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Isolation of Steroid Receptor Binding Protein from Chicken Oviduct and Production of Monoclonal Antibodies†

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ABSTRACT: Previous studies have shown that the molybdate-stabilized progesterone receptor from the chick oviduct contains a nonhormone binding component with a molecular weight of 90 000. This protein has also been shown to be associated with some other molybdate-stabilized steroid receptors of the oviduct. In order to access this larger pool of the receptor binding protein, we have developed an isolation procedure based on the observation that the protein is selectively shed from proteins adsorbed to heparin–agarose when molybdate is removed. The protein obtained by this procedure is shown to be the same as that isolated from affinity-purified progesterone receptor as compared by protease digestion and one-dimensional peptide mapping. Four immunoglobulin G secreting hybridoma cell lines were generated against the 90 000-dalton antigen. All of the antibodies recognize the 90 000-dalton protein obtained by electrophoretic transfer from sodium dodecyl sulfate–polyacrylamide gels. In addition, two of the antibodies complex the molybdate-stabilized progesterone receptor as demonstrated by sedimentation analysis on sucrose gradients. One of these antibodies was used to show the presence of the 90 000-dalton component in molybdate-stabilized glucocorticoid and androgen receptors and also to show its presence in brain, liver, and skeletal muscle, but not in serum.

Although the progesterone receptor from the avian oviduct has been studied extensively, the composition and structure of this protein have not yet been clearly defined. Early studies revealed two monomeric 4S receptor forms that have been termed receptors A and B (Schrader & O'Malley, 1972). These contain similar hormone binding sites but differ somewhat in size; the molecular weight of A is between 75 000 and 80 000, and that of B is about 110 000. More recent studies have shown that the large 8S, molybdate-stabilized, receptor also exists in two forms termed I and II (Dougherty & Toft, 1982). In this case, receptor I contains the A hormone binding component, and receptor II contains the B component (Dougherty et al., 1984). In addition, both 8S receptor forms contain a 90 000-dalton protein (90K protein)[1] that does not bind hormone (Dougherty et al., 1984; Renoir et al., 1984b).

The biological significance of the 90K protein is unknown. There is evidence that it exists not only as a component of the 8S progesterone receptor but also as a component of the ag-

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[1] Abbreviations: 90K protein, receptor binding protein of M_r 90 000; DOC–Sepharose, deoxycorticosterone–Sepharose; Mo, molybdate; Cl_3C-COOH, trichloroacetic acid; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum.

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gregate or molybdate-stabilized forms of the estrogen, androgen, and glucocorticoid receptors from the chicken oviduct (Joab et al., 1984).

To facilitate investigations on the 90K receptor binding protein, we have developed a rapid and convenient method for its isolation. We have also prepared four monoclonal antibodies for use in its characterization.

Experimental Procedures

Purification of Progesterone Receptor. Progesterone receptor purification by affinity chromatography has been described in detail by Puri et al. (1982). Briefly, 10 g of oviduct tissue was homogenized at 0–4 °C in 3 volumes of 50 mM potassium phosphate, 10 mM sodium molybdate, and 10 mM thioglycerol, adjusted to pH 7.0 at 23 °C (+Mo buffer). Cytosol containing 2 μM unlabeled cortisol was incubated with $^1/_6$th volume of affinity resin (deoxycorticosterone-derivatized Sepharose, DOC–Sepharose, G and K Biochemicals, Sommerset, NJ). After 2 h at 0–4 °C, the affinity resin was washed as described by Puri et al. (1982) and suspended in an equal volume of elution buffer (10 mM potassium phosphate, 10 mM sodium molybdate, 10 mM thioglycerol, and 10% glycerol, pH 7.0) containing 6 μM [^{3}H]progesterone (5 Ci/mmol) and incubated overnight at 0–4 °C. The eluate was run onto a heparin–agarose column (1 mL of gel/10 g of oviduct tissue, P-L Biochemicals) which was washed with 40 volumes of elution buffer and eluted with a 40-mL gradient of 0–500 mM KCl in elution buffer. A single peak of receptor was eluted at about 200 mM KCl. The proteins were separated by SDS–polyacrylamide gel electrophoresis, and the 90K protein was excised and used for protease digestion studies.

For some studies, the receptor purified by DOC–Sepharose chromatography was applied to two heparin–agarose columns. These were eluted in the presence or absence of molybdate as described in the text and figure legends.

Purification of 90K Protein. Oviducts from chicks stimulated with diethylstilbestrol for 2–4 weeks were homogenized in 4 volumes of +Mo buffer. The homogenate was centrifuged at 23000*g* for 5 min. The resulting supernatant was centrifuged at 110000*g* for 60 min, and lipids were aspirated. The clear supernatant (cytosol) was mixed with 0.1 volume of phosphocellulose (Whatman) which had been equilibrated with +Mo buffer and stirred on ice for 2 min. The phosphocellulose was removed by vacuum filtration.

Heparin–agarose (1 mL/g of oviduct tissue) equilibrated with +Mo buffer was added to the filtrate and stirred on ice for 20 min. The heparin–agarose was removed from the slurry and washed with 20 volumes of +Mo buffer aided by vacuum filtration. The gel was transferred to a 2.4-cm diameter column with an equal volume of +Mo buffer. The column was washed with 3 gel volumes of 50 mM potassium phosphate and 10 mM thioglycerol, pH 7.0 (–Mo buffer), at 1 mL/min. Buffer flow was stopped for 20 min. The 90K protein was eluted with an additional 3 gel volumes of –Mo buffer.

The heparin–agarose eluate was mixed with DEAE-A25 (0.3 mL/g of oviduct tissue, Pharmacia), equilibrated with –Mo buffer, and stirred for 20 min on ice. The DEAE-A25 was pelleted by centrifugation, the supernatant decanted, and the gel washed twice with 20 gel volumes of –Mo buffer. The gel was transferred to a 1-cm diameter column with an equal volume of –Mo buffer. The column was washed with 10 gel volumes of –Mo buffer plus 150 mM KCl. The 90K protein was eluted with 10 gel volumes of –Mo buffer plus 300 mM KCl, and 2-mL fractions were collected. The optical density at 280 nm was determined for each fraction, and the peak fractions were pooled.

Hydroxylapatite (1 mL/10 g of oviduct tissue, Bio-Rad) was equilibrated with 10 mM potassium phosphate and 10 mM thioglycerol, pH 7.0. Pooled fractions from the DEAE eluate were loaded onto the hydroxylapatite column at 0.5 mL/min. The column was washed with 20 volumes of 100 mM potassium phosphate and 10 mM thioglycerol, pH 7.0. The 90K protein was eluted with 10 bed volumes of 250 mM potassium phosphate and 10 mM thioglycerol, pH 7.0, and 1-mL fractions were collected. The optical density at 280 nm was determined, and peak fractions were pooled.

Electrophoresis. Following the addition of 5 μg of ovalbumin as carrier protein, samples were precipitated with Cl_3CCOOH at 20% (w/v) on ice for 30 min. The precipitate was pelleted by centrifugation at 6300*g* for 30 min. The pellet was washed twice with 2 mL of ether–ethanol (1:1 v/v). Samples were redissolved in 60 μL of 0.25 M Tris, 2% SDS (w/v), 0.05% Bromphenol blue, 20% glycerol (v/v), and 4% mercaptoethanol (v/v), pH 6.8 (SDS sample buffer). Samples were run on discontinuous polyacrylamide gels (3% stacking gel, 7.5% separating gel) under denaturing conditions as described by Laemmli (1970). Gels were stained with 0.1% Coomassie blue R-250 in 50% methanol and 10% acetic acid for 1 h and destained with 25% methanol and 10% acetic acid overnight.

Densitometry of Protein Samples. A 7.5% polyacrylamide gel was cast on Gelbond PAG (FMC Corp.) according to manufacturer's instructions. Acrylaide (1%) was used as the cross-linking agent to ensure bonding to the derivatized backing. Protein samples were concentrated, and electrophoresis was performed as above. Staining with Coomassie blue R-250 and destaining reagents were the same as noted, but incubation times were increased to compensate for decreased surface areas available for diffusion. In addition, the gel was incubated for 2 h in 5% glycerol and 5% acetic acid and then dried overnight at 37 °C.

Using a Cliniscan densitometer (Helena), the protein lanes were scanned at 645 nm, and the fractional percent of 90K protein in the lane was determined.

Peptide Mapping. Affinity-purified progesterone receptor and 90K protein (10 μg of each) were subjected to electrophoresis under denaturing conditions as described above. After electrophoresis, the gels were fixed, stained for 30 min in 0.25% Coomassie blue, 40% methanol, and 10% acetic acid, and then destained for 1 h or less. The protein bands at 90 kDa were cut out and trimmed to a width of 5 mm. Analysis of the proteins by limited proteolytic digestion was performed according to Cleveland et al. (1977). The 90K protein slices were equilibrated for 30 min in 10 mL of 0.125 M Tris-HCl and 0.1% SDS, pH 6.8. Digestion with *Staphylococcus aureus* V8 protease or α-chymotrypsin occurred in the stacking gel during electrophoresis. To obtain a reasonable level of digestion, the stacking gel was cast to a length of 5 cm. To accommodate the size and range of the generated peptides, a 12% separating gel was cast to a length of 18 cm. The sample wells were filled with the Laemmli running buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 6.8) and the gel pieces pushed to the bottom. Gel pieces were overlayed with 10 μL of 0.125 M Tris-HCl and 0.1% SDS, pH 6.8, containing 20% glycerol. *Staphylococcus aureus* V8 protease at the concentrations indicated in the figure legends was dissolved in 0.125 M Tris-HCl and 0.1% SDS, pH 6.8, containing 10% glycerol and 10 μL added to the wells. Electrophoresis was started at 30-mA constant current at 22 °C and continued until the dye front neared the separating gel. The current was switched off for 30 min. Electrophoresis resumed

at 130-V constant voltage at 0–4 °C and continued overnight. The gel was stained in 0.1% Coomassie blue, 50% methanol, and 10% acetic acid for 1 h and destained overnight in 25% methanol and 10% acetic acid, and silver stained according to the method of Morrissey (1981).

Monoclonal Antibody Production. To prepare antigen, the 90K protein was purified as described above, through the DEAE-Sephadex step. It was dialyzed against 250 volumes of 0.1 M ammonium bicarbonate and 0.1 M EDTA, pH 7.4, for 2 h and then against 250 volumes of distilled water for 4 h and lyophilized. Initially, the protein was dissolved in phosphate (5 mM) buffered saline, pH 7.0, but later was found to be more readily soluble in 5 mM Tris and 10% glycerol, pH 7.4. The first inoculum, containing 30–50 μg of 90K protein in 150 μL and an equal volume of complete Freunds adjuvant, was injected intraperitoneally into a Balb/c mouse (Jackson Laboratories). After 3–5 weeks, the mouse was injected as before except that incomplete Freunds adjuvant was used. After 6 weeks, the mouse was bled and the serum screened for the presence of antibody with ELISA. One week later, the mouse was given a final injection of antigen without adjuvant. Three days following the final injection, the mouse was killed and the spleen aseptically removed.

Hybridomas were prepared by fusion with the mouse plasmacytoma cell line P3NS-11-AG4-1 (NS-1) as previously described in detail (Vroman et al., 1985). Briefly, (8–10) × 10^7 spleen cells were fused with an equal number of NS-1 cells in the presence of 0.5 mL of 50% poly(ethylene glycol) 4000 for 2 min at 23 °C. The fusogen was diluted at 1-min intervals with 0.5-mL aliquots of serum-free medium. After the addition of four aliquots, the cells were diluted to 15 mL with serum-free medium and centrifuged. The cell pellet was resuspended to a concentration of (1–2) × 10^6 cells/mL in HAT medium (0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine in RPMI 1640 medium supplemented with 4.5 g of glucose/L, 10 mL of 200 mM glutamine, 10% FCS, 10 nM Na_2SeO_3, and 10% NCTC 135). Aliquots (0.1 mL/well) were plated into 96-well microtiter plates with mouse peritoneal macrophages added as feeder cells. Cultures were fed every 3 days with HAT medium and monitored for growing colonies. The conditioned media were tested for antibodies against the 90K protein by ELISA, and positive colonies were recloned at 0.5 and 1 cell/well by limiting dilution. After being screened, the antibody-positive clones were grown in Falcon flasks (75 cm^2) and injected into mice primed with 0.3 mL of pristane (2,6,10,14-tetramethylpentadecane) for the production of antibody containing ascitic fluid.

Ascitic fluid was clarified by centrifugation at 84000*g* for 30 min. To purify antibodies from ascites fluid, proteins precipitating from the supernatant between 30% and 50% ammonium sulfate were dialyzed against 1 L of 5 mM Tris, pH 7.5, overnight. The dialysate was loaded onto a DE-52 column (1 mL of gel/10 mg of protein, Whatman) and the column washed with 5 mM Tris, pH 7.5, until the absorbance at 280 nm was less than 0.05. The column was developed with a 0–100 mM NaCl gradient in 5 mM Tris, pH 7.5, and 2- mL fractions were collected. On the basis of the absorbance at 280 nm, fractions were pooled and tested for the presence of antibody to the 90-kDa protein by ELISA. The antibodies were isotyped by ELISA using affinity-purified antibodies to each immunoglobin subclass (from Southern Biotechnology Associates).

ELISA. The ELISA methods used were reported by Luka et al. (1984). DEAE-purified 90K protein was diluted 50-fold in 0.5 M sodium carbonate buffer, pH 9.5, and plated onto 96-well polystyrene microtiter plates (Linbro, Flow Laboratories) at 100 μL/well. After overnight incubation at 4 °C, the plates were washed twice in 20 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5 (ELISA buffer), containing 0.1% (w/v) ovalbumin or bovine serum albumin. The plates were held at 22 °C for 1 h and stored dry at 4 °C for up to 3 weeks. Prior to assay, a plate was washed twice with ELISA buffer containing 0.1% ovalbumin. For screening of hybridoma cultures, 50 μL of culture supernatant was added per well and allowed to incubate at 22 °C for 1 h. The plate was washed 4 times with ELISA buffer plus ovalbumin, and alkaline phosphatase conjugated anti-mouse IgG (Sigma) diluted 1:400 in the same buffer was added to 100 μL/well and allowed to bind for 15 min at 22 °C. After four washes with ELISA buffer plus ovalbumin, dinitrophenyl phosphate (Sigma) at 1.25 mg/mL in 1 M diethanolamine, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$, pH 9.8, was added at 100 μL/well. Absorbances were read at 410 nm by a Microplate MR600 reader (Dynatech).

Immunostaining. Following electrophoresis, as described above, the proteins were transferred to nitrocellulose by the Western blotting method (Burnette, 1981). Essentially the unstained gel was sandwiched between nitrocellulose paper (0.45 μm, Schleicher & Schuell) and Whatman 3 chromatography paper. The sandwich was assembled into the electrophoretic transfer apparatus (Transblot, Bio-Rad) containing 0.025 M Tris, 0.192 M glycine, and 20% methanol (v/v) at 4 °C. Proteins were transferred from the gel to the nitrocellulose paper at 200-mA constant current for 1 h at 4 °C. Following transfer, the proteins were visualized by staining sections of the nitrocellulose paper in ELISA buffer containing 0.1% (v/v) drawing ink (Hancock & Tsang, 1983). The remaining sections of the nitrocellulose paper were soaked for 1 h at 22 °C in buffer containing 0.02 M Tris, 0.15 M sodium chloride, 0.5% Tween 20 (v/v), and 0.1% bovine serum albumin (w/v), pH 7.4 (Western buffer), to block the remaining protein binding sites. The blocked nitrocellulose paper could be stored for several weeks at 20 °C. As needed, 3-mm-wide strips were cut from the nitrocellulose paper and incubated for 4 h at 22 °C with 4 mL of Western buffer containing 40 μg of antibody. The strips were washed in Western buffer and incubated in this buffer containing alkaline phosphatase conjugated rabbit anti-mouse IgG antibody. After being further washed in Western buffer, the 90K protein–antibody complexes were visualized with Fast Violet V, naphthol ASB1 in 0.1 M Tris, and 0.001 M magnesium chloride, pH 9.5. Negative control strips were not incubated with the first antibody but were carried through the remaining procedures as described above. Additional controls (not illustrated) included the use of an unrelated monoclonal antibody which did not react with 90K protein in this test and, also, the use of alkaline phosphatase conjugated goat anti-mouse IgG which provided results comparable to those with the rabbit antibody.

Sucrose Gradients. Chick oviduct cytosol was prepared as described above, and samples were incubated for 1 h at 0–4 °C with radiolabeled and unlabeled steroids as indicated in the figure legends. Aliquots (50 μL) of labeled cytosol were then incubated for 4 h with buffer (control) or antibody (10–40 μg) in a final volume of 200 μL. Aliquots (100 μL) were layered onto 5–20% linear sucrose gradients (Dougherty & Toft, 1982) containing homogenizatin buffer. After centrifugation for 16 h at 105000*g*, the gradients were fractionated into four-drop fractions, and 5 mL of scintillation cocktail (toluene and Fisher Scintiprep I, 95:4 v/v) was added to each fraction prior to counting. Ovalbumin (3.7 S) labeled with

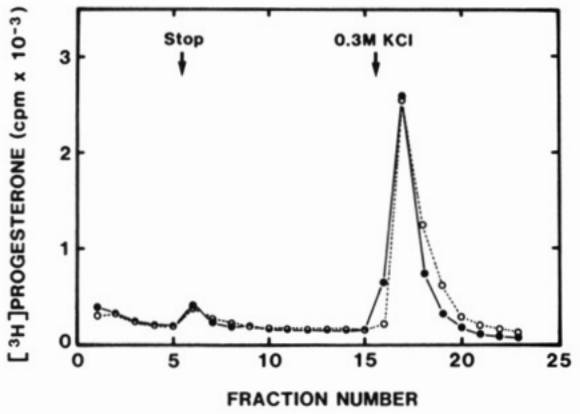

FIGURE 1: Molybdate-dependent heparin–agarose chromatography of progesterone receptor. Progesterone receptor purified by affinity chromatography on DOC–Sepharose was loaded equally onto two identical 1.0-mL heparin–agarose columns equilibrated in phosphate buffer (10 mM potassium phosphate and 10 mM thioglycerol, pH 7.0) plus 10 mM sodium molybdate. The columns were washed with 40 mL of phosphate buffer containing 0.12 M KCl and then further washed with 10 mL of phosphate buffer containing 0.12 M KCl plus (●) or minus (○) 10 mM sodium molybdate at 1 mL/min. Buffer flow was stopped for 20 min to allow further equilibration with buffer followed by a 20-mL wash with the same buffer solution. The columns were finally eluted with phosphate buffer containing 0.3 M KCl plus or minus 10 mM sodium molybdate at 0.5 mL/min. Two-milliliter fractions were collected throughout. Radioactivity was measured in aliquots (25 μL) from each fraction. The remaining volume of each wash fraction (1.975 mL) and the three peak fractions (pooled) from 0.3 M KCl elution were precipitated with Cl_3CCOOH.

^{14}C (Rice & Means, 1971) was used as sedimentation marker. Variations from the above are indicated in the figure legends.

Antigen Detection in Various Tissues. Cytosol extracts were obtained from chick oviducts, breast muscle, liver, and brain as described above. The tissues were homogenized in 4 volumes of 50 mM Tris and 10 mM thioglycerol, pH 8. Serum was also obtained by centrifugation of clotted blood and was diluted with an equal volume of the above buffer.

One-milliliter samples of cytosol or serum were incubated for 2 h in ice with 100 μg of antibody 4F3. Swollen protein A–Sepharose CL-4B (Pharmacia) (12 mg dry weight) was added for an additional 1 h in ice. The resin was pelleted by centrifugation and washed by suspension and centrifugation twice with homogenization buffer, twice with this buffer plus 0.3 M KCl, and then twice with homogenization buffer (2-mL volumes). The final pellet was extracted with an equal volume of 2× concentrated SDS sample buffer and the extract analyzed by electrophoresis.

In some experiments, the above fractionation method was modified by adsorption of antibody complexes to anti-mouse IgG–agarose (Sigma) rather than to protein A–Sepharose.

RESULTS

Molybdate-Dependent Fractionation of 90K Protein. The fractionation procedure described here is based on the observation that when the 90 k protein is dissociated from the 8S progesterone receptor complex it binds weakly, if at all, to heparin–agarose. In contrast, this resin is very effective in binding the 8S receptor forms as well as the A and B, 4S, progesterone receptors (Dougherty et al., 1984; Yang et al., 1982). It was, therefore, possible to adsorb the molybdate-stablized receptor to heparin–agarose and to then release the 90K protein by disruption of the 8S complex after the removal of molybdate. An experiment of this type is illustrated in Figures 1 and 2. The 8S progesterone receptor was first purified by affinity chromatography on deoxycorticosterone–Sepharose (Puri et al., 1982). It was then divided into two

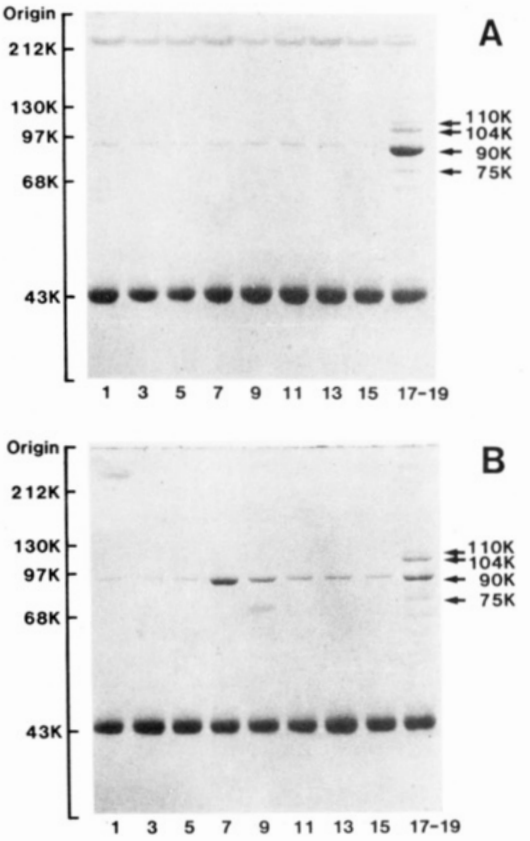

FIGURE 2: Effect of molybdate on heparin–agarose chromatography of progesterone receptor. Precipitated column fractions from Figure 1, plus sodium molybdate (A) and minus sodium molybdate (B), were electrophoresed on 7.5% SDS–polyacrylamide gels and stained with 0.1% Coomassie blue R-250. Ovalbumin (M_r 43 000) was added to all fractions as carrier before precipitation. Note that the stained bands in the 212K region represent an artifact caused by the presence of sodium molybdate. The number below each lane corresponds to the fraction number in Figure 1. The molecular weight standards were myosin (M_r 212 000), β-galactosidase (M_r 130 000), phosphorylase *b* (M_r 97 000), bovine serum albumin (M_r 68 000), and ovalbumin (M_r 43 000).

equal portions which were loaded onto two identical columns of heparin–agarose. Both columns were washed extensively in buffer containing molybdate and 0.12 M KCl to remove impurities and unbound hormone. They were then eluted with buffer containing 0.12 M KCl, with or without molybdate. After five fractions were collected, the columns were stopped for 20 min to allow further equilibration with the elution buffer, and then elution was continued for 10 more fractions. Finally, the columns were eluted with 0.3 M KCl. With both columns, the hormone binding components were eluted with the 0.3 M KCl step (Figure 1). The column fractions were analyzed by SDS–polyacrylamide gel electrophoresis to determine the elution of the 90K protein (Figure 2A,B). With the column maintained in molybdate buffers, very little protein eluted in the 0.12 M KCl step, and the 0.3 M KCl peak of receptor showed a polypeptide pattern as described previously (Dougherty et al., 1984). The 90K protein was predominant with smaller amounts of the A and B receptor proteins plus a 104 000-dalton contaminating protein. However, in the absence of molybdate (Figure 2B), approximately 50% of the 90K protein was eluted in the 0.12 M KCl step in essentially pure form. Thus, it appears that the 90K protein is retained on heparin–agarose by its molybdate-stabilized association with the A and B receptor components.

With the fractionation illustrated in Figure 2B, a portion of the 90K protein remained bound to heparin–agarose until

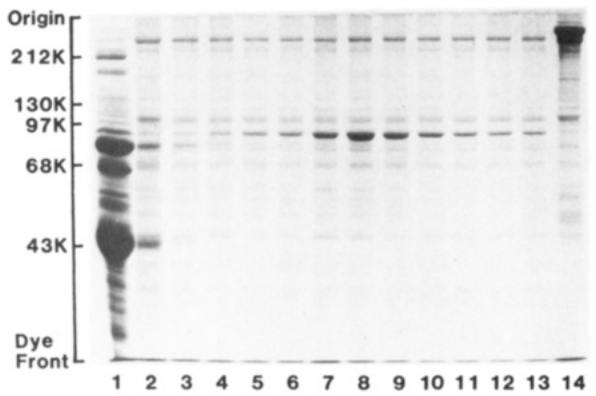

FIGURE 3: Electrophoresis of proteins released from heparin–agarose in buffer without sodium molybdate. Cytosol (25 mL) prepared with +Mo buffer and treated with phosphocellulose (see Experimental Procedures) was applied to a 10-mL heparin–agarose column, and 2-mL fractions were collected throughout. The column was washed with 200 mL of +Mo buffer. Buffer without sodium molybdate was then added, and five fractions were collected. After 20 min to allow equilibration, another 20 fractions were collected. The remaining proteins were eluted with 30 mL of −Mo buffer containing 300 mM KCl. Fractions were analyzed on a 7.5% polyacrylamide gel under denaturing conditions. Lane 1 represents 5 μL of unadsorbed flow-through proteins. Lanes 2–13 represent 250-μL aliquots of alternate fractions eluted with −Mo buffer. Lane 5 corresponds to the first fraction after resuming column flow. Lane 14 represents 8 μL of the protein peak eluted with 300 mM KCl.

the final elution step. Whether this was due to an incomplete equilibration of the column in the absence of molybdate or to heterogeneity in 90K complexes is still under investigation. Our immediate objective was to develop a facile method for purifying the 90K protein from oviduct cytosol rather than from purified receptor. Previous studies indicated that the cytosol content of 90K protein is much higher than that of the progesterone binding components (Baulieu et al., 1983; Dougherty et al., 1984). Therefore, greater yields of 90K protein could be obtained from cytosol should it fractionate in a molybdate-dependent fashion on heparin–agarose. Preliminary experiments showed this to be the case. After several experiments to optimize conditions, we adopted the following procedure. Oviduct cytosol in molybdate buffer was incubated with phosphocellulose to adsorb proteases (Birnbaumer et al., 1983) and other proteins that might also bind heparin. The flow-through as then fractionated on heparin–agarose essentially as described for Figure 1. The majority of cytosol protein was not adsorbed to heparin–agarose. About 90% of the protein adsorbed to the column was retained until the 0.3 M KCl elution step. However, analysis of the column fractions by gel electrophoresis clearly showed a preferential elution of 90K protein when the column was reequilibrated with molybdate-free buffer (Figure 3). Although the fractions containing the 90K protein also contained several additional proteins, the extent of 90K purification was very substantial. Some of the other proteins appeared to be slowly washing off the resin rather than eluting by a molybdate-dependent process. However, there were also some minor proteins which coeluted with the 90K protein. Whether these are related to the 90K protein or share its property of molybdate-dependent elution is not known.

Since we could not accurately measure the quantity of 90K protein in cytosol, it was not possible to calculate the percent recovery or fold purification. However, gel densitometry analysis of the 90K fractions (see Experimental Procedures) indicated that the 90K protein was about 15% pure. Approximately 3–4 μg of 90K protein was obtained per milliliter cytosol or 12 μg/g of oviduct.

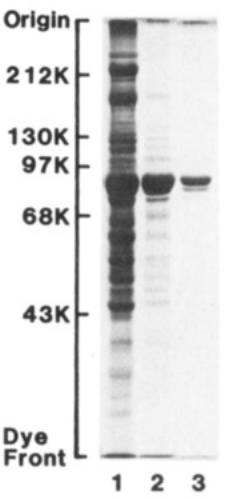

FIGURE 4: Profiles of proteins collected during subsequent steps in purification of the 90K protein from cytosol. During the purification of 90K protein from cytosol, aliquots representing $^1/_{40}$th peak volume were concentrated by Cl_3CCOOH precipitation. Represented are proteins eluted from heparin–agarose with −Mo buffer (lane 1), DEAE-A25-Sepharose with 300 mM KCl (lane 2), and hydroxylapatite at 250 mM potassium phosphate (lane 3).

We have found that the 90K protein can be further purified by chromatography on DEAE-Sephadex and hydroxylapatite (see Experimental Procedures). Using a KCl gradient, the protein was eluted from DEAE-Sephadex as a single peak at about 0.25 M KCl (results not shown). It also eluted from hydroxylapatite as a single peak at about 0.15 M potassium phosphate. Figure 4 illustrates the degree of purification achieved in these steps. The recovery of 90K protein from DEAE-Sephadex was close to 100% with a purity of 35% or better. Hydroxylapatite chromatography provided additional purification. However, the percent recovery from this step was variable and often quite low.

To confirm that the 90K protein from purified progesterone receptor and that obtained from cytosol are the same protein, they were compared by peptide mapping. Samples from each preparation were run on SDS gels. The 90K bands were cut out and placed on another gel with various amounts of protease to generate a pattern of peptide fragments. As shown in Figure 5, the peptide patterns from the two preparations were identical after treatment with V8 protease. The patterns were also the same after digestion with α-chymotrypsin (results not shown). Therefore, the 90K protein from cytosol and that of the receptor are structurally similar if not identical.

Preparation of Monoclonal Antibodies. The 90K protein isolated from cytosol was used to generate monoclonal antibodies as described under Experimental Procedures. Four separate clones of antibody-producing cells were obtained by using an ELISA screening assay. These were used to produce ascitic fluid, and the antibodies from the fluid were purified by ammonium sulfate precipitation and DEAE-cellulose chromatography. The antibodies are referred to by their ELISA assay codes as 7D11, 4F3, CB1, and CA4. The properties of these antibodies are summarized in Table I.

Since the 90K protein preparations used for injection and screening contained some contaminants, it was important to confirm the interaction of the antibodies with 90K protein. This was done by Western blotting as illustrated in Figure 6. A single 90 000-dalton band of interaction was obtained for all four antibodies when tested against 90K protein purified by heparin –agarose as described above. Similar results were obtained when the source of 90K protein was 8S progesterone receptor purified as described for Figure 2A.

Table I: Properties of Monoclonal Antibodies to 90K Protein

monoclonal antibody	class	protein A binding[a]	immunoblot	cytosol 90K protein binding[a]	8S receptor binding[b] +Mo	8S receptor binding[b] −Mo
7D11	$IgG_{1,\kappa}$	+	+	+	−	−
4F3	$IgG_{1,\kappa}$	+	+	+	+	−
CB1	$IgG_{1,\kappa}$	+	+	+	+	+
CA4	$IgG_{1,\kappa}$	+	+	+	−	−

[a] This was tested by incubation with protein A–Sepharose as described in Figure 10. [b] This was tested by sedimentation analysis.

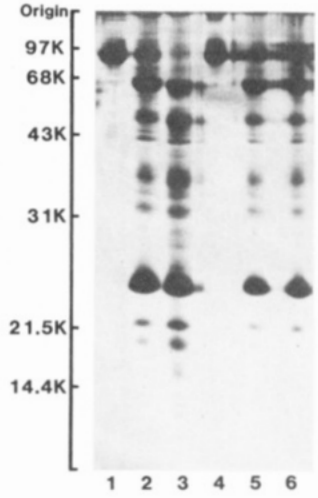

FIGURE 5: Comparison of peptide maps generated by V8 protease digestion of 90K protein purified from cytosol or from purified progesterone receptor. 90K protein from cytosol (lanes 1–3) was purified by chromatography on heparin–agarose and DEAE-Sephadex. Receptor-associated 90K protein (lanes 4–6) was from purified progesterone receptor. The proteins were first separated by SDS gel electrophoresis. The 90K proteins were excised from the gel, treated with V8 protease, and resolved by gel electrophoresis (see Experimental Procedures). The treatments were no enzyme (lanes 1 and 4) or 5 (lanes 2 and 5) or 25 ng (lanes 3 and 6) of V8 protease. The molecular weight standards were phosphorylase *b* (M_r 97 000) bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000) carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 000).

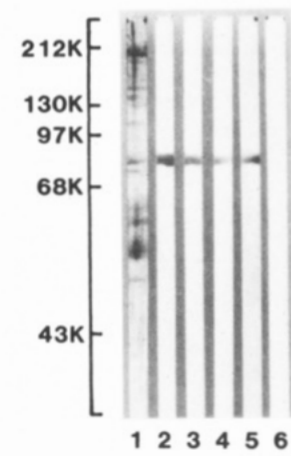

FIGURE 6: Immunoblotting of 90K protein with monoclonal antibodies. Proteins were eluted from heparin–agarose with −Mo buffer, concentrated by Cl_3CCOOH precipitation, and separated on a 7.5% polyacrylamide slab gel under denaturing conditions. The proteins were electrophoretically transferred to a nitrocellulose membrane. A 3-mm-wide strip was removed from the membrane and stained with drawing ink to visualize the proteins (lane 1). The active sites on the remainder of the membrane were blocked with bovine serum albumin. Additional 3-mm-wide strips were cut and individually incubated with the following antibody preparations: 7D11, 4F3, CB1, CA4, and no antibody (lanes 3, 4, 5, and 6, respectively). After the strips were washed, they were incubated with rabbit anti-mouse IgG antibody conjugated to alkaline phosphatase. The excess antibody was washed off, and the immunoreactive proteins were visualized with an enzyme-specific stain.

We next tested for the interaction of these antibodies with the progesterone receptor complex by sedimentation analysis on sucrose gradients. Aliquots of oviduct cytosol containing [^{3}H]progesterone bound to receptor were incubated with the different antibodies, and complex formation was determined by a shift in sedimentation. Antibodies 7D11 and CA4 did not alter the sedimentation of 8S progesterone receptor under any of the conditions tested (results not shown). However, an interaction of the other two antibodies with the receptor complex was observed. When tested in the presence of molybdate, antibody 4F3 shifted the receptor complex from 8 S to about 9.5 S, and antibody CB1 shifted the receptor to a larger complex sedimenting at about 15 S (Figure 7). Figure 7 also illustrates the combined effects of antibodies 4F3 and CB1. Receptor was first complexed with 4F3 and then with CB1 to form a complex that sedimented the same as CB1 complexes alone. This indicates that the receptor can react with both antibodies simultaneously, but it is also possible that 4F3 was completely replaced by CB1 in the complex. Whether the larger complex formed by CB1, compared to 4F3, is due to the number of interaction sites on the receptor or to some other type of aggregation is unknown.

The interaction of antibodies 4F3 and CB1 with the progesterone receptor was further characterized as illustrated in Figure 8. When stabilized by sodium molybdate, the receptor reacted with both antibodies in the absence or presence of 0.3 M KCl which was used to diminish nonspecific associations (compare panels A and B of Figure 8). Note that the longer sedimentation time used in these experiments caused a partial sedimentation of CB1 complexes to the tube bottom. The size of the CB1 complexes appears to be diminished and somewhat heterogeneous under the high-salt conditions. When cytosol receptor was prepared and sedimented in the absence of molybdate, both 6S and 8S receptor forms were observed (Figure 8C). Antibody CB1 removed receptor from both gradient regions, but no interaction was detected by using antibody 4F3, and apparently, this antibody has a preferential affinity for the molybdate-stabilized receptor forms. When the 90K protein was dissociated from the receptor by exposure to 0.3 M KCl without molybdate, no interaction of 4S receptor monomers with the antibodies was observed (Figure 8D).

In a recent study by Baulieu and co-workers (Joab et al., 1984), a monoclonal antibody to the 90K protein from chick oviduct was shown to react not only with the progesterone receptor but also with estrogen, androgen, and glucocoritcoid receptors. This indicated that a similar or identical 90K protein was associated with all four steroid receptors. To confirm this important observation, we tested the interaction of antibody CB1 with the androgen and glucocorticoid receptors from the chick oviduct. Sedimentation studies showed that both of these receptor complexes are recognized by the antibody (Figure 9).

Baulieu and co-workers (Baulieu et al., 1983) have also noted that their antibody to the 90K protein from the oviduct could interact with proteins from many other tissues of the

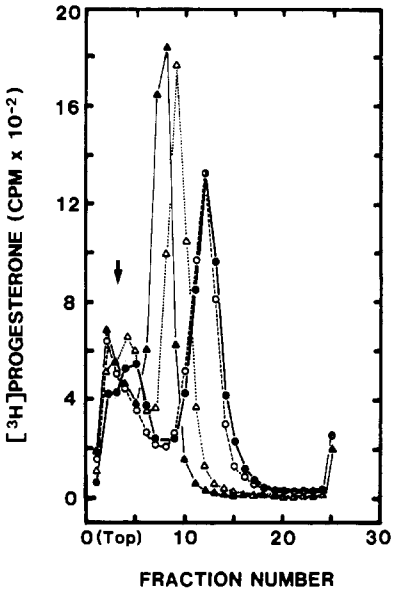

FIGURE 7: Effects of antibodies on sedimentation of progesterone receptor complex. Cytosol was prepared in 10 mM phosphate buffer (+Mo) and labeled for 1 h with [^{3}H]progesterone. Aliquots (50 μL) were incubated with buffer (▲), 4F3 antibody (△), or CB1 antibody (○) for 15 h; a 50-μL aliquot of labeled cytosol was incubated 5 h with 4F3 antibody followed by the addition of CB1 antibody for 10 more h (●). Final volume of all samples was 200 μL. Aliquots of 100 μL were layered on sucrose gradients containing 10 mM phosphate buffer (+Mo) and centrifuged at 105000g for 6 h. The arrow indicates [^{14}C]ovalbumin.

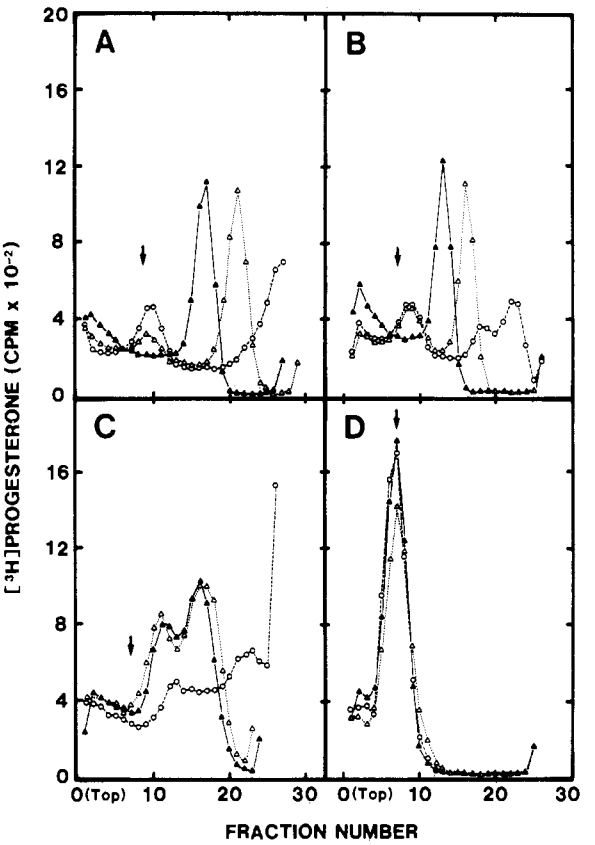

FIGURE 8: Effects of molybdate and KCl on antibody–receptor interactions. (A) +Mo, –KCl: Cytosol prepared in phosphate buffer (+Mo) was incubated for 1 h with 5 nM [^{3}H]progesterone plus 2.5 μM cortisol and then incubated for 2 h with buffer (control) (▲), 4F3 antibody (△), or CB1 antibody (○) (the same symbols for all panels). Aliquots were layered onto gradients containing phosphate buffer (+Mo). (B) +Mo, +KCl: Cytosol was prepared and labeled as in panel A. Aliquots were then incubated for 9 h with buffer or antibody and layered onto gradients containing 10 mM phosphate buffer (+Mo) plus 0.3 M KCl. (C) –Mo, –KCl: Cytosol in 10 mM phosphate buffer (–Mo) was labeled for 30 min and incubated with antibody or buffer for 1 h before being layered onto gradients containing 10 mM phosphate buffer (–Mo). (D) –Mo, +KCl: Cytosol was prepared in phosphate buffer (–Mo). KCl (3 M) was added to a final concentration of 0.3 M KCl at the time of labeling and incubated for 1 h. Aliquots were incubated with buffer or antibody and layered onto sucrose gradients containing phosphate buffer (–Mo) plus 0.3 M KCl. Arrows indicate [^{14}C]ovalbumin (3.7 S). Centrifugation for all samples was for 16 h at 105000g.

chicken, indicating that the 90K protein existed in most tissues. To confirm this by direct analysis, antibody 4F3 was added to cytosol fractions from chicken oviduct, liver, brain, skeletal muscle, and, also, serum. The antibody complexes were then adsorbed to protein A–Sepharose and recovered by centrifugation. After several washing steps, the adsorbed protein was solubilized in SDS sample buffer and analyzed by electrophoresis as shown in Figure 10. This procedure provides a rapid and very selective isolation of antigenic proteins which can then be compared with regard to size and relative quantities. Substantial amounts of the 90K protein were obtained from all four tissue cytosols, but not from serum. An alternate method of isolation was also used where the protein A–Sepharose was replaced with immobilized anti-mouse antibody (Figure 10, lanes 7 and 8). Again, no detectable 90K protein was obtained from serum even though this method was very effective in adsorbing the 90K protein from liver cytosol.

Discussion

While the 90K protein of the progesterone receptor was first thought to be a hormone binding component (Puri et al., 1982; Renoir et al., 1982), it is now clear that this protein does not bind hormone but is a component of the 8S receptor complex. It is observed in highly purified preparations of molybdate-stabilized receptor, but both immunological studies (Renoir et al., 1984b; Baulieu et al., 1983) and photoaffinity labeling studies (Baulieu et al., 1983; Dougherty et al., 1984) show that it lacks progesterone binding activity. The number of 90K protein molecules per 8S receptor is still unclear. With our purification procedures for the progesterone receptor, the 90K protein is always found in considerable excess over the A and B hormone binding proteins. Recent studies by Bauleau and co-workers indicate that there are two 90K molecules per 8S receptor (Renoir et al., 1984a). However, studies from this group (Renoir et al., 1984b) and our own (Dougherty et al., 1984) show that there is much additional 90K protein that is not bound to the progesterone receptor. Some of this 90K protein is bound to other steroid receptors, as indicated recently by Joab et al. (1984) and confirmed by the present study.

One purpose of the present study was to develop methods for isolation of the 90K protein, not only from progesterone receptor complexes but also from a larger pool of cytosol complexes that contain the 90K protein in a molybdate-stabilized form. This was readily accomplished by relying on the differential affinities of free and complexed 90K protein for heparin–agarose. The procedure is rapid and very effective. It also illustrates the reversibility and selectivity of molybdate action. It is clear that molybdate does not cause generalized aggregation of proteins but stabilizes associations of the 90K protein rather specifically. While the mechanism through which molybdate acts is unknown, it is believed to act directly on nontransformed steroid receptor complexes to enhance, in some way, the interaction between components (Puri et al., 1982; Dougherty et al., 1984). Molybdate has a similar action on essentially all steroid receptors tested, in a variety of organisms ranging from the primitive water mold *Achlya* (Riehl

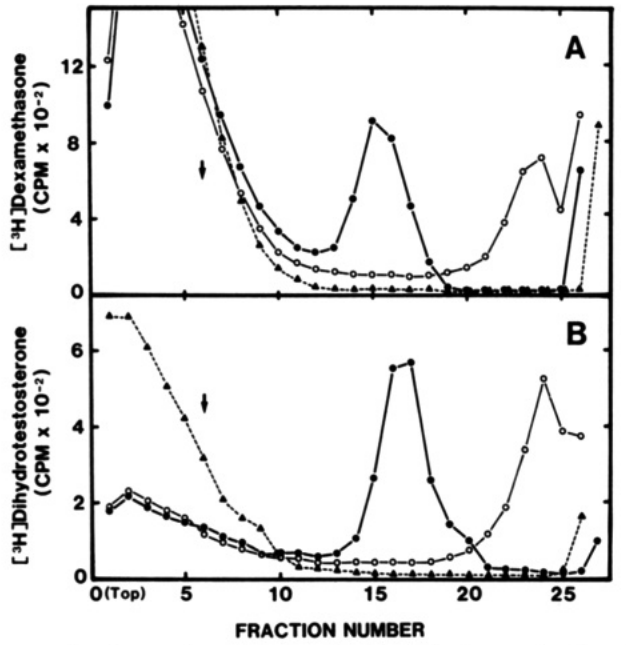

FIGURE 9: Interaction of CB1 antibody with glucocorticoid and androgen receptors. Cytosol was prepared from chick oviducts in 10 mM phosphate buffer +Mo. Glucocorticoid receptor (A) was labeled with 10 nM [^{3}H]dexamethasone with or without 5 μM cortisol as a competitor. Androgen receptor (B) was labeled with 10 nM [^{3}H]-dihydrotestosterone in the presence of 50 nM progesterone (to saturate progesterone receptor sites), and a background sample was prepared in the same way but with 5 μM dihydrotestosterone as a competitor. In each case, samples were incubated for 2 h with buffer alone (●), antibody CB1 (36 μg/100 μL) (○), or 5 μM unlabeled competitor (▲). Aliquots of 100 μL were centrifuged on sucrose gradients in phosphate buffer +Mo for 16 h at 105000g. Arrows indicate [^{14}C]ovalbumin (3.7 S).

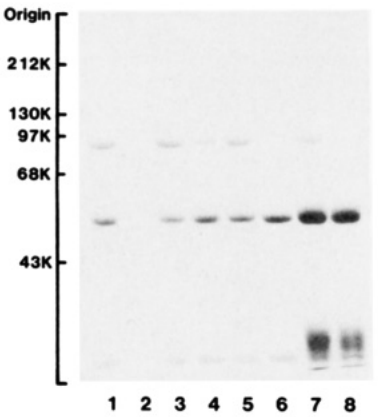

FIGURE 10: Detection of 90K protein in various tissues. Serum and cytosol fractions from oviduct, liver, brain, and breast muscle were prepared, and 1-mL samples were incubated with 100 μg of antibody 4F3 for 2 h in ice and then with protein A–Sepharose for 1 h in ice. The resin was washed, extracted with SDS sample buffer, and analyzed by SDS–polyacrylamide gel electrophoresis. Additional 1-ml samples of serum and liver cytosol were treated with antibody and fractionated by using anti-mouse IgG–agarose followed by extraction and electrophoresis. The samples and initial protein concentrations were as follows: lane 1, oviduct cytosol, 42 mg of protein/mL; lane 2, oviduct cytosol control without addition of antibody; lane 3, liver cytosol, 18 mg/mL; lane 4, muscle cytosol, 16 mg/mL; lane 5, brain cytosol, 13 mg/mL; lane 6, serum, 24 mg/mL; lane 7, liver cytosol; lane 8, serum. Samples for lanes 7 and 8 were fractionated by using anti-mouse IgG–agarose. For lanes 1–6, about one-third of the total extract was applied to the gel, and for lanes 7 and 8, one-twelfth of the total was applied.

et al., 1984) to humans (Miller et al., 1981). Therefore, while the 90K protein has only been demonstrated in the chicken, it seems likely that an anlogous protein exists in other organisms. In results to be reported elsewhere, we have used protein A–Sepharose plus gel electrophoresis (see Figure 10) to test for the presence of immunoreactive proteins in other species. Antibodies 7D11, 4F3, and CA4 were found to be quite specific for chicken 90K protein. However, a 90K protein could be isolated from rat liver cytosol by using antibody CB1, indicating that this protein does exist in mammals.

Two important features of the 90K protein are revealed from the present fractionation studies. First, the 90K protein isolated from cytosol appears to be very similar to or identical with that which is complexed with the purified progesterone receptor both by peptide mapping and by immunological analysis. The results also argue that some of the excess 90K protein, not bound to progesterone receptor, is not free in the cytosol but is bound to other components in molybdate-stabilized complexes that bind heparin–agarose and are subsequently disrupted by the fractionation procedure. It will be important to identify and characterize these presumed 90K complexes to see how they compare to the steroid receptor complexes. It is possible that steroid receptors represent only a portion of a larger group of proteins which bind the 90K protein and may be involved in cellular regulation. On the other hand, preliminary studies to fractionate 90K protein from cytosol by use of antibody affinity resins indicate much higher yields than obtained by using heparin–agarose. This suggests that a significant fraction of 90K protein exists in a free form. Efforts are being made to describe this more clearly by using quantitative immune assays.

We have prepared four monoclonal antibodies which react with the 90K protein. Although these antibodies are not fully characterized, they are not identical and may serve multiple purposes. Two of these antibodies, 4F3 and CB1, form complexes with 8S steroid receptors from the oviduct. The antibody studies provide important confirmation of observations reported recently by Baulieu and co-workers (Baulieu et al., 1983). First, they show that the 90K protein is actually a component of the 8S receptor both in the presence and in the absence of sodium molybdate. They also show it to be a component of other receptor complexes in the oviduct and to be a protein of wide tissue distribution.

Colvard & Wilson (1981) have identified a protein which interacts with the androgen receptor of rat prostate to convert the 4.5S form of this receptor to an 8S form. We had suggested previously that this protein might be similar to the 90K protein in the chicken (Dougherty et al., 1984). However, unlike our results, the protein studied by Colvard and Wilson, termed 8S androgen receptor promoting factor, was readily observed and fractionated from rat serum.

Two of the antibodies which we prepared, 7D11 and CA4, appear not to interact with progesterone receptor complexes. This could be because they interact with regions of the 90K protein that are not exposed until denaturation of the 90K protein. This appears not to be the case since we have found (results not shown) that these antibodies will react with much of the 90K protein in cytosol as tested by the protein A fractionation technique (as in Figure 10). Therefore, it seems likely that the antigenic sites for antibodies 7D11 and CA4 are masked when the 90K protein is complexed to receptor. These antibodies may prove useful in identifying regions of protein–protein interaction or indefining different conformational states of the 90K protein. They may also be useful for monitoring the proportion of free and bound 90K protein under various experimental conditions. Efforts are under way to develop specific quantitative radioimmune assays for this purpose.

Several important questions remain unanswered regarding the 90K protein. Foremost is the question of its biological significance. It clearly is not a specific component only of the progesterone receptor. Its broad tissue distribution suggests that it has a more general purpose in cellular processes. One could argue that the binding of progesterone receptor to the 90K protein is an in vitro artifact following cell disruption. However, while this possibility must be investigated, the selective nature of this interaction and the occurrence of apparently analogous interactions with essentially all steroid receptor systems indicate that this is not the case. The 90K protein may function to maintain steroid receptors in an inactive form. It could also act in providing directions for intracellular localization of receptors or in processing of receptors to various functional states. Only a few properties of the 90K protein are known. It is an acidic, relatively globular protein (M_r 90000; sedimentation = 6–7 S). It contains phosphoserine residues, but whether it exists in both phosphorylated and unphosphorylated states is unknown (Dougherty et al., 1984). The purification methods and antibodies described here should provide much assistance in further characterization of this protein.

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