Structural Analysis of Chicken Oviduct Progesterone Receptor Using Monoclonal Antibodies to the Subunit B Protein†

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ABSTRACT: Two monoclonal antibodies against the B subunit (M_r 108 000)[1] of chick oviduct progesterone receptor (PgR) were produced by immunizing rats and fusing spleen cells with NS-1 mouse myeloma cells. The hybridoma lines designated 9G10 and 3E8 produce rat IgG2a and IgG2b, respectively. Antibody–receptor interactions were demonstrated under protein denaturing conditions. Previous studies by Weigel et al. [Weigel, N. L., Tash, J. S., Means, A. R., Schrader, W. T., & O'Malley, B. W. (1981) *Biochem. Biophys. Res. Commun. 102*, 513–519] have shown that chick PgR can be phosphorylated in vitro. Both antibodies, 9G10 and 3E8, were shown to displace partially denatured ^{32}P-labeled PgR from its characteristic 4*S* position on high salt sucrose density gradients to a form with a higher sedimentation coefficient. Further specificity and sensitivity were demonstrated by protein immunoblotting experiments. In partially purified as well as electrophoretically pure receptor B subunit preparations antibodies reacted with the M_r 108 000 receptor B band. By immunoblot assay 9G10 was 20-fold more sensitive than 3E8, the former detecting down to 5 ng of receptor and the latter 100 ng. Because of its sensitivity 9G10 was able to detect the M_r 108 000 receptor as a single band in a crude oviduct fraction and did not cross-react with any other contaminating proteins. Receptor antigenic determinants were localized by immunoblot assay of receptor proteolytic digests. Under mild conditions of enzymatic proteolysis a characteristic series of stable peptides containing the hormone binding site are produced when homogeneous receptor B preparations are utilized. The smallest fragment containing the hormone binding site under these conditions is a M_r 31 000 peptide. Determinants for both antibodies were contained in all peptides down to M_r 34 000 but were lost when digesting from M_r 34 000 to M_r 31 000. This places the antigenic sites in a M_r 3000 region adjacent to the M_r 31 000 hormone binding domain. Since we have been able to detect antibody–antigen interactions only under denaturing conditions, indirect methods were used to demonstrate structural identity between the antigen and hormone-bound receptor. We conclude that antibodies react either with a denatured conformation of receptor or with a modified form of intracellular receptor unable to bind hormone.

Chicken oviduct progesterone receptor (PgR) is composed of two dissimilar hormone binding polypeptide subunits, A and B, having molecular weights of 79 000 and 108 000, respectively (Schrader et al., 1980a; Sherman et al., 1976; Boyd & Spelsberg, 1979).[1] On the basis of reversible cross-linking experiments (Birnbaumer et al., 1979), receptor subunits appear to exist in cytosol as a 6–7S dimer, and there is no evidence based on enzymatic conversion studies for a precursor–product relationship betwen A and B subunits (Schrader et al., 1980b). Although receptor A and B proteins display equivalent hormone binding kinetics and specificities, they display distinct functional properties. The receptor A protein binds strongly to DNA (Hughes et al., 1981; Compton et al., 1983), while the B protein binds preferentially to chromatin but only weakly to DNA (Schrader et al., 1972; Vedeckis et al., 1980). By use of the synthetic progestin [^{3}H]R5020 (17α,21-dimethyl-19-norpregna-4,9-diene-3,20-dione), photoaffinity labeling of receptor subunits has been achieved (Dure et al., 1980) which allows analysis of receptor structure by high-resolution denaturing techniques. By use of this technique, recent studies have established peptide maps of the chick receptor subunits by partial proteolytic digestion of the covalently labeled receptor–R5020 complex (Birnbaumer et al., 1983a). Peptide maps of the separated A and B proteins were found to be strikingly similar below the molecular weight of the smaller A subunit, indicating similar structural organization of the receptor subunits (Birnbaumer et al., 1983b; Gronemeyer et al., 1983). However, tryptic peptide mapping of purified receptor subunits under denaturing conditions revealed differences in significant portions of primary sequence of the two proteins, suggesting that the A and B subunits are in fact separate gene products that may have evolved through gene duplication (Birnbaumer et al., 1983b).

To study further the structural features of the chick receptor subunits, we have attempted to prepare antibodies to the receptor B subunit. Spontaneous antibodies in sheep were first used (Weigel et al., 1981b) to identify a common immunoreactive domain present in both subunits A and B. Subsequent in vitro immunization of mouse spleen cells with subunit B followed by fusion with mouse myeloma cells (Dicker et al., 1983) yielded a monoclonal antibody (class IgM) reactive with receptor. Both of these antibodies, however, were only weakly reactive against receptor proteins. Recently, an additional report of a monoclonal antibody to chick progesterone receptor has appeared (Radanyi, 1983). This antibody does not react with the 4S receptor subunits themselves but rather with another protein which appears to be associated in vitro with all classes of steroid receptors (Joab et al., 1983). Thus, it has been difficult to raise suitable antibodies for use in receptor structural studies. We have used as antigen the receptor B subunit purified from laying hen oviducts. In the present study, we report isolation of two monoclonal antibodies reacting with this antigen, derived from two independent hybridoma cell lines. We have used these antibodies to map unique structural

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[1] The molecular weight of the B protein appears to be 105 000 ± 3000 when measured by SDS gel electrophoresis. The protein will be referred to as having a molecular weight of 108 000 in this paper as previously reported (Birnbaumer et al., 1983a).

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determinants present in receptor B but not in subunit A.

Experimental Procedures

Animals. Balb/c female mice obtained from Charles Rivers Breeding Laboratories, Inc. (Wilmington, MA), were used as a source of thymus cells for feeder layers in cloning dishes. Inbred male Lewis rats (Microbiological Associates) were used for immunizations. Athymic nude mice (nu/nu) from Harlan Sprague-Dawley were used for growth of rat × mouse hybridomas as ascites tumors.

Reagents. $Na^{125}I$ (100 mCi/mL), [^{3}H]progesterone (43 Ci/mmol), and [^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham. [17α-*methyl*-^{3}H]R5020 (70–87 Ci/mmol) was obtained from New England Nuclear Corp. The catalytic subunit of bovine cardiac cAMP dependent protein kinase was a gift of Dr. Joseph Tash. Bovine pancreas trypsin (TPCK[2] treated to remove chymotryptic activity) and chymotrypsin were obtained from Worthington Enzymes. *Staphylococcus aureus* protease strain V8 (500–700 units/mg) was obtained from Pierce. Goat anti-rat immunoglobulin (IgG heavy and light chain specific) was obtained from Cappel Laboratories and Enzymobeads from Bio-Rad.

Immunization. The B subunit of the progesterone receptor was purified from laying hen oviducts as previously described (Schrader et al., 1977; Weigel et al., 1981a). Material for injections was estimated to be 50% pure on the basis of gel electrophoresis in sodium dodecyl sulfate (SDS). Preparations either were lyophilized and redissolved in phosphate-buffered saline (0.18 M NaCl, 3 mM KCl, 10 mM sodium phosphate, and 1.5 mM potassium phosphate, pH 7.4; PBS) containing 0.1% (w/v) sodium dodecyl sulfate or were frozen at the final step of purification and thawed immediately before injection. Two male Lewis rats were injected with approximately 100 μg of receptor B protein per injection, given either 4 times over a period of 4 months or 5 times over a period of 5 months. The primary immunization was given at multiple sites subcutaneously in complete Freund's adjuvant (Gibco). Booster injections were given subcutaneously in incomplete Freund's adjuvant (Gibco). Animals were bled from the tail vein during the course of the immunizations and by cardiac puncture after removal of the spleen. Antiserum was assayed for positive antibody titer against the partially purified receptor B antigen before taking for cell fusion. Three days prior to cell fusion, animals were injected intravenously with 50 μg of B receptor in 0.5 mL of PBS.

Cell Fusion. Single cell suspensions of rat spleen cells were mixed at a ratio of 4 to 1 (spleen/NS-1) with mouse P3 NS-1-Ag4-1 (NS-1) myelomas and fused with 50% (w/v) polyethylene glycol (PEG; M_r 1540 obtained from Koch Light) in serum-free RPMI-1640 as described by Oi & Herzenberg (1980). Culture medium for myeloma cells was RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Sterile Systems), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 units/mL penicillin–streptomycin (Gibco), 0.2% sodium bicarbonate. Fused cells were plated for 24 h in Petri dishes in NS-1 growth medium supplemented with 20% fetal bovine serum and then seeded into 96-well microtiter dishes (Costar) in HAT selection medium (the same medium described above with the addition of 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) at a density of 5×10^5 cells/well. Culture medium was removed for screening 1–2 weeks after fusion. After the first screening, positive antibody producing cultures were transferred and plated into larger 1-mL wells (Costar 24 well plates), allowed to grow for an additional 2–3 days, and then reassayed. At this point, cells from positive wells were cloned directly by limiting dilution into 96-well dishes seeded 24 h in advance with a feeder layer of Balb/c mouse thymus cells plated in 100 μL of growth medium (same medium as for hybrids minus aminopterin; HT) at 5×10^5 cells/well. Wells containing single hybridoma colonies (1–2 weeks after cloning) were assayed for antibody production. Positive clones were then expanded and maintained in suspension culture in 100-mm Petri dishes. Hybridoma lines were cryopreserved in 95% fetal bovine serum and 5% dimethyl sulfoxide (American Type Tissue Culture Collection) in liquid nitrogen and have been successfully retrieved and regrown without loss of antibody production. Further expansion of cloned hybridoma lines was achieved by growing cells as ascites tumors in pristane-primed athymic nude mice. Pristane (0.5 mL) was injected intraperitoneally 1 week prior to intraperitoneal injection of 2×10^6 hybridoma cells per animal. Ascites fluid obtained by abdominal puncture 1–2 weeks later was used as the source for antibody purification. The IgG fraction was purified by two successive precipitations of immunoglobulins at 40% saturation of ammonium sulfate follwed by gel filtration on a 1.5 × 20 cm Sephacryl S-300 column equilibrated and eluted with PBS. Eluted antibody was dialyzed against PBS containing 0.02% sodium azide and stored at 4 °C at a concentration of 1 mg/mL. Antibody typing for rat Ig class was performed by Ouchterlony double diffusion analysis using antisera specific for rat Ig subclasses (Miles) and by a solid-phase immunoadsorption assay coating plates with typing antisera (diluted 1:10 in PBS) and incubating with monoclonal antibodies followed by radioiodinated anti-rat immunoglobulin.

Screening Assays. Anti-receptor B protein activity was assayed by a solid-phase immunoadsorption assay. For initial screenings of rat antiserum and hybridoma cultures, the same partially purified B protein used as immunogen was bound to flexivinyl 96-well plates (Dynatech) by incubating each well overnight at 4 °C with 50 μL of a 10 μg/mL antigen solution. The antigen can be saved and reused several times since only a small portion binds to the plate. For secondary screenings, plates were coated with homogeneous receptor B protein obtained either by electroelution of the receptor band from SDS electrophoresis gels or by high-pressure liquid chromatography (HPLC) (described below). Remaining binding sites on the plates were blocked by incubation for 1 h at 4 °C with 1% (w/v) bovine serum albumin (BSA). Individual wells were then incubated overnight at 4 °C with 50 μL of either hybridoma media or purified antibody from ascites fluid. Unbound antibodies were removed by washing the wells 3 times with 200 μL of PBS. Bound antibody was then detected by incubation of each well for 4 h at 4 °C with 50 000 cpm of ^{125}I-labeled goat anti-rat immunoglobulin. After three final washes with 200 μL of PBS, individual wells were cut out with a hot wire device, dropped into a tube, and counted in a Nuclear Chicago γ counter. Goat anti-rat immunoglobulin (Cappel; heavy and light chain specific) was radioiodinated by a solid-phase lactoperoxidase method (Morrison et al., 1971). Nonspecific (or background) binding (~250 cpm) in this assay was determined by incubating receptor B protein coated plates with either normal rat serum, medium from NS-1 cultures, or an unrelated monoclonal antibody. Positive antibody producing cultures were taken as those showing binding 10-fold above background.

[2] Abbreviations: TPCK, tosylphenylalanine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Immunoelectroblotting Technique. Proteins were separated by SDS–polyacrylamide gel electrophoresis on a vertical slab gel apparatus as originally described by Laemmli (1970) and modified by Wykoff et al. (1977) using a gel matrix of 10% acrylamide/0.25% bisacrylamide (Bio-Rad) and a stacking gel of 3% acrylamide. For staining with 0.5% Coomassie blue R-250, gels were fixed in 10% acetic acid and 40% methanol and then destained in 7% acetic acid. Highly sensitive silver staining of gels was according to the method of Merril et al. (1981).

Immunological detection of antigen transferred from electrophoresis gels to paper was performed essentially as described by Renart et al. (1979) except that the transfer buffer was 20 mM sodium phosphate, pH 6.5, and the paper was aminophenyl thioether–cellulose (APT) (Schleicher & Schuell). The APT paper was converted to diazophenyl thioether (DPT) by a 30-min incubation in ice-cold nitrous acid (100 mL of 1.2 N HCl and 3 mL of 10 mg/mL sodium nitrate) immediately before use and then rapidly rinsed with ice-cold water. Gels were equilibrated for 1 h with three changes of the transfer buffer, and proteins were transferred with an E-C Corp. transfer apparatus at 0.65 A for 4 h at room temperature. After transfer, diazo groups were quenched by a 2-h incubation in 0.1 M Tris-HCl, pH 9.0, containing 0.25% gelatin, and the paper was then sealed in a plastic bag containing the monoclonal antibody purified from ascites fluids and incubated on a rocker platform overnight at 4 °C. Paper was then washed with binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM Na_2EDTA, 0.25% gelatin, and 0.05% NP-40) and reincubated overnight at 4 °C with ^{125}I-labeled goat anti-rat IgG at a concentration of 500 000 cpm/5 mL per gel lane. After extensive washing in a final wash buffer (50 mM Tris, pH 7.4, 1 M NaCl, 5 mM Na_2EDTA, and 0.4% sodium lauroylsarcosine), the paper was air-dried and exposed for 48 h to Kodak X-OMAT-XRP-1 film in a cassette with Du Pont Cronex Quanta III intensifying screens. Efficiency of protein transfer was monitored by staining the gels with Coomassie blue following transfer. Aliquots of the radioiodinated second antibody were routinely electrophoresed and transferred to paper as an internal control for protein transfer efficiency, as a monitor of the quality of the second antibody probe, and as a molecular weight marker for orientation on the autoradiogram.

Preparation of Crude Chick Receptor B Subunit. Chick oviduct cytosol (20 mL) was prepared as previously described (Schrader et al., 1977; Weigel et al., 1981b), labeled with 10 nM [^3H]progesterone for 2 h at 4 °C, precipitated at a final concentration of 35% saturation of ammonium sulfate, and centrifuged at 13200*g* for 30 min. The precipitate was resuspended in buffer A (10 mM Tris, pH 7.4, 1 mM Na_2 EDTA, and 12 mM 1-thioglycerol) and dialyzed for 2 h against the same buffer. The samples were then applied to a 2-mL DEAE-cellulose column, washed with buffer A containing 0.15 M NaCl to elute the A subunit, and finally the B-subunit was eluted with buffer A containing 0.3 M NaCl. This enriched receptor B preparation, separated from A protein, was used both for sucrose density gradient analysis and for immunoblotting experiments. Preparation of aporeceptor (chick receptor B protein lacking bound hormone) was prepared as previously described through the DEAE step (Schrader et al., 1977; Weigel et al., 1981b). The subunits were dissociated by treatment with 0.3 M NaCl overnight at 4 °C. The receptor preparation was adjusted to a final concentration of 0.1 M NaCl and passed through a 2-mL DNA–cellulose column to remove the A subunit. The flow through containing the B receptor was applied to a 2-mL DEAE column and eluted with 0.3 M NaCl. The crude DEAE receptor B protein used in immunoblot experiments contained approximately 1.4 μg of receptor (560 000 cpm of bound [^3H]progesterone) and 4.75 mg of total protein per mL.

Sucrose Gradient Ultracentrifugation. Receptor samples were incubated overnight at 4 °C with antibody (diluted as described in the text) in a final volume of 200 μL. Samples were layered on 5–20% sucrose gradients prepared in buffer A containing 0.3 M NaCl. Centrifugation was performed at 65 000 rpm for 2 h on a Beckman VTi 65 vertical rotor. Fractions (200 μL) were collected from each gradient and measured for radioactivity in 10 mL of ACS liquid scintillation fluid in a Beckman LS8000 scintillation counter with a counting efficiency of 25% for ^3H and 90% for ^{32}P.

Preparation of Homogeneous Receptor B Protein. Homogeneous receptor B protein from laying hen oviducts was prepared for use (1) in phosphorylation studies, (2) in proteolytic digestions, and (3) as a secondary screening antigen. Preliminary partial purification of the receptor B protein was as previously described (Weigel et al., 1981a). The final step in purification to electrophoretic purity was achieved by one of two methods. Method one was high-pressure liquid chromatography on a TSK-3000 gel filtration column. The eluted B protein at M_r 108 000 was determined to be homogeneous by highly sensitive silver stained SDS–polyacrylamide electrophoresis gels. Method 2 was electroelution of the B-protein band from an SDS electrophoresis gel. The partially purified B protein was subjected to single dimensional SDS gel electrophoresis, and the M_r 108 000 band was electroeluted from the gel. One gel lane was stained with Coomassie blue to identify the B protein band. The B protein bands in parallel unstained gel lanes were cut out, and the receptor protein was electroeluted by placing the gel pieces in an open-ended glass tube fitted with a porous plug (consisting of a small piece of Kimwipe soaked in SDS) to hold the gel pieces. A dialysis bag was then tied to the end of the tube. Tubes were placed in a tube gel electrophoresis apparatus in Tris–glycine containing 0.1% SDS and run at 3 mA/tube. The eluted protein, collected through the bottom of the tube in the dialysis bag, was exhaustively dialyzed against 0.3 M NaCl with 0.2% SDS followed by dialysis in buffer A containing 0.01% SDS to reduce the level of SDS. Protein eluted in this manner represents essentially homogeneous receptor B subunit as judged by reelectrophoresis.

Preparation of ^{32}P-Phosphorylated Receptor B Protein. Homogeneous receptor B protein prepared by HPLC was phosphorylated with the catalytic subunit of bovine cardiac cAMP-dependent protein kinase as previously described (Weigel et al., 1981a) except that the ATP concentration was 15 μM and the specific activity of [^{32}P]ATP was higher (80 000 cpm/pmol) than previously reported. The free [^{32}P]ATP was removed by exhaustive dialysis against buffer A. Under these conditions the receptor tended to aggregate so the preparation was incubated for 2 h at room temperature in buffer A containing 0.01% SDS. Buffer A containing 0.6 M sodium chloride was added to give a final concentration of 0.3 M, and receptor was then used for sucrose density gradient assays.

Limited Proteolytic Digestion of the Receptor B Protein. Homogeneous receptor B subunit was subjected to limited proteolytic digestion according to the following conditions. For each reaction the starting material was 0.6 μg of receptor, which was incubated with the following proteases: (1) 0.025 μg of chymotrypsin in 15 μL of 0.1 M sodium phosphate, pH

7.0, at room temperature for 3 and 16 h; (2) 0.125 μg of *S. aureus* protease V8 in 35 μL of 0.05 M sodium phosphate, pH 7.0, at 37 °C for 3 and 16 h; (3) 0.004 μg of trypsin in 11 μL of 0.1 M sodium phosphate, pH 7.0, at room temperature for 3 and 16 h. Digestion was stopped at the appropriate time by quick freezing the samples in dry ice–methanol and storing at −70 °C. Samples to be run on gels were thawed at 4 °C and immediately boiled for 5 min in SDS sample buffer.

Photoaffinity Labeling and Two-Dimensional Gel Electrophoresis. Covalent labeling of receptor proteins with [^{3}H]R5020 by UV irradiation was as previously described (Birnbaumer et al., 1983a). Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (1975). Isoelectric focusing was used as the first dimension, and sliced duplicate gels were used for determination of pH gradient. Electrophoresis in 7.5% polyacrylamide in the presence of 1% SDS (w/v) was used as the second dimension. Fluorographic analysis of dried slab gels was a previously described (Birnbaumer et al., 1983a).

Results

Production and Detection of Receptor Monoclonal Antibodies. Immunization of Lewis rats was performed by using partially purified receptor B antigen of the degree of purity shown in the left panels of Figure 2. Spleen cells from two rats were fused separately with mouse NS-1 myelomas, and after propagation of hybridomas in HAT selection medium, cultures were first screening for antibody by the solid-phase radiometric assay described under Experimental Procedures, using the partially purified B antigen. Of 1090 wells containing hybridomas, 267 (24%) gave positve results on first screening against the partially pure antigen. The cultures (168) showing the highest binding were split to larger 1-mL wells (Costar 24-well plates) and reassayed 2–3 days later against the homogeneous B antigen of the purity shown in Figures 5A and 9. Fifty cultures remained positive against pure antigen and were cloned by limiting dilution on T-cell feeder layers, and from this, two stable independently derived clones were isolated. One clone designated as 9G10 secretes rat IgG2a, and the other clone, 3E8, secretes rat IgG2b. Both clones have proven to be monoclonal lines, since repeated subcloning resulted in 100% of the isolated colonies producing antibody to the B antigen. Both cell lines have also been grown successfully in pristane-primed athymic nude mice and produce high antibody titers. As shown in Figure 1 antibody titers in ascites fluids, determined by solid-phase immunoadsorption assay, were considerably higher than in unfractionated culture medium. The titer of antibody 3E8 in both culture medium and ascites fluid is higher than that of 9G10. Antibody 9G10, however, appears to have higher affinity for receptor (as shown in subsequent experiments); no binding above background was observed at any dilution with ascites fluids from NS-1 myeloma cells grown in Balb/c mice. All subsequent experiments described are with antibodies 9G10 and 3E8 purified from ascites fluids.

Specificity was initially examined by the solid-phase binding assay with the appropriate control incubations. No binding above background (~250 cpm) was observed when the receptor antigen was omitted or when plates were coated instead with unrelated antigens such as bovine serum albumin, purified human estrogen receptor, estrogen-induced proteins from MCF-7 cells (Edwards et al., 1980), or ovalbumin. Replacing the primary antibody with buffer alone, unrelated rat antibodies, normal rat serum, or NS-1 spent medium also gave no binding above background (data not shown).

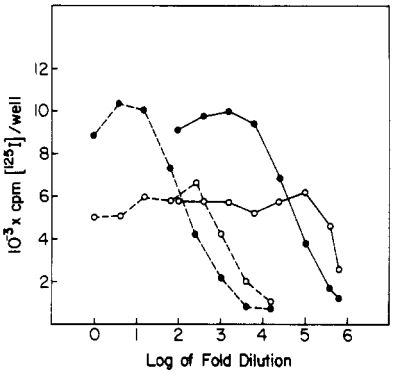

FIGURE 1: Antibody titer in culture medium vs. ascites fluids. Receptor antibody binding to PgR-coated plates was measured by the solid-phase immunoabsorption assay utilizing ^{125}I-labeled second antibody as described under Experimental Procedures. Unfractionated culture medium from 9G10 (●---●) and 3E8 (○---○) or ascites fluid from 9G10 (●—●) and 3E8 (○—○) grown in nude mice were diluted in PBS buffer containing 1% bovine serum albumin.

Immunoblot Analysis of Progesterone Receptor. Further characterization of antibody–receptor interaction was by protein immunoblot experiments. Varying amounts of partially purified hen receptor B protein were electrophoresed and either stained with Coomassie blue or transferred to activated APT paper and incubated with monoclonal antibodies 9G10 and 3E8 as described under Experimental Procedures. The left panels in Figure 2 show the stained gels of the partially purified receptor B protein used for these experiments. The predominant band at M_r ~108 000 has been previously identified as authentic receptor by photoaffinity labeling with [^{3}H]R5020 (Birnbaumer et al., 1983a). IgG's in the stained gel and in the autoradiograms in the right panels are included as molecular weight markers. The autoradiogram in Figure 2A shows reactivity of 9G10 with the receptor B protein band, detecting down to 5 ng of antigen. Some smaller molecular weight bands were also reactive. These are a result of degradation of the B protein during storage and increase (as judged by silver-stained SDS gels) with time. No other bands in the preparation show reaction. The autoradiogram in Figure 2B shows reactivity of antibody 3E8 also with the receptor B protein. This antibody is 20-fold less sensitive than 9G10 by immunoblot assay since its lower limit of detection is 100 ng of antigen.

A more rigorous test of specificity was examined in Figure 3 by immunoblotting a crude oviduct protein fraction. The crude receptor used was a DEAE-cellulose fraction of chick oviduct cytosol (as described under Experimental Procedures), in which receptor A protein has been removed. On the basis of the number of specific hormone binding sites, receptor represents less than 0.1% of total protein in this fraction. A stained SDS gel of the crude DEAE fraction and, for reference, the partially purified receptor B protein is shown in the left panel of Figure 3. As shown in the autoradiogram in the center panel, 9G10 reacted with a single band in the crude preparation at the same position as the M_r 108 000 receptor B protein. No cross-reaction was detected with the numerous contaminating bands. As an internal control, 400 ng of purified receptor B protein was added to an aliquot of the crude sample (far right lane of center panel) and was also immunoblotted with 9G10. The presence of some smaller molecular weight radioactive bands in this lane, compared with the single radioactive band observed in crude samples, further indicates that smaller molecular weight bands in the purified preparation represent receptor degradation products. In the experiment shown in Figure 3, 9G10 was able to detect down to 20 ng of

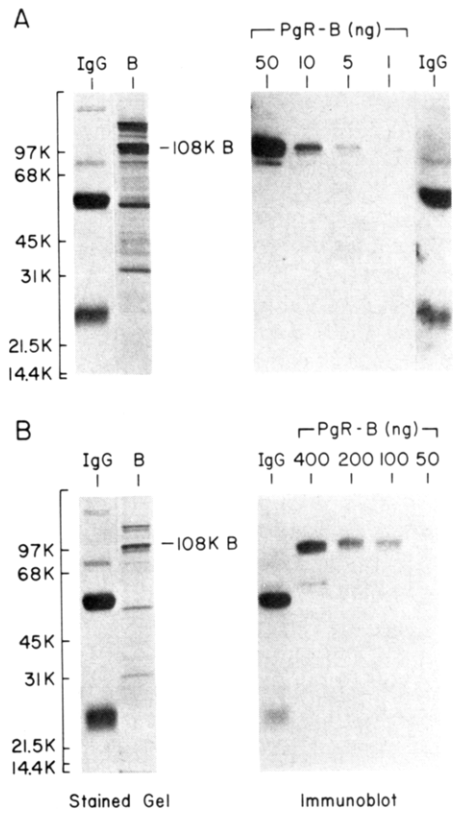

FIGURE 2: Immunoblot analysis of partially purified hen progesterone receptor B protein. Proteins to be analyzed were electrophoresed on 10% SDS–polyacrylamide gels and either stained with Coomassie blue (left panel) or transferred to activated APT paper (autoradiogram/right panel) and incubated with purified monoclonal antibody followed by ^{125}I-labeled second antibody as described under Experimental Procedures. The IgG's shown in the stained gels and autoradiographs are the second antibody used for radioiodination. (A) Decreasing amounts of the partially purified receptor B protein (50–1 ng) were transferred and incubated with 9G10 (40 μg/mL). The band indicated at 108K represents receptor B protein identified in previous studies as receptor by photoaffinity labeling with [^{3}H]R5020. Standard proteins used were low molecular protein standards from Bio-Rad; phosphorylase B (M_r 97 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000) and soybean trypsin inhibitor (M_r 21 500). (B) Immunoblot analysis of partially purified hen progesterone receptor B protein with monoclonal 3E8. Conditions were the same as described in panel A except that decreasing amounts of partially purified receptor B protein, ranging from 400 to 50 ng were transferred to activated paper, and the paper was incubated with purified 3E8 (110 μg/mL).

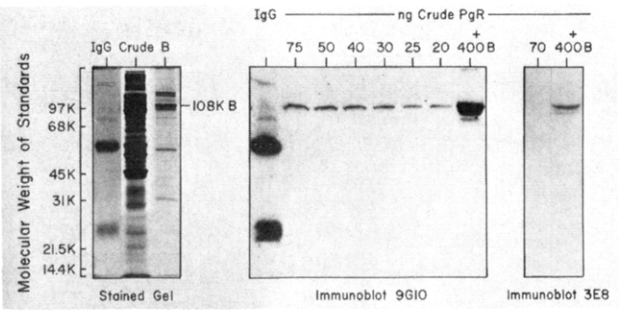

FIGURE 3: Immunoblot analysis of crude chick oviduct progesterone receptor with monoclonals 9G10 and 3E8. Chick oviduct cytosol was fractionated on DEAE as described under Experimental Procedures. This material was resolved on SDS electrophoresis gels and either stained with Coomassie blue (left panel) or transferred to activated APT paper and incubated with purified 9G10 (40 μg/mL), center panel, or purified 3E8 (110 μg/mL), right panel, followed by ^{125}I-labeled second antibody. For reference, a sample of partially purified receptor B protein (B) was included in the stained gel. Decreasing amounts of crude receptor B protein (estimated from the number of hormone binding sites in the sample) ranging from 75 to 20 ng were immunoblotted with antibody 9G10. 3E8 was blotted against 70 ng of crude DEAE receptor only. As an internal control 400 ng of partially purified receptor B protein (+400B) was mixed with aliquots of the crude DEAE fraction and immunoblotted with antibody 9G10 (far right lane of center panel) and 3E8 (right lane of right panel).

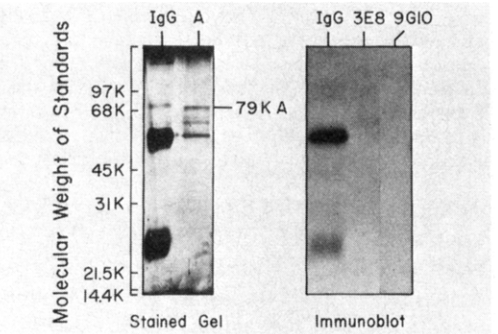

FIGURE 4: Immunoblot analysis of partially purified progesterone receptor A protein with monoclonal 3E8 and 9G10. Partially purified M_r 79 000 receptor A protein (~0.6 μg) was resolved on SDS electrophoresis gels and either silver stained (left panel) or transferred to activated APT paper and incubated with purified 3E8 (110 μg/mL) or 9G10 (40 μg/mL) followed by ^{125}I-labeled second antibody (autoradiogram, right panel).

receptor. In subsequent experiments (not shown), the lower limit of detection with this antibody in crude preparations was about 5–10 ng of B protein. Also shown in the autoradiogram in the far right panel, the less sensitive antibody 3E8 was not able to detect the M_r 108 000 B protein in the crude DEAE preparation. This antibody did, however, selectively react with 400 ng of purified receptor B protein mixed with a crude sample without evidence of cross-reaction with contaminating bands in the preparation (far right lane). The 3E8 antibody, therefore, is of equivalent specificity as antibody 9G10 but is less sensitive by immunoblot assay.

A further test of specificity of the antibodies is shown in Figure 4. We tested both monoclonal antibodies for cross-reaction with authentic receptor subunit A, a closely related subunit of the intact receptor complex. Earlier studies had shown at least one common immunological determinant on the A and B subunits (Weigel et al., 1981b). However, as evidenced by the lack of a radioactive band in the autoradiogram of Figure 4, at M_r 79 000, monoclonal 9G10 and 3E8 failed to react with subunit A. Thus, by this test, both monoclonal antibodies are specific for the receptor B subunit and are sufficiently discriminating to be useful for receptor peptide identificaiton studies.

Identification of Antigenic Sites by Immunoblotting of Receptor Proteolytic Peptides. Partial proteolytic peptide maps of the receptor B protein, covalently labeled with [^{3}H]R5020, have been previously published and have resulted in identification of distinctive hormone binding peptides. Birnbaumer et al. (1983b) identified peptide maps using three enzymes, trypsin, chymotrypsin, and *S. aureus* protease. These previous peptide mapping experiments have allowed us to compare hormone binding peptides with immunoreactive peptides and identify antigenic determinants (epitopes) in relationship to the hormone binding domain of the receptor molecule. This was done by Western immunoblot analysis of partial proteolytic digests of a homogeneous preparation of the receptor B protein. Figure 5 shows SDS–polyacrylamide silver stained gels of the peptides generated from the digestion of homogeneous receptor B protein with the three enzymes shown. Paired with the stained gels are the corresponding autoradiographs (the right panel of each pair) of the immunoreactive peptides identified by monoclonal 9G10. Panel A

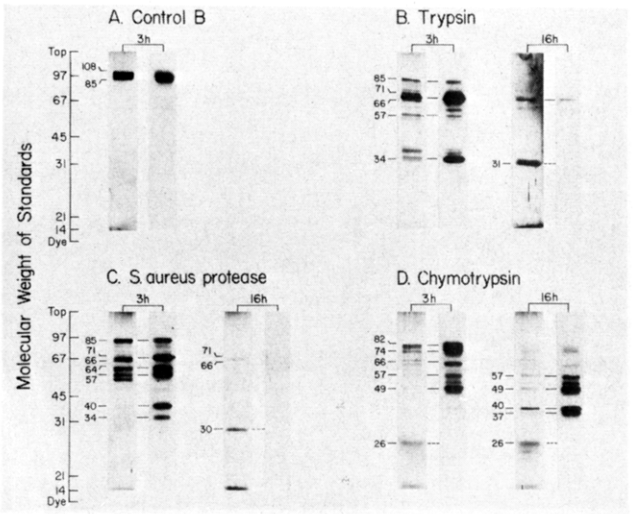

FIGURE 5: Mapping of receptor antigenic determinants for monoclonal 9G10. Homogeneous receptor B protein was subjected to mild proteolytic digestion for 3 and 16 h as described under Experimental Procedures, with trypsin, *S. aureus* protease V8, and chymotrypsin. Proteolytic peptides as well as control undigested B protein (B) were electrophoresed on 10% SDS–polyacrylamide gels and either silver stained or transferred to activated APT paper and incubated with purified 9G10 (40 μg/mL) followed by ^{125}I-labeled second antibody. (A) Control undigested receptor B protein, (B) tryptic peptides, (C) *S. aureus* protease V8, and (D) chymotrypsin. Silver-stained gels (left panel) and corresponding autoradiograms (right panel) for each sample were paired together. Molecular weights were determined from the standard proteins on the stained gel. Some distortion occurred during transfer to the paper. Therefore, the stained bands corresponding to the immunoreactive bands are indicated by solid lines. Broken lines denote peptides which were not immunoreactive.

shows the control undigested B protein and panel B the results of digestion with trypsin for 3 and 16 h. At 3 h, a series of protein-stained bands are present, which correspond with the hormone binding fragments found by Birnbaumer et al. (1983b). After 16 h of digestion, most of the larger fragments have disappeared, leaving a predominant 31K peptide. This fragment had also been detected in the [^{3}H]R5020 photolabeling study (Birnbaumer et al., 1983b) and thus under these conditions is the smallest fragment containing the hormone binding site. An examination of the corresponding autoradiograph reveals that all of the peptides larger than 31K are immunoreactive whereas the 31K fragment is not.

The receptor B protein purified by our large-scale isolation procedures contains the native B protein and some partially degraded B ranging in sizes down to M_r ~85 000. Sequence analysis of these fragments revealed that all of these degradative products had a blocked amino-terminus as did the undegraded M_r 108 000 B protein. Thus, endogenous proteolysis occurs at the carboxyl-terminal end of the molecule (Weigel et al., 1983). We have also performed sequence analysis of the 31K hormone binding peptide generated exogenously with trypsin and found that this fragment also had a blocked amino-terminal. Thus, trypsin appears to cleave receptor sequentially from the carboxyl terminus which places the 31K peptide at the amino-terminal end of the molecule. Therefore, of particular interest in the tryptic peptide map was the immunoreactive 34K peptide seen at 3 h of digestion which was absent at 16 h. This distinction between the immunoreactive 34K band and the nonreactive 31K band therefore places the antigenic site between 34 000 and 31 000 from the amino-terminal end of the molecule.

By use of this information it was then possible to identify peptides in the less well-characterized *S. aureus* protease and chymotrypsin digests. Previous experiments have suggested that *S. aureus* protease also cleaves sequentially from the carboxyl-terminal end (Birnbaumer et al., 1983b). The pattern of immunoreactive peptides in panel C (Figure 5) confirms this observation. A series of bands ranging from the native size of 108K to 34K, which are immunoreactive, appear when the receptor is incubated with *S. aureus* protease for 3 h. Bands smaller than 34K are not reactive. More extensive digestion (16 h) results in complete loss of the larger molecular weight immunoreactive bands and the appearance of smaller nonreactive peptides. Thus, the digestion pattern with *S. aureus* protease is similar to but not identical with the trypsin digest pattern. The *S. aureus* digest in contrast to the tryptic fragments contained a number of immunoreactive peptides not previously identified by Birnbaumer et al. (1983b) as containing R5020 binding sites. This may be due to the reported differences in the specificity of the *S. aureus* protease in the presence and absence of ammonia-containing buffers (Houmard & Drapeau, 1972). The experiments reported here were performed in sodium phosphate, whereas the previous experiments of Birnbaumer et al. (1983b) were performed in ammonium bicarbonate.

Partial digestion of the [^{3}H]R5020-covalently labeled receptor B protein with chymotrypsin reported by Birnbaumer et al. (1983b) resulted in production of two predominant hormone binding fragments at 33K and at 28K. No larger hormone-bound intermediates were seen, which suggests that the pattern of digestion with chymotrypsin differs from that of the other enzymes studied. We saw in this study that limited digestion of the purified B protein with chymotrypsin under mild conditions also produced a peptide at about 28K (e.g., 26K) which should also be derived from the N-terminus of the protein, based upon the explanation above. If the fragment were entirely bounded by the 31K trypsin fragment sequence, then the 26K peptide would not be expected to be immunoreactive. Figure 5D shows that this idea is correct; the 26K peptide is abundant in the 3- and 16-h digest but is not immunoreactive. Although we do not know from amino acid sequencing whether the 26K peptide has a blocked N-terminus, it is likely to represent the first 26K portion of the protein. In any event, it cannot arise from a portion extending up to 34K, since it would then be immunoreactive on the basis of the trypsin data.

As shown in Figure 5D, larger molecular weight chymotryptic peptides were also produced in this experiment which contained antigenic sites. On the basis of relative protein staining intensities, we predict that these larger molecular weight fragments represent the C-terminal fragments liberated when the 26K peptide is excised. Since the starting receptor B preparation contained a mixture of both the intact 108K protein and smaller proteolytic forms as small as 85K (panel A), some of the larger molecular weight bands in panel D may reflect the C-terminal heterogeneity of the starting material. Fragment sizes of 82K, 74K, and 66K present in the 3-h digest would sum in molecular weight with the major 26K band to equal the known molecular sizes of the B starting material mixture. Further analysis will be needed to prove this point.

We conclude from this family of digestions that these three proteases all can liberate N-terminal fragments which lack immunoreactivity. Thus, the immunologic site on receptor B must lie to the C-terminal side of the 31K trypsin fragment. This finding is supported by both the lack of immunoreactivity with *S. aureus* peptides smaller than 31K and lack of reactivity with the chymotrypsin 26K band.

The C-terminal limit of the antigenic site is less well-defined. Since the smallest peptides containing the antigenic sites are

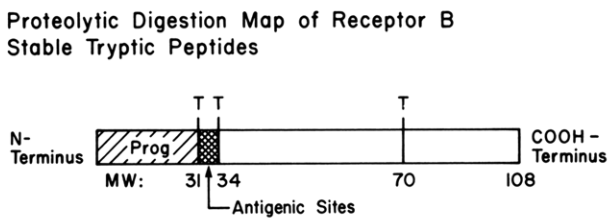

FIGURE 6: Schematic of receptor antigenic determinants for monoclonal 9G10 and 3E8.

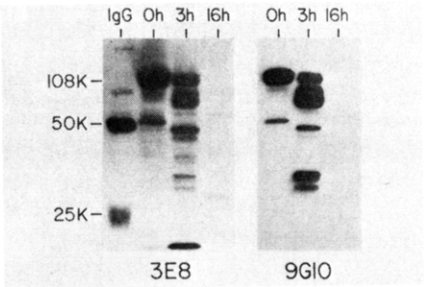

FIGURE 7: Mapping of receptor antigenic determinants for monoclonal 3E8. Partially purified receptor B protein was subjected to mild proteolytic digestion with trypsin for 3 and 16 h as described under Experimental Procedures. Proteolytic peptides as well as undigested B protein (Oh) were electrophoresed on 10% SDS–polyacrylamide gels, transferred to activated APT paper, and incubated with purified 3E8 (110 μg/mL), left panel, or purified 9G10 (40 μg/mL), right panel, followed by ^{125}I-labeled second antibody.

the 34K trypsin and *S. aureus* protease fragments, it appears that the immunogenic domain must lie on the N-terminal side of the 34K peptide. If true, then these data can be used to construct the linear receptor B structural map shown in Figure 6, with the epitope located near the 31K peptide which contains the hormone binding domain. Since a strict precursor–product relationship has not been proven for the trypsin 34K and 31K bands, nor for the *S. aureus* 34K and 30K bands, we cannot exclude the possibility that the antigenic site is displaced more toward the C-terminus. However, from relative band intensities during the time courses of digestion here and in the previous [^{3}H]R5020 photolabeling work (Birnbaumer et al., 1983b), it is unlikely that the fragments can arise independently.

Because of the lower sensitivity of antibody 3E8, it was difficult to obtain with each of the three enzymes the quantity of receptor fragments required to interpret immunoreactive peptide maps with this antibody. Thus, we compared antibody 9G10 and 3E8 against tryptic peptides only, utilizing a 20-fold excess of receptor B protein for analysis with 3E8 (compared with 9G10) to compensate for the difference in sensitivity. As shown in Figure 7, both antibodies reacted with similar tryptic peptides with the exception that 3E8 reacted also with a small peptide in the dye front. Neither antibody recognized the 31K peptide generated by 16-h digestion, and immunoreactive sites for both are contained in all peptides down to M_r ~34 000. The control M_r ~108 000 B protein in this experiment was partially degraded, which accounts for the smaller molecular weight radioactive bands in the control lane of Figure 7. Although both antibodies appear to react with a similar region of the receptor molecule, differences in sensitivity suggest they may react with separate determinants.

Antibody Detection of Phosphorylated Receptor. We have shown that hen PgR can be phosphorylated in vitro (Weigel et al., 1981a). To further analyze antibody interactions with structural features of receptor, we have examined whether these monoclonals can detect phosphorylated receptor. Homogeneous receptor B protein was labeled covalently with

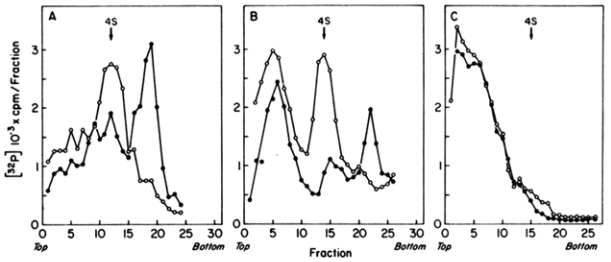

FIGURE 8: Sucrose density gradient analysis of phosphorylated receptor B protein. Homogeneous hen receptor B protein was labeled with [^{32}P]ATP as described under Experimental Procedures and centrifuged through a linear 5–20% sucrose gradient prepared in buffer A containing 0.3 M NaCl. Prior to gradient centrifugation phosphorylated receptor was (A) incubated overnight at 4 °C with 100 μL of purified monoclonal 3E8 (2.0 mg/ml) (●) or 100 μL of unrelated rat IgG control antibody (○). (B) Phosphorylated receptor incubated overnight at 4 °C with 100 μL of purified monoclonal 9G10 (1 mg/mL) (●) or 100 μL of unrelated rat IgG (○). The arrow indicates the position of the 4S albumin marker. (C) Incubation of free [^{32}P]ATP overnight at 4 °C (80 000 cpm/mL) with 100 μL of 9G10 (1 mg/mL) (●) or 100 μL of unrelated rat IgG (○) followed by sucrose gradient analysis.

[^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase and then exposed to 0.1% SDS to cause partial denaturation. The phosphorylated receptor retains its 4.2 S sedimentation coefficient after this treatment when analyzed by sucrose density gradient centrifugation in high salt buffer.

As shown in Figure 8A, addition of an unrelated rat IgG does not affect the sedimentation of phosphorylated receptor, while addition of antibody 3E8 displaced the 4S receptor peak toward a form with a higher sedimentation coefficient. The sedimentation coefficient of phosphorylated receptor in the presence of buffer alone was the same as with the unrelated rat IgG (not shown). Addition of antibody 9G10, shown in Figure 8B, also displaced the 4S phosphorylated receptor peak to a form with a higher sedimentation rate. The counts at the top of the gradient in Figure 8B represent residual free [^{32}P]ATP. Figure 8C shows that antibody 9G10 does not recognize free [^{32}P]ATP.

Structural Comparison of Antigenic Sites and Hormone Binding Sites. These immunoblot experiments demonstrated interaction of antibodies with highly denatured receptor B protein. We next examined antibody recognition of native receptor B protein from chick and from laying hen oviducts. These two preparations were labeled with [^{3}H]progesterone, and the antibody was tested for its ability to shift the sedimentation of [^{3}H]progesterone–receptor from its characteristic 4.2S position on high-salt sucrose density gradients. This method has been successfully applied to detect estrogen receptor interaction with monoclonal antibody (Greene et al., 1980a,b). Repeated experiments with various ratios of receptor to antibody and times of incubation failed to produce the expected shift (data not shown) in receptor sedimentation rate on sucrose density gradients. Considering that antibodies might be directed against a determinant near or at the hormone binding site, and steroid binding might interfere with the binding of antibody, we also assayed for antibody interaction with aporeceptor (no bound hormone) by labeling the collected gradient fractions with [^{3}H]progesterone after ultracentrifugation. This method also failed to produce a shift in receptor migration (not shown). Since neither antibody was able to detect native hormone-bound receptor, we had to consider the possiblity that our purified preparations contain an unrelated protein of M_r 108 000 which copurifies with receptor B protein and which preferentially reacts with antibody. Although antigenic sites for 9G10 and 3E8 are contained in a M_r 108 000 band in electrophoretically pure

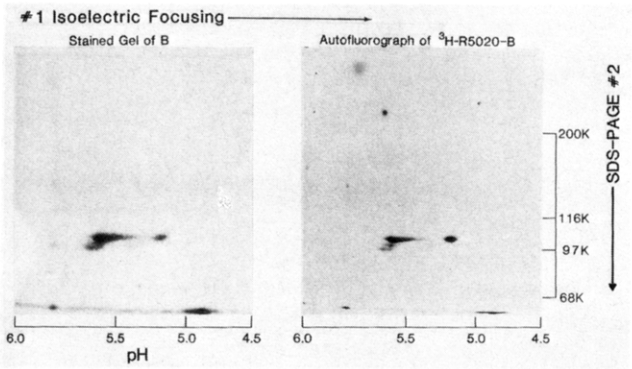

FIGURE 9: Two-dimensional gel analysis of receptor B protein. The B protein was purified from a preparation of hen oviduct receptor labeled with [^{3}H]R5020 (as described under Experimental Procedures). The purified B was then covalently labeled by exposure to an ultraviolet lamp and analyzed by two-dimensional electrophoresis. (Left panel) 10 μg of B protein stained with Coomassie blue. (Right panel) Autofluorograph of the gel from the left panel.

preparations of the B protein as judged by silver-stained single-dimensional gels (as shown in Figure 5A), it is possible that another protein of the same size as receptor but a different charge may be contained within this M_r 108 000 band.

To examine this possibility, we labeled a hen receptor crude extract with [^{3}H]R5020 (20 nM) and carried this material through purification steps to electrophoretic purity as determined by single-dimension SDS gels. After purification, the preparation was UV irradiated to covalently couple ligand to receptor sites, and an aliquot was analyzed by the two-dimensional polyacrylamide gel method of O'Farrell (1975).

Results of the experiment are shown in Figure 9. The left panel shows a gel stained with Coomassie brilliant blue. A series of protein spots at M_r 108 000 were visible as well as a smaller molecular weight proteolytic degradation product. To test this material for receptor content, the gel was soaked in EnHance (New England Nuclear) and fluorographed. Significantly, the tritium fluorogram (right panel of Figure 9) is identical with the staining pattern. All the protein spots contained covalently labeled receptor. Moreover, labeling intensities were proportional to the staining intensities. Similar findings were reported with the chick receptor covalently labeled with [^{3}H]R5020. On two-dimensional gels the hormone binding sites of the chick B protein correspond with the staining of the purified receptor protein (Birnbaumer et al., 1983b). Thus, Figure 9 demonstrates that the hen receptor B protein used as antigen has the same molecular size and charge as does [^{3}H]R5020–receptor complexes prepared in vitro. The specific radioactivity was very low, less than 1% of the theoretical maximum if one R5020 were bound per protein molecule. Thus, the experiment of Figure 9 does not exclude the possibility that not all receptor B antigen contains functional hormone-binding sites. Thus, antibody could be interacting with either nonfunctional forms of receptor or recognition may require denaturing and unfolding of the receptor molecule. Since hybridomas in this study were screened by solid-phase immunosorption assay, independent of hormone binding activity, we may have selected for antibodies reactive only with partially denatured antigen.

Discussion

Two independently derived monoclonal antibodies to the hen progesterone–receptor B protein were utilized for probing structural domains of the receptor. Previous studies (Birnbaumer et al., 1983b) with photoaffinity-labeled receptor have defined hormone binding peptide maps. These peptide mapping studies along with partial sequence analysis (Weigel et al., 1983) have revealed that receptor is cleaved sequentially from the carboxyl-terminus and that the hormone binding domain is contained within a 31K peptide at the N-terminus of the molecule.

In our current study, Western blot analysis of proteolytic peptides has allowed us to map antigenic sites in relationship to the hormone binding domain. We found that antigenic peptides correspond with most, but not all, hormone binding polypeptides. The smallest peptide containing antigen sites was a 34K fragment. The 9G10 antibody did not reconize anything below 34K including 31K and 26K peptides which, under the conditions used in these experiment, are the smallest fragments containing hormone binding sites. These peptide maps revealed that antigenic sites for both monoclonal antibodies are localized within a M_r 3000 region on the C-terminal side of the 31K fragment. Thus, both antibodies appear to interact with a similar locus on receptor adjacent to the hormone binding domain.

Neither antibody in this study showed cross-reaction with the PgR A subunit. This was determined both by coating 96-well plates with purified receptor A subunit (not shown) and by immunoblot assay (Figure 4). This lack of cross-reactivity gives further support to the evidence that receptor A and B subunits are separate gene products. The monoclonal antibodies produced in this study appear to be highly specific for a determinant on the B subunit of the hen receptor. To further analyze similarities and differences between receptor A and receptor B subunits, we have studies in progress to produce separate monoclonal antibodies to the receptor A protein.

We previously demonstrated in vitro phosphorylation of purified hen oviduct receptor B protein using physiological amounts of cAMP-dependent protein kinase. The phosphorylation site as determined by peptide mapping was found to be localized at the carboxyl-terminal region of the receptor (Weigel et al., 1983). Both monoclonal antibodies produced in this study were found to interact with phosphorylated receptor B protein. These antibodies, therefore, may prove to be important probes for studying the possible role of phosphorylation in receptor function.

The present studies with the 9G10 and 3E8 monoclonal antibodies have produced some unexpected results. We found that neither antibody was capable of interaciton with the native hormone binding form of receptor. Thus, we have analyzed antibody–receptor antigen interactions in this study by Western blot analysis and by sucrose density gradient analysis of ^{32}P-labeled receptor. This has forced us to use indirect methods for demonstrating the identity of antigen with the receptor molecule. Using high resolution techniques, we have been able in this study to demonstrate that antigen and receptor are structurally similar molecules. First, two-dimensional gel analysis revealed that highly purified receptor B protein which contains antigenic sites (for both 9G10 and 3E8) and the B protein covalently bound with [^{3}H]R5020 are the same size and charge and thus appear to be the same protein. Second, partial proteolysis of highly purified B antigen (with three different enzymes) produced immunoreactive peptide maps which correspond with hormone binding peptide maps produced through proteolysis of photoaffinity-labeled receptor. Thus, receptor and antigen appear also to be the same protein based on comparison of their peptide maps.

As an attempt to demonstrate directly antibody interaction with hormone-bound receptor, we have also reacted antibody 9G10 with SDS-denatured receptor B protein, covalently

coupled with [^{3}H]R5020. We have not been able to detect such an interaction by sucrose gradient ultacentrifugation analysis. One possible explanation for this observation is that receptor B protein exhibits microheterogeneity in the hormone binding site as well as at or near antigenic sites. We have tested this possibility by purifying the [^{3}H]progesterone–receptor B complex to homogeneity by an alternate strategy. In another work (S. Peleg et al., unpublished results), we have found that the denatured hormone-binding receptor B protein is not immunoreactive and can be separated chromatographically from the bulk of the antigenic protein. Both proteins, however, have identical proteolytic peptide maps. Thus, we conclude that the two monoclonal antibodies described in the present study react with a form of receptor unable to bind hormone, probably due to posttranslational modification and not to differences in primary sequence. We cannot exlcude the unlikely possibility that two unrelated proteins are synthesized, which are indistinguishable by peptide mapping and two-dimensional gel analysis. Further investigations by direct sequencing of the cloned gene for hormone binding receptor and antigen will be needed finally to answer this question.

It should also be possible to address the question of biologically inactive forms of receptor by isolation and purification of the receptor B–antigen with an antibody affinity column and then to use the purified antigen for immunization and production of further monoclonal antibodies. If antigen recognized by 9G10 and 3E8 represents a chemically modified form of receptor unable to bind hormone, it should be possible to select wtih appropriate screening procedures, antibodies cross-reactive with native hormone bound receptor.

The potential applications for the antibodies produced in this study are numerous. The fact that antibody recognizes denatured antigen may be an advantage. For example, some applications will require denaturation of antigen including histochemical localization of receptor, identification of receptor mRNA in cell-free translation assays, screening of cDNA libraries for the receptor gene, and further structural mapping. The utility of the present antibodies as probes (especially 9G10 because of its high sensitivity) for mapping structural and functional domains of the receptors molecule was demonstrated in this study by the immunoblot experiments with receptor proteolytic digests. By continuing with immunizations, it should be possible to select further antibodies against a variety of receptor determinants (hormone binding domain and the carboxyl-terminal region of receptor) to be used for a more complete structural analysis of both the receptor A and receptor B subunits.

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