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

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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

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analysis to contain a protein of molecular weight about 90 000 (90K protein)[1] that has no steroid binding activity. This common 90K protein has, hitherto, been found only in avian tissues due to a lack of cross-reactivity by the monoclonal antibodies employed as probes (Renoir & Mester, 1984).

A protocol (Sullivan et al., 1985) similar to that employed for isolation of the 90K protein component of molybdate-stabilized steroid receptors from the chick oviduct was applied to the antheridiol receptor system in *Achlya*. A protein of about 88 kDa was isolated from *Achlya*, and a monoclonal antibody having a broad range of cross-reactivity with avian and mammalian 90K proteins was obtained.

Experimental Procedures

Preparation of Cytoplasmic Extracts. Mycelia of *A. ambisexualis* Raper strain E87 (male) were grown in suspension culture and fungal cytosol prepared in 25 mM K_2HPO_4, 25 mM KH_2PO_4, 25 mM Na_2MoO_4, 10 mM 2-mercaptoethanol, and 10 mM MES adjusted to pH 7.4 at 0 °C as previously described (Riehl & Toft, 1984). Hamster, mouse, and rat tissues were collected and placed in ice-cold saline for removal of adherent fat and connective tissues. Subsequently, tissues were minced with scissors, and a 4 mL/g wet weight solution of 40 mM MOPS, 20 mM Na_2MoO_4, 1 mM Na_2EDTA, and 1.5 mM dithiothreitol containing 10% (v/v) glycerol, pH 7.4 at 0 °C, was added. Homogenization was performed within an ice/methanol slush by four separate bursts of a Polytron (P20 probe, Brinkmann Instruments) at half-maximum speed with a 1-min rest period at 0 °C between bursts. Homogenates were filtered through two layers of Miracloth (Calbiochem), and the filtrate was centrifuged at 250000*g* for 1 h at 0 °C. The resultant supernatants were passed through a Millex-HA filter unit (0.45 μm, Millipore Corp.), and the cytosol was adjusted to pH 7.5 at 0 °C. Spinner cultures of HeLa cells (S3 cells) were harvested by centrifugation (500*g*, 5 min) and washed twice in saline. Approximately 2 g of packed cells was covered with 10 mL of homogenization buffer and stored frozen at −20 °C prior to thawing and preparation of the cytosol as described for rodent tissues. Human endometrial tissue (2 g wet weight) was obtained after curettage, immediately frozen in liquid nitrogen, and stored at −70 °C until preparation of the cytosol as described for other tissues. Porcine ovarian follicular granulosa cells were harvested from aspirated follicular fluid by centrifugation (500*g*, 10 min), washed twice in saline, covered with homogenization buffer, and stored frozen at −20 °C prior to preparation of the cytosol as described for HeLa cells. Cytosolic fractions from all tissues and cells were stored at −70 °C until used.

Isolation of the 88K Protein from Achlya. The method of Sullivan et al. (1985) for purification of the 90K protein from chick oviducts was employed to isolate a simlar protein from *Achlya* that, in contrast, was found to have a molecular weight of roughly 88 000 upon SDS–polyacrylamide gel electrophoresis (see Results, Figure 2). All steps were performed at 4 °C. *Achlya* cytosol, prepared as previously described (Riehl & Toft, 1984), was mixed with $^1/_{10}$th volume of phosphocellulose that had been equilibrated with 20 mM KH_2PO_4, 10 mM Na_2MoO_4, and 10 mM thioglycerol, pH 7.0 at 23 °C (+Mo buffer). The suspension was stirred for 2 min and the phosphocellulose removed by vacuum filtration in a fritted glass funnel. The filtrate was mixed with a 10-mL bed volume of heparin–agarose (P-L Biochemicals) that had been equilibrated in +Mo buffer. After being stirred for 20 min, the gel was collected on a fritted glass funnel by vacuum filtration and washed with 50 mL of +Mo buffer. The gel was then transferred in +Mo buffer to a 2.4-cm diameter column and washed with 20 mL of 20 mM KH_2PO_4 and 10 mM thioglycerol, pH 7.0 (−Mo buffer), at a flow rate of 1 mL/min. After collection of three 2-mL fractions, buffer flow was stopped for 20 min. Subsequently, the column was eluted with an additional 20 mL of −Mo buffer.

The heparin–agarose eluate was mixed with a 5-mL bed volume of DEAE-Sephadex A25 previously equilibrated in −Mo buffer and stirred for 20 min. The gel was collected by centrifugation, washed twice in 45 mL of −Mo buffer, and transferred to a 1-cm diameter column containing −Mo buffer. The gel was washed with 10 volumes of −Mo buffer containing 150 mM KCl. The 88K protein was eluted with 10 volumes of 350 mM KCl in −Mo buffer. The absorbance at 280 nm was determined for each 2-mL fraction collected, and the peak fractions were pooled.

Production of Monoclonal Antibody. The pooled DEAE-Sephadex fractions were first 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. The dialyzed fractions were lyophilized. The protein (approximately 50 μg) was dissolved in 5 mM Tris-HCl, pH 7.4, containing 10% glycerol, and this solution was mixed with an equal volume (150 μL) of complete Freund's adjuvant. The preparation was injected intraperitoneally into two Balb/c mice (Jackson Laboratory). After 4–5 weeks, a second injection was given of incomplete Freund's adjuvant, and 1 week later, the serum was tested by ELISA. After 7 weeks, a final injection of antigen alone was given to the most highly reactive mouse, and 3 days later, the mouse was sacrificed and the spleen removed. Hybridomas were prepared by poly(ethylene glycol)-mediated fusion of spleen cells with the mouse plasmacytoma cell line P3NS-11-AG4-1 (NS-1) as previously described (Vroman et al., 1985). Hybridomas were selected by growth in RPMI 1640 medium supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, 2 mM glutamine, 10 nM Na_2SeO_3, 10% (v/v) fetal calf serum, 10% (v/v) NCTC135, and 0.45% (w/v) glucose by using 96-well microtiter plates [(1–2) × 10^5 cells per well] with mouse peritoneal macrophages added as a feeder layer. Cultures were fed at 3-day intervals, and the withdrawn conditioned medium was tested for the presence of antibodies by an enzyme-linked immunosorbent assay (ELISA; Luka et al., 1984) using freshly prepared DEAE-Sephadex purified 88K protein as the adsorbed antigen. Positive cultures were subjected to limiting dilution analysis at 0.5 and 1 cell per well of a 96-well microtiter plate. The positive reclones were then grown to a density of (1–2) × 10^7 cells per 75-cm^2 flask and washed 3 times in Dulbecco's phosphate-buffered saline, and approximately (2–3) × 10^6 cells were injected intraperitoneally into mice previously primed with 0.3 mL of pristane.

After 5–7 days, ascitic fluid was collected at daily intervals, clarified by centrifugation at 6000*g* for 15 min, and stored at 4 °C. Finally, the fluids were pooled and centrifuged at 100000*g* for 30 min. The supernatant was adjusted to 30% of saturation with $(NH_4)_2SO_4$, and the precipitated proteins were discarded. The supernatant was then adjusted to 50% of saturation with $(NH_4)_2SO_4$. The precipitated proteins were

[1] Abbreviations: 90K protein, the 90 000 molecular weight protein component of molybdate-stabilized steroid receptor complexes; kDa, kilodalton(s); Mo, molybdate; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; ELISA, enzyme-linked immunosorbent assay; IgG_1, immunoglobulin G_1; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

resuspended in 6 mM Tris-HCl, pH 7.5, and dialyzed for 16 h against 1 L of the same buffer. The dialysate was applied to a DEAE-cellulose column (1 mL of gel/10 mg of protein) previously equilibrated with 6 mM Tris-HCl, pH 7.5, and washed with 10 gel volumes of the same buffer. Proteins were eluted from the gel with a linear 0–120 mM NaCl gradient in 6 mM Tris-HCl, pH 7.5, and 2-mL fractions were collected. The absorbance at 280 nm of each fraction was determined, and the peaks and troughs were pooled. Pooled fractions were screened for antibodies to the 88K protein by an ELISA, and the positive peak (30–60 mM NaCl) was dispensed in 1-mL aliquots (2.4 mg of protein/mL) that were stored at −70 °C. The antibody (IgG_1) used in this study was isotyped by ELISA using purified antibodies to each immunoglobin subclass (Southern Biotechnology Associates). This mouse anti-*Achlya* 88-kDa protein monoclonal antibody is hereafter referred to as AC88.

Slab Gel Electrophoresis. Samples were solubilized in 125 mM Tris-HCl, pH 6.8, containing 2-mercaptoethanol (5% v/v), SDS (1% w/v), glycerol (10% v/v), and Bromophenol Blue (0.025% w/v) by heating at 100 °C for 5 min. Samples were subjected to electrophoresis in a 7.5% resolving/3% stacking acrylamide gel system essentially according to the method of Laemmli (1970). A constant current of 15 mA was applied until the samples penetrated the resolving gel; the current was then increased to 30 mA until the dye front had migrated to within 2 cm of the bottom of the gel. Staining was accomplished by 12–15 h in 0.05% (w/v) Coomassie Brilliant Blue R250 in methanol/acetic acid/water (5:1:4 by volume), and destaining was done in methanol/acetic acid/water (2.5:1:6.5 by volume).

Immunoreplication of Slab Gels on Nitrocellulose. Electrophoresis was performed as described above, and the proteins were transferred to sheets of nitrocellulose membranes (0.45 μM; Schleicher & Schuell) by the method of Burnette (1981). Portions of the nitrocellulose sheet were stained with 0.1% (v/v) drawing ink to visualize the transferred proteins. The remainder of the sheet was incubated for 16 h at 4 °C in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% (v/v) Tween 20, and 0.1% (w/v) bovine serum albumin (Wester buffer) followed by 30 min at 37 °C in fresh buffer. Subsequently, incubation was performed for 1 h at 22 °C in Western buffer containing 10 μg/mL antibodies. The strips were then washed in Western buffer and incubated for 1 h in this buffer containing alkaline phosphatase–conjugated rabbit anti-mouse IgG antibody (Sigma). After being washed in Western buffer, the immunocomplexes were visualized with Fast Violet V and Napthol ASB1 in 0.1 M Tris-HCl, pH 9.5, containing 1 mM $MgCl_2$. Control strips were processed in the same manner without inclusion of the first antibody.

Preparation of Immunoaffinity Gel. AC88 was covalently coupled to Affi-Gel 10 (Bio-Rad), an agarose gel bead with 10-atom spacer arm, according to the manufacturer's specifications for aqueous coupling. Briefly, purified AC88 was dialyzed against 0.1 M $NaHCO_3$, pH 8, for 3 h, and 30 mg of AC88 was incubated with the washed gel (5 mL) for 4 h at 4 °C. The gel was collected by centrifugation, and the unoccupied coupling sites were blocked with 0.1 mL of 1 M ethanolamine, pH 8, per milliliter of gel for 1 h. Subsequently, the gel was washed 5 times with 50 mL of saline and twice with 50 mM potassium phosphate buffer, pH 7.0. Finally, the gel was resuspended in 5 mL of phosphate buffer, pH 7, containing 0.02% NaN_3 and stored at 4 °C until used. The efficiency of AC88 coupling ranged from 93% to 98% (three preparations).

Immunosorbent Assays. Tissue cytosols, prepared as described, were incubated in the presence of 200 μg of AC88 for 1 h at 22 °C in siliconized conical glass centrifuge tubes. Subsequently, 0.5 mL of a 1:4 (w/v) suspension of protein A–Sepharose (Pharmacia) in 25 mM potassium phosphate buffer, pH 7.2, containing 10% (v/v) glycerol and 0.02% NaN_3 was added. Incubation was continued at 22 °C for 1 h with resuspension of the settled gel at 15-min intervals. The gel was collected by centrifugation (2300*g*, 5 min) and washed twice with 8 mL of 25 mM potassium phosphate buffer, pH 7.2, containing 10% (v/v) glycerol. The gel was then washed twice with 8 mL of phosphate buffer plus 0.3 M KCl and twice with phosphate buffer plus 0.2% Nonidet P40. The gel was transferred to a centrifugal filter unit (Centrex, Schleicher & Schuell) using phosphate buffer plus 0.2% Nonidet P40 and collected on the filter by centrifugation (2300*g*, 5 min). The filtrate was discarded from the receiver tube, and 0.5 mL of 4 M NaSCN in 10 mM Tris-HCl, pH 8, was added to the gel in the upper chamber. After 30 min at 22 °C, the unit was centrifuged (2300*g*, 5 min); 0.5 mL of distilled water was added to the upper chamber, and the unit was again centrifuged. The filtrate (1 mL), containing the eluted proteins, was adjusted to contain 0.02% sodium deoxycholate, incubated at 22 °C for 10 min, and then adjusted to 20% (w/v) trichloroacetic acid. After incubation at 4 °C for 12–16 h, the precipitated proteins were collected by centrifugation, washed with 3 mL of 95% ethanol/5% water, and finally washed with acetone. The proteins in the pellet were then solubilized for electrophoresis as described under Experimental Procedures.

Demonstration of Immunological Similarities of 90K and 88K Proteins. Cytosol from diethylstilbestrol-primed chick oviducts was prepared as previously described (Sullivan et al., 1985) and incubated with AC88 affinity gel for 1 h at 4 °C. After washing, the eluted proteins were subjected to electrophoresis and transferred to nitrocellulose sheets as described. Immunoreplication was performed with AC88 and three other monoclonal antibodies directed against the chick oviduct 90K protein (4F3, CA4, and 7D11) that have been described in detail elsewhere (Sullivan et al., 1985).

Results and Discussion

In comparison to other well-characterized steroid hormone receptor systems, little is known concerning the steroid receptor forms in *Achlya*. The antheridiol receptor is very unstable, and highly purified preparations have not yet been obtained. The results of our previous studies on the properties of the molybdate-stabilized antheridiol receptor indicated that the procedure used for isolation of the 90K protein from chick oviduct might be applicable to the *Achlya* system as well. This procedure is based on the observation that the progesterone receptor–90K protein complex binds to heparin–agarose whereas free 90K protein does not. Thus, when molybdate-stabilized receptor was adsorbed to heparin–agarose, the 90K protein could be selectively eluted in buffer lacking molybdate. Therefore, *Achlya* cytosol containing the molybdate-stabilized receptor form was treated with phosphocellulose to remove the majority of the proteins that are relatively basic in *Achlya* cytosols (Riehl & Toft, 1984). The nonadsorbed acidic proteins were then incubated with heparin–agarose. After extensive washing of the gel with buffer containing molybdate to remove nonadsorbed and low-salt elutable proteins, the column was equilibrated in low-salt buffer without molybdate and then developed with the same buffer to collect proteins that elute from the gel in the absence of molybdate. A result of these experiments is shown in Figure 1, which depicts electrophoretic analysis of the proteins in fractions from the

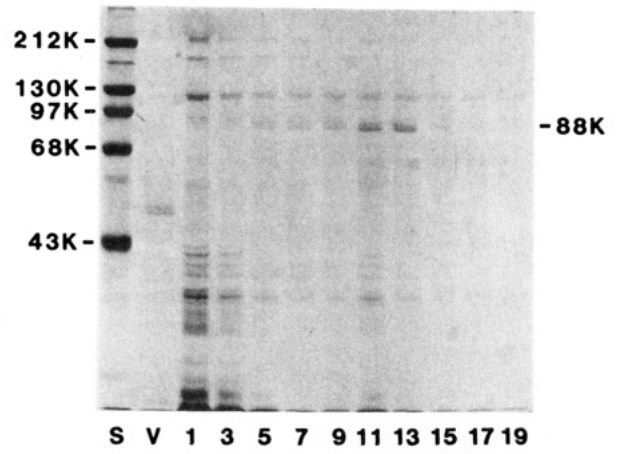

FIGURE 1: Electrophoretic profile of proteins eluted from heparin–agarose in buffer without molybdate. *Achlya* cytosol (250 mL), pretreated with phosphocellulose, was incubated with heparin–agarose and washed with buffer containing molybdate as described under Experimental Procedures. Molybdate-free buffer (20 mL) was then added to the column, and three 2-mL fractions were collected prior to stopping the buffer flow for 20 min. An additional 20 mL of molybdate-free buffer was added, and the flow (1 mL/min) was resumed with collection of the remaining 17 fractions. The proteins in each odd-numbered fraction were precipitated with TCA, solubilized, and subjected to electrophoresis under denaturing conditions. Gel lanes labeled with letters represent the following samples: S represents molecular weight standards composed of myosin heavy chain (212K), β-galactosidase (130K), phosphorylase B (97K), bovine serum albumin (68K), and ovalbumin (43K); V represents the column wash in buffer containing molybdate. Gel lanes labeled with numbers indicate the eluate fraction number in molybdate-free buffer. The column flow was stopped for 20 min between fractions 3 and 4. This gel was stained with Coomassie Blue.

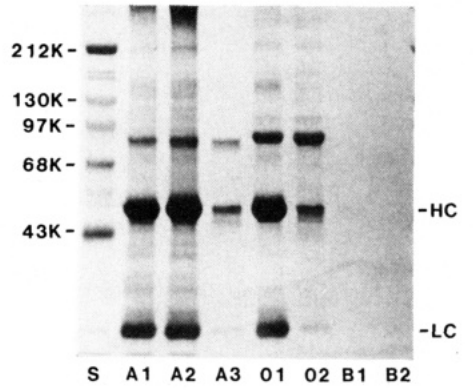

FIGURE 2: Affinity isolation of fungal 88K and oviductal 90K proteins using monoclonal antibody AC88. Cytosol preparations from *Achlya* males (A1) and *Achlya* females (A2), 10 mL of each, and from chick oviduct (O1), 0.5 mL, were incubated with 200 μg of monoclonal antibody AC88 for 1 h at 23 °C. The protein–antibody complexes were then adsorbed to protein A–Sepharose, washed, eluted with 4 M NaSCN, and subjected to electrophoresis as described under Experimental Procedures. Similar aliquots of *Achlya* male and oviduct cytosols were also incubated for 1 h at 22 °C with agarose-linked AC88 (lanes A3 and O2, respectively) and prepared for electrophoresis as described for the protein A–Sepharose-treated samples. Lanes B1 (*Achlya* male) and B2 (oviduct) represent aliquots of the respective cytosols incubated with protein A–Sepharose alone. Lane S represents the molecular weight standards as described in the legend to Figure 1. HC and LC indicate antibody heavy and light chains, respectively. This gel was silver stained according to the method of Morrissey (1981).

heparin–agarose eluate in molybdate-free buffer. These results indicate the presence of a protein in *Achlya* that is similar to the chick oviduct 90K protein with respect to its molybdate-dependent association with proteins that bind to heparin–agarose and its electrophoretic mobility in the molecular weight region of 90 000 (Figure 1, lanes 11 and 13). Because the antheridiol receptor has yet to be purified, it is not conclusive that this protein was dissociated from the adsorbed steroid binding components of the antheridiol receptor complex as is the case with the progesterone receptor in chick oviduct (Baulieu et al., 1983; Sullivan et al., 1985). Experiments designed to purify the molybdate-stabilized antheridiol receptor are in progress and should provide the information necessary to determine whether or not the 88K protein is indeed associated with the antheridiol receptor in *Achlya*.

In order to further characterize the similarity between these proteins found in *Achlya* and the chick oviduct, a monoclonal antibody (AC88) to the *Achlya* protein was produced as described under Experimental Procedures. AC88 was added to cytosol preparations from both *Achlya* and chick oviduct, and the mixture was incubated, washed, and adsorbed to protein A–Sepharose as described. In addition, the antibody was coupled to Aff-Gel-10 and the antibody immunosorbent gel added to each cytosol preparation. As shown in Figure 2, chick oviduct 90K protein (lanes O1, and O2) and the *Achlya* protein antigen (lanes A1–A3) were both recognized by AC88. The small difference in molecular weight between the two proteins is clearly shown in Figure 2. The *Achlya* protein migrated just ahead of the 90K protein from chick oviduct, corresponding to a molecular weight of approximately 88 000. These results could mean that the antibody is recognizing a similar determinant on two unrelated molecules that have similar molecular weights. Although such a coincidence is possible, it seems highly improbable. In this regard, we investigated the possibility that AC88 recognizes and binds to a 90K protein in chick oviduct that is different from the 90K protein associated with the progesterone receptor in chick oviduct. Chick oviduct cytosol was incubated with AC88 coupled to Affi-Gel 10. The immunocaptured proteins were subjected to electrophoresis, Western blotting, and immunoreplication using AC88 and anti-chick 90K monoclonal antibodies as described under Experimental Procedures. A result of these experiments is shown in Figure 3. The mouse anti-chick 90K monoclonal antibodies 7D11, 4F3, and CA4 (lanes 2, 3, and 4, respectively) did indeed recognize the same 90K protein bound by AC88 (lane 5). The three antibodies to chick oviduct 90K protein have been described elsewhere (Sullivan et al., 1985). Antibody 4F3 was shown to react with molybdate-stabilized progesterone receptor whereas the other two antibodies react only with uncomplexed 90K protein. These results support a hypothesis that the 88K protein from *Achlya* and the 90K protein from chick oviduct are antigenically related and are likely to be the same type of protein. The differences in electrophoretic mobilities could then be due to species differences in molecular weights of analogous proteins or in degrees of phosphorylation, acetylation, or glycosylation. The appearance of a doublet in the 88K region on the gel in Figure 1 could also be indicative of these latter processes or to microheterogeneity of the 88K protein. However, the appearance of this doublet band is not consistent among preparations, and as shown in Figure 2, the antibody recognizes a single protein.

Previous studies from our and other laboratories (Renoir & Mester, 1984; Sullivan et al., 1985) reported a lack of general cross-reactivity of anti-chick 90K protein monoclonal antibodies with 90K proteins from mammalian tissues. We investigated the cross-reactivity of AC88 with a variety of mammalian tissues by adding AC88 to cytosolic preparations from rat, mouse, hamster, pig, and human tissues as described under Experimental Procedures. A result of these experiments is shown in Figure 4. AC88 was found to recognize 90K

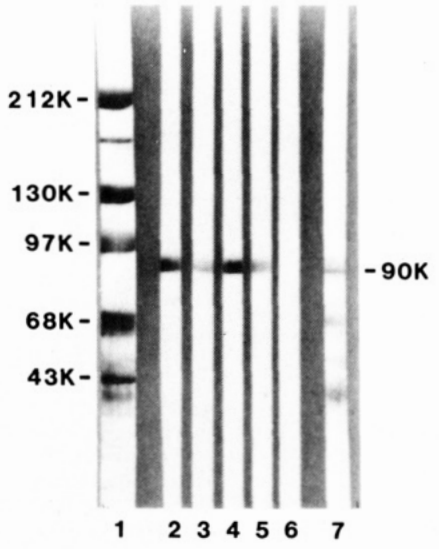

FIGURE 3: Immunoblots of oviductal 90K proteins using different monoclonal antibodies. Chick oviduct cytosol was incubated with agarose-linked AC88 antibody, and the adsorbed proteins were eluted, subjected to electrophoresis, and transferred to nitrocellulose sheets as described under Experimental Procedures. Individual strips of the nitrocellulose sheets were incubated with anti-chick 90K monoclonal antibodies 7D11 (lane 2), 4F3 (lane 3), and CA4 (lane 4) and with anti-*Achlya* 88K monoclonal antibody AC88 (lane 5). Lane 6 represents a strip incubated without antibody. Strips in lanes 2–6 were subsequently incubated with alkaline phosphatase–conjugated rabbit anti-mouse IgG antibody and immunospecific bands visualized with Fast Violet V and Napthol ASB1. Strips in lanes 1 and 7 were incubated with 0.1% drawing ink to stain the molecular weight standards and total transferred proteins, respectively.

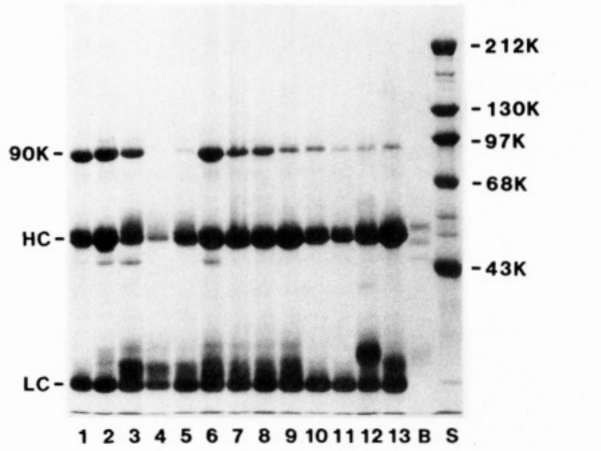

FIGURE 4: Electrophoresis of proteins isolated with AC88 from cytosol preparations of various tissues. Cytosols were prepared, incubated with 200 μg of AC88, adsorbed to protein A–Sepharose, eluted, and subjected to electrophoresis as described under Experimental Procedures. Lane S represents the molecular weight standards as denoted in Figure 1. HC and LC represent the antibody heavy chain and light chain, respectively. The tissue source and volume of cytosols used were the following: lane 1, chick oviduct (0.5 mL); lane 2, rat liver (0.5 mL); lanes 3–5, mouse liver (0.5 mL), uterus (2 mL), and skeletal muscle (2 mL), respectively; lanes 6–10, hamster liver (0.5 mL), lung (1 mL), kidney (1 mL), uterus (5 mL), and skeletal muscle (5 mL), respectively; lane 11, HeLa cells (1 mL); lane 12, human endometrium (1 mL); lane 13, porcine granulosa cells (1 mL); lane B, rat liver (0.5 mL), incubated without antibody. This gel was stained with Coomassie Blue.

proteins in all the mammalian tissues included in this study (see legend to Figure 4). Among these tested, the highest levels of 90K protein appear to be present in liver samples (Figure 4, lanes 2, 3, and 6). The apparent lack of a 90K protein in mouse uterus (Figure 4, lane 4) is due in part to poor photographic resolution of the band that is indeed present and in part to poor recovery of protein from this one aberrant sample. It can be seen that the remainder of the lanes depicted in Figure 4 contain approximately the same amount of heavy and light antibody chains (indicating parity in recovery of protein) and that a distinct 90K band is present in the hamster uterine sample (Figure 4, lane 9) from which the relative recovery of proteins was consistent with that of the other samples shown in this figure. In addition, preliminary trials were performed individually on all tissues and cells to confirm the presence of the 90K protein before consolidation onto the gel shown in Figure 4. These preliminary trials were also positive for all samples including mouse uterus.

Interestingly, all the vertebrate proteins recognized by AC88 have electrophoretic mobilities (i.e., 90 kDa) that are the same, which is slightly different than that of the 88K protein in *Achlya*. From these results and those demonstrating a broad avian tissue distribution of 90K protein (Gasc et al., 1984; Sullivan et al., 1985), it is concluded that the 90K protein is highly conserved and is found in many cell types.

The cellular function of these 90K protein is unknown. It has been shown that a large pool of 90K protein exists in avian cells that is not associated with steroid hormone receptors (Baulieu et al., 1983). The *Achlya* 88K protein is also found in cells of the female strain (Figure 2, lane A2), which do not possess detectable levels of the antheridiol receptor (Riehl & Toft, 1984). Although female cells do not respond to antheridiol, they do respond to the steroid oogoniol (Horgen, 1981) and therefore probably possess a receptor for oogoniol. In contrast to those anti-chick 90K antibodies that have been shown to alter the sedimentation coefficient of molybdate-stabilized steroid receptors (Joab et al., 1984; Sullivan et al., 1985), AC88 recognizes only the noncomplexed 90K proteins. That is, AC88 failed to shift the sedimentation coefficient of either the stabilized antheridiol receptor or the chick oviduct progesterone receptor (data not shown). This lack of recognition of the steroid receptor associated 90K protein has also been observed with some of the anti-chick 90K protein antibodies as well (Sullivan et al., 1985). These results indicate that the monoclonal antibodies employed are specific for a limited domain of the 90K protein that is either masked or undergoes a conformational change upon association with the steroid binding components of the receptors.

In conclusion, this antibody, with its broad cross-reactivity, should prove to be a useful probe for further studies on the biological role of the 90K proteins. These results also clearly demonstrate that this nonsteroid binding component associated with steroid receptor complexes in the chick oviduct is not peculiar to avian tissues but exists in a variety of tissues from a number of species. Whether or not 90K proteins are found in plant and prokaryotic cell types is as yet unknown, but experiments addressing this question are now in progress.

One final note: The molecular weight, tissue concentration, and broad species distribution of the 90K protein indicate a resemblance to a 90-kDa protein that is stimulated in a variety of cells by heat shock treatment [see Schlesinger et al. (1982) for review]. This protein is also known to bind the oncogene product of Rous sarcoma virus (Brugge et al., 1981). After submission of this paper, this possibility was investigated in collaboration with the laboratory of J. Brugge (Schuh et al., 1985). Our results demonstrate that the two proteins are indistinguishable. A similar conclusion has been recently reached by Catelli et al.[2] These studies provide new insight

[2] M. G. Catelli, N. Binart, I. Jung-Testas, J. M. Renoir, E. E. Baulieu, J. R. Feramisco, and W. J. Welch, unpublished results.

on the properties and significance of the 90K protein beyond an involvement in steroid hormone action.

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Multiple Sites of Steroid Hydroxylation by the Liver Microsomal Cytochrome P-450 System: Primary and Secondary Metabolism of Androstenedione†

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ABSTRACT: To investigate the potential interaction of the various pathways of androgen hydroxylation, we have conducted studies to identify the profile of products formed during the time course of metabolism of androst-4-ene-3,17-dione (AD). Incubates containing AD, NADPH, and liver microsomes (from rats pretreated with phenobarbital) were sampled at times between 0 and 20 min and the metabolites resolved by reverse-phase (C_{18}) high-performance liquid chromatography. By this method, the pattern of formation and of utilization of eight major primary and secondary metabolites of AD was determined. We report here the formation of two previously unidentified major metabolites of AD: 6β,16α-dihydroxyandrost-4-ene-3,17-dione and 6β,16β-dihydroxyandrost-4-ene-3,17-dione. We propose that liver microsomal cytochromes P-450 can sequentially hydroxylate a single molecule of AD at multiple sites. These hydroxylase activities are presumably a result of multiple cytochrome P-450 isozymes acting on AD resulting in a transient time course for the appearance of some monohydroxylated metabolites. In addition, a unidirectional conversion of the metabolite 16α-hydroxyandrost-4-ene-3,17-dione to 16β-hydroxyandrost-4-ene-3,17-dione is described. Evidence is provided to support the role of cytochrome P-450 in catalyzing this reaction.

The cytochromes P-450 associated with the microsomal fraction of liver catalyze the NADPH- and oxygen-dependent metabolism of a variety of lipophilic foreign and endogenous compounds (Conney et al., 1968a, Cooper et al., 1979). It is generally considered that steroids and fatty acids constitute endogenous substrates of liver microsomal cytochromes P-450 (Conney et al., 1965; Kupfer, 1982). Studies with androst-4-ene-3,17-dione (AD)[1] or testosterone have shown that site-specific hydroxylation reactions are catalyzed by unique liver microsomal cytochromes P-450. These can occur at the

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[1] Abbreviations: HPLC, high-performance liquid chromatography; GC, gas chromatography; testosterone, 17β-hydroxyandrost-4-en-3-one; AD, androst-4-ene-3,17-dione; *X*αOH-AD, *X*α-hydroxyandrost-4-ene-3,17-dione, where *X* indicates the number of the hydroxyl-substituted carbon atom; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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