Molybdate-Stabilized Glucocorticoid Receptor: Evidence for a Receptor Heteromer†

Sam Okret, Ann-Charlotte Wikström,* and Jan-Åke Gustafsson

Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital F69, S-141 86 Huddinge, Sweden

Received March 20, 1985

ABSTRACT: The composition of the molybdate-stabilized glucocorticoid receptor (GR) complex has been investigated with a monoclonal antibody against the steroid-binding M_r 94 000 (94K) GR protein. It was concluded that one antibody molecule binds one 94K GR molecule. This finding constituted the basis for calculating the number of antibodies bound to the molybdate-stabilized nonactivated GR complex, which has an M_r of 302 000 (302K). Gel filtration on Sephacryl S-400 and density gradient centrifugation showed that only one antibody molecule bound to the molybdate-stabilized GR complex (calculated relative molecular mass for the antibody–molybdate-stabilized GR complex, 456 000; relative molecular mass for one antibody molecule, 157 000). Furthermore, experiments performed with a second antibody immunoprecipitation assay in the presence of an excess of both antibody and GR confirmed the above results. The possibility of steric hindrance not allowing more than one antibody molecule to bind to the molybdate-stabilized GR complex could be excluded. These results suggest that the molybdate-stabilized GR complex with an M_r of 302K only contains one steroid-binding 94K GR molecule and therefore represents a heteromeric complex.

A crucial step in the mechanism of action of steroid receptors is the activation[1] process, i.e., the process whereby steroid receptors acquire the ability to bind to cell nuclei or DNA. In the case of the glucocorticoid receptor (GR),[2] this activation process requires that the hormone is bound to GR (Milgrom et al., 1973). By comparison of the structure of nonactivated GR to that of activated GR, more can be learned about the activation process. However, the pronounced instability of GR in its nonliganded state (Leach et al., 1979; Sando et al., 1979), as well as its liability to become activated after ligand binding during analysis (Atger & Milgrom, 1976), have significantly hampered efforts to characterize nonactivated GR. Several groups have tried to overcome this problem by using 10–20 mM sodium molybdate, which has remarkable effects on both functional and structural characteristics of GR. Pratt and collaborators (Leach et al., 1979) initially described that molybdate could stabilize the hormone-binding ability of GR. Molybdate was also shown to inhibit the activation of GR (Leach et al., 1979) as well as binding of GR to both specific and unspecific DNA sequences (Scheidereit et al., 1983). Similar findings were also obtained with other classes of steroid receptors when studied in the presence of molybdate (Nishigori & Toft, 1980; Noma et al., 1980; Chong & Lippman, 1981). Addition of sodium molybdate to cytosolic preparations after activation of steroid receptors, however, did not reverse the activation process (Leach et al., 1979).

Addition of molybdate to cytosol prior to receptor activation also had a profound effect on the size of GR as well as of other steroid receptors. Sherman et al. (1982) have described that the presence of molybdate during fractionation of cytosol from various tissues stabilized a form of GR with a Stokes radius (R_S) of 7–8 nm and a sedimentation coefficient ($s_{20,w}$) of 9–10S (calculated M_r ~300 000–330 000). This is in contrast to the activated GR, which exists as a monomer with an R_S of ~6 nm and an $s_{20,w}$ value of ~4S (calculated M_r ~90 000–100 000; Carlstedt-Duke et al., 1977; Wrange et al., 1984). The observation that molybdate inhibits activation, together with the finding of a GR complex with a calculated M_r of 300 000–330 000, has led several groups to assume that the molybdate-stabilized GR complex may represent the native nonactivated GR form in vivo (Holbrook et al., 1983; Raaka & Samuels, 1983). Activation might therefore involve a dissociation of a receptor oligomer to a receptor monomer (Raaka & Samuels, 1983; Vedeckis 1983). However, dissociation of a receptor oligomer to a monomer is not the sole mechanism behind activation since we have previously shown that nonactivated GR can also exist as a receptor monomer (Radojcic et al., 1985).

The chemical nature of the molybdate-stabilized GR complex is still unclear, as well as the mechanism behind the inhibition of activation by molybdate. The relative molecular mass of the molybdate-stabilized GR complex is approximately 3–4 times that of the monomeric activated GR (M_r ~90K). However, the number of steroid binding sites of the molybdate-stabilized GR complex and the structural relationship between its subunits have not yet been established. Contradictory data exist, suggesting either a single class of steroid binding subunits or nonidentical subunits in the molybdate-stabilized GR complex (Raaka & Samuels, 1983; Joab et al., 1984). Using monoclonal antibodies against the steroid-binding GR molecule, forming stoichiometrically well-defined complexes with their antigen, recognizing a single antigenic determinant, we undertook an investigation of the subunit composition of the molybdate-stabilized GR complex. Our

† This work was supported by a grant from the Swedish Medical Research Council (13X-2819) and Magnus Bergwalls Stiftelse. S.O. has a postdoctoral position financed by the Swedish Medical Research Council.

[1] Activation is referred to as the process whereby steroid receptors acquire the ability to bind to cell nuclei or polyanions such as DNA^- or phosphocellulose.

[2] Abbreviations: GR, glucocorticoid receptor; TA, triamcinolone acetonide, 9α-fluoro-11β,21-dihydroxy-16α,17α-[(1-methylethylidene)-bis(oxy)]-1,4-pregnadiene-3,20-dione; M_r, relative molecular mass; R_S, Stokes radius; S, svedberg unit; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

0006-2960/85/0424-6581$01.50/0

data suggest that the molybdate-stabilized GR complex is a receptor heteromer containing a single 94K steroid-binding GR moiety.

Materials and Methods

Materials

[6,7-^{3}H]Triamcinolone acetonide (specific radioactivity 30–50 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and unlabeled triamcinolone acetonide was obtained from Squibb (Princeton, NJ). Protein A–Sepharose, Sephacryl S-400, thyroglobulin, ferritin, and bovine serum albumin were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), while catalase, ovalbumin, and myoglobin were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-mouse immunoglobulin was obtained from Dako Immunoglobulin Ltd. (Copenhagen, Denmark). Rabbit immunoglobulin G was prepared by ourselves by purifying normal rabbit serum on protein A–Sepharose (see below). Sodium molybdate was obtained from Merck AG (Darmstad, FRG). Sources of other materials have been given previously (Okret et al., 1981; Okret, 1983; Okret et al., 1984).

Methods

Preparation and Specificity of a Monoclonal Antibody against Rat Liver GR. Preparation and characterization of the monoclonal anti-GR antibodies have been described in detail in a previous paper (Okret et al., 1984). The monoclonal IgG2a antibody [designated 7 in Okret et al. (1984)] was produced by growing the hybridoma cells as ascites. Purification of the ascites fluid was performed by ammonium sulfate precipitation (50%, w/v) followed by dialysis against EPG buffer (20 mM sodium phosphate, pH 7.4, 1 mM Na_2EDTA, 10% (w/v) glycerol, 2 mM dithiothreitol) containing 0.15 M NaCl and 0.02% (w/v) sodium azide. The monospecificity of monoclonal antibody 7, recognizing the 94K GR protein, has previously been described (Okret et al., 1984). Control ascites fluid was obtained by injecting the non-Ig-producing mouse myeloma cell line Sp 2/0 (Shulman et al., 1978) (which was originally used as fusion partner in the hybridoma production) intraperitoneally into Balb/C mice. Purification of the control ascites was performed as described above.

In some cases (see below), protein A purified hybridoma culture supernatant containing monoclonal antibody 7 was used. Purification using protein A–Sepharose was performed as previously described (Okret et al., 1981). Protein was determined according to Lowry et al. (1951). Purity of the protein A purified monoclonal antibody was ≥98% when analyzed by denaturing and reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Preparation of GR. Cytosol was prepared from livers of male rats, adrenalectomized 4–5 days prior to experiments. Cytosol was prepared in EPG buffer as previously described (Okret et al., 1981). Prior to incubation with [^{3}H]triamcinolone acetonide ([^{3}H]TA), 1 M sodium molybdate was added to a final concentration of 20 mM followed by addition of 5 M NaCl to a final concentration of 0.15 M NaCl. To samples where molybdate was not required, an equal volume of NaCl was added to a final concentration of 0.18 M. The inclusion of 0.18 M NaCl in samples not containing sodium molybdate was sufficient to dissociate the GR to a monomer with an M_r of 94K (Radojcic et al., 1985). The two cytosolic preparations were then incubated for 1 h with 100 nM [^{3}H]TA (9 or 37 Ci/mmol) at 0 °C. The number of specific binding sites was determined by incubating cytosol with [^{3}H]TA in the presence or absence of a 200-fold molar excess of unlabeled TA in order to calculate the nonspecific and total binding, respectively. The specific binding was obtained as the difference between the total and the nonspecific binding after dextran-coated charcoal adsorption as previously described (Okret et al., 1981). Following incubation with [^{3}H]TA and, in indicated cases (see below), dextran-coated charcoal adsorption (cf. above), the cytosol was incubated with anti-GR antibody or control antibody for 2–3 h at 4 °C prior to analysis.

Gel Filtration. Gel filtration was carried out at 4 °C in 80 × 2.6 cm columns of Sephacryl S-400 equilibrated in EPG buffer containing 0.15 M NaCl and 0.02% (w/v) sodium azide with or without 20 mM sodium molybdate. The elution rate was 6 mL cm^{-2} h^{-1}. The columns were calibrated with the following standard proteins: thyroglobulin (Thgb), horse spleen ferritin (Fe), bovine liver catalase (Cat), bovine serum albumin (BSA), hen egg white ovalbumin (Ov), and whale skeletal muscle myoglobin (Myo). Thgb, Cat, BSA, and Ov were ^{14}C-labeled according to Rice & Means (1971). The Stokes radii (R_S) of these proteins were 8.61, 6.15, 5.13, 3.59, 2.86, and 2.01 nm, respectively. References for the R_S values of the standard proteins are given elsewhere (Edelhoch, 1960; Sherman et al., 1980). The void volume (V_0) was determined with Dextran blue and the total liquid volume of the column (V_t) with ^{14}C-labeled leucin. No major difference in the elution profile of the standard proteins was seen when calibration was performed in the presence or in the absence of 20 mM sodium molybdate.

Plotting the distribution coefficient $K_d^{1/3}$ [$K_d = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume] vs. the R_S of the standard proteins, a straight line with a correlation coefficient of $r = 0.95$ was obtained. In the case of determination of the R_S of the antibody–molybdate-stabilized GR complex, a linear extrapolation from the standard curve was performed.

Glycerol Density Gradient Centrifugation. Five-milliliter linear glycerol density gradients, 12–50% (w/v), were prepared in EPG buffer, pH 7.4, containing 0.15 M NaCl and, in indicated cases, 20 mM sodium molybdate. Unbound hormone was removed from the cytosol by dextran-coated charcoal adsorption prior to incubation with the antibodies (Okret et al., 1981). Aliquots, 0.15 mL, of cytosolic [^{3}H]TA-labeled GR–antibody complex were layered on the gradients and centrifuged for 16–18 h at 0–2 °C in an SW 50.1 rotor at 200000g. Fractions, 200 μL, were collected from the bottom of the gradient with a Beckman fraction recovery system.

The sedimentation coefficients ($s_{20,w}$) for the various complexes of [^{3}H]TA-labeled GR were calculated according to Martin & Ames (1961) using internal controls consisting of methylated [^{14}C]thyroglobulin ([^{14}C]Thgb), catalase (Cat), rabbit immunoglobulin G (IgG), bovine serum albumin (BSA), and ovalbumin (Ov) for calibration. Sedimentation coefficients for the standard proteins were as follows: 19.2, 11.3, 6.6, 4.4, and 3.5 S, respectively (Sherman et al., 1980; Sober, 1970).

Calculation of Relative Molecular Mass (M_r). From the obtained sedimentation coefficients ($s_{20,w}$) and from the Stokes radius values (R_S), relative molecular mass for the different complexes were calculated according to Siegel & Monty (1966) with the equation $M_r = 4224 R_S s_{20,w}$ (R_S in nanometers and $s_{20,w}$ in svedberg units), assuming a partial specific volume of 0.732 cm^3/g (Sherman et al., 1983).

Second Antibody Immunoprecipitation Assay. [^{3}H]TA–GR complex from rat liver cytosol (2.4 pmol) was, after dextran-coated charcoal adsorption, incubated with various amounts of protein A purified monoclonal anti-GR antibody (0.5–8 pmol of antibody) and 5 μL of normal mouse serum in a final volume of 0.23 mL of EPG buffer containing either

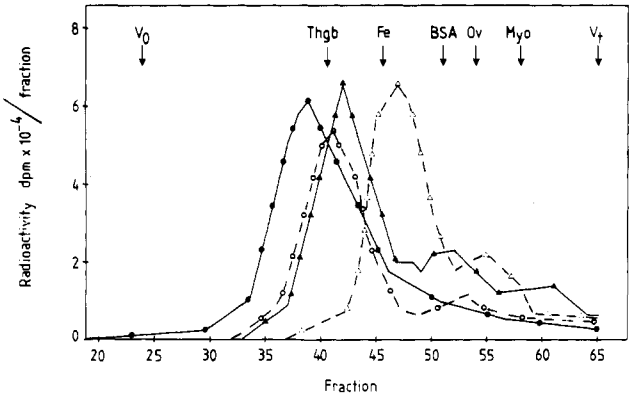

FIGURE 1: Gel filtration analysis of GR in the presence or absence of anti-GR antibody and molybdate. Two milliliters of rat hepatic cytosol in the presence (+Mo) or absence (-Mo) of 20 mM sodium molybdate was incubated with [^{3}H]TA (37 Ci/mmol) for 1 h at 0 °C followed by another 2–3 h in the presence of 0.5 mL of purified ascites fluid containing anti-GR antibody (+Ab) or control ascites fluid (-Ab). The incubation mixture was then applied to Sephacryl S-400 columns equilibrated in EPG buffer containing 0.15 M NaCl and 20 mM sodium molybdate in cases where molybdate was present in the incubation mixture or in the absence of molybdate when incubation mixtures did not contain molybdate. (●) Cytosol, +Mo, +Ab; (○) cytosol, +Mo, -Ab; (▲) cytosol, -Mo, +Ab; (△) cytosol, -Mo, -Ab. Stokes radii for the standard proteins and further details are given under Materials and Methods.

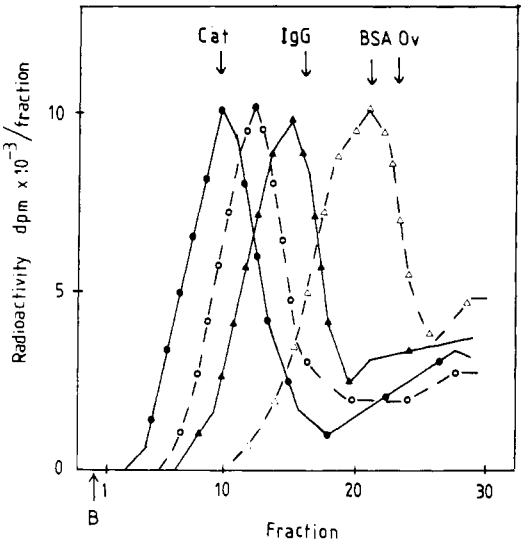

FIGURE 2: Glycerol density gradient analysis of GR in the presence or absence of anti-GR antibody and molybdate. Cytosol was prepared and incubated exactly as described in the legend to Figure 1 with the only exception that the cytosol was treated with dextran-coated charcoal (Okret et al., 1981) prior to incubation with antibody. Aliquots, 0.15 mL, of cytosolic [^{3}H]TA-labeled GR complex were layered on 5-mL linear glycerol gradients, 12–50% (w/v), in EPG buffer, 0.15 M NaCl, with or without 20 mM sodium molybdate. (●) Cytosol, +Mo, +Ab; (○) cytosol, +Mo, -Ab; (▲) cytosol, -Mo, +Ab; (△) cytosol, -Mo, -Ab. For sedimentation coefficients for the standard proteins and further details, see Materials and Methods. "B" represents the amount of radioactivity adhered to the bottom of the centrifuge tube.

0.15 M NaCl and 20 mM sodium molybdate or 0.18 M NaCl alone for 1 h at 4 °C. GR–antibody complex was precipitated by addition of 25 μL of undiluted rabbit anti-mouse immunoglobulin. After incubation for 5 h at 4 °C, precipitates were pelleted by centrifugation (10000*g*, 10 min) and washed 3 times with 1 mL of ice-cold EPG buffer containing 0.15 M NaCl, 100 μg of insulin/mL, and 20 mM sodium molybdate. Following dissolution of the pellets in 1 M NaOH and neutralization with 1 M HCl, the radioactivity was measured. Nonspecific binding was determined in tubes containing [^{3}H]TA–GR complex and normal mouse serum.

Radioactivity. ^{3}H and ^{14}C were measured in a 1216 Rackbeta II liquid scintillation counter (LKB-Wallac, Stockholm, Sweden). Samples were mixed with 20 volumes or more of Scintillation 299 (United Technologies, Packard, IL). Efficiency for tritium was 40–45%.

RESULTS

Gel Filtration and Sedimentation Analysis of the Antibody–GR Complex in the Presence or Absence of Sodium Molybdate. In order to analyze the composition of the molybdate-stabilized GR complex, the number of an IgG monoclonal antibody [7 in Okret et al. (1984)] binding to the steroid binding GR molecule with an M_r of 94K (Wrange et al., 1984) was determined. The relative molecular mass of the antibody–GR complex in the presence or absence of sodium molybdate was calculated by measuring the Stokes radius (R_S) and the sedimentation coefficient ($s_{20,w}$) by gel filtration and density gradient centrifugation, respectively. Analysis of [^{3}H]TA–GR under isotonic conditions (0.15 M NaCl) in the absence of sodium molybdate but in the presence of an excess of monoclonal anti-GR antibody by gel filtration on Sephacryl S-400 gave an R_S of 7.3 ± 0.6 nm (n = 3) (Figure 1). Experiments performed under identical conditions, but in the presence of purified control ascites fluid, gave an R_S of 5.0 ± 0.3 nm (n = 3). When parallel incubations were analyzed by glycerol density gradient centrifugation with or without monoclonal anti-GR antibody in the absence of sodium molybdate, $s_{20,w}$ values of 8.0 ± 0.2 S (n = 3) and 4.4 ± 0.2 S (n = 3), respectively, were observed (Figure 2). With the obtained data from gel filtration and density gradient centrifugation, the relative molecular mass of the antibody–GR complex was calculated to be 247K (Table I). In the absence of anti-GR antibody, an M_r of 94K for GR was calculated, in accordance with a previous determination for the purified GR (Wrange et al., 1984). Assuming two heavy and two light chains, the relative molecular mass of the antibody was 157K when analyzed on reducing and denaturing SDS–PAGE (not shown). From this information it could be calculated that one antibody molecule bound to one steroid-binding 94K GR molecule: subtraction of the relative molecular mass of one antibody molecule, 157K, from the relative molecular mass of the antibody–GR complex, 247K, gave a relative molecular mass for the steroid-binding GR moiety of 90K, which is very close to the experimentally obtained M_r of 94K. Performing identical experiments with GR and anti-GR antibody in the presence of 20 mM sodium molybdate gave an R_S of 9.7 ± 0.3 nm (n = 5) (Figure 1) and an $s_{20,w}$ of 11.1 ± 0.7 S (n = 3) (Figure 2); relative molecular mass was calculated to be 456K (Table I). In the presence of control ascites fluid, an R_S of 7.6 ± 0.4 nm (n = 3) (Figure 1) and an $s_{20,w}$ of 9.4 ± 0.4 S (n = 3) (Figure 2) were obtained, giving an M_r of 302K

Table I: Summary of Hydrodynamic Parameters of GR in the Presence or Absence of Anti-GR Antibody and Sodium Molybdate, Respectively[a]

	-anti-GR antibody		+anti-GR antibody	
	-molybdate	+molybdate	-molybdate	+molybdate
R_S (nm)	5.0 ± 0.3 (n = 3)	7.6 ± 0.4 (n = 3)	7.3 ± 0.6 (n = 3)	9.7 ± 0.3 (n = 5)
$s_{20,w}$ (S)	4.4 ± 0.2 (n = 3)	9.4 ± 0.4 (n = 3)	8.0 ± 0.2 (n = 3)	11.1 ± 0.2 (n = 3)
M_r	94 000	302 000	247 000	456 000

[a] The values are mean ± SD; the number of experiments are given within parentheses. Calculation of relative molecular mass was performed as described under Materials and Methods.

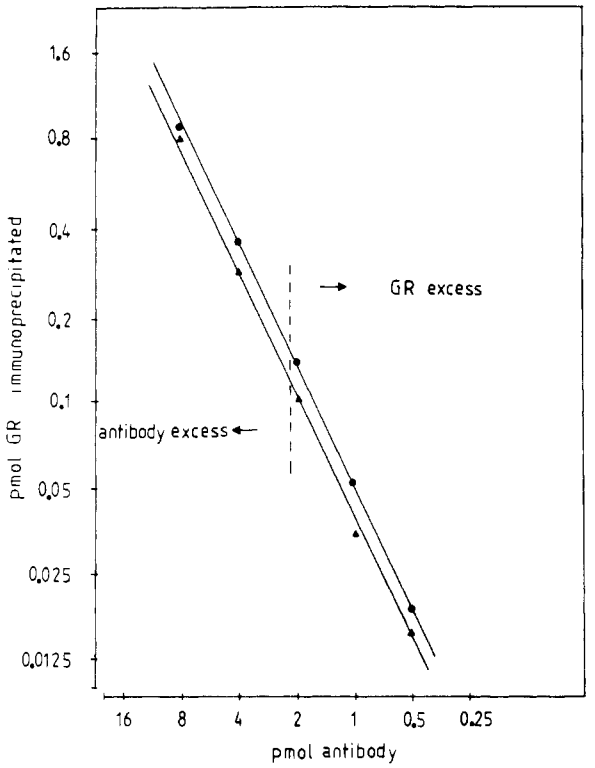

FIGURE 3: Second antibody immunoprecipitation assay of GR in the presence or absence of molybdate. Cytosolic rat liver [^{3}H]TA–GR complex (2.4 pmol) was incubated with various amount of protein A purified hybridoma culture supernatant containing monoclonal anti-GR antibody (0.5–8 pmol) in a final volume of 0.23 mL of EPG buffer containing 0.15 M NaCl and 20 mM sodium molybdate (●) or 0.18 NaCl alone (▲) for 1 h at 4 °C. After addition of rabbit anti-mouse immunoglobulin and another 5-h incubation at 4 °C, the reaction mixture was centrifuged, the pellets were washed, and the radioactivity in the pellets was determined. For further details, see Materials and Methods.

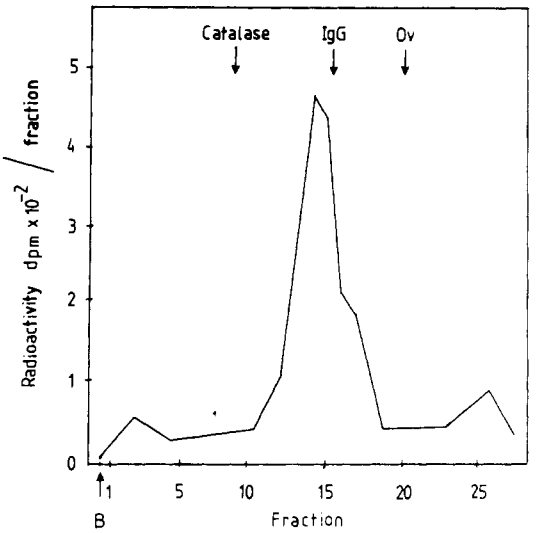

FIGURE 4: Glycerol density gradient centrifugation of antibody–molybdate-stabilized GR complex after removal of molybdate. Following gel filtration of the antibody–molybdate-stabilized GR complex on Sephacryl S-400, the fraction containing the highest amount of radioactivity (fraction 38, Figure 1) was chromatographed on a Sephadex G-25 column in the absence of sodium molybdate in order to remove the molybdate. Following chromatography, 0.3 mL of the void volume was analyzed by glycerol density gradient centrifugation as described in the legend to Figure 2 and under Materials and Methods. "B" represents the amount of radioactivity adhered to the bottom of the centrifuge tube. The sedimentation coefficients for the reference proteins are given under Materials and Methods.

(Table I). Comparison of the relative molecular mass of molybdate-stabilized GR in the presence of anti-GR antibody (456K) with the relative molecular mass in the absence of anti-GR antibody (302K) indicates that only one monoclonal antibody molecule bound to the molybdate-stabilized GR complex.

Second Antibody Immunoprecipitation Assay. The ability of the monoclonal antibody to react with the monomeric steroid-binding GR molecule (M_r 94K) compared to molybdate-stabilized GR complex was also investigated with a second antibody immunoprecipitation assay, under conditions of both excess antibody and excess GR, respectively (Figure 3). The results showed that the same amount of radioactivity could be precipitated with a defined antibody concentration, whether the receptor was present in a molybdate-stabilized GR complex or not. Since the same amount of radioactivity was precipitated at the same antibody concentration independently of the oligomeric organization of the receptor, this result is in line with the contention of a single steroid-binding protein in the molybdate-stabilized complex. This is based on the concept that the binding of a single antibody molecule to a complex is sufficient to precipitate the whole complex in the second antibody immunoprecipitation assay.

Exclusion of Steric Hindrance in Antibody Binding to the Molybdate-Stabilized GR Complex. In order to exclude the possibility that the inability of more than one antibody molecule to bind to the molybdate-stabilized GR complex could be due to steric hindrance or to concealment of antigenic determinants in the complex, the following experiment was performed. The molybdate-stabilized GR complex was incubated with monoclonal anti-GR antibody and analyzed on Sephacryl S-400 in the presence of 0.15 M NaCl and 20 mM sodium molybdate. As described above, this antibody–molybdate-stabilized GR complex eluted with an R_S of 9.7 nm, separated from free antibody and molybdate-stabilized GR complex, respectively. The fraction containing the highest amount of radioactivity (fraction 38, Figure 1), which did not contain any free antibody, was chromatographed on a Sephadex G-25 column equilibrated in EPG with 0.15 M NaCl without sodium molybdate in order to remove the molybdate. The removal of molybdate enables the GR to become activated (Leach et al., 1979). Furthermore, chromatography of GR on Sephadex G-25 in the absence of sodium molybdate activates GR (Litwack et al., 1980). Activated GR exists as a monomer with an M_r of 94K (Wrange et al., 1984). The void volume of the Sephadex G-25 column containing the excluded proteins ($M_r \geq 5000$) was then analyzed by glycerol density gradient centrifugation under isotonic conditions in the absence of sodium molybdate. If the molybdate-stabilized GR complex contains several steroid-binding 94K molecules and only one of these binds an antibody molecule due to steric hindrance, chromatography on Sephadex G-25 in the absence of sodium molybdate will cause the molybdate-stabilized GR complex to dissociate into a radioactive antibody–GR complex sedimenting at 8.0 S as well as to GR molecule(s) free from antibody and sedimenting at 4.4 S (cf. above). In contrast, a molybdate-stabilized GR heteromer consisting of a single 94K steroid-binding molecule will give rise to a single 8.0S radioactive peak, corresponding to the antibody–GR complex, when analyzed by density gradient centrifugation. The result of the experiment (Figure 4) was in line with the latter alternative; i.e., a single 8S radioactive peak was obtained suggesting the presence of a single 94K steroid-binding molecule in the molybdate-stabilized GR complex. Furthermore, binding of an antibody molecule to nonliganded GR in 0.15 M NaCl, i.e., monomeric 94K GR molecules (Radojcic

et al., 1985), did not inhibit the formation of the molybdate-stabilized GR complex (data not shown).

Discussion

The observation that molybdate stabilizes an oligomeric nonactivated complex of GR has stimulated the interest to explore the supramolecular organization of nonactivated GR. However, only a few research groups have been able to address this issue. In the present investigation we have approached this problem by utilizing monoclonal antibodies that have been raised against the steroid-binding 94K rat hepatic GR (Okret et al., 1984) and that form stoichiometrically well-defined complexes with GR. On the basis of the results obtained, we suggest that the molybdate-stabilized GR complex is a heteromer consisting of a single steroid-binding 94K moiety.

The basic requirement for concluding that one single steroid-binding moiety is present in the molybdate-stabilized GR complex is the exclusion of the possibility that steric hindrance prevents more than one antibody binding to the molybdate-stabilized GR complex. This was performed by dissociating the antibody–molybdate-stabilized GR complex into its subunit components by gel filtration in the absence of molybdate followed by sedimentation analysis. This revealed that all radioactivity sedimented as a single 8S antibody–GR complex. No steroid-binding GR, to which no antibody had bound, was detected. This excluded the possibility of steric hindrance. This conclusion was also supported by the second antibody immunoprecipitation assay, which showed linear and parallel receptor amounts precipitated in the presence or absence of molybdate under conditions of both antibody excess and GR excess, respectively. The experiment also suggests that antibody binding to the molybdate-stabilized GR complex does not cause a change in the composition of the molybdate-stabilized GR complex. Furthermore, preformation of antibody–GR complex prior to addition of molybdate did not inhibit the formation of the molybdate-stabilized GR complex. This was possible to demonstrate since nonliganded (=nonactivated) GR exists as a monomer under isotonic conditions after gel filtration in the absence of molybdate (Radojcic et al., 1985).

The determination of relative molecular mass performed here by gel filtration and sedimentation analysis appears to be very accurate in relation to the theoretically calculated relative molecular mass for the respective component. This could be demonstrated by comparing the relative molecular mass value calculated by these techniques, for the GR and the antibody–GR complex to the relative molecular mass values of purified antibody and GR, respectively, obtained by SDS–PAGE [see Results and Wrange et al. (1984)]. By use of a gel-filtration matrix (Sephacryl S-400) allowing separation of molecules in a range up to M_r 8×10^6, true R_S values should be obtained also for analysis of antibody–GR complexes in the presence of molybdate. On the basis of these considerations and the results obtained, we believe that it is warranted to conclude that a single steroid-binding 94K GR molecule is present in the molybdate-stabilized GR complex.

The presence of nonligand-binding component(s) of the molybdate-stabilized GR complex is also supported by an indirect observation by Joab et al. (1984), who raised a monoclonal antibody against the chick molybdate-stabilized progesterone receptor complex. This monoclonal antibody cross-reacted with 8S molybdate-stabilized nonactivated forms of chick glucocorticoid, estrogen, and androgen receptors but not with the activated steroid-binding 4S molecules of these receptors. This excluded the presence of identical steroid-binding subunits in the molybdate-stabilized steroid receptor complexes. The monoclonal antibody of Joab et al. seemed to recognize a nonhormone binding protein common to several molybdate-stabilized steroid receptors but only present in the molybdate-stabilized 8S forms. This protein recognized by the monoclonal antibody was shown to have an M_r of ~90K. Birnbaumer et al. (1984) showed that such a protein is present in molybdate-stabilized progesterone receptor preparations from chick tissue purified by ligand affinity chromatography. Furthermore, Grandics et al. (1984), who partially purified nonactivated rat hepatic GR also using ligand affinity chromatography in the presence of sodium molybdate, observed a few smaller receptor-specific bands in addition to a major 90K GR protein, when analyzing the purified material under denaturing conditions by SDS–PAGE. Due to the presence of several, possibly proteolytic, receptor-specific bands, no definitive conclusion could be drawn concerning the subunit composition of nonactivated GR. In contrast, Raaka & Samuels (1983) suggested a homomeric composition of the nonactivated molybdate-stabilized GR complex. By the use of dense amino acid labeling of whole GH_1 cells, a model with an equilibrium between the 4S and 10S GR, where the 10S GR is a tetramer of homologous 4S subunits, was proposed.

A GR complex has also been detected in the absence of molybdate when purified activated GR has been studied by electron microscopy (Payvar et al., 1983) and density gradient centrifugation (Ö. Wrange and J. Carlstedt-Duke, unpublished observations). No information concerning the subunit composition of this complex is available at present. However, similarities do not necessarily exist between oligomeric forms of purified GR and molybdate-stabilized GR complex, respectively.

Acknowledgments

We thank Dr. Lorenz Poellinger for fruitful discussions, Kenneth Spetz for excellent technical assistance, and Leslie Morgan for typing the manuscript.

Registry No. Molybdate, 14259-85-9.

References

Atger, M., & Milgrom, E. (1976) *J. Biol. Chem. 251*, 4758–4762.

Birnbaumer, M., Bell, R. C., Schrader, W. T., & O'Malley, B. W. (1984) *J. Biol. Chem. 259*, 1091–1098.

Carlstedt-Duke, J., Gustafsson, J.-Å., & Wrange, Ö. (1977) *Biochim. Biophys. Acta 497*, 507–524.

Chong, M. T., & Lippman, M. E. (1981) *J. Recept. Res. 2*, 575–600.

Edelhoch, H. (1960) *J. Biol. Chem. 235*, 1326–1334.

Grandics, P., Miller, A., Schmidt, T. J., Miltman, D., & Litwack, G. (1984) *J. Biol. Chem. 259*, 3173–3180.

Holbrook, N. J., Bodwell, J. E., Jeffries, M., & Munck, A. (1983) *J. Biol. Chem. 258*, 6477–6485.

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M.-G., Binart, N., Mester, J., & Baulieu, E.-E. (1984) *Nature (London) 308*, 850–853.

Leach, K. L., Dahmer, M. K., Hammond, N. D., Sando, J. J., & Pratt, W. B. (1979) *J. Biol. Chem. 254*, 11884–11890.

Litwack, G., Schmidt, T. J., Markovic, R. D., Eisen, H. J. Barnett, C. A., DiSobro, D. M., & Phelps, D. S. (1980) in *Perspectives in Steroid Receptor Research* (Bresciani, F., Ed.) pp 113–131, Raven Press, New York.

Lowry, O. H., Rosebrough, N. J., Fall, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.

Milgrom, E., Atger, M., & Baulieu, E. E. (1973) *Biochemistry 12*, 5198-5205.
Nishigori, H., & Toft, D. (1980) *Biochemistry 19*, 77-83.
Noma, K., Nakao, K., Sato, B., Nishizaha, Y., Matsumoto, K., & Yamamura, Y. (1980) *Endocrinology (Baltimore) 107*, 1205-1211.
Okret, S. (1983) *J. Steroid Biochem. 19*, 1241-1248.
Okret, S., Carlstedt-Duke, J., Wrange, Ö., Carlström, K., & Gustafsson, J.-Å. (1981) *Biochim. Biophys. Acta 677*, 205-219.
Okret, S., Wikström, A.-C., Wrange, Ö., Andersson, B., & Gustafsson, J.-Å. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1609-1613.
Payvar, F., DeFranco, D., Firestone, G. L., Edgar, B., Wrange, Ö., Okret, S., Gustafsson, J.-Å., & Yamamoto, K. R. (1983) *Cell (Cambridge, Mass.) 35*, 381-392.
Raaka, G. A., & Samuels, H. H. (1983) *J. Biol. Chem. 258*, 417-425.
Radojcic, M., Okret, S., Wrange, Ö., & Gustafsson, J.-Å. (1985) *J. Steroid Biochem. 23*, 1-8.
Rice, R. H., & Means, G. E. (1971) *J. Biol. Chem. 246*, 831-832.
Sando, J. J., LaForest, A. C., & Pratt, W. B. (1979) *J. Biol. Chem. 254*, 4772-4778.
Scheidereit, C., Geisse, S., Westphal, H. M., & Beato, M. (1983) *Nature (London) 304*, 749-752.
Sherman, M. R., Tuazon, F. B., & Miller, L. K. (1980) *Endocrinology (Baltimore) 106*, 1715-1727.
Sherman, M. R., Moran, M. C., Neal, R. G., Niu, E.-M., & Tuazon, F. B. (1982) in *Progress in Research and Clinical Applications of Corticosteroids* (Lee, H. J., & Fitzgerald, T. J., Eds.) pp 45-66, Heyden, Philadelphia.
Sherman, M. R., Moran, M. C., Tuazon, F. B., & Stevens, Y.-W. (1983) *J. Biol. Chem. 258*, 10366-10377.
Shulman, M., Wilde, C. D., & Köhler, G. (1978) *Nature (London) 276*, 269-270.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346-362.
Sober, H. A., Ed. (1970) *Handbook of Biochemistry. Selected Data for Molecular Biology*, Chemical Rubber Co., Cleveland, OH.
Vedeckis, W. V. (1983) *Biochemistry 22*, 1983-1989.
Wrange, Ö., Okret, S., Radojcic, M., Carlstedt-Duke, J., & Gustafsson, J.-Å. (1984) *J. Biol. Chem. 259*, 4534-4544.

Immunological Evidence That the Nonhormone Binding Component of Avian Steroid Receptors Exists in a Wide Range of Tissues and Species†

Robert M. Riehl,* William P. Sullivan, Benjamin T. Vroman, Vickie J. Bauer, Gary R. Pearson,‡ and David O. Toft
Department of Cell Biology, Mayo Medical School, Rochester, Minnesota 55905
Received March 21, 1985

ABSTRACT: A monoclonal antibody to a fungal protein has been used to demonstrate the presence of the nonhormone binding component of molybdate-stabilized steroid receptors in a variety of vertebrate tissues. We recently identified a steroid receptor in the aquatic fungus *Achlya ambisexualis* where sexual morphogenesis of the male is directed by the steroid antheridiol. This receptor resembles receptors of higher organisms in exhibiting an 8S, molybdate-stabilized form. In the chick oviduct, a 90 000 molecular weight protein has previously been shown to be associated with the molybdate-stabilized complex of the progesterone receptor. We have isolated a similar protein of molecular weight about 88 000 from *A. ambisexualis* and have obtained a hybridomal-derived monoclonal antibody directed against it. This mouse anti-*Achlya* immunoglobulin G_1 (IgG_1) cross-reacts with the 90 000 molecular weight protein in chick oviduct cytosol and was used to detect analogous 90 000 molecular weight proteins in mammalian tissues. Tissue cytosols were incubated with antibody, and the complexes were isolated onto protein A–Sepharose. The resin-bound proteins were then analyzed by gel electrophoresis. This procedure revealed the presence of 90 000 molecular weight proteins in several mammalian tissues including rat liver, mouse liver and uterus, pig ovarian granulosa cells, human endometrium, and HeLa cells. These results demonstrate that the 90 000 molecular weight protein is not peculiar to the chick oviduct but is present in several different tissues from a variety of animals. This antibody should be a useful probe for further studies on the biological role of these proteins.

In the water mold *Achlya*, male sexual differentiation and morphogenesis are induced by the steroid pheromone antheridiol that is released from the female cell (Horgen, 1981). A cytosolic protein found in male, but not female, cells of *Achlya ambisexualis* has been identified (Riehl et al., 1984) that probably represents the steroid receptor protein among these primitive eukaryotes. The antheridiol receptor has biochemical properties that are similar to those of higher organisms. Notably, the antheridiol binding activity that is stabilized in the presence of sodium molybdate and low ionic strength is associated with a 192 000 molecular weight protein complex that has a sedimentation coefficient of 8.3 S but which, under conditions of high ionic strength, appears as a 4S form (Riehl & Toft, 1984).

The molybdate-stabilized forms of the progestin (Dougherty et al., 1984; Joab et al., 1984), estrogen, androgen, and glucocorticoid receptors (Joab et al., 1984) within the chick oviduct have been shown by immunological and biochemical

† These studies were supported by NIH Grants AM 25625 and HD 9140.
* Author to whom correspondence should be addressed.
‡ Present address: Department of Microbiology, Georgetown University Medical Center, Washington, DC 20007.

0006-2960/85/0424-6586$01.50/0