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Activation and Chromatographic Properties of the AtT-20 Mouse Pituitary Tumor Cell Line Glucocorticoid Receptor†

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ABSTRACT: The physicochemical properties of the glucocorticoid receptor (GC-R) from the mouse AtT-20 pituitary tumor cell line were studied. Analyses involved ion-exchange (diethylaminoethylcellulose, phosphocellulose), gel filtration (Sephadex G-150), and adsorption (DNA–cellulose, hydroxylapatite) chromatography. The receptor was characterized in four different states: native (unactivated), activated, nuclear, and Na_2MoO_4 stabilized. The unactivated receptor had chromatographic properties which were very similar to those of other steroid hormone receptor proteins; that is, it was not adsorbed to phosphocellulose (PC) or DNA–cellulose but did adsorb to diethylaminoethylcellulose (DEAE-cellulose) and eluted at 0.2 M KCl. In addition, a single peak, which eluted at 0.11 M phosphate, was obtained upon hydroxylapatite (HAP) chromatography. Because of aggregation, it was not possible to estimate the size of the unactivated GC-R. After activation by Sephadex G-25 gel filtration or by precipitation at 40% saturated $(NH_4)_2SO_4$, the receptor adsorbed to both PC and DNA–cellulose, eluting in a single, symmetrical peak at 0.17 and 0.14 M KCl, respectively. The fact that the activated receptor was a more basic molecule was confirmed by DEAE-cellulose chromatography; the activated GC-R did adsorb but eluted earlier as a sharp peak at 0.08 M KCl. The physicochemical properties of the receptor which are responsible for adsorption to HAP were unchanged after activation, since the activated GC-R eluted identically with the unactivated GC-R at 0.11 M phosphate. Sephadex G-150 gel filtration of the activated GC-R yielded a Stokes radius of 6.8 nm. The characteristics of the nuclear receptor were identical with those of the in vitro activated cytosol receptor. Finally, Na_2MoO_4 appeared to stabilize the unactivated form of the receptor. The Stokes radius of the MoO_4^{2-}-stabilized receptor was 7.7 nm. No heterogeneous forms were detected after activation on any chromatographic system utilized. That is, there was no evidence of dissimilar steroid-binding subunits. Also, limited proteolysis of the glucocorticoid receptor was not seen in this cell line. Thus, it is most likely that activation of the glucocorticoid receptor in this mouse pituitary tumor cell line is due to a conformational change in the protein or the dissociation of identical subunits.

All steroid hormones are now believed to act via a similar scheme (Yamamoto & Alberts, 1976). This involves the uptake of the hormone into the cell followed by a specific, saturable, noncovalent binding of the hormone to an intracellular, soluble receptor protein. Subsequent to ligand binding, the receptor undergoes a poorly understood process called "activation" [sometimes referred to as "transformation" (Sando et al., 1979)]. This results in the accumulation of the hormone–receptor complex in the nucleus, which is believed to be its primary site of action. It is not yet clear whether nuclear translocation involves the acquisition of the ability to penetrate the nuclear membrane or is due to a dramatic switch

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in the equilibrium of receptor partitioning in favor of the nuclear compartment. In any event, the hormone–receptor complex, presumably via an interaction with specific nuclear "acceptor sites", becomes located proximal to the appropriate hormone-responsive genes in that target tissue. The acceptor sites are most likely composed of DNA or a DNA–nonhistone protein complex. More recently, it has been suggested that the three-dimensional conformation of the hormone-responsive genes in the interphase chromatin may dictate their availability to hormonal regulation (Johnson et al., 1980; Schrader et al., 1981). Although other effects have been reported, it appears that the primary consequence of hormone–receptor complex localization in the nucleus is the increased transcription of messenger RNA from the hormone-responsive genes. After mRNA is processed and exported, new functional protein is synthesized which results in the observed hormonal effect.

It is clear from this model that the structural properties of the activated receptor are essential for its functional role as the gene regulatory protein. Despite the fact that the phenomenon of receptor activation has been known for years (Brecher et al., 1970; Jensen et al., 1971), it is as yet still unclear what alterations in protein structure occur during this process. Indeed, it was not until the recent work of Munck & Foley (1979) that any data existed ruling out the possibility of activation being merely an in vitro artifact. These researchers, investigating glucocorticoid receptor activation in intact rat thymocytes, demonstrated in vivo cytoplasmic receptor activation prior to receptor translocation to the nucleus. Therefore, a knowledge of the molecular mechanism of receptor activation is crucial for our understanding of steroid hormone action. From earlier studies, evidence exists for each of the following as the molecular mechanism of steroid activation: (1) the dissociation of receptor multimers into the constitutive subunits; (2) dimerization of receptor subunits (Notides & Nielsen, 1974; Yamamoto, 1974); (3) limited proteolysis of the unactivated receptor (Puca et al., 1972; Sica et al., 1976); and (4) a conformational change in the receptor (Samuels & Tomkins, 1970; Rousseau et al., 1972). Also, in at least one system, the chick oviduct progesterone receptor, substantial evidence exists for the occurrence of native, dissimilar, hormone-binding subunits, which may have different functional roles in the stimulation of gene expression [for recent reviews, see Vedeckis et al. (1978) and Schrader et al. (1981)].

The experiments presented here clarify the structural alterations which occur during activation of the glucocorticoid receptor from the murine AtT-20 pituitary tumor cell line. Because these cells are of a single type, the possibility of artifacts due to the release of components from other cell types during homogenization (as occurs in whole tissue extracts) was eliminated. The results obtained do not support the concepts that receptor activation is due to the association of subunits or proteolytic cleavage of the unactivated receptor. In addition, no data were obtained which were consistent with the receptor being composed of dissimilar, hormone-binding subunits. Rather, the activation process most likely involves a conformational change in the receptor or the dissociation of identical receptor protein subunits.

Materials and Methods

Cells. The cell line utilized has been designated AtT-20 and was originally isolated from a mouse anterior pituitary tumor. This cell line responds in an apparently physiological manner when exposed to glucocorticoids. Thus, glucocorticoids suppress the production of adrenocorticotropic hormone (ACTH)[1] (Watanabe et al., 1973; Herbert et al., 1978) as well as endorphins (Simantov, 1979; Sabol, 1980), which are normally synthesized as an ACTH–β-lipotropin–endorphin precursor in corticotroph cells. The inhibition has been shown to occur at the level of ACTH mRNA transcription (Nakanishi et al., 1977; Roberts et al., 1979). Therefore, this cell line has retained the normal negative feedback of glucocorticoids on ACTH production.

Cell Culture. Cells were grown in Dulbecco's modified Eagle's (DME) medium (KC Biological) containing 10% fetal calf serum (Reheis Chemical Co.) which had been heat inactivated at 56 °C for 1 h. Suspension culture was performed in a 1-L spinner flask with constant stirring at 60 rpm at 37 °C. The medium and overlying atmosphere were equilibrated in 5% CO_2–95% air prior to sealing the flask. Cells were seeded at (1–2) $\times 10^5$/mL. Half of the medium was changed twice a week, or the cells were reseeded into a new spinner flask after they reached a density of about 2×10^6 cells/mL.

Harvesting of Cells. Cells were harvested in two ways. The first method involved tilting the spinner flask at about a 60° angle to the horizontal and allowing the cells to settle in the corner of the flask. Approximately one-half of the cellular pellet was removed with a sterile pipet. One-half of the spent medium was then aspirated and replaced with fresh medium. The collected cells were pelleted by centrifugation at 100*g* for 5 min, and the medium was aspirated. This was followed by two 30-mL rinses with cold Tris–saline (10 mM Tris-HCl, pH 7.4, at 25 °C and 0.148 M NaCl), with intervening centrifugations to pellet the cells. Cells were then suspended in 3–10 volumes of TETG buffer (20 mM Tris-HCl, pH 7.4, at 25 °C, 1 mM Na_2EDTA, and 12 mM 1-thioglycerol) and homogenized as described below.

The second method used to harvest cells involved transferring 30–100 mL of the cell suspension (2×10^6 cells/mL) to a fresh, sterile spinner flask for propagation of the culture. The remaining 900–1000 mL of cell suspension was transferred to 350-mL plastic centrifuge bottles and spun at 10000*g* in a Beckman JA-10 rotor for 15 min at 4 °C. The medium was removed, and the cellular pellets were combined and washed twice in 30 mL of Tris–saline as described above. The cellular pellet was then suspended in 3–10 volumes of TETG buffer.

Preparation of Cytosol (Cytoplasmic Supernatant Fraction). After suspension in TETG buffer, the cells were allowed to swell at 0–4 °C for 15 min. All subsequent procedures were performed at 0–4 °C.

In initial experiments, the cells were homogenized by three 10-s bursts (with 30-s intervening cooling periods) using a Polytron PT10 (Brinkmann) homogenizer, followed by three series of three strokes each with a Teflon–glass homogenizer. Subsequently, two other methods were found to yield better receptor extraction (unpublished observations). Thus, some experiments utilized either three 15-s sonications using a Quaracell sonifier (with 1-min intervening cooling periods) or 12 strokes using a stainless-steel Dounce homogenizer (Kontes). The homogenates were centrifuged at 190000*g* for 1 h at 2 °C, and any floating lipid layer was removed by aspiration. The remaining supernatant fraction was used as cytosol. The glucocorticoid receptor was labeled with (3–5) $\times 10^{-8}$ M 1,2,4-[^{3}H]triamcinolone acetonide (TA) (20 Ci/mmol, Amersham) for 16–18 h. This time was found to be

[1] Abbreviations used: DEAE, diethylaminoethyl; PC, phosphocellulose; HAP, hydroxylapatite; GC-R glucocorticoid receptor; ACTH, adrenocorticotropic hormone (corticotropin); DME medium, Dulbecco's modified Eagle's medium; TA, triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone); Na_2EDTA, disodium ethylenediaminetetraacetate.

optimal for hormone binding to the receptor (unpublished observations).

Preparation of Nuclear Receptor. Nuclei were prepared from AtT-20 cells according to a previously published method (Garroway et al., 1976). Cells were harvested from spinner flasks as described above. A total of 2 mL of cells (packed volume) was resuspended in 30 mL of DME medium containing 10% fetal calf serum. [^{3}H]Triamcinolone acetonide was added to a final concentration of about 5×10^{-8} M. After a 2-h incubation at 0 °C, the cells were warmed to 37 °C for 1 h to activate the receptor and drive it into the nucleus. The cells were centrifuged, the supernatant medium was aspirated, and the cells were washed once in 30 mL of cold Tris–saline. All subsequent steps were performed at 0–4 °C. The cells were suspended in 20 mL of TKM buffer (50 mM Tris-HCl, pH 7.4, at 25 °C, 5 mM KCl, and 1.6 mM $MgCl_2$) containing 0.5 M sucrose and homogenized with 25 strokes of a motor-driven Teflon–glass homogenizer with a tight-fitting pestle. The volume was adjusted to 100 mL with TKM–0.5 M sucrose and the homogenate centrifuged at 4800*g* for 10 min. The supernatant was decanted, and the pellets were resuspended in 12 mL of TKM–1.8 M sucrose and dispersed by using a motor-driven Teflon–glass homogenizer. Four 3.5-mL aliquots of this were layered on top of 30 mL of TKM–1.8 M sucrose and centrifuged at 30000*g* for 15 min. After the supernatant was decanted, the tube walls were wiped dry. The four purified nuclear pellets were resuspended in 12 mL total of TETG buffer containing 0.4 M KCl. The resuspended pellets were homogenized with ten strokes of the stainless-steel Dounce homogenizer. After the preparation sat for 30 min, the extracted nuclei were centrifuged at 190000*g* for 15 min, and the supernatant was used as the nuclear receptor. This preparation was desalted over a 60-mL (packed volume) Sephadex G-25 column equilibrated in TETG buffer. Virtually 100% of the extracted radioactive TA was bound to receptor. Column chromatography of the nuclear receptor was performed immediately after desalting exactly as described below for the cytoplasmic receptor.

Ion-Exchange and Adsorption Chromatography. DEAE-cellulose (Whatman DE-52) was suspended in 0.2 M Tris-HCl, pH 7.4, for 5–10 min, filtered dry, and resuspended for 5–10 min in 0.1 M Tris-HCl, pH 7.4. This was filtered, and the resin washed extensively with TETG buffer until the pH and conductivity of the eluate were the same as those of the washing buffer. DEAE-cellulose was stored as a 50% (v/v) slurry in TETG buffer containing 0.04% NaN_3.

Phosphocellulose (Whatman P-11) was prepared as suggested by the manufacturer. This included treating the resin sequentially with 0.5 N NaOH and 0.5 N HCl. This was followed by suspension of the resin in TETG buffer containing 1 M KCl. The resin was then washed extensively with TETG buffer until the pH and conductivity of the eluate were the same as those of the washing buffer. Storage was the same as for the DEAE-cellulose.

Hydroxylapatite (HAP) (Bio-Rad HTP) was suspended repeatedly in distilled, deionized water with intervening aspiration of fines. Storage of this resin was in distilled, deionized water containing 0.04% NaN_3.

DNA–cellulose was prepared according to a modification of the method of Alberts & Herrick (1971). A 1-g sample of highly polymerized calf thymus DNA (Sigma) was dissolved in 417 mL of 0.1 × SSC buffer (0.015 M NaCl–0.0015 M sodium citrate, pH 7.0, at 25 °C). This was then divided into two batches. To each 208-mL batch was added 40 g of dried Bio-Rad Cellex N-1 cellulose, which had been prewashed successively with boiling 95% ethanol, 0.01 N NaOH, 0.001 M Na_2EDTA, and 0.01 M HCl and then extensively with distilled water until the pH of the eluate was 5.5. The cakes of DNA and cellulose were placed into petri dishes and allowed to dry thoroughly. After clumps were broken up with a mortar and pestle, 208 mL of 100% ethanol was added to each batch. The suspensions were stirred for 15–20 min under a short-wavelength UV light which was 15 cm from the surface of the slurry. The batches were combined, and the ethanol was removed by filtration. The DNA–cellulose was then washed twice with 833 mL of 0.1 × SSC, with 670 mL of TETG containing 1 M KCl, and then extensively with TETG buffer. DNA–cellulose was stored frozen as a 50% (v/v) slurry in TETG buffer.

Routine chromatography was performed as follows. Columns (4 mL) (packed volume) were poured and equilibrated in TETG buffer. Cytosol (1–2 mL) was applied and the column washed until 25 mL of dropthrough was collected. Receptor was then eluted with a linear salt gradient (80-mL total volume), and 1-mL fractions were collected. Aliquots (0.5 mL) were analyzed by liquid scintillation counting, and the conductivity of every fifth fraction was determined. Since there was some variability among the gradients, the column profiles were standardized by converting the fraction number to the concentration of salt in that particular fraction. This facilitated comparisons between experiments. The salt gradients (all in TETG buffer) utilized were as follows: DEAE-cellulose (up to 0.5 M KCl); phosphocellulose (up to 0.6 M KCl); DNA–cellulose (up to 0.4 M KCl). The hydroxylapatite columns were eluted with a 0.005–0.35 M potassium phosphate gradient.

Receptor Activation. Three methods of receptor activation were utilized. Most of the results presented below were obtained by using Sephadex G-25 gel filtration to activate the receptor. The receptor (excluded volume) is believed to be separated from a small molecular weight endogenous inhibitor of activation (Cake et al., 1976; Bailly et al., 1977; Goidl et al., 1977; Sato et al., 1980). Activation was virtually complete after letting the gel-filtered receptor stand at 0–4 °C for 4 h. Precipitation of the receptor at 40% $(NH_4)_2SO_4$ was also found to activate the receptor and gave virtually the same results as Sephadex G-25 activation (see below). When gel filtration chromatography was to be performed, receptor was activated by incubation at 0–4 °C for 2 h in 0.3 M KCl.

Sephadex G-150 Gel Filtration Chromatography. The Stokes radius of the glucocorticoid receptor was estimated by using Sephadex G-150 (Pharmacia) gel filtration chromatography in TETG buffer containing 0.3 M KCl. The column dimensions were 2.6 cm × 63 cm. Samples were pretreated with 0.3 M KCl for 1–3 h to ensure that nonspecific receptor aggregates were minimized. Typically, a 2-mL sample was applied, and 3-mL fractions were collected by using gravity flow at a rate of 18 mL/h. Aliquots (1 mL) of the fractions were counted for radioactivity. Proteins used as standards were obtained from Sigma Chemical Co.

Liquid Scintillation Counting. Aqueous scintillation cocktail (4 or 8 mL) was added to 0.5- or 1-mL aliquots, respectively. The cocktail used was either Budget-Solve (Research Products International, Corp.) or Ready-Solve EP (Beckman), both of which gave virtually identical results under the conditions used. Counting was performed on a Beckman LS 7500 liquid scintillation spectrometer at a counting efficiency of about 35%.

Results

Glucocorticoid Receptor Quantitation. In all of the experiments performed, it was essential that the amount of re-

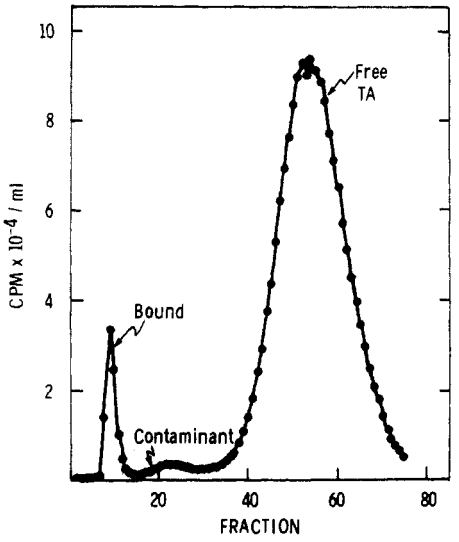

FIGURE 1: Sephadex LH-20 chromatography of the AtT-20 cytosol glucocorticoid receptor. [^{3}H]TA-labeled AtT-20 cytosol (2 mL) was applied to a 20-mL Sephadex LH-20 column equilibrated in TETG buffer. Fractions (1 mL) were collected and counted for radioactivity.

ceptor which adsorbed to the various resins be carefully quantitated (see figure legends). This was also necessary in comparing the quantity of receptor which eluted as multiple peaks on the chromatograms, so that accurate assignments of their physical states (activated, unactivated) could be made. In order to quantitate the total amount of receptor-bound hormone in the starting cytosol, Sephadex LH-20 column chromatography was performed on an aliquot of cytosol. This method was first applied successfully to rat liver and kidney glucocorticoid receptors by Sherman and her colleagues (Sherman et al., 1979). A typical example of the results obtained by using [^{3}H]TA-labeled AtT-20 cytosol is shown in Figure 1. The first peak which eluted was [^{3}H]TA which was bound to the hydrophilic glucocorticoid receptor (GC-R). Because of the nature of the protein, the radioactive receptor-bound hormone passed through the column unretarded. The second peak observed (which was usually about 8% of the total radioactivity) was a contaminant present in the [^{3}H]TA as obtained from the manufacturer. It was somewhat more hydrophilic than authentic TA. The last peak obtained was uncomplexed (free) [^{3}H]TA, which, due to its hydrophobic nature, interacted with the LH-20 matrix and was retarded. The advantages of LH-20 chromatography are that the column can be run at a relatively rapid rate and there is very little dilution of the receptor protein (Sherman et al., 1979). Typically, receptor-bound [^{3}H]TA comprised 5–10% of the total radioactivity applied to the LH-20 column.

Unactivated AtT-20 Glucocorticoid Receptor. When AtT-20 cytosol was applied to a DEAE-cellulose column in TETG buffer, greater than 80% of the GC-R was adsorbed. Upon elution with a KCl gradient, two peaks of GC-R were obtained (Figure 2A). The first peak, which eluted at 0.08 M KCl, was always present in lesser amounts than the second major peak, which eluted at 0.2 M KCl. In most instances, the first peak comprised less than 30% of the total receptor-bound hormone. As was confirmed later, the first peak (at 0.08 M KCl) was activated GC-R. This either was present in the whole cells initially or was generated by the experimental procedures. The second peak (at 0.2 M KCl) was the unactivated form of the GC-R and thus appears to be a relatively acidic protein.

When cytosol was applied to either phosphocellulose (PC) or DNA–cellulose, very little receptor was adsorbed. That

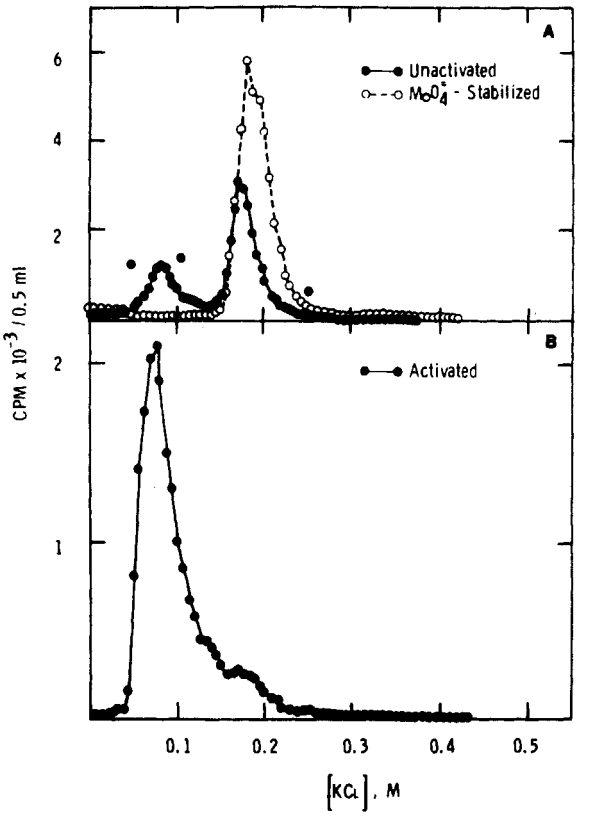

FIGURE 2: DEAE-cellulose chromatography of the AtT-20 cytosol glucocorticoid receptor. (A) AtT-20 cytosol (10.5 mL) was labeled with [^{3}H]TA, and 3 mL was removed and adjusted to 20 mM Na_2MoO_4. After 18 h, 1 mL of cytosol and 1 mL of MoO_4^{2-}-treated cytosol were chromatographed on identical 4-mL DEAE-cellulose columns as described under Materials and Methods. Aliquots (0.5 mL) were counted from each 1-mL fraction. (B) Cytosol (3 mL) was activated by chromatography on a 35-mL Sephadex G-25 column and allowing the excluded receptor to stand at 0–4 °C for 4 h. One-third (2.7 mL) of the pooled, excluded receptor peak (activated receptor) was chromatographed on a 4-mL DEAE-cellulose column as described in (A). The total amounts of applied receptor which adsorbed to the DEAE-cellulose columns were as follows: unactivated, 82%; MoO_4^{2-} stabilized, 100%; activated, 88%.

which was adsorbed (Figure 3A) was found to be quantitatively identical with the amount which eluted at 0.08 M KCl from DEAE-cellulose. The elution position of the receptor absorbed to PC was identical with that obtained when the receptor was activated fully (see below). Since most of the unactivated cytosol receptor did not adsorb to PC or DNA–cellulose (data not shown), its designation as an acidic protein was supported.

Hydroxylapatite (HAP) adsorption chromatography was performed because of its unique mechanism of protein adsorption. One advantage of this column is that receptor proteins can be applied at fairly high KCl concentrations and still be adsorbed (Schrader, 1975). In addition, this column binds all of the GC-R present in AtT-20 cytosol and mouse liver cytosol (unpublished data). Elution with a linear potassium phosphate gradient yielded a single symmetrical peak of hormone-binding activity at 0.11 M phosphate (Figure 4A). Since, as mentioned above, some activated receptor was present in this preparation, it appeared that both the unactivated and the activated receptor eluted at 0.11 M potassium phosphate.

Activated AtT-20 Glucocorticoid Receptor. When Sephadex G-25 activated receptor was applied to a DEAE-cellulose column, greater than 80% of the GC-R was adsorbed. Salt gradient elution revealed a single peak of hormone-binding activity which eluted at 0.08 M KCl (Figure 2B). This result confirmed that the minor component observed in the cytosol

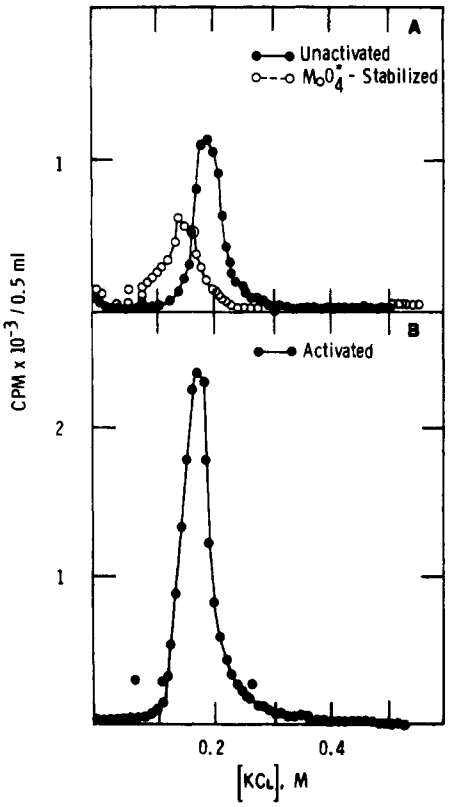

FIGURE 3: Phosphocellulose chromatography of the AtT-20 cytosol glucocorticoid receptor. Equal aliquots from the same samples analyzed on DEAE-cellulose (Figure 2) were applied to 4-mL phosphocellulose columns as described under Materials and Methods. (A) Unactivated and MoO_4^{2-}-stabilized receptor. (B) Activated receptor. The total amounts of applied receptor which adsorbed to the PC columns were as follows: unactivated, 21%; MoO_4^{2-} stabilized, 14%; activated, 74%.

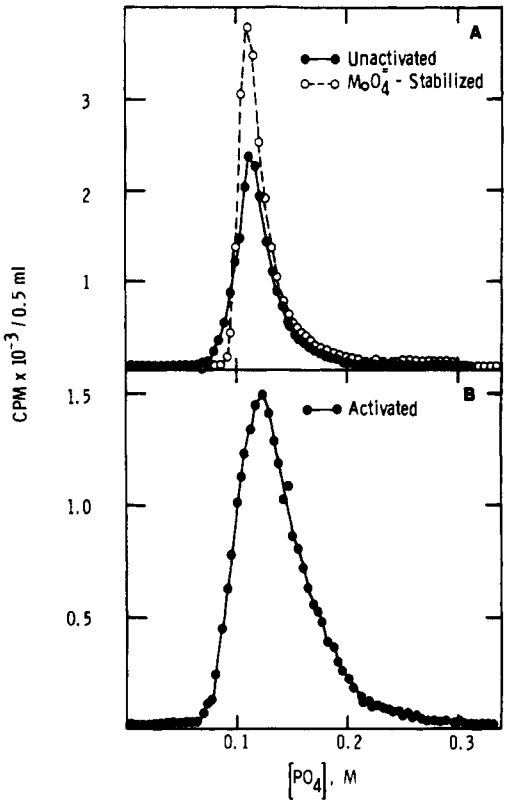

FIGURE 4: Hydroxylapatite chromatography of the AtT-20 cytosol glucocorticoid receptor. Equal aliquots from the same samples analyzed on DEAE-cellulose (Figure 2) were applied to 4-mL hydroxylapatite columns and chromatographed as described under Materials and Methods. (A) Unactivated and MoO_4^{2-} stabilized receptor; (B) activated receptor. The total amounts of applied receptor which adsorbed to the HAP columns were as follows: unactivated, 90%; MoO_4^{2-} stabilized, 100%; activated, 87%.

(Figure 2A) was indeed contaminating activated receptor present in the cytosol. Two points were noted. First, the activated receptor appeared to be more basic than the unactivated form. Second, all of the activated receptor eluted from DEAE-cellulose in a single, symmetrical peak with quantitative conversion of the unactivated receptor to the apparent single activated species. No evidence of dissimilar, heterogeneous molecular forms of the activated receptor was ever obtained. Thus, with the assay method used, the activated AtT-20 GC-R appeared to be a single molecular species.

As has been documented for steroid hormone receptors in general, activation of the AtT-20 GC-R resulted in the acquisition of the ability to bind to polyanions. Thus, the activated AtT-20 GC-R bound to PC and eluted at a KCl concentration of 0.17 M (Figure 3B). Again, this corresponded to the same elution position of the minor component present in the unactivated cytosol (Figure 3A). As was observed with DEAE-cellulose chromatography, the activated GC-R eluted as a single, symmetrical peak on PC; no dissimilar subunits were in evidence. In a like manner, the activated AtT-20 GC-R adsorbed to DNA–cellulose and eluted as a single, symmetrical peak at 0.14 M KCl (Figure 5B). Multiple activated forms of the GC-R were not present.

When activated GC-R was applied to HAP, greater than 80% of the receptor was adsorbed. The activated AtT-20 GC-R eluted at exactly the same potassium phosphate concentration (Figure 4B) as did the unactivated receptor (Figure 4A). Thus, whatever molecular characteristics of the GC-R are responsible for its adsorption to HAP are apparently unaltered by receptor activation.

Finally, the identity of the activated AtT-20 GC-R was

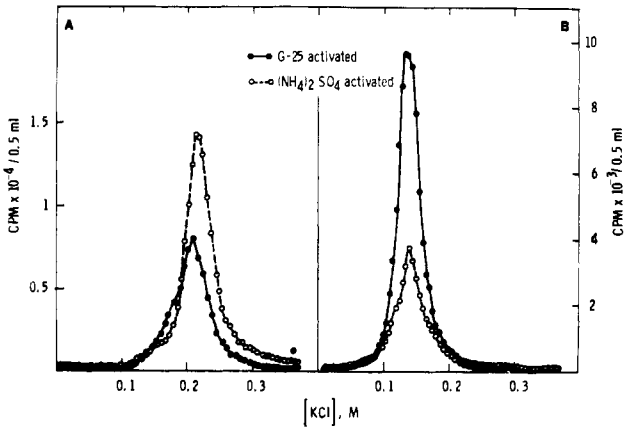

FIGURE 5: Comparison of AtT-20 cytosol glucocorticoid receptor activated by Sephadex G-25 gel filtration chromatography and $(NH_4)_2SO_4$ precipitation. [^{3}H]TA-labeled cytosol (3 mL) was applied to a 20-mL Sephadex G-25 column equilibrated in TETG buffer and the excluded receptor peak allowed to sit at 0–4 °C for 4 h. After the receptor peak was pooled, equal aliquots were applied to 4-mL phosphocellulose (A) and DNA–cellulose (B) columns, and chromatography was performed as described under Materials and Methods. An additional 3 mL of [^{3}H]TA-labeled cytosol was precipitated at 40% saturated $(NH_4)_2SO_4$, the receptor pelleted by centrifugation, and the pellet redissolved in 3 mL of TETG buffer. Equal aliquots were then subjected to PC (A) and DNA–cellulose (B) chromatography. G-25 activated GC-R (●); $(NH_4)_2SO_4$-activated GC-R (○).

confirmed by sequential chromatography. Receptor was activated by Sephadex G-25 gel filtration and the void volume pooled. This was then applied to a 7-mL PC column, the dropthrough fraction was collected, and the adsorbed receptor

was eluted by using a KCl gradient of up to 0.7 M KCl in TETG buffer. Because of the adsorption of the receptor to phosphocellulose, this preparation contained, by definition, activated GC-R (Atger & Milgrom, 1976). The fractions containing the activated receptor were pooled and desalted by passage over a Sephadex G-25 column equilibrated in TETG buffer. After the void volume was pooled (desalted, activated GC-R), equal aliquots were chromatographed on columns of DEAE-cellulose, DNA–cellulose, and HAP. The activated receptor eluted from these columns at 0.08 M KCl, 0.15 M KCl, and 0.10 M potassium phosphate, respectively (data not shown). In addition, the Stokes radius of this partially purified, activated receptor was found, by Sephadex G-150 gel filtration chromatography, to be 6.6 nm, which is very similar to the value obtained for crude, activated receptor (6.8 nm) and identical with that of the nuclear receptor (see below). Therefore, the properties of both crude and partially purified activated receptor were identical.

Comparison of Glucocorticoid Receptor Activated by Sephadex G-25 Gel Filtration and $(NH_4)_2SO_4$ Precipitation. A wide variety of activation methods have been used with steroid hormone receptors. It was important, therefore, to determine whether the chromatographic properties of the activated AtT-20 GC-R were dependent upon the method of activation utilized. This is of particular significance when comparing the structure of steroid hormone receptors from different systems (see Discussion).

AtT-20 cell cytosol was prepared, and one-half was activated by passage over Sephadex G-25 and allowing the excluded receptor peak to stand at 0–4 °C for 4 h. Meanwhile, the other half was brought to a final concentration of 40% saturated $(NH_4)_2SO_4$ by the dropwise addition of a 100% saturated $(NH_4)_2SO_4$ solution. After this preparation stood at 0–4 °C for 30 min, the precipitated protein was pelleted by centrifugation in a clinical centrifuge (30 min, maximal speed). The supernatant was decanted, and the tube walls were rinsed with distilled water and wiped dry. The pellet was dissolved in a volume of TETG equivalent to that in the initial cytosol aliquot. The receptors which were activated by Sephadex G-25 gel filtration and $(NH_4)_2SO_4$ precipitation were then analyzed on DNA–cellulose and PC. These results are shown in Figure 5. As can be seen, both samples behaved identically on these two resins. This was true even though the $(NH_4)_2SO_4$-precipitated receptor contained much less total protein than the G-25-filtered preparation. Thus, the chromatographic properties of the activated AtT-20 GC-R were independent of the method of activation used. As before, no heterogeneous activated receptor forms were observed in these experiments.

Sodium Molybdate Stabilized AtT-20 Glucocorticoid Receptor. A number of studies have demonstrated that Na_2MoO_4 stabilizes both the hormone-binding activity of cytosol preparations (Nielsen et al., 1977; Leach et al., 1979; Grody et al., 1980; McBlain & Shyamala, 1980) and the unactivated form (John & Moudgil, 1979; Toft & Nishigori, 1979; Leach et al., 1979; Grody et al., 1980; McBlain & Shyamala, 1980; Nishigori & Toft, 1980; Schmidt et al., 1980; Shyamala & Leonard, 1980; Wolfson et al., 1980) of the steroid receptor. In these studies, the analyses of MoO_4^{2-} stabilization of the unactivated form of the receptor consisted of measurements of the dissociation rate of hormone from the receptor (which is different for the unactivated and activated forms), the *s* value of the receptor in sucrose gradients, and the apparent Stokes radius obtained upon gel filtration chromatography. It was of interest in the present study to determine if Na_2MoO_4 altered the chromatographic patterns obtained, and if its use would confirm the assignments made to the various peaks obtained above.

AtT-20 cytosol was prepared, and 1 M Na_2MoO_4 was added to a final concentration of 20 mM. After the receptor was labeled overnight with [^{3}H]TA, the MoO_4^{2-}-stabilized receptor was analyzed on the ion-exchange and adsorption columns described above. Na_2MoO_4 clearly and strikingly preserved the unactivated form of the AtT-20 GC-R. Thus, only the second peak, at 0.2 M KCl, was observed on the DEAE-cellulose column (Figure 2A); no activated receptor (at 0.08 M KCl) was present. Thus, it appears that the activated receptor observed in the original cytosol preparation was due to spontaneous activation which occurred during the overnight incubation, rather than being present originally in the intact cell. Likewise, the amount of activated GC-R which was observed on PC was substantially decreased (Figure 3A), confirming that unactivated receptor does not adsorb to this resin. Finally, MoO_4^{2-}-stabilized GC-R eluted at 0.11 M potassium phosphate from the HAP column, identical with both the unactivated (cytosol) and activated receptor, confirming that receptor activation does not alter the biochemical properties of the receptor required for HAP interaction.

Sephadex G-150 Gel Filtration Chromatography of the AtT-20 Glucocorticoid Receptor. The Stokes radii of the MoO_4^{2-}-stabilized and the activated AtT-20 GC-R were determined by chromatography of the samples on a Sephadex G-150 column in TETG buffer containing 0.3 M KCl. The buffer included 20 mM Na_2MoO_4 when MoO_4^{2-}-treated cytosol was analyzed. The high KCl concentration was required to prevent nonspecific receptor aggregation, which resulted in the exclusion of the receptor from the column. Therefore, since high salt concentrations activate steroid hormone receptors, it was not possible to obtain an estimate for the Stokes radius of the unactivated (cytosol) receptor.

The Stokes radius of the MoO_4^{2-}-stabilized receptor was 7.7 nm, while that of the activated receptor was 6.8 nm (Figure 6). MoO_4^{2-} treatment of AtT-20 cytosol resulted in a substantial amount of receptor eluting in the excluded volume of the column. Therefore, it is not yet known whether the 7.7-nm Stokes radius obtained for the MoO_4^{2-}-stabilized receptor is the same as that for authentic unactivated receptor, or if it represents aggregation of the receptor with other cytosolic protein(s).

Properties of the AtT-20 Nuclear Glucocorticoid Receptor. It was important to determine if the activated receptor characterized above was indeed representative of the authentic, activated (that is, nuclear) receptor. Therefore, nuclear receptor was prepared as described under Materials and Methods and analyzed by using ion-exchange, adsorption, and gel filtration chromatography. The nuclear receptor behaved identically with the in vitro activated cytosolic receptor upon DEAE-cellulose, phosphocellulose, hydroxylapatite, and DNA–cellulose chromatography (Figure 7). Additionally, the Stokes radius of the nuclear receptor was determined to be 6.6 nm by using Sephadex G-150 gel filtration chromatography (Figure 8), which is very similar to that obtained for the activated cytosol receptor (6.8 nm, see Figure 6). Therefore, the in vitro activated cytosolic GC-R had properties virtually identical with those of the in vivo nuclear receptor.

Discussion

The characterization of the glucocorticoid receptor from the murine AtT-20 pituitary tumor cell line has provided considerable information about the physicochemical properties of this important gene regulatory protein. Most importantly, these experiments have narrowed down the possible molecular

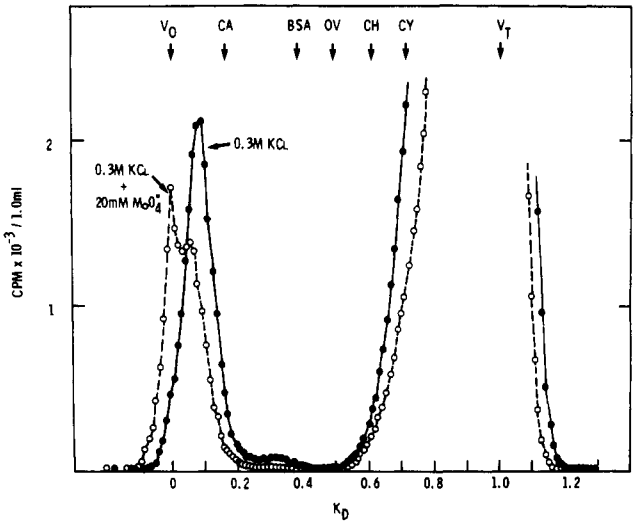

FIGURE 6: Sephadex G-150 gel filtration chromatography of the AtT-20 cytosol glucocorticoid receptor. Activated receptor was prepared by treating [^{3}H]TA-labeled cytosol with 0.3 M KCl for 2 h. Salt-treated cytosol (2 mL) was applied to a 2.6 × 63 cm Sephadex G-150 column equilibrated in TETG buffer containing 0.3 M KCl (●) and chromatographed as described under Materials and Methods. A 2-mL sample of cytosol containing 20 mM Na_2MoO_4 was salt treated for 2 h prior to chromatography on a Sephadex G-150 column equilibrated in TETG buffer containing 0.3 M KCl and 20 mM Na_2MoO_4 (○). The elution positions labeled in the figure are as follows: catalase (CA), R_s = 5.75 nm; bovine serum albumin (BSA), R_s = 3.63 nm; ovalbumin (OV), R_s = 2.8 nm; chymotrypsinogen A (CH), R_s = 2.21 nm; cytochrome *c* (CY), R_s = 1.74 nm. $K_D = (V_e - V_0)/(V_t - V_0)$, where V_e = elution position of the receptor or standard proteins, V_0 = void volume determined by using blue dextran (M_r 2 000 000), and V_t = total volume of the column determined by the elution position of KCl. The Stokes radius of the receptor was determined by plotting K_D vs. R_s (data not shown).

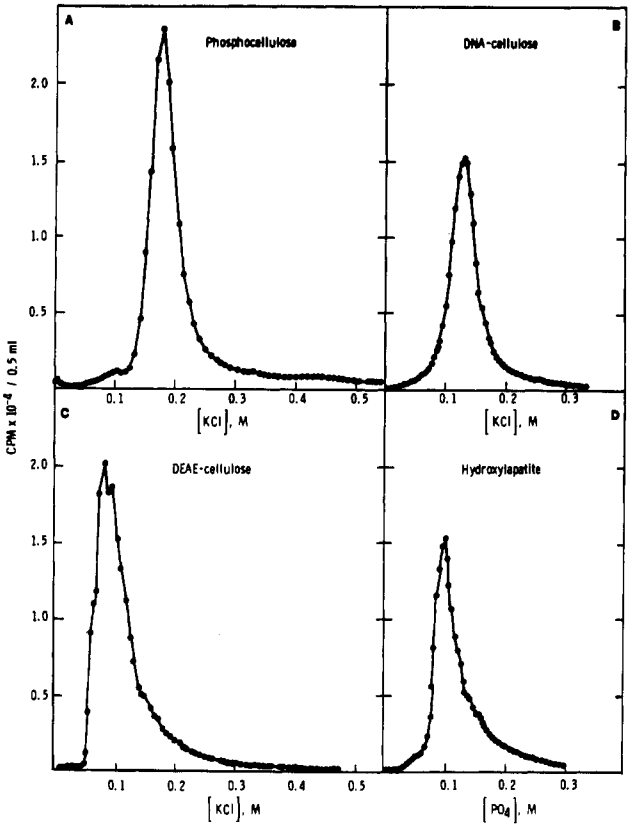

FIGURE 7: Ion-exchange and adsorption chromatography of the AtT-20 nuclear glucocorticoid receptor. Nuclear receptor was prepared and ion-exchange chromatography performed as described under Materials and Methods. Aliquots (2 mL) of the desalted nuclear receptor were applied to 4-mL columns of phosphocellulose (A), DNA–cellulose (B), DEAE-cellulose (C), and hydroxylapatite (D). The total amounts of applied nuclear receptor which adsorbed to these columns were as follows: PC, 89%; DNA–cellulose, 87%; DEAE-cellulose, 95%; HAP, 98%.

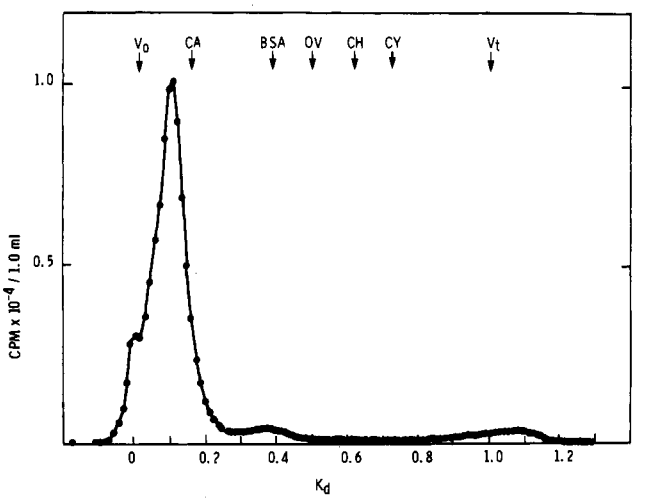

FIGURE 8: Sephadex G-150 gel filtration chromatography of the AtT-20 nuclear glucocorticoid receptor. Nuclear receptor (1.2 mL) was treated with 0.3 M KCl for 2 h prior to chromatography. The protein standards used and the determination of the K_D and Stokes radius were as described in the legend for Figure 6.

mechanisms involved in the process of glucocorticoid receptor activation.

The unactivated AtT-20 GC-R had properties very similar to those observed for other steroid receptors, as well as to the glucocorticoid receptor in other systems. The unactivated receptor was an acidic protein as evidenced by its elution at 0.2 M KCl from DEAE-cellulose and its failure to adsorb to phosphocellulose. The use of Na_2MoO_4 was particularly important for the confirmation of these conclusions. Thus, the more basic species present in the unactivated cytosol was due to spontaneous receptor activation which occurred during the 16–18-h labeling period. Slow, spontaneous activation of rat liver GC-R at 0–4 °C has been described previously (Milgrom et al., 1973). Molybdate almost completely inhibited this spontaneous activation.

Activation of the AtT-20 GC-R resulted in the generation of a more basic receptor protein. This was evidenced by an earlier elution from DEAE-cellulose (at 0.08 M KCl) and the acquisition of the ability to adsorb to PC and DNA–cellulose. The elution positions of the activated GC-R on these resins were independent of the method of activation used, and the fact that the nuclear GC-R had identical properties further substantiates this interpretation.

Importantly, there was no evidence of heterogeneous activated AtT-20 GC-R forms. In previous studies performed on the glucocorticoid receptor in other systems, it was concluded that the receptor consisted of only one major protein species (Beato & Feigelson, 1972; Litwack et al., 1973; Eisen & Glinsmann, 1975; Sakaue & Thompson, 1977; Wrange et al., 1979; Eisen, 1980; Govindan, 1980; Govindan & Manz, 1980), as opposed to being comprised of dissimilar hormone-binding subunits. In those instances where receptor heterogeneity was observed, it appeared to be due to limited receptor proteolysis (Wrange & Gustafsson, 1978; Carlstedt-Duke et al., 1979; Govindan, 1980; Govindan & Manz, 1980; Stevens & Stevens, 1981; Tsawdaroglou et al., 1981). However, in at least one instance (Litwack & Rosenfield, 1975; Markovič et al., 1980), a second species of glucocorticoid receptor, designated binder IB, which did not appear to be a proteolytic fragment of the

major receptor form (binder II; Litwack et al., 1973), was found to occur in a tissue-specific fashion. In the present study, the activated receptor eluted as a single, symmetrical peak on all the columns tested, regardless of the activation method used. Receptor homogeneity was observed even when very shallow salt gradients were used to elute the columns. The absence of dissimilar receptor subunits in the AtT-20 glucocorticoid receptor system is of considerable interest. When I performed the exact same experiments in the laboratories of W. T. Schrader and B. W. O'Malley, equivalent amounts of two dissimilar chick oviduct progesterone receptor proteins, designated A and B, were obtained. Thus, the differences between these two systems cannot be ascribed to differences in the experimenter or the method utilized. Although there are many similarities in the structure and hormone-binding properties of the A and B receptor proteins (Vedeckis et al., 1979, 1980a), extensive attempts to demonstrate the interconversion of these proteins were unsuccessful (Schrader et al., 1980). More recently, detailed biochemical analyses of the chick oviduct A and B receptor proteins and their proteolytic products have added further support to the concept that the A and B receptors are distinct proteins, that is, that they are the products of separate receptor genes (Schrader et al., 1981). Therefore, at the present time, it is not clear if the dissimilar subunits proposed for the chick oviduct progesterone receptor are peculiar to the avian system, to progesterone receptors, or are, indeed, common to all receptors. If dissimilar subunits are characteristic of all receptors, then the putative dissimilar subunits for the AtT-20 glucocorticoid receptor were so alike that the differences are undetectable with all of the assay methods used.

The unactivated and activated AtT-20 GC-R's have differing properties on all the chromatographic systems tested save one, hydroxylapatite. Thus, the receptor eluted at 0.11 M potassium phosphate, regardless of its state. Studies on mouse liver glucocorticoid receptor revealed that limited proteolysis of the GC-R can alter its elution from HAP (unpublished data). Thus, there is a region of the GC-R, distinct from the hormone-binding proteolytic fragment, which is involved in the binding of the protein to hydroxylapatite.

Sodium molybdate, as has been observed previously, preserved the unactivated form of the receptor. Since both phosphocellulose (Atger & Milgrom, 1976) and hydroxylapatite (unpublished data) promote activation and alter the state of the GC-R, molybdate was particularly useful in allowing a definitive determination of the behavior of the unactivated AtT-20 GC-R on these chromatographic systems. The MoO_4^{2-}-stabilized receptor had a slightly larger Stokes radius (7.7 nm) the activated receptor. Although resolution of these two forms was not striking using the G-150 column, the differences in elution positions were consistent and statistically significant (7.7 ± 0.1 nm, $n = 3$; 6.8 ± 0.1 nm, $n = 7$). Because a substantial amount of MoO_4^{2-}-stabilized receptor eluted in the void volume of the column, it is not yet clear whether the Stokes radius of authentic unactivated AtT-20 GC-R is truly 7.7 nm or if molybdate promoted an artifactual receptor aggregation. However, MoO_4^{2-}-stabilized mouse liver GC-R also had a Stokes radius of 7.7 nm, and no receptor in this system eluted in the void volume (Vedeckis, 1981). Since the protein composition and concentration vary widely in AtT-20 and liver cytosol, it seems likely that the 7.7-nm MoO_4^{2-}-stabilized form is physiologically significant and probably represents the unactivated receptor. The activated AtT-20 GC-R had a Stokes radius of 6.8 nm.

Limited receptor proteolysis has been observed in a number of systems for progesterone (Sherman et al., 1974, 1976, 1978; Vedeckis et al., 1979, 1980a,b), estrogen (Puca et al., 1972, 1977; Sica et al., 1976; Sherman et al., 1978), androgen (Wilson & French, 1979), aldosterone (Sherman et al., 1978), and glucocorticoid (Carlstedt-Duke et al., 1977; Sherman et al., 1978; Wrange & Gustafsson, 1978) receptors. In general, the proteolytic fragments have Stokes radii of about 4 and 2 nm. The first of these (4 nm) was designated form IV by Sherman et al. (1976) for the chick oviduct progesterone receptor. In most systems, evidence exists for the retention of the DNA-binding domain on this fragment (Wrange & Gustafsson, 1978; Schrader et al., 1981; Stevens & Stevens, 1981). The 2-nm form, called meroreceptor by Sherman et al. (1976), contains the hormone-binding domain but does not contain the DNA-binding region of the protein. Although similar forms have been observed for the mouse liver GC-R (Vedeckis, 1980, 1981), only a very minor proportion of the AtT-20 GC-R was ever observed to exist in these forms. In addition, no proteolytic GC-R fragments were observed in the nuclear extracts of the AtT-20 cells, although a 3.2-nm form has been found in nuclear extracts of mouse L cells (Middlebrook & Aronow, 1977). Previous experiments with the chick oviduct progesterone receptor demonstrated that the addition of nuclei to the cytoplasmic receptor and subsequent warming to 25 °C caused an almost complete conversion of the native A and B receptor proteins to form IV and meroreceptor [W. V. Vedeckis, W. T. Schrader, and B. W. O'Malley, published in Schrader et al. (1981)]. Cytosol receptor warmed in the absence of nuclei did not undergo this proteolysis. These results supported the speculation that a high concentration of receptor-specific protease in the nucleus, and the resultant receptor cleavage, might be a mechanism for turning off hormone-induced gene expression (Vedeckis et al., 1980a). This does not appear to be a likely mechanism in AtT-20 cells. Finally, in preliminary experiments using the mouse lymphoma WEHI-7 cell line (W. V. Vedeckis, unpublished experiments), proteolytic receptor fragments were absent, or present in a very low amount. Therefore, limited receptor proteolysis does not appear to be absolutely required for glucocorticoid hormone action, since two hormone-responsive cell lines (AtT-20 and WEHI-7) do not exhibit significant limited proteolysis of the GC-R.

In conclusion, dissimilar receptor subunits or limited receptor proteolysis does not appear to play a significant role in the AtT-20 GC-R system. Rather, activation of the receptor probably involves a conformational change in the receptor, the dissociation of identical receptor subunits, or, perhaps, the dissociation of a low molecular weight inhibitor of receptor activation (probably resulting in a conformational change in the receptor). Further studies are planned to distinguish between these possibilities.

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