Immunologic Analysis of Human Breast Cancer Progesterone Receptors. 1. Immunoaffinity Purification of Transformed Receptors and Production of Monoclonal Antibodies†

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ABSTRACT: A monoclonal antibody (MAb), designated PR-6, produced against chick oviduct progesterone receptors [Sullivan, W. P., Beito, T. G., Proper, J., Krco, C. J., & Toft, D. O. (1986) *Endocrinology (Baltimore) 119*, 1549-1557] cross-reacts with the M_r 120 000 human B receptors. An immunomatrix prepared with PR-6 was used to purify progesterone receptors (PR) from T47D human breast cancer cells. Single-step immunoaffinity chromatography results in enrichment of B receptors (identified by immunoblot with PR-6 and by photoaffinity labeling with [^{3}H]promegestone) to a specific activity of 1915 pmol/mg of protein (or 23% purity) and with 27% yield. Purity and yields as judged by gel electrophoresis and densitometric scanning of the B protein were approximately 1.7-fold higher due to partial loss in hormone binding activity at the elution step. A second purification step by diethylaminoethyl chromatography gives further enrichment to 3720 pmol/mg of protein (or 44% purity) to yield essentially two proteins, 120-kilodalton (kDa) B receptors and a 76-kDa non-steroid binding protein, each in approximately equivalent amounts. B receptors purified under these conditions are transformed and biologically active. They were maintained as undegraded 120-kDa doublets and retained both hormone and DNA binding activities. Isolated B receptors were free of the 90-kDa non-steroid binding protein observed to be associated with 8S untransformed receptors in other systems and were free also of the non-hormone binding 105-108-kDa B antigen described previously to copurify with chick PR. These purified B receptors were used as immunogen for production of four monoclonal antibodies against human PR. Three of the MAbs, designated as B-30 (IgG_1), B-64 (IgG_1), and B-11 (IgM), are specific for B receptors. The fourth MAb, A/B-52 (IgG_1), reacts with both A and B receptors. The IgG MAbs are monospecific for human PR since they recognize and absorb native receptor-hormone complexes, displace the sedimentation of 4S receptors on salt containing sucrose gradients, and, by immunoblot assay of crude T47D cytosol, react only with receptor polypeptides. Although mice were injected with B receptors only, production of A/B-52 which recognized both A and B receptors provides evidence that these two proteins share regions of structural homology. These new MAbs are valuable reagents for further studies of human receptor structure and function and for clinical immunodetection of PR in breast tumors.

The mechanism of action of progesterone receptors (PR)[1] at the molecular level is not well understood. Further progress in this area requires purification of receptors as well as development of specific antibodies as direct probes for detection of receptors. Hormone-responsive breast cancer cells containing PR provide an excellent model system for such studies. The T47D human breast cancer cell line (Keydar et al., 1979) is a particularly good model since these cells contain an unusually high PR content (i.e., 250 000 molecules/cell) compared to normal target tissues, and by several measurable parameters contain a functional receptor system (Horwitz et al., 1982; Mockus & Horwitz, 1983; Vignon et al., 1983; Chalbos & Rochefort, 1984a,b; Horwitz & Freidenberg, 1985). Studies on structure and function of human PR in breast cancer cells have been limited, however, to work with crude receptor preparations. In situ photoaffinity labeling of T47D cells with the synthetic progestin [^{3}H]R5020 and analysis of cell extracts on denaturing SDS-polyacrylamide gels have shown that human PR are composed of two hormone binding proteins of 94 000 and 120 000 daltons (Horwitz & Alexander, 1983). These are analogous, although larger in apparent molecular weight, to chick oviduct A and B receptors (Horwitz et al., 1985a,b) which are ≈79 000 and 108 000 daltons, respectively (Birnbaumer et al., 1983a,b). It has not been possible as yet to assign structural relationships or

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[1] Abbreviations: PR, progesterone receptor(s); MAb, monoclonal antibody; MEM, minimal essential medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; DCC, dextran-coated charcoal; HAP, hydroxylapatite; BSA, bovine serum albumin; PBS, phosphate-buffered saline; hsp, heat shock protein; DEAE, diethylaminoethyl; SDS, sodium dodecyl sulfate; R5020, promegestone (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione); DTT, dithiothreitol; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene; GR, glucocorticoid receptor(s).

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functional properties to A and B receptors in breast cancer cells, since neither has been purified and characterized.

Purification of PR and production of specific antibodies have been difficult problems for a number of reasons. In most target tissues, PR are present in low amounts and are susceptible to proteolytic degradation, and most purification methods, including steroid affinity chromatography, generally give low receptor yields. Moreover, receptor purifications, at least with chick oviduct PR, have been complicated by the copurification of other abundant cellular non-steroid binding proteins. Steroid affinity chromatography in the presence of sodium molybdate, to stabilize PR in the untransformed state, has resulted in the copurification of a 90-kDa non-steroid binding protein which has now been identified as a heat shock protein (hsp). The 90-kDa hsp has been proposed to be a component of 8S untransformed PR (Dougherty et al., 1984; Joab et al., 1984; Catelli et al., 1985; Schuh et al., 1985) and has been found to be associated with molybdate-stabilized forms of other steroid receptors as well (Joab et al., 1984; Housley et al., 1985; Sanchez et al., 1985). Purification of chick B receptors by conventional chromatographic procedures has resulted in copurification of another major cellular protein termed B antigen because of its structural similarity in charge and size to chick B receptors (Peleg et al., 1985). Despite these problems, a few antibodies reactive with PR have been produced. Polyclonal serum antibodies have been raised against partially purified preparations of rabbit uterine PR (Logeat et al., 1981), B receptors of chick oviduct (Renoir et al., 1982; Touhimaa et al., 1984), and partially purified PR from rabbit uterus (Feil, 1983). Gronemeyer et al. (1985) produced specific rabbit antisera to chick oviduct A and B receptors isolated separately from electrophoresis gels. Monoclonal antibodies have also been prepared against rabbit uterine PR, but their cross-reaction with human breast cancer receptors has not been characterized (Logeat et al., 1983). More recently, Toft and co-workers (Sullivan et al., 1986) were successful in producing several MAbs against chick oviduct PR, one (PR-6) of which cross reacts with human B receptors.

In the present and following paper (Wei et al., 1987), we have characterized in detail the cross-reaction of this anti-chick MAb with human breast cancer PR. This antibody was used to study human receptor structure and covalent modification by phosphorylation and for large-scale immunoaffinity purification of transformed receptors. Purified B receptors were utilized as immunogen for production of four MAbs against human PR. One MAb was produced which reacts with both A and B receptors; the others are B specific. These antibodies are valuable reagents not only for further structural and functional studies of human PR but also for clinical immunodetection of PR in breast tumors.

Experimental Procedures

Materials. [^{3}H]R5020 [[17α-*methyl*-^{3}H]promegestone (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione); 87 Ci/mmol] and unlabeled R5020 were obtained from New England Nuclear Corp. (Boston, MA). Unlabeled progesterone, 17β-estradiol, dihydrotestosterone, and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). DEAE-cellulose (DE-52) was obtained from Whatman, DNA-grade hydroxylapatite from Bio-Rad (Richmond, CA), and protein A–Sepharose C1-4B from Sigma Chemical Co. DNA–cellulose was prepared by the methods of Alberts and Herrick (1971) using calf thymus DNA at a substitution of 2 mg of DNA/mL of resin. Proteolysis inhibitors leupeptin, aprotinin, pepstatin A, and bacitracin were purchased from Sigma Chemical Co. Dimethyl pimelimidate dihydrochloride was obtained from Pierce Chemical Co. (Rockford, IL). Nitrocellulose filters, pore size 0.45 μm, were obtained from Scheicher & Schuell (Keene, NH). Rabbit anti-mouse IgG (heavy and light chain specific) and goat anti-mouse IgG–peroxidase conjugate (heavy and light chain specific) were obtained from Cappel Cooper Biomedical (Malvern, PA). Bovine serum albumin, fraction V (96–99% purity), 4-chloro-1-naphthol, dextran (clinical grade), and activated charcoal (250–350 mesh) were from Sigma Chemical Co.

Cell Culture. T47D human breast cancer cells (Keydar et al., 1979) were grown in Eagle's MEM (Gibco) supplemented with 2 mM L-glutamine (Gibco), insulin (6 ng/mL), 1% nonessential amino acids (Gibco), 10 mM HEPES buffer, pH 7.3, 0.2% sodium bicarbonate, 25 μg/mL Gentamicin (Irvine Scientific), and 10% fetal bovine serum (HyClone Labs, Inc.). Cells were plated either in Corning 150 cm^2 T flasks at a density of 3×10^6 cells/flask and grown in a 5% CO_2 incubator at 37 °C or, for large-scale production, in Corning 850 cm^2 roller bottles plated at 40×10^6 cells/vessel. Cells were harvested by a 10-min incubation in 1 mM EDTA and were washed once in serum-free MEM and once in homogenization buffer.

Cell Fractionation. Cells were homogenized at 0 °C in a Teflon–glass Potter–Elvehejm homogenizer at a cell/buffer ratio of 1/2 (v/v) in either of two buffers: TEDG (10 mM Tris–OH, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) or TEG (10 mM Tris–OH, pH 7.4, 1 mM EDTA, and 10% glycerol). A cocktail of proteolysis inhibitors described by Loosfelt et al. (1984) was added to the homogenization buffers which included pepstatin A (1 μg/mL), leupeptin (47 μg/mL), bacitracin (100 μg/mL), and aprotinin (77 μg/mL). All subsequent procedures were at 4 °C. Homogenates were centrifuged at 105000g for 30 min in a Beckman 50Ti rotor to yield cytosol supernatant.

Progesterone Receptor Assays. In crude cell extracts, a single saturating dose dextran-coated charcoal (DCC) method was used (Edwards et al., 1980). Briefly, crude PR preparations were incubated for 16 h at 4 °C with 20 nM [^{3}H]-R5020 in the presence or absence of 2 μM unlabeled R5020. Free hormone was absorbed by DCC and pelleted by centrifugation, and the supernatant which contains protein-bound R5020 was counted for radioactivity.

In purified PR preparations where protein concentrations were low (<100 μg/mL), bound hormone and free hormone were separated with hydroxylapaptite (HAP) as described by Powell et al. (1979) to which 1% carrier bovine serum albumin (BSA) was added. Since PR concentration was enriched after purification, [^{3}H]R5020 was increased to 80 nM to ensure saturation of receptor sites. Parallel incubations with BSA alone were included as a measure of nonspecific binding.

Radioactivity in DCC and HAP assays was counted in 5.0 mL of 3A70B complete liquid scintillation cocktail (Research Products International Corp.) in a Beckman LS-230 liquid scintillation counter at a counting efficiency of 33% for tritium.

Sucrose Density Gradient Analysis of PR. Linear 5–30% sucrose density gradients were formed either in TEDG or in TEDG containing 0.3 M NaCl. Receptor samples of 200 μL were layered on the gradients along with [^{14}C]ovalbumin (4 S) and ^{14}C-immunoglobulins (7 S) as internal markers. Gradient tubes were centrifuged at 46 000 rpm for 18 h in a Beckman SW50Ti rotor at 4 °C, and 200-μL fractions were collected from the bottom. Fractions were counted for radioactivity in 5.0 mL of 3A70B liquid scintillation fluid.

Gel Electrophoresis and Immunoblot Procedures. Proteins were electrophoresed in SDS–polyacrylamide on a vertical slab

gel apparatus as described by Laemmli (1971) but with modifications. The separating gel contained 7.5% acrylamide/0.1% bis(acrylamide) while the stacking gel contained 3% acrylamide/0.25% bis(acrylamide) (Bio-Rad). Other modifications were inclusion of 0.1% SDS in the gel buffers and in the upper and lower reservoir buffers. Silver staining of SDS gels was according to the methods of Merril et al. (1981). Molecular weight standards (Bio-Rad) for silver-stained gels were the following: myosin, 200 000; β-galactosidase, 116 000; phosphorylase B, 97 000; bovine serum albumin, 68 000; and ovalbumin, 45 000. Gel scanning was performed with an LKB Model 2022 laser densitometer interfaced to a Varian CD5401 HPLC integrator.

Immunoblots for PR were performed as described by Towbin et al. (1979) with modifications. Proteins separated on SDS–polyacrylamide gels were electrotransferred to nitrocellulose filters with a Hoeffer Transphor apparatus at 0.4 A for 5 h (with a cooling coil) in transfer buffer containing 20 mM sodium phosphate, pH 6.8, 20% methanol, and 0.05% SDS. Unreacted sites on the nitrocellulose were blocked by a 2-h incubation at room temperature with 3% bovine serum albumin (BSA) in wash buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 30 mM NaCl, 0.05% Triton X-100, and 0.25% gelatin). The blocked nitrocellulose filters were then incubated overnight at 4 °C with 10 μg/mL monoclonal antibody in dilution buffer (wash buffer containing 1% BSA), washed 3 times for 5 min each on a rocker platform with excess wash buffer, and incubated for another 3 h at room temperature with goat anti-mouse peroxidase (Cappel) diluted 1/1500. After the filters were washed to remove unbound IgG peroxidase, immunoreactive bands were visualized by incubation of the nitrocellulose with a solution of 4-chloronaphthol (0.5 mg/mL)/0.025% hydrogen peroxide prepared in substrate buffer (50 mM HEPES, pH 7.4, and 150 mM NaCl/15% methanol). Molecular weight standards (Bio-Rad) were transferred from parallel gel lanes to nitrocellulose and visualized by staining with 0.2% Coomassie Blue R250 in 90% methanol/5% acetic acid.

Photoaffinity Labeling of PR. In vitro photoaffinity labeling was accomplished by photoirradiation of preformed PR–[^{3}H]R5020 complexes in solution for 2 min at 4 °C directly on the surface of a 300-nm UV transilluminator (Lessey et al., 1983). Photoaffinity-labeled PR were submitted to denaturing SDS–polyacrylamide gel electrophoresis, and covalently linked receptors were detected either by gel slicing and liquid scintillation counting of the slices (Edwards et al., 1981) or by fluorography (Bonner & Laskey, 1974). Gel pieces (1-mm slices from each lane) were processed for scintillation counting by incubation for 16 h at 40 °C in 10 mL of scintillation fluid containing 5% Protosol (New England Nuclear), 0.25% H_2O, 4.0 g of PPO, and 0.05 g of POPOP/L of toluene. Visualization of PR bands by fluorography was performed as previously described (Horwitz & Alexander, 1983). Gels were fixed in 40% methanol/10% acetic acid, stained with Coomassie Blue, and destained in 7.5% acetic acid. They were then soaked in En^{3}Hance (New England Nuclear) for 1 h and in water for 1 h, dried under vacuum, and exposed to Kodak X-Omat XAR-5 film in a cassette with Dupont Cronex Quanta III intensifying screens. Molecular weight ^{14}C-labeled standards on gel fluorograms were (Bethesda Research Laboratories) the following: myosin, 200 000; phosphorylase B, 97 000; bovine serum albumin, 68 000; ovalbumin, 43 000; α-chymotrypsin, 25 700; β-lactalbumin, 19 000.

Immunoprecipitation of PR. Aliquots of T47D cytosols labeled with 20 nM [^{3}H]R5020 (either reversibly or covalently) were incubated, without removal of excess free hormone, for 16 h at 4 °C with purified preparations of MAbs at the antibody and receptor concentrations indicated in experimental results. A 20–30-fold excess of rabbit anti-mouse IgG (over the primary MAb concentration) was then added to each assay and incubated for an additional 4 h at 4 °C. Receptor–antibody complexes were then adsorbed by incubation with protein A–Sepharose beads (1/1 v/v in suspension with PBS) for 1 h at 4 °C. Beads were washed 3 times in TEDG, once in TEDG containing 0.3 M NaCl, and once more in TEDG. Nonspecific immunoprecipitation was determined by incubation with a mouse MAb (IgG_1) reactive with an unrelated breast cancer antigen (Edwards et al., 1986), and values obtained were subtracted from total binding. Depending on the experiment, receptors were eluted from the protein A–Sepharose beads by two different methods. For direct counting of immunoabsorbed [^{3}H]R5020 complexes, beads were extracted in absolute alcohol for 1 h at 37 °C and centrifuged, and aliquots of the supernatant were counted directly for radioactivity. For SDS–polyacrylamide gels, beads were boiled in 1% SDS sample buffer (Laemmli, 1971) and centrifuged, and the supernatant was applied directly to electrophoresis gels.

Preparation of Monoclonal Antibody Affinity Matrix. The PR-6 MAb was chemically cross-linked directly to protein A–Sepharose by the method of Schneider et al. (1982). PR-6 used in cross-linking was purified from ascites fluids and dialyzed against borate buffer, (0.1 M boric acid/0.025 M sodium borate, pH 8.2). The MAb was first absorbed to protein A–Sepharose noncovalently by incubation in suspension for 45 min at room temperature using 10 mg of PR-6/mL of beads. Cross-linking was performed as described by Schneider et al. (1982) using 20 mM dimethyl pimelimidate. Approximately 5–6 mg of PR-6 was stably coupled per milliliter of beads and was resistant to elution with 2 M sodium thiocyanate.

Immunoaffinity Purification of PR. Cytosols (80 mL) were prepared from 40 confluent roller bottles (850 cm^2) of T47D cultures in TEG containing proteolysis inhibitors and were incubated for 4 h at 4 °C with 20 nM [^{3}H]R5020. Free hormone was not removed so that receptors would be in equilibrium with excess hormone during purification steps. The radiolabeled cytosols were circulated at 4 °C over a 2.0-mL column of the immunomatrix at a flow rate of 5–6 mL/h. The number of receptors absorbed by the column was determined by the difference between receptors in the cytosol and the column flow-through as measured by DCC assay. After passage of the cytosol over the column, the immunomatrix was washed sequentially in the column at a flow rate of 1 mL/min with the following buffers: TEG (100 mL), TEG containing 0.3 M NaCl (100 mL), and TEG (100 mL). The beads were then suspended in TE (10 mM Tris–OH, pH 7.4, 1 mM EDTA), transferred to a plastic centrifuge tube, washed twice in 10 mL of TE by end-over-end rotation of the tube for 15 min at 4 °C, and pelleted by low-speed centrifugation. In some experiments, a wash step with 0.2% Triton X-100, 1 M NaCl, and TEG at pH 9.0 was also included. Receptors were then eluted from the washed MAb beads by suspension in 3.5 mL of pH 11.5 buffer (50 mM Tris–OH) for 15 min at 4 °C on an end-over-end rotator. The eluate was collected as the supernatant after low-speed centrifugation of the beads. The above step was repeated, supernatants were combined, and the eluate was immediately neutralized at pH 7.4 by

addition of 5.6 mL of acidic buffer (0.2 M Tris-HCl, pH 4.7, and 1 mM EDTA). Residual receptors were eluted from the beads by suspension in 1 M sodium thiocyanate (prepared in 10 mM Tris, pH 7.4, and 1 mM EDTA) for 15 min at 4 °C, centrifugation, and collection of the supernatant. The immunomatrix was regenerated by successive washes with TEG, TEG containing 1.0 M NaCl, TEG, and sodium borate buffer. Protein concentrations in cytosols and purification fractions were measured by the method of Bradford (1976) using bovine plasma γ-globulin as the protein standard (Bio-Rad).

Immunizations. A Balb/cby mouse (male, age 8 weeks) obtained from Jackson Labs was immunized by injection of immunoaffinity-purified B receptors that were concentrated by vacuum centrifugation. The first injection was given subcutaneously in complete Freund's adjuvant and contained 35 μg of B receptors at 20–30% purity. Three booster injections were given in incomplete Freund's adjuvant at approximately 2-week intervals using between 50 and 60 μg of B receptors, of the same purity. The first and third booster injections were given subcutaneously and the second intraperitoneally. The mouse was allowed to rest for 3 weeks and was then given a final intraperitoneal boost 3 days before fusion with 28 μg of B receptors in PBS.

Cell Fusion and Hybridoma Production. Fusions and hybridoma techniques were performed as previously described (Edwards et al., 1984, 1986). A single cell suspension of the mouse spleen was mixed at a 4/1 ratio (spleen/myeloma) with Fox-NY mouse myelomas (Taggart & Samloff, 1983) and fused with 50% (w/v) poly(ethylene glycol) (M_r 1540 from Koch Light) in serum-free RPMI-1640 medium. The mouse myelomas were cultured for cell fusions in RPMI-1640 (Irvine) supplemented with 10% fetal bovine serum (Hyclone Sterile Systems), 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, penicillin–streptomycin (50 units/mL), 10 mM HEPES, pH 7.4, and 0.2% sodium bicarbonate. The postfusion cell mixture was plated for 24 h in 100-mm petri dishes in myeloma growth medium, except supplemented with 20% fetal bovine serum, and then seeded into 96-well microtiter dishes (COSTAR) in selection medium at a density of 3×10^5 cells/well. Selection medium was the same as that described above with the addition of 0.75 μM adenine, 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine. Culture medium was removed 12 days after fusion for screening by the enzyme-linked immunoabsorption assay (ELISA). Those cultures positive by ELISA were transferred to larger 1-mL culture wells (COSTAR; 24-well plates), allowed to grow an additional 6 days, and then reassayed for receptor–hormone absorbing activity by using 1.0 mL of culture medium. Selected hybridoma cultures were then cloned directly from 1-mL culture wells by limited dilution into 96-well dishes seeded 24 h in advance with a feeder layer of Balb/c thymus cells plated at a density of 5×10^5 cells/well. Wells containing single hybridoma colonies were reassayed, and positives were subcloned a second time. Clonal cell lines were cryopreserved in 95% fetal bovine serum and 5% dimethyl sulfoxide (American Type Culture Collection) in liquid nitrogen.

Screening Assays. An ELISA procedure was used as the initial screening method. Polyvinyl 96-well microtiter plates (Dynatech) were coated with immunoaffinity-purified B receptors by incubation overnight at 4 °C with 40 μL/well of a 50 μg/mL receptor solution. B receptors used as coating antigen were of the same purity (20–30%) as the immunogen. Prior to the coating of ELISA plates, purified receptors were desalted, and the buffer was exchanged with 0.125 M sodium borate, pH 8.2, by centrifugation in an Amicon Centricon-30 microconcentrator. Receptor-coated ELISA plates were blocked by incubation for 2 h at 4 °C with 1% BSA (200 μL/well). Each well was then washed 3 times with PBS and incubated overnight at 4 °C with 50 μL of hybridoma culture medium taken directly from 96-well fusion plates. ELISA plates were next washed 3 times with PBS (200 μL/well) and incubated for 3 h at room temperature with 150 μL/well of goat anti-mouse IgG peroxidase (Cappel) diluted 1/5000 in BSA–PBS buffer. Plates were then washed 5 times with PBS and reacted for 10 min at room temperature with 150 μL of enzyme substrate (2.5 mg/mL *o*-phenylenediamine/0.05% H_2O_2) prepared in citric acid buffer (50 mM citric acid monohydrate/0.2 M sodium phosphate dibasic, pH 4.9). Reactions were stopped by addition of 50 μL of 1 M sulfuric acid, and wells were read in a Dynatech Minireader II at a wavelength of 490 nm. Antibody typing for mouse immunoglobulin class and subclass was by ELISA using the Bio-Rad mouse subtyping kit.

Cultures that were positive by ELISA were rescreened for absorbing activity against crude receptor–hormone complexes by a modification of the protein A–Sepharose assay described above. Incubations were in reverse order to allow assay with 500-μL aliquots of the culture medium. We find that this increases the sensitivity of the assay by severalfold. Protein A–Sepharose was precoated with rabbit anti-mouse IgG (Cappel) by incubation in suspension for 1 h at room temperature with 1 mg of rabbit anti-mouse IgG/mL of beads. Precoated beads were washed twice with PBS, and then 100-μL aliquots (1/1 v/v suspension in PBS) were incubated with 500 μL of hybridoma medium for 4 h at 4 °C with constant shaking to keep the beads in suspension. The beads were washed once in PBS and once in TEDG to remove nonabsorbed proteins and then incubated on a shaker overnight at 4 °C with 100 μL of T47D cytosol receptor–[^{3}H]R5020 complexes (20 nM receptor). Beads were then pelleted by centrifugation at 1500 rpm for 5 min and washed 3 times in 500 μL of TEDG. Washed beads were extracted for 1 h at 37 °C with 1.0 mL of ethanol, and 800-μL aliquots of the extract were counted for radioactivity in 5.0 mL of 3A70B liquid scintillation fluid.

Purification of Monoclonal Antibodies. Hybridomas were grown as ascites tumors in Balb/cby mice. Tetramethylpentadecane was injected (0.5 mL) intraperitoneally 1 week prior to injection of 2×10^6 live hybridoma cells per mouse. Ascites fluid was drained 1–2 weeks later by abdominal puncture and was used as the source for MAb purification. The IgG fraction of ascites fluids was purified by precipitation at 50% saturation with ammonium sulfate followed by DEAE-cellulose (DE-52) chromatography. The IgG fraction eluted from DEAE was approximately 95% pure on the basis of Coomassie-stained SDS gel electrophoresis.

RESULTS

Characterization of Monoclonal Antibody Reaction with Human PR. One of five monoclonal antibodies prepared against partially purified chick oviduct progesterone receptors cross-reacts with human PR. This MAb, designated PR-6, is a mouse IgG2a, and details of its preparation have been previously described (Sullivan et al., 1986). This MAb absorbs native receptor–hormone complexes from cytosols of T47D human breast cancer cells by use of a secondary anti-mouse IgG and protein A–Sepharose as an immunoabsorbent. Maximum absorption of ≈40% of total cytosol receptors was achieved at 10 μg/mL PR-6 (Figure 1A); thus, ≈80% of B receptors were bound by using this MAb. Negligible ab-

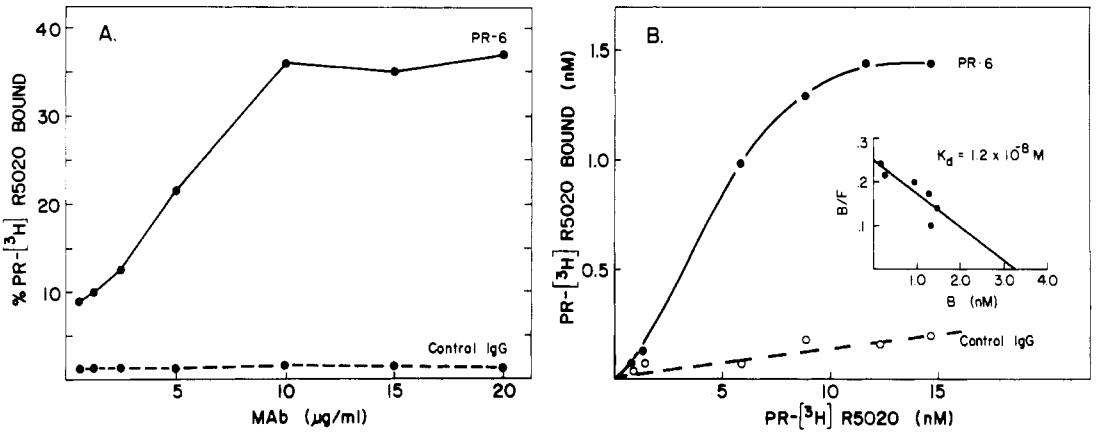

FIGURE 1: Immunoabsorption of T47D receptor–[^{3}H]R5020 complexes with PR-6 monoclonal antibody. (A) Cytosol receptor–[^{3}H]R5020 complexes (15 nM) were incubated overnight at 4 °C with the concentrations of PR-6 indicated, followed by incubation with rabbit anti-mouse IgG secondary antibody and absorption of immune complexes to protein A–Sepharose. Data are expressed as the percentage of total cytosol receptor–hormone complexes bound to protein A–Sepharose, and values are average determinations from triplicate incubations. Control IgG was a mouse monoclonal IgG reactive with a breast tumor associated surface antigen (Edwards et al., 1986). (B) Increasing concentrations of cytosol receptor–[^{3}H]R5020 complexes were incubated with a constant amount of PR-6 (20 mg/mL), and samples were processed as described above. The data are expressed as nanomolar concentrations of receptors bound to protein A–Sepharose at each input concentration. The inset is a Scatchard plot of the same data with the estimated dissociation constant (K_d) for PR-6 binding of receptors.

sorption of PR was obtained by using a control mouse MAb reactive with an unrelated breast cancer antigen. The binding affinity of PR-6 for human receptors was determined by maintaining the MAb concentration constant and varying the concentration of receptor–hormone complexes (Figure 1B). Saturation of antibody occurs between 12 and 15 nM receptors, and Scatchard analysis (inset) demonstrates that PR-6 has a single class of receptor binding sites with a K_d of 1.2×10^{-8} M.

T47D cells contain A and B receptors of ≈94 and 120 kDa, respectively, which have previously been identified by photoaffinity labeling with the synthetic progestin [^{3}H]R5020 (Horwitz & Alexander, 1983). To further examine the specificity of PR-6, photoaffinity-labeled cytosol receptors were absorbed with PR-6 and protein A–Sepharose, and aliquots of the absorbed and nonabsorbed fractions were subjected to SDS–polyacrylamide electrophoresis. With a control MAb, neither A nor B receptors were bound by protein A–Sepharose (Figure 2, panels A and C). Incubation with PR-6, however, resulted in depletion of B receptors from cytosol and their absorption to protein A–Sepharose, leaving A receptors in the supernatant (Figure 2, panels B and D). Thus, under these conditions, PR-6 is specific for hormone binding B receptors and does not recognize A receptors.

To examine the monospecificity of PR-6 for B receptors and to contrast the immunoreactivity of human and chick PR, crude cytosols obtained from T47D cells and chick oviducts were compared by immunoblotting (Figure 3). PR-6 reacts with a single band at ≈110 kDa in chick cytosol but with a doublet at ≈120 kDa in T47D cells. No reaction was observed at the position of A receptors in either cytosol. Previous photoaffinity labeling studies have shown that human A and B receptors are larger than the corresponding receptors of chick oviduct which are 79 and 110 kDa, respectively (Horwitz et al., 1985b). Thus, in chick and human cells, PR-6 cross-reacts with antigens of the correct size for their respective B receptors and have no apparent affinity for other proteins. B receptors from T47D cells characteristically migrate on SDS gels as doublets of 120 kDa which differ in the upper and lower bands by a few thousand daltons in apparent molecular mass. Further analysis of B-receptor doublets and their covalent modification by phosphorylation are described in the following paper (Wei et al., 1987).

Immunoaffinity Purification of B Receptors. Since PR-6 is monospecific for B receptors, efficiently immunoprecipitates the native receptor–hormone complex, and has a receptor binding affinity ideal for antigen purification (Figures 1–3), we sought to use this MAb for large-scale purification of PR from human breast cancer cells. We first prepared an immunoaffinity matrix by cross-linking PR-6 to protein A–Sepharose using the bifunctional cross-linker dimethyl pimelimidate (Schneider et al., 1982). This produces a stable linkage which allows multiple uses of the immunomatrix and also prevents contamination of the final product with MAb. Approximately 5 mg of MAb was coupled/mL of beads, and the matrix was determined to have a receptor binding capacity of ≈250 pmol/mL. Several methods for receptor elution were tested. The optimal method was found to be exposure to alkaline buffer (pH at 11.5) followed by immediate neutralization. This releases almost all receptors from the beads (determined by immunoblot) with minimal loss in biological activity. As a control experiment to estimate the effects of alkaline pH elution on the biologic activity of receptors, crude cytosol receptor–[^{3}H]R5020 complexes were exposed to pH 11.5 at 4 °C for 15 min and then neutralized. This resulted in ≈30% irreversible loss of hormone binding activity. If purified receptors are equally sensitive to pH 11.5, comparable losses would be expected in the immunoaffinity eluates.

A two-step procedure for purification of B receptors was developed which utilizes MAb affinity chromatography as the first step, followed by DEAE ion-exchange chromatography. We routinely start with 80 mL of concentrated cytosol receptor–[^{3}H]R5020 complexes which is passed over 2.0 mL of the PR-6 immunomatrix packed into a column. To prevent hormone dissociation and stabilize receptors during purification, excess free hormone was not removed during immunoaffinity chromatography. In 12 purifications, an average of 35% of total cytosol receptor–hormone complexes were absorbed by the immunomatrix, which represents 70% of B receptors. Analysis of the purification product obtained by alkaline pH elution of the MAb affinity column is shown in Figure 4. Silver staining of SDS electrophoresis gels shows four major proteins of 120 (which is a doublet), 76, 62, and 58 kDa. These 4 proteins were observed reproducibly in 12 separate purifications. The purified product was analyzed further by immunoblotting with PR-6 (Figure 4, middle panel)

Table I: Purification of B Receptors by Single-Step Immunoaffinity Chromatography

	protein		B receptors (hormone binding)				B receptors (gel scans)[c]			
	mg/mL	total mg	pmol[a]	yield (%)	sp act. (pmol/mg)	% purity[b]	pmol	yield (%)	sp act. (pmol/mg)	% purity
cytosol[d]	14.40	1152.0	814		0.71					
pH eluate	0.021	0.132	221	27.1	1915	23	405	49	3173	38

[a] B receptors in starting cytosol were estimated to be 50% of total receptors measured by DCC assay. [b] Theoretical purity for B receptors based on a molecular weight of 120000 = 8352 pmol/mg of cytosol protein. [c] Estimated from fraction of B receptors in gel scans of the immunoaffinity eluate compared with total protein. [d] Values are average determination from three separate purifications.

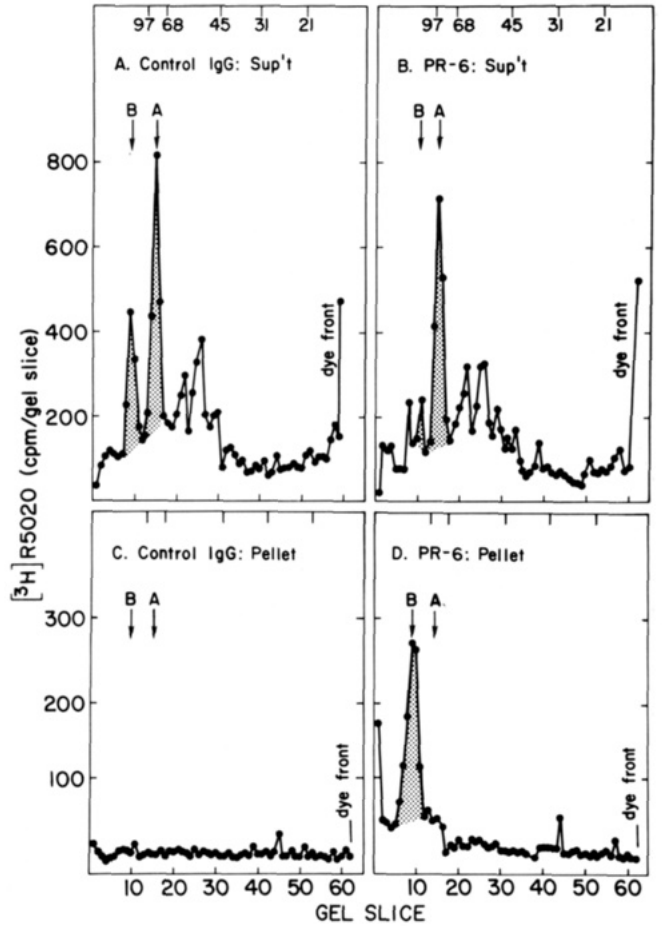

FIGURE 2: Immunoabsorption of photoaffinity-labeled progesterone receptors. T47D cytosol progesterone receptors were photoaffinity labeled in vitro with [^{3}H]R5020 as described under Experimental Procedures. Aliquots of these affinity-labeled cytosols were incubated as described in Figure 1 with either PR-6 or control IgG followed by a secondary anti-mouse IgG and absorption of the immune complexes to protein A–Sepharose. Aliquots of nonabsorbed proteins in the supernatant, and immune complexes absorbed to protein A–Sepharose and extracted by boiling of the beads in Laemmli (1971) SDS sample buffer, were submitted to denaturing SDS–polyacrylamide gel electrophoresis. The SDS gels were analyzed by counting of gel slices for protein-bound radioactivity. (A and C) Nonabsorbed supernatant fractions (sup't) and protein A–Sepharose-absorbed fractions (pellet) obtained with control IgG. (B and D) Nonabsorbed supernatant fractions (sup't) and absorbed fractions (pellet) obtained with PR-6.

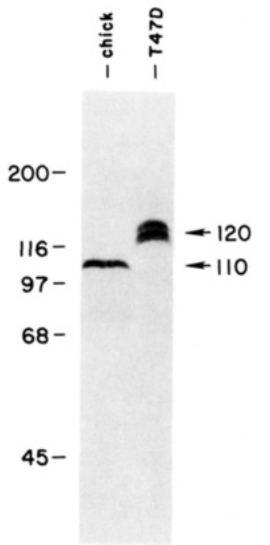

FIGURE 3: Immunoblot of crude cytosols with PR-6. Cytosols were prepared from chick oviduct and T47D cells in TEDG buffers containing proteolysis inhibitors. Samples (chick cytosol = 600 μg of total protein/gel lane; T47D cytosol = 780 μg/gel lane) were electrophoresed on denaturing SDS–polyacrylamide gels and electrotransferred to nitrocellulose filters, and the nitrocellulose filters were reacted with 10 μg/mL PR-6 and probed with secondary anti-mouse IgG peroxidase as described under Experimental Procedures. Molecular weight standards were run on a parallel gel lane, transferred to nitrocellulose, and stained with Coomassie blue.

and by photoaffinity labeling with [^{3}H]R5020 (Figure 4, right panel). The immunoblot of the starting cytosol and column flow-through demonstrates absorption of cytosol B receptors by the immunomatrix. The eluted 120-kDa doublet was identified as B receptors since it binds PR-6 by immunoblotting and also binds [^{3}H]R5020 as detected by fluorography. The 76-, 62-, and 58-kDa proteins, on the other hand, are not immunoreactive with PR-6 nor do they bind [^{3}H]R5020. Specific activities and yields of B receptors after the first-step MAb affinity column are given in Table I. These data were obtained by two methods: by hormone binding measurements and by densitometric scans of silver-stained gels. By hormone binding measurements, B receptors were enriched from 0.71 pmol/mg of protein in starting cytosol to a specific hormone binding activity of 1915 pmol/mg of protein. This represents a 2700-fold purification over the starting cytosol, making the receptor 23% pure based on a maximal theoretical specific activity of 8352 pmol/mg of protein. With densitometric gel scanning to estimate the B-receptor fraction of total protein, yields and specific activities were ≈1.7-fold higher (Table I). If we correct for a 30% irreversible loss of hormone binding activity due to exposure of receptors to alkaline pH, estimates of B-receptor purity by both methods are in close agreement. Thus, we can account for most of the 120-kDa doublet as having hormone binding capacity, ruling out the presence of an abundant non-hormone binding contaminant of size identical with the B receptors.

Subsequent fractionation of immunopurified receptors by DEAE ion-exchange chromatography is shown in Figure 5. Prior to application to DEAE, receptors eluted from the MAb column were reincubated with [^{3}H]R5020 to rebind available hormone binding sites that may have dissociated during immunoaffinity chromatography. Of these receptor–hormone complexes, 67% bound to DEAE and were released as a single peak at 0.2 M NaCl (left panel of Figure 5) by salt gradient elution. Silver-stained SDS gel analysis of the DEAE salt fractions 13–19 shows that the 120-kDa doublet fractionates with the hormone binding activity and that the peak receptor

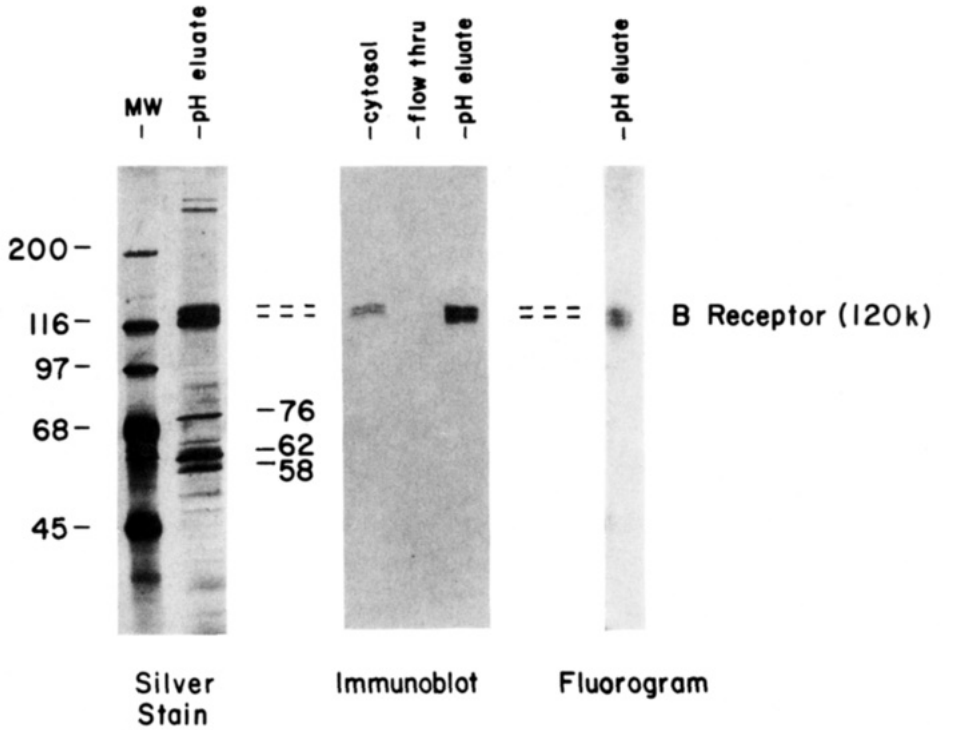

FIGURE 4: Single-step immunoaffinity purification of B receptors. B receptors were purified from 80 mL of T47D cytosols by single-step immunoaffinity chromatography as described under Experimental Procedures. The product eluted from the immunomatrix was analyzed by silver staining of SDS–polyacrylamide electrophoresis gels (left panel), PR-6 immunoblot (middle panel), and photoaffinity labeling with [^{3}H]R5020 and fluorography (right panel). Approximately 10 μg of total purified protein was applied per gel lane.

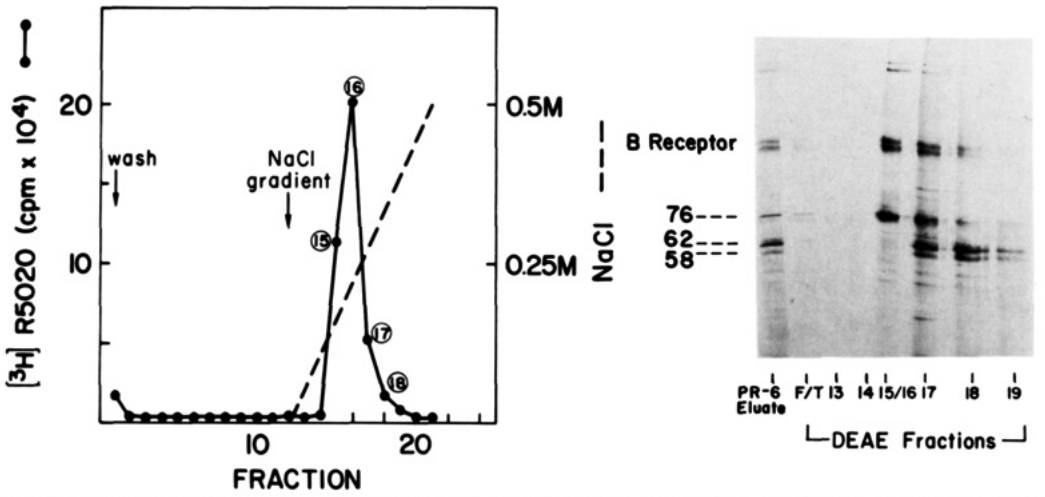

FIGURE 5: Two-step purification of B receptors. Immunoaffinity chromatography and DEAE ion exchange. B receptors eluted from the PR-6 immunomatrix by alkaline pH were neutralized by titration with acidic buffer, reincubated with [^{3}H]R5020 for 2 h at 4 °C, and then applied to a DEAE column (DE-52) equilibrated in 0.1 M Tris/1 mM EDTA, pH 7.4. The DEAE column was washed with TEDG, and bound receptor–hormone complexes were eluted with a linear 0–0.5 M salt gradient (NaCl) prepared in TEDG buffer. Aliquots of the wash and salt-eluted fractions were counted for radioactivity (left). The first-step immunoaffinity purification product PR-6 eluate (≈6 μg of total protein/gel lane), DEAE flow-through F/T (≈2.4 μg of total protein/gel lane), and DEAE salt-eluted fractions (≈9 μg of total protein in gel lanes 15 and 16) were also analyzed by SDS–polyacrylamide gel electrophoresis and silver staining (right).

fractions off of DEAE (15 and 16) have separated from two of the lower molecular mass non-steroid binding proteins (i.e., 58 and 62 kDa). The 76-kDa protein, however, continued to cofractionate with B receptors. Further attempts to separate B receptors from the 76-kDa protein have not been successful (i.e., DNA–cellulose chromatography, chromatography on hydroxylapatite, and rebinding to a freshly regenerated PR-6 resin). As judged by scanning densitometry of the silver-stained SDS gels, B receptors in the peak fractions off the DEAE column (15 and 16) were 44% pure; the remainder of protein was primarily in the 76-kDa band. This two-step method, therefore, yields highly purified receptors which contain essentially two proteins: B-receptor doublets and a non-steroid binding 76-kDa protein. Each protein is present in roughly equivalent amounts.

Physicochemical Properties of Purified B Receptors. Single-step immunoaffinity-purified receptors were used in the following studies because of higher total receptor yields. As indicated above, a large fraction of immunopurified receptors have retained hormone binding activity. Steroid binding specificity therefore was evaluated by testing the ability of unlabeled competing hormones to displace [^{3}H]R5020 binding (Table II). The purified product was found to display the correct steroid specificity for PR since progesterone and R5020 were the most effective competitors. Other steroid hormones were much less effective in displacing bound [^{3}H]R5020.

Table II: Relative Steroid Binding Specificity of Immunoaffinity-Purified B Receptors

unlabeled hormone	[^{3}H]R5020 binding (%)
none[a]	100
R5020	11
progesterone	15
17β-estradiol	72
dexamethasone	88
dihydrotestosterone	67

[a] B receptors were purified by single-step immunoaffinity purification as described in Figure 4. [^{3}H]R5020 binding in absence of unlabeled hormone was 14 295 cpm/assay. Unlabeled hormone was added in 200-fold excess simultaneously with 20 nM [^{3}H]R5020 and incubated overnight at 4 °C. [^{3}H]R5020 binding was measured by HAP assay.

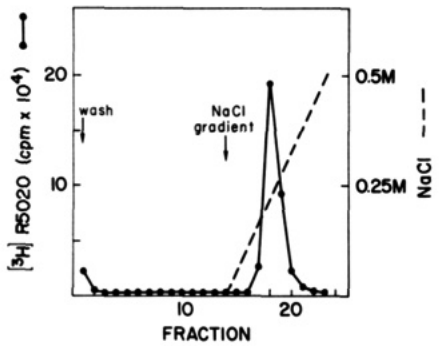

FIGURE 6: DNA–cellulose chromatography of immunoaffinity-purified B receptors. B receptors eluted from the PR-6 immunomatrix by alkaline pH were neutralized, reincubated with [^{3}H]R5020 for 2 h at 4 °C, and then applied to a DNA–cellulose column, equilibrated in 0.1 M Tris, pH 7.4, and 1 mM EDTA. The column was washed with TEDG, and bound receptor–hormone complexes were eluted by a linear 0–0.5 M NaCl gradient. Aliquots of wash and salt-eluted fractions were counted for [^{3}H]R5020.

The ability of purified B receptors to bind DNA was tested by DNA–cellulose chromatography. Of receptor–hormone complexes that eluted from the immunomatrix with alkaline pH, 60% were subsequently bound by DNA–cellulose. They were released as a single peak at ≈0.2 M salt by gradient elution (Figure 6). We observed also by silver staining of SDS gels and immunoblotting with PR-6 that both bands of the B-receptor doublet were bound by DNA–cellulose and coeluted with the hormone binding peak. The 76-kDa protein also coeluted with B receptors on DNA–cellulose (data not shown). Thus, human B receptors bind DNA efficiently and appear to have been transformed during purification.

Sucrose density gradient analyses in low- and high-salt-containing buffers are shown in Figure 7. In high salt, purified B-receptor–hormone complexes sedimented as a single peak at 4 S. When salt was omitted from the gradient, receptor aggregates sediment as a slightly broader 6S peak. Purified B receptors did not form larger 8–10S complexes, further indicating they were isolated in the transformed state.

Before using purified B receptors as immunogens, we tested these preparations for the presence of two non-hormone binding proteins of 90 and 105–108 kDa that in previous studies have copurified with chick PR and interfered with production of receptor-specific antibodies. The 90-kDa protein has been identified as a heat shock protein (hsp) and appears to associate with the molybdate-stabilized 8S form of PR and other steroid receptors (Joab et al., 1984; Catelli et al., 1985; Schuh et al., 1985; Pratt et al., 1985). The 105–108-kDa protein, on the other hand, copurified with 4S hen B receptors purified in the absence of molybdate. Because of its close similarity in charge and size with hen B receptors, this protein has been termed B antigen (Peleg et al., 1985). Neither

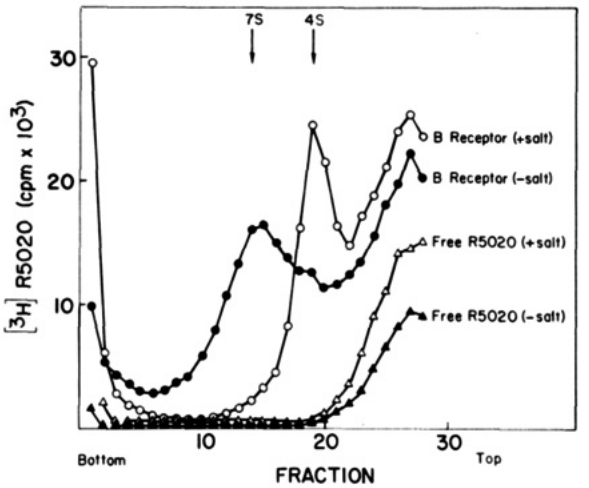

FIGURE 7: Sucrose density gradient centrifugation of immunoaffinity-purified B receptors. B receptors obtained by single-step immunoaffinity purification were reincubated for 2 h at 4 °C with [^{3}H]-R5020, and 200-μL aliquots were layered on 5–20% linear sucrose gradients prepared in either TEDG (●) or TEDG containing 0.4 M NaCl (○). Free [^{3}H]R5020 in buffer was also layered on parallel sucrose density gradients prepared in TEDG (▲) or TEDG containing 0.4 M NaCl (△). Gradient tubes were centrifuged at 46 000 rpm for 18 h in an SW50.1 rotor, and fractions were collected through the bottom of the tube and counted for radioactivity. Marker proteins are [^{14}C]IgG (7 S) and [^{14}C]ovalbumin (4 S).

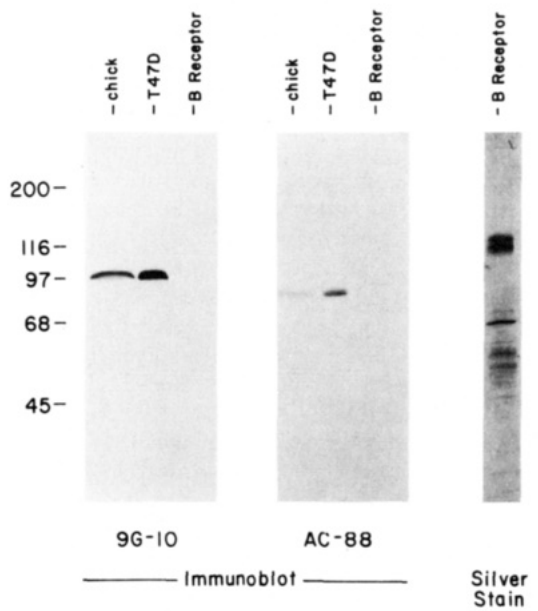

FIGURE 8: Immunoblot of cytosol and purified B receptors with monoclonal antibodies to 90- and 105–108-kDa non-hormone binding proteins. 9G-10 monoclonal antibody raised against 105–108-kDa chick B antigen was used for immunoblot of chick oviduct cytosol (lane 1), T47D cytosol (lane 2), and single-step immunoaffiniy-purified B receptors (lane 3). AC-88 monoclonal antibody raised against receptor-associated 90-kDa heat shock protein was used for immunoblot of chick oviduct cytosol (lane 4), T47D cytosol (lane 5), and immunoaffinity-purified B receptors from T47D cells (lane 6). Approximately 780 μg of total T47D cytosol protein, 600 μg of chick cytosol protein, and 5 μg of purified protein were applied per gel lane. For reference, the immunoaffinity-purified B receptors immunoblotted in lanes 3 and 6 were submitted to SDS–polyacrylamide gel electrophoresis and silver staining (lane 7).

90-kDa nor 105–108-kDa proteins (Figure 8) were observed by silver-stained SDS gels in our preparations of purified human receptors. To conclusively rule out their presence, however, we probed these purified receptors with the AC-88 MAb to 90-kDa hsp produced by Riehl et al. (1985) and with the 9G-10 MAb raised against the chick B antigen (Edwards

Table III: Monoclonal Antibodies to Human PR

MAb	mouse subtype	reactivity[a] A	B
B-30	IgG_1	-	+
B-64	IgG_1	-	+
A/B-52	IgG_1	+	+
B-11	IgM	-	+

[a] Reactivity with 94-kDa A or 120-kDa B receptors was determined by immunoblotting.

et al., 1984). Immunoblots (Figure 8) show that AC-88 reacts with a 90-kDa protein present in cytosols from chick oviduct and T47D cells, and 9G-10 with 105–108-kDa proteins that are identical in size in chick and human cells. Neither the 90-kDa hsp nor the B antigen is detectable immunologically in purified PR preparations (Figure 8). We conclude that immunoaffinity-purified human B receptors are free of the 8S receptor associated 90-kDa hsp and B-antigen contaminant.

Production of Monoclonal Antibodies to Purified B Receptors. One mouse was injected with B receptors purified by single-step immunoaffinity chromatography. Mouse serum was assayed for anti-PR titer by immunoabsorption of T47D cytosol receptor–hormone complexes using a secondary anti-mouse IgG and protein A–Sepharose. Serum antibodies against human PR were not detected until after the third injection. A fourth injection resulted in an increased anti-PR titer.

Hybridomas were initially screened by solid-phase ELISA using purified B receptors as the coating antigen. By this method, 55 of 191 hybridoma cultures were considered positive. These cultures were split from 96-well dishes to larger 1-mL culture wells and grown for an additional 5 days to reach confluence. Each culture was reassayed for production of antibodies capable of absorbing cytosol receptor–hormone complexes. By this assay, four cultures were positive. Each was subcloned twice and determined to be a cloned stable cell line since all colonies gave identical anti-PR absorbing activities. The identifying codes for each MAb are given in Table III along with immunoglobulin type and subclass.

Immunoabsorption of human receptor–[^{3}H]R5020 complexes with each of these MAbs is shown in Figure 9 along with PR-6 for comparison. PR-6, B-30, and B-64 each absorbs 35–45% of receptor–hormone complexes from T47D cytosol while A/B-52 absorbs twice this number of receptors (75–85%). The IgM antibody, B-11, is less efficient and absorbs ≈10% of receptors.

All four hybridoma cell lines have been grown as ascites tumors in mice (the MAbs purified) and further assessed for their interactions with receptors by shifts in sedimentation on sucrose density gradients and by immunoblot assays. In salt-containing sucrose gradients, cytosol receptor–[^{3}H]R5020 complexes sediment at 4 S in the presence of a control MAb. Incubation with A/B-52 shifts 4S receptors to the 8–9S region of the gradient (Figure 10A). Incubation with B-30 and B-64 also shifts the sedimentation of 4S receptors but does so less efficiently than A/B-52 (Figure 10B). Crude cytosols from T47D cells were assayed by immunoblotting to determine the monospecificity of each MAb for receptors. Antibodies B-11, B-30, and B-64 react with a doublet at 120 kDa that comigrates with hormone binding B receptors detected by PR-6 and by photoaffinity labeling with [^{3}H]R5020 (Figure 11). No other immunoreactive bands were detected in cytosol with B-30 and B-64, but the IgM B-11 cross-reacts with some higher molecular weight bands. The A/B-52 antibody reacts with the same 120-kDa doublet but detects an additional band at 94 kDa corresponding to the hormone binding A receptors

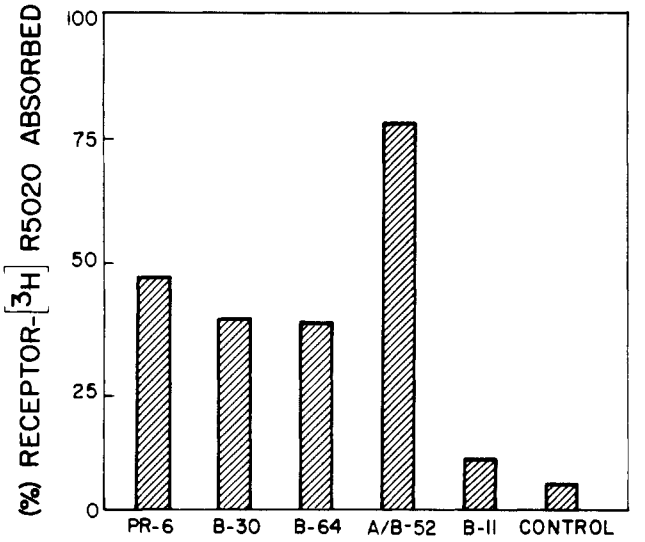

FIGURE 9: Immunoabsorption of receptor–hormone complexes with MAbs raised against purified human B receptors. T47D cytosol receptor–[^{3}H]R5020 complexes were prepared in TEDG containing protease inhibitors and incubated overnight at 4 °C with protein A–Sepharose which had been precoated sequentially with rabbit anti-mouse secondary antibody and undiluted spent growth medium from each of the hybridoma cultures, B-30, B-64, A/B-52, and B-11. The control incubation substituted growth medium from myeloma cell cultures. Purified PR-6 was diluted in growth medium at 10 μg/mL. Data are expressed as the percentage of cytosol receptor–hormone complexes absorbed by protein A–Sepharose.

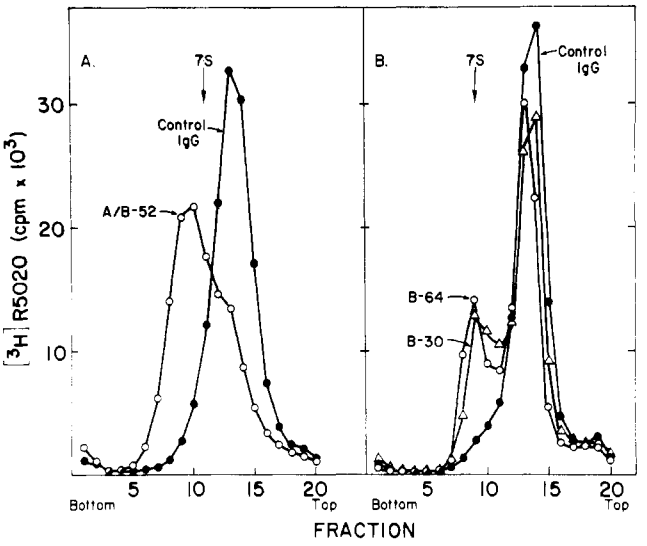

FIGURE 10: Effect of MAbs on sedimentation of human PR in salt-containing sucrose density gradients. A 200-μL aliquot of T47D cytosol receptor–[^{3}H]R5020 complexes (treated with DCC to remove excess free [^{3}H]R5020) was incubated overnight at 4 °C with 40 μg/mL of purified MAbs, A/B-52, B-30, B-64, or control IgG (mouse ascites fluid produced with a myeloma cell line). Samples (200 μL) were layered on 5–20% sucrose gradients prepared in TED containing 0.3 M NaCl and centrifuged at 53 000 rpm for 16 h in an SW60Ti rotor. (A) Control IgG (●) A/B-52 MAb (○). (B) Control IgG (●); B-64 MAb (○); B-30 MAb (△).

(Figure 11). A/B-52 cross reactivity with A and B receptors explains why this MAb absorbs twice the number of cytosol receptors as the B-specific MAbs, and why it is more efficient in shifting 4S receptors on sucrose gradients. Since mice were injected with B receptors only, the production of an antibody which cross reacts with both A and B proteins provides evidence that the two receptor forms share regions of structural homology.

The three IgG antibodies were tested for cross-reaction with other steroid receptors and with PR from other tissues and

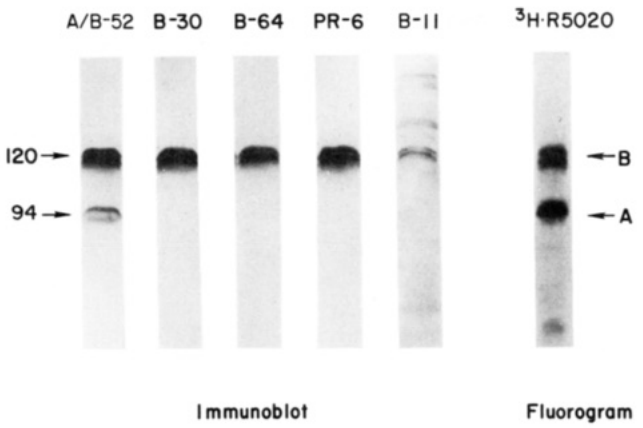

FIGURE 11: Immunoblot of T47D cytosol with MAbs raised against purified human B receptors. Cytosol was prepared in TEDG containing protease inhibitors, and aliquots (containing ≈700 μg of cytosol protein) were submitted to electrophoresis on SDS–polyacrylamide gels. Separated proteins were electrophoretically transferred to nitrocellulose; nitrocellulose was incubated overnight at 4 °C with 10 μg/mL purified MAbs followed by reaction with goat anti-mouse peroxidase conjugate as the detection probe (lanes 1–5, from left to right). As a reference marker for [^{3}H]R5020-bound A and B receptors, T47D cytosol receptors were photoaffinity labeled in situ with [^{3}H]R5020, electrophoresed on a parallel SDS–polyacrylamide gel lane, and transferred to nitrocellulose, and the nitrocellulose was treated with En^{3}Hance spray and exposed to X-ray film (lane 6).

species. None of the MAbs was able to bind with and shift sucrose density sedimentation of receptors for estrogens, glucocorticoids, or androgens extracted from MCF-7 human breast cancer cells. Limited tissue and species specificity was examined by immunoblot assay. No cross-reaction was detected with PR in cytosols of chick oviduct, mouse, rat, or rabbit uterus, with the exception that B-64 reacted with a doublet in rabbit uterus at the same size (120 kDa) as human T47D receptors (data not shown). These MAbs, therefore, are specific for PR among the steroid receptors and appear highly species restrictive for human PR.

Discussion

A two-step procedure was developed for purification of progesterone receptors from human breast cancer cells which involves monoclonal antibody affinity chromatography followed by DEAE ion-exchange chromatography. Purifications were performed under conditions which promote receptor transformation; receptors were bound with hormone, sodium molybdate was omitted from all buffers, and the MAb affinity column was washed extensively in salt-containing buffers prior to receptor elution. That receptors had been transformed during purification was indicated by their efficient binding to DNA–cellulose (Figure 6) and by sucrose density gradient analysis (Figure 7). This procedure yields highly purified receptors which contain essentially two proteins: a 120-kDa steroid binding doublet (B receptors) and a non-hormone binding 76-kDa protein. The two proteins are present in roughly equal amounts, and we have been unable by subsequent chromatographic steps to achieve their separation. The copurifying 76-kDa protein appears not to be a degradation product of receptors but rather is a structurally unrelated protein. Evidence for this is provided by the fact that we have identified the 76-kDa protein as a member of the 70-kDa family of stress proteins (Welch & Faramisco, 1984, 1985). This was accomplished by immunoblot assay of purified PR with a MAb to the 72–73-kDa stress proteins. This MAb showed strong immunoreactivity with the 76-kDa band but had no apparent affinity for B receptors (unpublished data).[2] We also observed induced synthesis of the 76-kDa protein but not of B receptors in response to exposure of T47D cells to elevated temperatures (2 h at 42 °C). Heat-induced synthesis is a property characteristic of the 70-kDa stress proteins. On the basis of these data, we have since revised the apparent molecular mass of the 76-kDa protein to 72 kDa. Copurification of the 76-kDa protein appeared to be dependent on the presence of receptors. This was observed in "mock" immunoaffinity purifications with receptor-depleted cytosols. B-receptor depletion was accomplished by passage of T47D cytosols (twice) over the PR-6 MAb column. The flow-through fraction which contains essentially all other cytosol proteins except absorbed B receptor was analyzed by immunoblot for loss of B protein and then used as a depleted cytosol. Depleted cytosols were then submitted to immunoaffinity purification, just as with PR-containing cytosols. These mock purification products lacked the 76-kDa protein and contained only the two lower molecular mass contaminants at 58 and 62 kDa. These two proteins were purified in amounts equivalent to that of untreated cytosols, suggesting they are abundant proteins that merely bind the immunomatrix nonspecifically (data not shown). These data, taken together with the fact that the 76-kDa protein and B receptors are purified in roughly equal amounts (see Figure 5), suggest the possibility that transformed PR and the 76-kDa protein copurify as a result of binding interactions between the two proteins. It was recently reported by Gustafsson et al. (1986) that purified transformed glucocorticoid receptors remain associated with a non-hormone binding protein of 72 kDa. Since the 94-kDa glucocorticoid receptor (GR) and this 72-kDa protein were isolated in roughly equivalent amounts, these investigators hypothesized that transformed GR may be heterodimers composed of one 94-kDa hormone binding subunit and one 72-kDa non-hormone binding subunit. Furthermore, the 72-kDa protein was reported to affect GR binding with sequence-specific DNA of the mouse mammary tumor virus gene, suggesting a physiological function for this protein. As a working model [presented in the following paper (Wei et al., 1987)], we propose a similar heterodimer structure for transformed human B progesterone receptors and suggest that the 72-kDa protein observed to be associated with GR is the same protein we obtain with human PR.

We have also performed immunoaffinity purification of molybdate-stabilized B receptors with the PR-6 immunomatrix. Purifications were the same as described for isolation of transformed PR except that 20 mM sodium molybdate was included in all buffers. Molybdate-stabilized B receptors contained the same protein components as transformed PR (see Figures 4 and 5) plus an additional non-hormone binding 90-kDa protein. By immunoblot assay with the AC-88 MAb to chick receptor-associated 90K hsp, we have been able to confirm that molybdate-stabilized human PR are also associated with a 90K hsp (data not shown). Thus, it appears that human B receptors associate with either one (72-kDa hsp) or two different (72- and 90-kDa hsp's) non-steroid binding proteins depending on whether they are isolated in transformed or untransformed states. Further investigation will be required to determine whether associations of 90- and 72-kDa hsp's with human PR are merely artifactual interactions that occur in vitro after cell lysis or whether they are meaningful associations that occur in vivo.

The purification method described in this paper is a rapid and relatively mild process which retains biological activity

[2] W. J. Welch and D. P. Edwards, unpublished observations.

of receptors. We find, for example, that a major fraction (>60%) of immunoreactive purified protein retains hormone binding activity. This is measurable by hydroxylapatite (Table I) and sucrose density gradient analysis (Figure 7). The inability of all immunoreactive B protein to bind hormone may be due to partial loss of hormone binding activity during purification (i.e., 30% loss due to exposure to pH 11.5) or in part to the existence of cellular receptors that lack hormone binding capacity. The immunoaffinity purification step would not distinguish between receptors bound with hormone and receptors without hormone binding capacity. Logeat et al. (1985), using similar methods for immunopurification of rabbit PR, reported that about half of the immunoreactive purified protein was capable of binding hormone. We find that a similar fraction (≥60%) of purified human B protein retains the ability to bind tightly to DNA–cellulose (Figure 6). That B receptors bind efficiently to DNA is of interest since there are conflicting reports regarding the DNA binding capacity of B receptors. Several studies with chick oviduct PR have reported that A receptors bind tightly to DNA whereas B receptors bind DNA weakly but show preference for binding with chromatin (Minghetti et al., 1983; Schrader et al., 1981). Studies with rabbit uterine PR (Lamb & Bullock, 1984; Von der Ahe et al., 1985) and other reports with chick oviduct PR (Gronemeyer et al., 1985; Von der Ahe et al., 1986) have demonstrated that B receptors bind DNA as well as A receptors. At least under the conditions used in these studies, we find that purified human B receptors bind DNA–cellulose with affinities similar to those previously reported for chick A receptors. We cannot rule out that the 76-kDa protein may participate in DNA binding since the B-receptor doublets and 76-kDa protein cofractionate by DNA–cellulose chromatography (not shown). The ability to obtain purified receptors in a biologically active form is important for future studies. This will allow us to examine the DNA binding properties of different receptor forms, in particular the effects of receptor-associated hsp's and the effects of ligands on receptor–DNA interactions.

Previous purifications of PR have for the most part used the rabbit uterus (Lamb et al., 1982; Lamb & Bullock, 1984; Logeat et al., 1985) and the chick oviduct as the receptor source (Kuhn et al., 1977; Coty et al., 1979; Puri et al., 1982; Renoir et al., 1984). There are two reports of purification of PR from human uterus by use of steroid affinity chromatography (Smith et al., 1981; Manz et al., 1982). Human uterine receptors, however, appeared to have been extensively degraded by endogenous proteolysis since both studies reported molecular weights of 42K–43K for the purified protein. In the study by Smith et al. (1981), photoaffinity labeling even of crude uterine cytosol failed to detect intact 94- and 120-kDa A and B receptors, suggesting that human uterus contains proteases for PR that are not present in T47D cells. This is the first report of purification of an intact, biologically active PR from human breast cancer cells. Purified B receptors used in this study as immunogen were free of the receptor-associated 90-kDa hsp and the 105–108-kDa B antigen, two proteins which have interfered in the past with the production of receptor-specific antibodies (Joab et al., 1984; Edwards et al., 1984). The B antigen is of particular interest to us since it copurified with hen B receptors isolated by conventional chromatographic procedures under conditions which yield transformed PR. When this material was used as immunogen, MAbs were raised against the non-hormone binding B antigen only (Edwards et al., 1984) but none to receptors. This protein copurified with hen B receptors as a result of its similarity in size and charge with B receptors and not as a result of forming molecular complexes with receptors (Peleg et al., 1985). The B antigen does not coelute with immunoaffinity-purified human receptors in these studies (Figure 8). The chick B antigen was recently identified as a novel hsp (Sargan et al., 1986) based on sequence homology of the B-antigen cDNA with other hsp's. By immunologic cross-reaction, however, we have identified the chick B antigen as indistinguishable from a previously described mammalian 100-kDa glucose-regulated stress protein (unpublished data).[2] This is a glycoprotein localized in Golgi and characterized by its ability to undergo induced synthesis in response to glucose deprivation rather than exposure to elevated temperature (Welch et al., 1983).

The three IgG MAbs produced in this study appear to be monospecific for steroid binding polypeptides of human PR. They do not cross-react with receptor-associated hsp's or with other potential contaminants including the 105–108-kDa B antigen. Furthermore, each MAb detects B receptors as doublets which is characteristic of the human B protein detected by photoaffinity labeling with [^{3}H]R5020 (Horwitz et al., 1985a). B receptors only were used as immunogen, yet one MAb (A/B-52) was produced which recognizes an epitope on both A and B proteins. This provides strong evidence that A and B receptors are structurally related. Gronemeyer et al. (1985) obtained similar results using chicken A and B receptors purified separately from SDS gels and used as immunogens in rabbits. Polyclonal antisera reactive with both A and B receptors were obtained in each case. Partial proteolytic peptide mapping studies of photoaffinity-labeled human PR have also demonstrated that A and B proteins are structurally related (Horwitz et al., 1985a). Definitive conclusions regarding the molecular origins and structural relationships of A and B proteins should soon be possible since the cDNAs coding for chick PR have recently been cloned (Conneely et al., 1986; Jeltsch et al., 1986).

The MAbs produced in this study are valuable reagents for further analysis of human PR structure and function. By preparing immunomatrices with B-specific MAbs and with the A–B specific MAb, it will now be possible to purify human A and B receptors separately or together which will permit study of the functional properties of these two receptor forms. These MAbs will also be of value for clinical immunodetection of PR in breast tumors.

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Immunologic Analysis of Human Breast Cancer Progesterone Receptors. 2. Structure, Phosphorylation, and Processing†

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ABSTRACT: We have used a monoclonal antibody (MAb) directed against chick oviduct progesterone receptors (PR), that cross-reacts with human PR, to analyze PR structure and phosphorylation. This MAb, designated PR-6, interacts only with B receptors (M_r 120 000) of T47D human breast cancer cells; it has no affinity for A receptors (M_r 94 000) or for proteolytic fragments from either protein. The antibody immunoprecipitates native B receptors and was used to study the structure of native untransformed 8S and transformed 4S receptors, using sucrose density gradient analysis, photoaffinity labeling, and gel electrophoresis. On molybdate-containing low-salt gradients, PR-6 complexes with 8S B receptors, causing their shift to the bottom of the gradient while A receptors remain at 8 S. Therefore, A and B receptors form separate 8S complexes, and we conclude that A and B do not dimerize in the holoreceptor. Similar gradient studies using salt-containing, molybdate-free buffers show that there are two forms of salt-transformed 4S receptors, comprising either A proteins or B proteins, suggesting that A and B are also not linked to one another in transformed PR. The independence of A- and B-receptor complexes was confirmed by the finding that purified, transformed B receptors bind well to DNA–cellulose. Since PR-6 cross-reacts with nuclear PR, it was used to analyze nuclear PR processing—a down-regulation step associated with receptor loss as measured by hormone binding. Insoluble nuclear receptors and soluble cytosol receptors were measured by immunoblotting following treatment of T47D cells for 5 min to 48 h with either R5020 or progesterone. From 8 to 48 h after R5020 treatment, immunoassayable receptors decreased in nuclei and were not recovered in cytosols. Nuclear receptors also decreased after progesterone treatment but replenished in cytosols between 8 and 24 h after the start of treatment. Thus, processing involves a true loss of nuclear receptor protein, and not just loss of hormone binding activity, and occurs after progesterone or R5020 treatment. This loss is chronic, however, only in R5020-treated cells. Additional studies focused on the covalent modifications of receptors. We previously described shifts in apparent molecular weight of nuclear PR following R5020 treatment using in situ photoaffinity labeling. To show whether these shifts can be explained by receptor phosphorylation, untreated cells and hormone-treated cells were metabolically labeled with [^{32}P]orthophosphate, and the B receptors were isolated by immunoprecipitation with PR-6 and analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis. In both treatment states, B receptors were labeled in vivo with ^{32}P, thus demonstrating directly that human PR are phosphoproteins. Since B receptors were labeled in the absence of hormone and also after their in vivo transformation by hormone, they appear to be substrates for two phosphorylation reactions, one in the untransformed state and another after they are tightly bound to chromatin. The second phosphorylation may account for the mobility shift of the receptors on SDS gels. On the basis of these data and those in the preceding paper [Estes, P. A., Suba, E. J., Lawler-Heavner, J., Elashry-Stowers, D., Wei, L. L., Toft, D. O., Sullivan, W. P., Horwitz, K. B., & Edwards, D. P. (1987) *Biochemistry* (preceding paper in this issue)], a model of human PR structure and subcellular receptor dynamics is presented.

Two hormone binding forms have been described for the progesterone receptors (PR) of human breast cancer cells: A receptors of 94 000 daltons and B receptors which are doublets of approximately 120 000 daltons (Horwitz et al., 1985a). Their origins and relationship to one another are still unclear as is the manner in which they are organized in the 250 000–300 000-dalton native holoreceptor complex (Horwitz et al., 1985b).

Evidence is accumulating that active[1] holoreceptors contain not only a hormone binding subunit(s) but also additional proteins that do not bind steroids. At least one non-hormone binding protein (M_r 90 000) has been identified (Dougherty et al., 1984; Joab et al., 1984), and others have been implicated (Wrange et al., 1984). Moreover, the structural organization of the receptors is not immutable. Transformation of the active receptors by hormone binding and warming may involve not

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