the glucocorticoid receptor of rat liver is subjected to a purification procedure, involving ammonium sulphate precipitation and Sephadex chromatography, the purified receptor can be activated completely (i.e. 100% of the complexes transfer to nuclei), a degree of activation not seen with unfractionated cytosol.

The hepatic glucocorticoid receptor can also be activated by gel filtration on Sephadex G-15, G-25 and G-50 (Table 1). These particular columns were run more conventionally with a 500 µl aliquot of [^{3}H]dexamethasone labeled cytosol applied to a buffer-equilibrated column and the macromolecular-bound radioactivity eluted and assayed for dexamethasone binding and binding to DNA-cellulose. Since the steroid-receptor complexes underwent dilution, a condition shown to cause activation of the complex (unpublished observations), a sample of the [^{3}H] dexamethasone-labeled cytosol was diluted equivalently. It can be seen that with all types of Sephadex, gel filtration caused activation to a level significantly greater than that seen with simple dilution. The fact that Sephadex G-15 caused activation suggests that the low molecular weight component(s) involved in this activation process might have a molecular weight less than 1,500 daltons.

Table 1. Activation of the glucocorticoid receptor by Sephadex chromatography

Treatment	DNA-cellulose binding (% bound)	
	Exp. 1	Exp. 2
None	2.0	2.0
Dilution, 1:5	10.4	11.3
Sephadex G-15	46.4	53.6
Sephadex G-25	42.6	43.1
Sephadex G-50	47.7	51.0

Adrenalectomized rat liver cytosol was incubated for 2 hours at 0° with 3×10^{-8} M [^{3}H]dexamethasone. Aliquots (500 µl) were applied to Sephadex G-15, G-25 and G-50 minicolumns (1.5 x 6.0 cm) equilibrated and eluted with homogenization buffer. The macromolecular-bound radioactivity (2.5 ml) was collected and assayed for dexamethasone binding and binding to DNA-cellulose. The results are expressed as the percent of receptor-bound radioactivity which binds to DNA-cellulose.

However, several compounds are known to behave anomalously on gel filtration columns. In particular, it has been shown (14) that a 4,000-7,000 molecular weight component of rat liver cytosol behaves as an included molecule on columns of Bio-Gel P-2, which have an exclusion limit of 1,800 daltons. This same molecule has also been shown (J. Steeger and G. Litwack, unpublished observations) to behave as an included molecule on columns of Sephadex G-15.

Since the presence of a small molecule(s) appears to regulate the degree of activation of the receptor, the possibility that a small molecule might also influence steroid binding to receptor was investigated. When adrenalectomized rat liver cytosol, in the absence of exogenous steroid, is subjected to gel filtration on a minicolumn of Bio-Gel P-4, the undiluted macromolecular fractions separated from the small molecules of that cytosol have much reduced binding activity towards [^{3}H]dexamethasone (Fig. 2). If elution was continued then this binding activity was restored as the concentration of low molecular weight

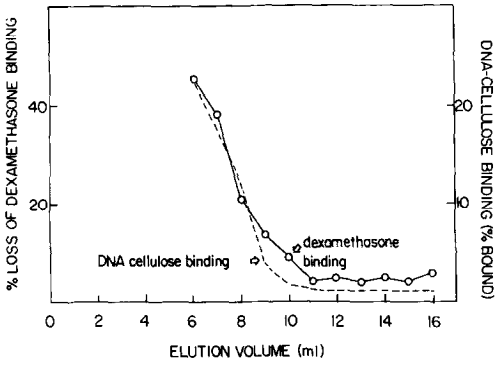

Figure 2. Effect of gel filtration on unlabeled cytosol

Adrenalectomized rat liver cytosol was applied to a Bio-Gel P-4 minicolumn equilibrated and eluted with homogenization buffer. Fractions (1.0 ml) were collected and incubated with [^{3}H]dexamethasone ± cold dexamethasone for 2 hours at 0° and assayed for specific binding of [^{3}H]dexamethasone. The data are presented as the percent decrease in specific binding as compared to untreated cytosol. Each point represents the mean of two separate experiments. For comparison, the DNA-cellulose binding data of [^{3}H]dexamethasone-labeled cytosol which was fractionated in a similar manner is presented.

components approached the level of the applied cytosol. Thus a low molecular weight component(s) is required either to prevent denaturation of the hepatic glucocorticoid receptor or to maintain it in a form which is capable of binding steroids. In addition, once the receptor has bound the steroid molecule to form a complex a low molecular weight component(s) must be removed in order to allow binding of the steroid-receptor complex to DNA. Both Munck et al. (15) and Ishii et al. (16) have concluded that when the receptor re-enters the cytosol from the nucleus the receptor is in an inactive form and must undergo a conformational change by some ATP-dependent process before being capable of binding another steroid molecule. Certain sugar phosphates, such as glucose-1-phosphate,

glucose-6-phosphate and fructose-1,6-diphosphate, stabilize the glucocorticoid receptor present in fibroblasts (17) and in our laboratory, cyclic AMP has been shown to have a definite stabilizing effect during the purification of the rat liver cytosol receptor (18). Whether or not the low molecular weight component(s) active in our system corresponds to one or other of these remains to be determined. Current work in our laboratory is aimed at identifying the nature of this component(s).

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