Limited Proteolysis of the Mouse Liver Glucocorticoid Receptor†

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ABSTRACT: Activation of the mouse liver glucocorticoid receptor resulted in the generation of a protein of very different characteristics from that found previously in the mouse AtT-20 pituitary tumor cell line [Vedeckis, W. V. (1981) *Biochemistry 20*, 7237–7245]. Ion-exchange and adsorption chromatography showed that the activated liver receptor was a more basic protein—it eluted earlier from a DEAE-cellulose column, while a later elution was observed upon phosphocellulose and DNA–cellulose chromatography. Further experiments showed that this was due to proteolysis of the liver receptor to a smaller form (3.2 S; R_s = 3.9 nm; M_r = 53 000; f/f_0 = 1.45) after activation. Mero-receptor (2.4 S; R_s = 2.4 nm; M_r = 24 000; f/f_0 = 1.15) was detectable when cytosol was chromatographed on hydroxylapatite or was treated with trypsin. These proteolytic fragments are similar to those obtained for various other steroid hormone receptors. Mixing experiments with liver cytosol showed that the AtT-20 glucocorticoid receptor could also be cleaved to these fragments. Endogenous proteases are apparently in low concentration in this cell line. Finally, activation appears to result in the exposure of the protease-sensitive regions, since sodium molybdate, which prevents receptor activation, also renders the receptor resistant to proteolysis.

Previous experiments with the mouse AtT-20 pituitary tumor cell line glucocorticoid receptor (GC-R)[1] seemed to indicate that there were no dissimilar hormone-binding subunits (Vedeckis, 1981a). It was further suggested that receptor activation (acquisition of nuclear- and DNA-binding activity) resulted from either the dissociation of a multimeric complex into subunits or a conformational change. The smallest receptor form obtained had a Stokes radius of 6–7 nm, as analyzed by Sephadex G-150 gel filtration. In contrast to this, limited receptor proteolysis has previously been readily observed for the progesterone (Sherman et al., 1974, 1976, 1978; Vedeckis et al., 1979, 1980a,b; Hazato & Murayama, 1981), estrogen (Puca et al., 1972, 1977; Sica et al., 1976; Sherman et al., 1978; Miller et al., 1981; Tilzer et al., 1981), androgen (Wilson & French, 1979), aldosterone (Sherman et al., 1978), and glucocorticoid (Carlstedt-Duke et al., 1977, 1979; Sherman et al., 1978, 1979, 1981; Wrange & Gustafsson, 1978; Stevens et al., 1979) receptors.

In the present report, the physicochemical properties of the mouse liver GC-R are examined. Unlike results obtained in the AtT-20 cell line, limited receptor proteolysis was facile in liver cytosol. The mouse liver glucocorticoid receptor is composed of three functional protein domains: hormone-binding domain, DNA-binding domain, and a third domain which may be involved in specifying the site of interaction of the receptor in the nucleus. The GC-R from AtT-20 cells is also composed of these three domains, which are separated by protease-sensitive regions, but cytosol from these cells apparently contains less of the protease. The studies support a generalized structure for steroid receptors. Together with the results presented in the following paper (Vedeckis, 1983), a model is proposed for the structure and mechanism of activation of the mouse liver and AtT-20 cell glucocorticoid receptor.

Materials and Methods

Animals. Male Swiss Webster mice were obtained from the Tulane University School of Medicine Vivarium.

AtT-20 Cell Line. The mouse AtT-20 pituitary tumor cell line was obtained from Dr. Frank Svec, Division of Endocrinology, Department of Medicine, Louisiana State University Medical Center, New Orleans, LA. Maintenance of these cells in culture and the harvesting of the cells were as described previously (Vedeckis, 1981a).

Chemicals. [1,2,4-$^3H_3$]Triamcinolone acetonide ([^{3}H]TA) was obtained from Amersham (20 Ci/mmol) and New England Nuclear (31.3 and 37 Ci/mmol). Tris and sucrose were both "Ultra Pure" grade from Schwarz/Mann. Bovine pancreatic trypsin (type XI), egg white trypsin inhibitor (type II-0), and iodoacetamide (IAM) were from Sigma Chemical Co. All other chemicals were reagent grade and obtained from J. T. Baker.

Buffers. The buffers used were as follows: TETg (20 mM Tris-HCl, pH 7.4 at 25 °C, 1 mM EDTA, and 12 mM 1-thioglycerol); TE (20 mM Tris-HCl, pH 7.4 at 25 °C, and 1 mM EDTA); 5 mM potassium phosphate and 0.4 M potassium phosphate, both pH 7.4 at 25 °C. Potassium chloride was added to the Tris buffers to achieve the final salt concentration as indicated. When Na_2MoO_4 was used, the final concentration was made 20 mM by using a 1 M stock solution.

Preparation of Cytosol (Cytoplasmic Supernatant Fraction). Mice were killed by cervical dislocation, and the livers were removed and perfused with ice-cold 0.9% NaCl. All subsequent procedures were performed at 0–4 °C. The livers were blotted dry, weighed, and minced with scissors in 3 mL of TETg or TE buffer per g of tissue. The tissue was homogenized by three 10-s bursts (with 30-s cooling intervals) at setting 7 with a Polytron PT10 (Brinkmann) homogenizer, followed by three series of three strokes each with a Teflon–glass homogenizer. The homogenate was centrifuged at $8000g_{av}$ for 15 min and the supernatant centrifuged at $190000g_{av}$ for 1 h at 2 °C. The floating lipid layer was aspirated after each centrifugation. The high-speed supernatant (cytosol) was then labeled for 18 h with (3–5) × 10^{-8} M

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[1] Abbreviations: TA, triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone); EDTA, ethylenediaminetetraacetate; IAM, iodoacetamide; DEAE, diethylaminoethyl; PC, phosphocellulose; HAP, hydroxylapatite; GC-R, glucocorticoid receptor; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.

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[^{3}H]TA. Na_2MoO_4 was added to a final concentration of 20 mM after 2 h of labeling with hormone. Although most of the glucocorticoid receptor was labeled after 2 h, the apparent binding affinity of the receptor for the hormone was 2-fold higher after an 18-h incubation, with no change in the number of binding sites (data not shown).

In the experiment which involved IAM treatment of the cytosol, TE buffer was used. The receptor was labeled for 2 h with [^{3}H]TA followed by a 30-min treatment with 10 mM IAM. The excess IAM was then scavenged by the addition of 1-thioglycerol to a final concentration of 12 mM.

Cytosol preparation from the AtT-20 cell line was as described previously (Vedeckis, 1981a).

Ion-Exchange and Adsorption Chromatography. DEAE-cellulose (Whatman DE-52), phosphocellulose (PC) (Whatman P-11), hydroxylapatite (HAP) (Bio-Rad HTP), and DNA–cellulose chromatographies were as previously published (Vedeckis, 1981a). The DNA–cellulose was prepared by a modification (Vedeckis, 1981a) of the procedure of Alberts & Herrick (1971). The salt concentrations at which the various receptor forms eluted from these columns were determined by measuring the conductivity (at room temperature) of every fifth fraction with a Radiometer Model CDM 3 conductivity meter equipped with a Radiometer CDC 314 conductivity cell. The conductivities of these fractions were then compared to the standard curves of TETg buffer plus various concentrations of KCl or a standard curve obtained with various concentrations of potassium phosphate.

Gel Filtration. Gel filtration was performed with a 2.6 cm × 63 cm column of Sephadex G-150 (Pharmacia). Samples were treated for 1–3 h with 0.3 or 0.15 M KCl prior to chromatography to minimize receptor aggregation. The column was equilibrated in TETg buffer containing 0.3 or 0.15 M KCl, a 2-mL sample was applied, and 3-mL fractions were collected at a flow rate of 18 mL/h. One-milliliter aliquots were counted for radioactivity. Proteins used to calibrate the columns and the sucrose gradients (see below) were all from Sigma Chemical Co. except for ferritin, which was from Boehringer/Mannheim.

Sucrose Gradient Ultracentrifugation. Two hundred microliter samples were layered onto 5-mL 5–20% linear sucrose gradients in TETg buffer with or without 0.3 M KCl. Centrifugation was at 190000g_{av} and 2 °C for 16 h. Two hundred microliter fractions were collected and counted for radioactivity. Standard proteins run in parallel gradients were located by their absorbance at 280 nm.

Receptor Activation. Receptors were activated by gel filtration on Sephadex G-25 as described previously (Vedeckis, 1981a). This procedure is believed to remove a low molecular weight inhibitor of activation [reviewed in Milgrom (1981) and Sekula et al. (1981)]. For gel filtration, the cytosol receptor was activated by adjusting the KCl concentration to 0.3 M, a procedure which also prevents the formation of large receptor aggregates which are excluded from the column.

Liquid Scintillation Counting. Four or eight milliliters of aqueous scintillation fluid (Ready-Solv EP, Beckman) was added to 0.5- or 1-mL aliquots, respectively. Counting efficiency was about 35% with a Beckman LS 7500 liquid scintillation spectrometer.

Results

Ion-Exchange and DNA–Cellulose Chromatography. In all of the experiments described below, the amount of receptor was quantitated by using Sephadex LH-20 chromatography (Sherman et al., 1979; Vedeckis, 1981a). This allowed an accurate estimate of the total amount of receptor-bound hormone which adsorbed to the columns and the proportion which eluted in various peaks.

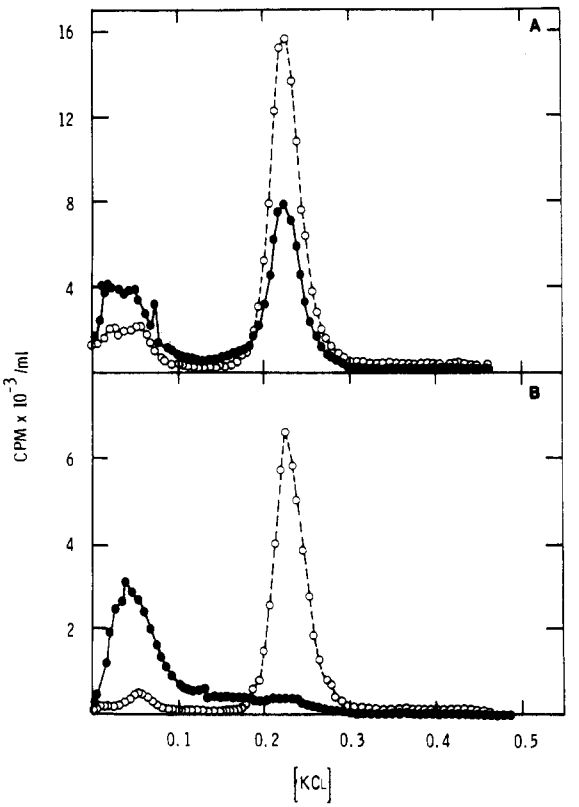

FIGURE 1: DEAE-cellulose chromatography of the mouse liver glucocorticoid receptor. Fourteen milliliters of mouse liver cytosol was labeled with [^{3}H]TA for 18 h in TETg buffer (closed circles), and 16 mL of the same cytosol was treated identically except for the addition of 20 mM Na_2MoO_4 to the sample (open circles). (A) Two milliliters of both cytosols was chromatographed on 4-mL DEAE-cellulose columns as described under Materials and Methods. Half-milliliter aliquots were counted from each 1-mL fraction. The amount of total receptor applied to these columns which adsorbed was 100% for cytosol alone (●) and 100% for MoO_4^{2-}-stabilized cytosol (○). (B) Five milliliters of cytosol was activated on a 35-mL Sephadex G-25 column and 6.4 mL of the void volume (receptor) pooled and allowed to stand at 0–4 °C for 4 h (activated receptor; closed circles). Two milliliters of the activated receptor was then chromatographed on a 4-mL DEAE-cellulose column as described above. Five milliliters of the MoO_4^{2-}-stabilized receptor was applied to a 35-mL Sephadex G-25 column equilibrated in TETg–20 mM Na_2MoO_4, and chromatography was carried out in the same buffer. The void volume (6.2 mL) was pooled and allowed to stand for 4 h at 0–4 °C. Two milliliters of this sample (MoO_4^{2-}-stabilized receptor subjected to activating conditions; open circles) was chromatographed on DEAE-cellulose as described above. The amount of total receptor applied to these columns which was adsorbed was 71% for the activated receptor (●) and 72% for the MoO_4^{2-}-stabilized receptor subjected to activating conditions (○).

When DEAE-cellulose chromatography was performed on unactivated mouse liver cytosol, most of the receptor eluted at 0.22 M KCl (Figure 1A), similar to results obtained previously for the AtT-20 glucocorticoid receptor (GC-R) (Vedeckis, 1981a). However, a substantial amount of liver GC-R eluted earlier, at 0.03–0.08 M KCl. This was in contrast to previous results on the AtT-20 GC-R, in which a discrete, symmetrical peak of activated receptor was observed at 0.08 M KCl. Furthermore, upon activation of the liver GC-R via Sephadex G-25 gel filtration, most of the receptor eluted at 0.03 M KCl (Figure 1B); that is, the adsorption to DEAE-cellulose was weaker than that obtained for the AtT-20 receptor. Two possible explanations for this discrepancy were that the activated forms of the liver and AtT-20 cell receptors were different or that (after activation) the receptor in liver

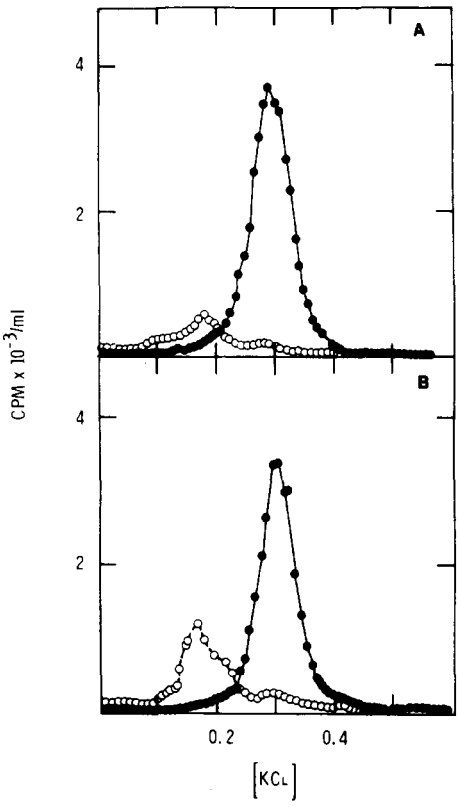

FIGURE 2: Phosphocellulose chromatography of the mouse liver glucocorticoid receptor. Two-milliliter aliquots of samples identical with those analyzed in Figure 1 either were chromatographed on 4-mL PC columns as cytosol (A) or were chromatographed after being subjected to activating conditions with Sephadex G-25 gel filtration (B). The amount of the total receptor applied to each column which adsorbed was 38% for the unactivated receptor (●) and 6% for the MoO_4^{2-}-stabilized receptor (○) in panel A and 53% for the activated receptor (●) and 15% for the MoO_4^{2-}-stabilized receptor subjected to activating conditions (○) in panel B.

cytosol was undergoing limited proteolysis to a smaller form. That the latter was probably the case was shown by adding a sulfhydryl protease inhibitor (10 mM iodoacetamide) to the cytosol and then chromatographing the cytosol on DEAE-cellulose. No 0.03–0.08 M KCl-eluting form was obtained, showing that IAM inhibited the conversion of the liver GC-R to this form (data not shown). Further experiments presented below confirmed that partial proteolysis of the activated liver receptor was occurring. Thus, the following experiments were performed to characterize the partially proteolyzed receptor and its generation.

It appeared that activation of the receptor was a prerequisite for proteolysis by the endogenous liver enzyme(s). That is, proteolysis was rather slow in unactivated cytosol. This was confirmed by adding 20 mM Na_2MoO_4 (which prevents receptor activation) to the cytosol. Thus, MoO_4^{2-} prevented spontaneous receptor activation (Figure 1A), as well as inhibiting activation via Sephadex G-25 chromatography (Figure 1B). Very little receptor proteolysis to the 0.03 M KCl-eluting DEAE-cellulose peak occurred.

Because the adsorption of the partially proteolyzed receptor from liver cytosol to DEAE-cellulose was very weak, the chromatographic behavior of the liver GC-R was further analyzed by phosphocellulose chromatography (Figure 2). When Na_2MoO_4 was used to stabilize the unactivated liver GC-R, there was very little adsorption to PC, consistent with previous observations that unactivated steroid receptors do not adsorb to polyanionic exchange resins [reviewed in Milgrom (1981)]. The small amount which did adsorb eluted exactly as did activated AtT-20 GC-R, at 0.17–0.20 M KCl (Vedeckis, 1981a). Chromatography of unactivated (but not MoO_4^{2-}-stabilized) liver GC-R on PC resulted in significant receptor adsorption (Figure 2A). This can be attributed to the fact that contact with phosphocellulose promotes receptor activation (Atger & Milgrom, 1976). Activated liver GC-R adsorbed strongly to PC (Figure 2B) while MoO_4^{2-} again effectively inhibited receptor activation by G-25 chromatography (Figure 2B).

Most significantly, the partially proteolyzed liver GC-R eluted from PC at a higher salt concentration (0.3 M KCl) than that obtained for the AtT-20 GC-R (0.17–0.20 M KCl) (Vedeckis, 1981a). Thus, as had been suggested by DEAE-cellulose chromatography, the partially proteolyzed GC-R appeared to be a more basic protein than the activated AtT-20 GC-R. Additionally, the partially proteolyzed GC-R eluted as a sharp, symmetrical peak from PC. No evidence of dissimilar hormone-binding subunits or fragments was obtained.

Similar experiments were performed by using DNA–cellulose adsorption chromatography (data not shown), with similar results. The unactivated liver GC-R did not absorb, and the partially proteolyzed GC-R eluted at a higher salt concentration (0.20 M KCl) than did the activated GC-R from the AtT-20 cell line (0.14 M KCl) (Vedeckis, 1981a).

Hydroxylapatite Chromatography. Previous studies on the AtT-20 glucocorticoid receptor showed that the unactivated and activated forms eluted identically from hydroxylapatite, at 0.11 M potassium phosphate (Vedeckis, 1981a). Because the liver GC-R behaved differently from the AtT-20 receptor on the columns described above, it was of interest to analyze the liver GC-R on HAP.

When unactivated cytosolic liver GC-R was chromatographed on HAP, a complex pattern was obtained, with four peaks being evident (Figure 3A). Peak 3, at 0.11 M phosphate, appeared to be the same as that obtained with the AtT-20 GC-R. The fact that the unactivated liver GC-R was similar to that in AtT-20 cells was confirmed by preserving the unactivated receptor with MoO_4^{2-}. Thus, if MoO_4^{2-}-treated mouse liver GC-R was chromatographed directly (Figure 3A) or after being subjected to activating conditions in the presence of MoO_4^{2-} (Figure 3B), the majority of the receptor eluted at 0.11 M phosphate. However, when the liver GC-R was fully activated, most of the radioactivity eluted as peak 4 (Figure 3B, at 0.17 M phosphate), rather than remaining as peak 3. Treatment of the liver cytosol with 10 mM IAM completely inhibited the appearance of peaks 1, 2, and 4; the liver receptor eluted exclusively as peak 3 (data not shown). This, again, suggested that receptor proteolysis was responsible for the various forms of liver receptor seen upon HAP chromatography. Further experiments, described below, demonstrated that peak 4 was actually the partially proteolyzed form of the GC-R. Thus, the partially proteolyzed receptor elutes at a higher phosphate concentration from HAP than both the unactivated GC-R and the activated receptor analyzed from AtT-20 cells.

Hydroxylapatite Promotion of Receptor Activation. During these experiments, it was noted that the relative amount of receptor which eluted in the four peaks varied among preparations. In addition, the amount of time which the preparation was exposed to HAP also affected the results. It was suspected that exposure to HAP might actually promote receptor activation during the chromatography. Since activation of the receptor appeared necessary for proteolysis (see above), a HAP-induced activation could be responsible for the variations in the peaks obtained from this column. Therefore, the fol-

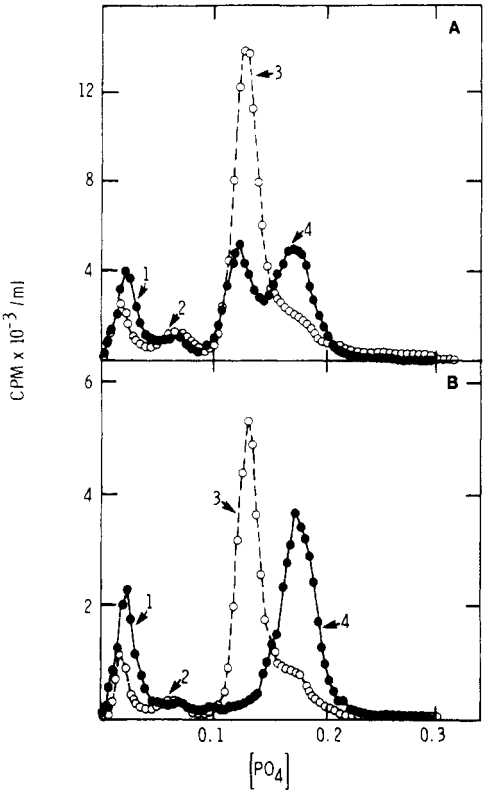

FIGURE 3: Hydroxylapatite chromatography of the mouse liver glucocorticoid receptor. Two-milliliter aliquots of samples identical with those analyzed in Figure 1 either were chromatographed on 4-mL HAP columns as cytosol (A) or were chromatographed after being subjected to activating conditions with Sephadex G-25 gel filtration (B). The amount of the total receptor applied to each column which adsorbed was 94% for the unactivated receptor (●) and 79% for the MoO_4^{2-}-stabilized receptor (○) in panel A and 86% for the activated receptor (●) and 69% for the MoO_4^{2-}-stabilized receptor subjected to activating conditions (○) in panel B.

lowing experiments were performed.

Cytosol was prepared and allowed to stand (aged) at 0–4 °C for varying periods of time. Additional aliquots (1 mL) were applied to 4-mL HAP columns and allowed to drain into the resin bed. The columns were then stopped and the samples allowed to stand in contact with HAP for varying times. These columns were washed with TETg buffer until 25 mL of dropthrough was collected and then eluted with a potassium phosphate gradient. The aged samples which were standing in the absence of HAP were applied to separate columns and chromatographed as rapidly as possible. These results are shown in Figure 4.

As can be seen (Figure 4A), the amount of peak 3 decreased, while the amount of peak 4 concomitantly increased, with longer aging of the cytosol. Two points should be noted. First, the rate of apparent conversion of peak 3 to peak 4 was relatively slow (an 18% conversion over the first 22 h of aging). Second, the level of peak 4 obtained at 2 h of aging was relatively high (32%). This is despite the fact that no activation was performed prior to chromatography. The apparent reason for this can be seen in Figure 4B. When mouse liver cytosol was allowed to incubate in contact with HAP, a rapid conversion of peak 3 to peak 4 occurred (10% within the first hour). Thus, HAP presumably promotes a rapid activation of the GC-R, followed by proteolysis. Again, the amount of partially proteolyzed receptor (peak 4) was high at "zero time". This probably indicates that HAP-induced receptor activation was occurring during the short time (~30 min) required to perform the chromatography. Thus, as had been observed

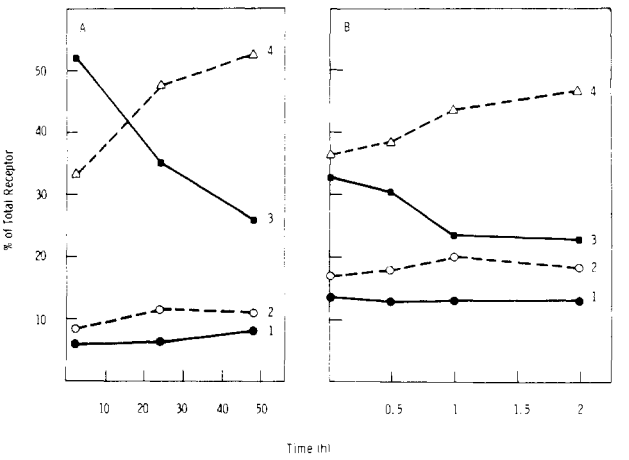

FIGURE 4: Hydroxylapatite promotion of mouse liver glucocorticoid receptor activation. [^{3}H]TA-labeled mouse liver cytosol (2 mL) was allowed to stand at 0–4 °C for the indicated times prior to HAP chromatography (A) or was incubated in the HAP resin for the indicated times prior to chromatography (B). The percent of the total amount of receptor which eluted in the various peaks as shown in Figure 3 was then determined after phosphate gradients were run through each column. Further details are described under Results.

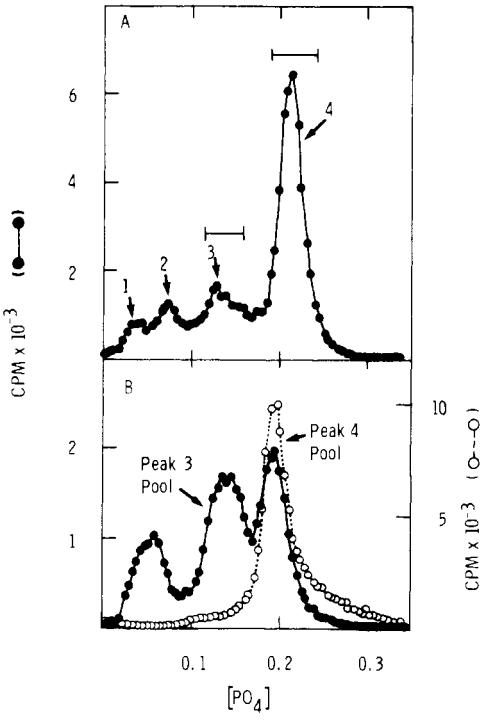

FIGURE 5: Rechromatography of peaks 3 and 4 on HAP. Mouse liver cytosol (24 mL) was chromatographed on a 20-mL HAP column (A), and peak 3 and peak 4 (fractions indicated by the solid bar) were pooled and dialyzed for 2 h vs. three changes of TETg buffer. Aliquots (6 mL) of the dialyzed pools were then rechromatographed on 5-mL HAP columns. The amount of the applied receptor in each pool which adsorbed to the second HAP column was 73% for the peak 3 pool (●) and 69% for the peak 4 (○) pool.

previously using polyanionic chromatography resins (Milgrom, 1981), HAP can apparently also cause receptor activation.

Rechromatography was performed to confirm that peak 3 was truly converted to peak 4. Mouse liver cytosol (24 mL) was chromatographed on a 20-mL HAP column (Figure 5A). Peaks 3 and 4 were pooled and dialyzed vs. TETg buffer, and aliquots were rechromatographed on HAP (Figure 5B). Peak 4 (partially proteolyzed receptor) continued to chromatograph at 0.17 M potassium phosphate. However, when peak 3 was rechromatographed, it also gave rise to peaks 1 and 4. Thus,

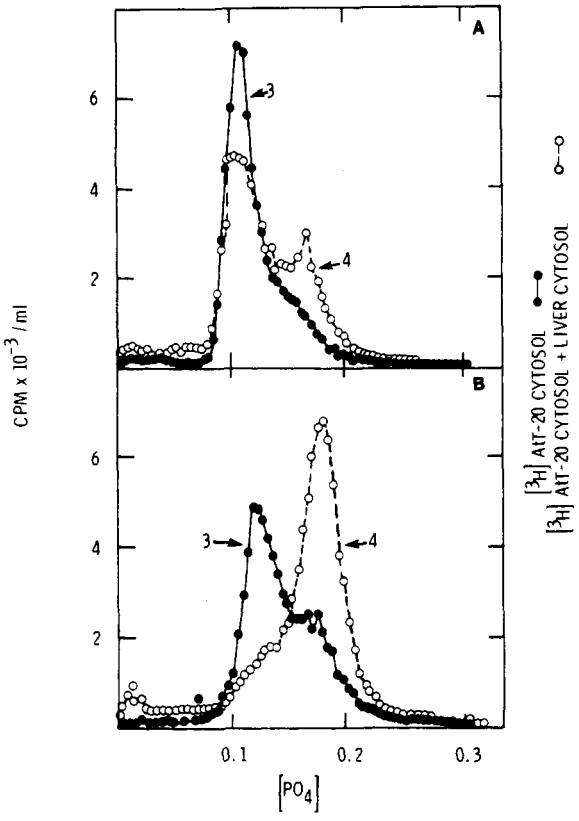

FIGURE 6: HAP chromatography of mixed cytosols. Cytosol from AtT-20 cells was labeled with [^{3}H]TA, and mouse liver cytosol was blocked with radioinert TA as described under Results. Equal volumes of [^{3}H]TA-labeled AtT-20 cytosol were mixed with either TETg buffer (closed circles) or radioinert TA-blocked liver cytosol (open circles). These samples were then chromatographed immediately (A) or after activation by Sephadex G-25 gel filtration (B). The amount of the total receptor applied to each column which adsorbed was 100% for AtT-20 cytosol alone (●) and 100% for AtT-20 cytosol plus liver cytosol (○) in panel A and 90% for activated AtT-20 cytosol alone (●) and 93% for activated AtT-20 cytosol plus liver cytosol (○) in panel B.

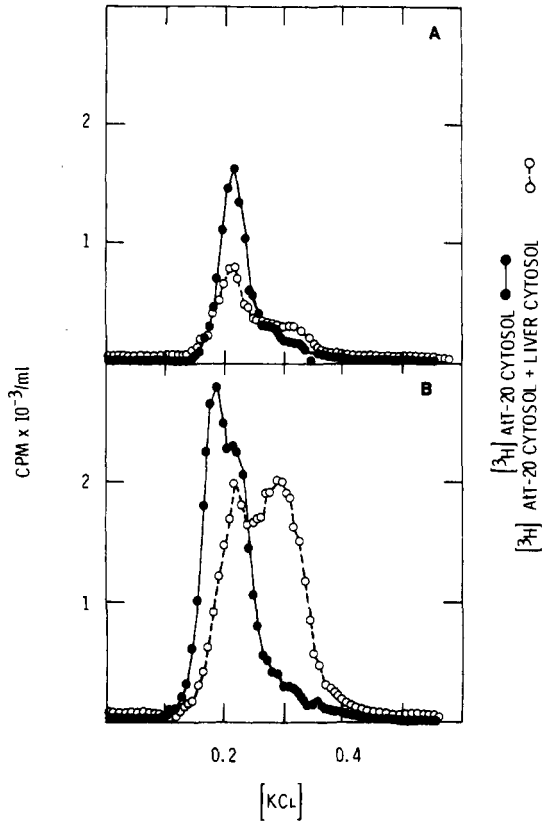

FIGURE 7: PC chromatography of mixed cytosols. Samples identical with those analyzed on HAP (Figure 6) were subjected to PC chromatography. The amount of the total receptor applied to each column which adsorbed was 21% for AtT-20 cytosol alone (●) and 13% for AtT-20 cytosol plus liver cytosol (○) in panel A and 59% for activated AtT-20 cytosol (●) and 55% for activated AtT-20 cytosol plus liver cytosol (○) in panel B.

peak 3 was converted to both peak 1 (see below) and peak 4, the partially proteolyzed GC-R.

Limited Proteolysis of the Activated AtT-20 Glucocorticoid Receptor. It was not yet clear whether the AtT-20 GC-R and that of liver had similar or different structures. Thus, mixing experiments were performed to determine if the AtT-20 receptor could be cleaved to the same proteolytic fragments, and if the relative lack of proteolysis in these cells was due to a lower protease concentration than that found in liver cytosol.

Cytosol was prepared from both mouse liver and the AtT-20 cell line. The liver cytosol was then incubated with 9×10^{-8} M radioinert TA, while 3×10^{-8} M [^{3}H]TA was added to the AtT-20 cytosol. The ^{3}H-labeled AtT-20 cytosol was then split into two portions. To one was added an equal volume of radioinert TA-blocked liver cytosol, while the other received the same amount of TETg buffer. These two samples (mixed cytosols and control) were then chromatographed immediately (unactivated) on HAP and PC columns, or were activated by chromatography on a Sephadex G-25 column, followed by an incubation of 4 h prior to HAP and PC chromatography. On the basis of the kinetic properties of hormone dissociation at a 1000 molar excess of cold ligand, exchange of ligand between liver receptor ($t_{1/2}$ of dissociation = 8 h) (Mirás & Harrison, 1979) and the AtT-20 receptor ($t_{1/2}$ of dissociation = 7 days) (Yeakley et al., 1980) did not occur.

As had been seen previously (Vedeckis, 1981a), both the unactivated and activated AtT-20 glucocorticoid receptors eluted from HAP as peak 3 at 0.11 M potassium phosphate (Figure 6). The shoulder seen in the peak 4 region for the activated AtT-20 represents the largest amount of this form ever observed in AtT-20 cytosol and suggests that although protease activity is not completely absent in AtT-20 cell cytosol, it is present at much lower levels than that found in liver cytosol. When liver cytosol was added to the ^{3}H-labeled AtT-20 cytosol, and HAP chromatography performed, a substantial portion eluted as peak 4 (Figure 6A). However, when the mixed cytosols were activated first, the vast majority of the ^{3}H-labeled AtT-20 GC-R eluted as peak 4, that is, identically with that normally obtained for the activated liver GC-R (Figure 3). Phosphocellulose chromatography gave similar results. Thus, the control ^{3}H-labeled AtT-20 GC-R eluted at 0.17–0.20 M KCl (Figure 7A,B). In contrast, when the ^{3}H-labeled AtT-20 GC-R was activated in the presence of blocked liver cytosol, two distinct peaks were obtaned (Figure 7B). The first, at 0.17–0.20 M KCl, represented native, activated AtT-20 GC-R (Vedeckis, 1981a) while the second, at 0.3 M KCl, resembled activated liver GC-R (Figure 2).

In most experiments performed to date, the activated receptor eluted from PC at about 0.17 M KCl (Vedeckis, 1981a, and Figure 2). However, it should be noted that, in the experiment shown here, the activated AtT-20 GC-R (Figure 7B, closed circles) seemed to show a doublet of receptor in the 0.17–0.20 M KCl-eluting region. This may represent a slight difference in elution characteristics for the intermediate (dimeric?) activated receptor form and the monomeric, activated receptor [see Vedeckis (1983)]. This possibility is currently under investigation.

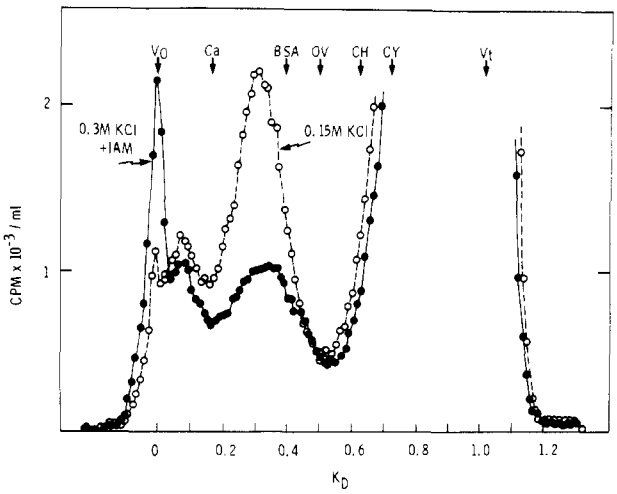

FIGURE 8: Sephadex G-150 chromatography of the mouse liver glucocorticoid receptor. Chromatography was performed on a Sephadex G-150 column (2.6 cm × 63 cm) as described under Materials and Methods. One sample of cytosol (○) was pretreated for 3 h with 0.15 M KCl in TETg buffer prior to chromatography in the same buffer. Also, a sample of cytosol was prepared in TE buffer and labeled for 2 h with [^{3}H]TA. Iodoacetamide (10 mM) was then added for 30 min, followed by 12 mM 1-thioglycerol for 30 min. The sample was made 0.3 M in KCl and allowed to stand at 0–4 °C for 3 h. Chromatography was then performed in TETg buffer containing 0.3 M KCl (●). The elution positions labeled in the figure are as follows: catalase (Ca), R_s = 5.13 nm; bovine serum albumin (BSA), R_s = 3.59 nm; ovalbumin (OV), R_s = 2.8 nm; chymotrypsinogen A (CH), R_s = 2.21 nm; cytochrome *c* (CY), R_s = 1.79 nm. $K_D = (V_e - V_0)/(V_t - V_0)$, where V_e = the elution position of the receptor or standard protein, V_0 = the void volume determined by using blue dextran ($M_r = 2 \times 10^6$), and V_t = the total volume of the column determined by the elution position of KCl. The Stokes radii of the receptor proteins were determined from linear correlations of $K_D^{1/3}$ and R_s (data not shown).

These studies confirmed that the structures of the GC-R from both sources were probably identical but that the concentration of protease(s) was lower in the AtT-20 cytosol than in liver cytosol. The fact that the observed differences were due to receptor proteolysis was shown by using gel filtration.

Gel Filtration. When activated mouse liver GC-R was chromatographed on a Sephadex G-150 column in TETg–0.3 M KCl, a single species with a Stokes radius of 3.9 nm was obtained (Vedeckis, 1981b). Because limited proteolysis of other steroid receptors seemed to be caused by a thiol protease (Puca et al., 1977; Vedeckis et al., 1979, 1980a; Wilson & French, 1979), it was desirable to find conditions which would inhibit liver GC-R proteolysis and, thus, preserve the activated (but nonproteolyzed) form of the GC-R found previously in the AtT-20 cells. Thus, mouse liver cytosol was chromatographed on Sephadex G-150 at an isotonic salt concentration (0.15 M KCl), or at high salt concentration after treatment of the cytosol with 10 mM iodoacetamide (IAM). These results are shown in Figure 8. A substantial amount of receptor eluted in the void volume (aggregated receptor) and as the partially proteolyzed receptor (just prior to the BSA standard). The Stokes radius obtained for the latter on the G-150 column was 3.9 nm. Significantly, in both of these experiments, a third peak of receptor was obtained between the void volume and the elution position of catalase. This receptor form corresponded exactly to that obtained for the native, activated AtT-20 GC-R on this column (Vedeckis, 1981a). The result obtained with IAM-treated cytosol is significant, since chromatography performed in high salt concentration causes complete activation of the receptor, with the dissociation of the multimeric receptor complex into monomeric subunits [see Vedeckis (1983)]. Thus, this implies that IAM was irreversibly inactivating the activity (protease) responsible for the conversion of the 6-nm monomeric subunit [see Vedeckis (1983)] to the 3.9-nm partially proteolyzed form. In addition, phosphocellulose chromatography of IAM-treated cytosol demonstrated a significant amount of receptor eluting at 0.17–0.20 M KCl, identically with that obtained for native, activated AtT-20 receptor (data not shown). Thus, chromatography of liver GC-R in isotonic salt, or after iodoacetamide treatment, resulted in the preservation of a nonproteolyzed activated GC-R from mouse liver, which was probably identical with that obtained normally from AtT-20 cells. That is, proteolysis of the mouse liver GC-R can be partially inhibited under certain conditions.

Finally, it was confirmed that peak 4 from HAP and the 0.3 M KCl-eluting form from PC, which are obtained under activating conditions from liver cytosol, represented partially proteolyzed receptor. Pools of these peaks, when chromatographed on the G-150 column, gave a single peak with a Stokes radius of 3.9 nm (data not shown). Thus, although the native, activated receptor can be detected in the mouse liver cytosol (Figure 8), the majority is rapidly converted to the partially proteolyzed receptor.

Generation of the Mero-receptor. The 3.9-nm partially proteolyzed liver receptor closely resembled limited cleavage products of other steroid hormone receptors, which contain both the hormone- and DNA-binding protein domains. Except for a very minor peak of radioactivity (with an apparent Stokes radius of 2.4 nm) seen on some Sephadex G-150 columns of activated liver GC-R (data not shown), no dramatic evidence was obtained for a mero-receptor-like fragment. Mero-receptors, which have been observed for many steroid receptors, contain the hormone-binding, but not the DNA-binding, protein domain (Sherman et al., 1974, 1976, 1978, 1979, 1981; Carlstedt-Duke et al., 1977, 1979; Wrange & Gustafsson, 1978; Vedeckis et al., 1979, 1980a,b; Wilson & French, 1979; Hazato & Murayama, 1981; Schrader et al., 1981; Stevens & Stevens, 1981). Because trypsin treatment of receptors can generate a hormone-binding fragment of very similar size to the mero-receptor, GC-R receptor from AtT-20 cells was activated by G-25 gel filtration and treated with 50 μg/mL trypsin for 1 h. The reaction was stopped by the addition of 500 μg/mL egg white trypsin inhibitor. After 30 min, aliquots of this sample were chromatographed on Sephadex G-150, HAP, and PC. Trypsin treatment converted all of the AtT-20 GC-R to mero-receptor, which had a Stokes radius of 2.4 nm (Figure 9A). Most significantly, trypsin-generated mero-receptor eluted as peak 1 on the HAP column (Figure 9B). The fact that the 2.4-nm, HAP peak 1 material was, indeed, the mero-receptor was shown by PC chromatography. None of this receptor form adsorbed to PC, indicating that the DNA-binding receptor domain had been removed (data not shown).

Thus, it was possible to assign all four receptor forms to the peaks obtained on HAP. The unactivated and activated receptors eluted at 0.11 M phosphate (Vedeckis, 1981a), the 3.9-nm partially proteolyzed receptor at 0.17 M phosphate, and the mero-receptor at 0.02 M phosphate. The identity of peak 2 (a minor form) has not yet been resolved. Finally, these results confirm that the mero-receptor can be generated without the addition of exogenous protease. Chromatography on HAP apparently promotes receptor activation and proteolysis to both the 3.9-nm partially proteolyzed receptor and to peak 1, mero-receptor (Figure 3).

Table I: Physicochemical Properties of Steroid Receptor Proteolytic Fragments

	s (s^{-13})	R_s (nm)	M_r[a]	f/f_0[b]	axial[c] ratio
Partially Proteolyzed					
glucocorticoid					
mouse liver[f]	3.2	3.9	53 000	1.45	8
mouse mammary tumor[g]	3.8	2.9	46 000	1.13[d]	3
mouse fibroblastic cell line[h]	4.0	3.2	54 000	1.18	4
mouse P1798 lymphoma[i]	3.3	2.9	40 000	1.25	5
rat liver[j]	3.2	3.6	46 000	1.38	7[d]
estrogen					
calf uterus[k]	4.5	3.3	61 000	1.25	3.2[e]
progesterone					
chick oviduct[l]	3.63	2.74	43 000	1.09	3
androgen					
rat ventral prostate[m]	3.6	3.7	62 000	1.45	8[d]
Mero-receptor					
glucocorticoid					
mouse liver[f]	2.4	2.4	24 000	1.15	3
mouse P1798 lymphoma[n]	2.75	1.9	22 000[d]	0.94[d]	1[d]
rat liver[j]	2.5	1.9	19 000	1.00	1[d]
estrogen					
calf uterus[k]	2.8	2.7	31 000	1.42	5.5[e]
human breast tumor[o]	2.9	2.4	29 000	1.07	2.4
progesterone					
chick oviduct[l]	2.6	2.08	23 000	1.02	1
androgen					
rat ventral prostate[m]	3.0	2.3	28 500	1.1	3[d]

[a] Calculated by using the equation $M_r = 424sR_s$ (Siegel & Monty, 1966). [b] Calculated by using the equation $f/f_0 = 1.393(R_s/M_r^{1/3})$ (Sherman, 1975). [c] Determined from the frictional ratios, assuming the shape of a prolate ellipsoid (Schachman, 1959). [d] These do not appear in the original work but have been calculated by using the equation shown above. [e] The discrepancy between these values for each f/f_0 and the rest given in the table may be due to the source used to obtained these values for the calf uterine receptor (Oncley, 1941). [f] This study. [g] Costello & Sherman (1979). [h] Middlebrook & Aronow (1977). [i] Stevens & Stevens (1979). [j] Carlstedt-Duke et al. (1977). [k] Sica et al. (1976). [l] Sherman et al. (1976). [m] Wilson & French (1979). [n] Stevens & Stevens (1981). [o] Miller et al. (1981).

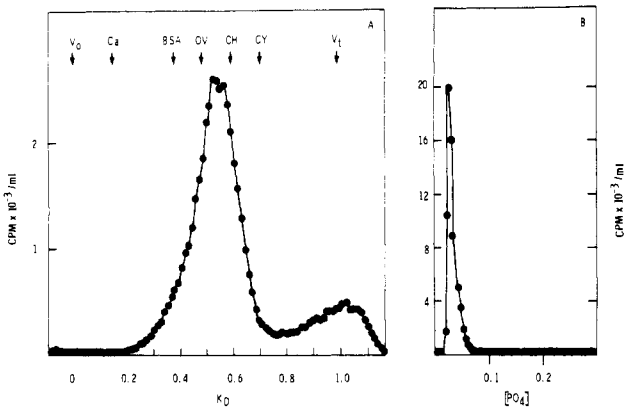

FIGURE 9: Chromatographic properties of the mero-receptor. The mero-receptor was generated by trypsin treatment of AtT-20 glucocorticoid receptor as described in the text. Aliquots were then chromatographed on a Sephadex G-150 column (panel A; see Figure 8 legend for the details of the chromatography procedure) and a HAP column (panel B; chromatographic protocol was as described in the text and the legend for Figure 3). The amount of the applied receptor which adsorbed to HAP was 89%.

Sucrose Gradient Ultracentrifugation. Sucrose gradient ultracentrifugation was performed in order to obtain molecular weight estimates of the proteolytic GC-R fragments. Activated mouse liver GC-R (that is, the partially proteolyzed form) sedimented at 3.2 S, while trypsin-treated receptor had a sedimentation coefficient of 2.4 S (data not shown). Using the following formula (Siegel & Monty, 1966)

$$M_r = 424sR_s$$

where R_s is the Stokes radius in angstroms and s is the sedimentation coefficient, it was determined that the apparent molecular weights of the partially proteolyzed receptor and the mero-receptor are 53 000 and 24 000, respectively. If a solvation of 0.2 g/g of protein and a partial specific volume of 0.735 cm^3/g are assumed, the frictional ratios were calculated to be 1.45 for the partially proteolyzed receptor and 1.15 for the mero-receptor. Finally, on the basis of a prolate ellipsoid, the axial ratios were found to be 8 and 3 for the partially proteolyzed receptor and mero-receptor, respectively. The physicochemical properties of the mouse GC-R proteolytic fragments are summarized in Table I.

Discussion

Three major conclusions can be drawn from the study presented above. First, the receptor heterogeneity observed in liver can be completely accounted for by limited receptor cleavage due to an endogenous protease of an apparently single, homogeneous, hormone-binding species. Second, similar quantities of protease are not present in all glucocorticoid-responsive cells. However, the receptors present in cytosols lacking in substantial protease activity contain the same protein domains. These domains are separated by protease-sensitive regions, which are susceptible to cleavage when an exogenous source of enzyme is supplied. Third, all steroid hormone receptors may have the same basic structure, three protein domains separated by protease-sensitive regions.

Previous studies on the AtT-20 GC-R demonstrated that the activated receptor is a single hormone-binding species (Vedeckis, 1981a). Extensive attempts to show dissimilar hormone-binding subunits (as has been clearly demonstrated for the chick oviduct progesterone receptor) (Schrader et al., 1980, 1981) were unsuccessful. When the same experiments were performed with mouse liver cytosol, a much more complex situation was observed. While the unactivated receptor appeared to be homogeneous, heterogeneous forms were obtained when the receptor was activated. A combination of HAP, PC, and gel filtration chromatography, sucrose gradient ultracentrifugation, rechromatography, MoO_4^{2-} stabilization,

and cytosol mixing experiments clarified the situation. Whereas the AtT-20 cells contain only low levels of endogenous, receptor-cleaving proteases, the liver contains considerable quantities of such an enzyme(s). Thus, the heterogeneous receptor forms seen in liver cytosol were due to limited proteolysis of the native protein.

Exposure to HAP resulted in receptor activation. This was not immediately evident when AtT-20 GC-R was analyzed, as both unactivated and activated receptor eluted at 0.11 M phosphate. However, when HAP induced the activation of the liver GC-R, subsequent proteolysis occurred, giving rise to peak 4 (the 3.9-nm partially proteolyzed receptor) and peak 1 (the mero-receptor). It is not yet known if the mero-receptor was only observed upon HAP chromatography because the calcium phosphate matrix activated calpain (Murachi et al., 1981), the ubiquitous, calcium-activated, neutral, thiol protease (Ishiura, 1981) found to cleave steroid hormone receptor proteins. These studies also showed that only the activated receptor is a substrate for the protease, since MoO_4^{2-} (which inhibits receptor activation) inhibited proteolysis. Additionally, unactivated receptor in liver cytosol [which contains the enzyme(s)] appears to be resistant to proteolysis. Therefore, activation of the glucocorticoid receptor converts the protease-sensitive regions from a protected to a susceptible state. MoO_4^{2-} inhibition of receptor proteolysis (Hazato & Murayama, 1981; Miller et al., 1981; Sherman et al., 1981, 1982; Tilzer et al., 1981) may, thus, be due to the stabilization of the receptor in its unactivated, multimeric form [see Vedeckis (1983)], which is not a substrate for the enzyme.

The apparent identity of the liver and AtT-20 GC-R was confirmed by mixing experiments, in which the AtT-20 form was converted to the partially proteolyzed liver form, and by Sephadex G-150 chromatography of the liver GC-R at isotonic salt or after IAM treatment, in which the native, activated (nonproteolyzed) receptor form was preserved in liver cytosol. Because the protease activity is low in the cytosol from hormone-responsive cells, such as the AtT-20 cells and the mouse lymphoma WEHI-7 cell line (unpublished experiments), it may be that its involvement is not essential for steroid hormone action.

Gel filtration and sucrose gradient ultracentrifugation elucidated the sizes of the various receptor forms. The native, activated receptor has a molecular weight of 81 000 [see Vedeckis (1983)] and consists of three functional domains, separated by two protease-sensitive regions. The smallest form, represented by the mero-receptor (M_r = 24 000), contains only the hormone-binding domain. The partially proteolyzed receptor (M_r = 53 000) contains the hormone- and DNA-binding protein domains. Thus, the DNA-binding domain has a molecular weight of about 29 000. The native, activated receptor contains both of these domains plus a third, which has a molecular weight of about 28 000. Besides its effect on the interaction of the receptor with HAP, this protein domain alters DNA–cellulose binding, resulting in an earlier elution from this column than that seen for the partially proteolyzed receptor. This protein domain appears to be essential for effecting hormone action. Naturally occurring variant forms, which lack this domain, have been found for the glucocorticoid receptor in the S49 mouse lymphoma cell line (nuclear transfer increased mutant, nt^i) (Yamamoto et al., 1974, 1976) and in the P1798 mouse lymphoma (resistant CR P1798 mutant) (Stevens & Stevens, 1979, 1981). These cells are resistant to killing by glucocorticoids. In addition, androgen-resistant, testicular-feminized (Tfm) mice also may contain a receptor lacking this protein domain (Wieland & Fox, 1979). Therefore, because this third receptor domain apparently specifies the proper interaction of the receptor with the physiologically relevant hormone-responsive genes, I propose that it be designated the "specifier domain".

Finally, the structure of all steroid hormone receptors appears to be basically the same—three protein domains separated by protease-sensitive regions. Representative examples of receptor proteolytic fragments are given in Table I. These similarities, plus those which exist for the nonproteolyzed forms of the protein [see Vedeckis (1983)], allow some general conclusions to be drawn about the overall structure of these gene regulatory proteins. This is done for the glucocorticoid receptor from mouse liver and the AtT-20 pituitary tumor cell line in the following paper.

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Subunit Dissociation as a Possible Mechanism of Glucocorticoid Receptor Activation†

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ABSTRACT: For the elucidation of the mechanism of steroid hormone receptor activation, the hydrodynamic properties of the unactivated and activated forms of the nonproteolyzed glucocorticoid receptor from the mouse AtT-20 pituitary tumor cell line were determined. The unactivated, molybdate-stabilized receptor has the following properties: sedimentation coefficient = 9 S; R_s = 8.3 nm; M_r = 317 000; f/f_0 = 1.70; axial ratio (prolate ellipsoid) = 14. The activated monomeric receptor has a sedimentation coefficient of 3.2 S, a Stokes radius of 6 nm, a molecular weight of 81 000, a frictional ratio of 1.93, and an axial ratio (prolate ellipsoid) of 18. A receptor species of intermediate size was detected when the analysis was performed in buffer containing both 0.3 M KCl and 20 mM Na_2MoO_4. Its characteristics are as follows: sedimentation coefficient = 5 S; R_s = 8.3 nm; M_r = 176 000; f/f_0 = 2.06; axial ratio (prolate ellipsoid) = 22. A preliminary study seemed to indicate that this is an activated form of the receptor. On the basis of the molecular weights, it is likely that the unactivated receptor is a tetramer of identical hormone-binding subunits (M_r = 81 000) while the intermediate form is a homodimer. Alternatively, non-hormone-binding components (receptor-binding factors) may be involved in forming the multimeric, nonactivated receptor complex. In either case, the dissociation of a multimeric, nonactivated receptor into subunits appears to be a possible mechanism of receptor activation. Finally, the addition of high concentrations of 1-thioglycerol promoted activation. Thus, sulfhydryl groups may be involved in receptor subunit interaction.

Ever since their discovery, steroid hormone receptor proteins have been the subject of intense investigation. Early studies [reviewed in Milgrom (1981)] demonstrated that the receptor, which is usually localized in the cell cytoplasm, undergoes a process called activation (or transformation) which allows its accumulation in the nucleus. Recent studies have shown that activation does occur in the cell and is not just an in vitro artifact (Munck & Foley, 1979; Markovič & Litwack, 1980). Concomitant with receptor activation, there seems to be some alteration in receptor structure. Results obtained for the estrogen receptor (Notides & Nielsen, 1974; Notides et al., 1981) implicated subunit association (as evidenced by a 4S → 5S transformation) as the structural mechanism for receptor activation. A similar alteration has not been demonstrated

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