Coleman, R., Lowe, Ph. J., & Billington, D. (1980) *Biochim. Biophys. Acta 599*, 294.
Crabbe, M. J. C. (1985) *Comput. Biol. Med. 15*, 111.
Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399.
De Kruijff, B., Verkleij, A. J., van Echteld, C. J. A., Gerritsen, W. J., Mombers, C., Noordam, P. C., & de Gier, J. (1979) *Biochim. Biophys. Acta 555*, 200.
Demel, R. A. & de Kruijff, B. (1976) *Biochim. Biophys. Acta 457*, 109.
Erlinger, S. (1981) *Hepatology (Baltimore) 1*, 352.
Gregory, D. H., Vlahcevic, Z. R., Prugh, M. F., & Swell, L. (1978) *Gastroenterology 74*, 93.
Jones, A. L., Schmucker, D. L., Mooney, J. S., Ockner, R. K. & Adler, R. D. (1979) *Lab. Invest. 40*, 512.
Kashchiev, D., & Andexerowa, D. (1983) *Biochim. Biophys. Acta 732*, 133.
Kawato, S., Kinosita, K., Jr., & Ikegami, A. (1977) *Biochemistry 16*, 2319.
Klotz, I. M. (1982) *Science (Washington, D.C.) 217*, 1247.
Kremmer, T., Wisher, M. H., & Evans, W. H. (1976) *Biochim. Biophys. Acta 455*, 655.
Lakowicz, J. R., Prendergast, F. G., & Hogen, D. (1979) *Biochemistry 18*, 508.
Matern, S., & Gerok, W. (1979) *Physiol. Biochem. Pharmacol. 85*, 126.
Mazer, N. A., Kwasnick, R. F., Carey, M. C., & Benedek, G. B. (1976) *Micellization, Solubilization, Microemulsions 1*, 383.
Meier, P. J., Gordon, E., Moseley, R., & Boyer, J. L. (1974) *Ann. N.Y. Acad. Sci. 435*, 309.
Miller, R. G. (1980) *Nature (London) 287*, 166.
Milsmann, M. H. W., Schwendener, R. A., & Weder, H.-G. (1978) *Biochim. Biophys. Acta 512*, 147.
Müller, K. (1981) *Biochemistry 20*, 404.
O'Connor, Ch. J., Wallace, R. G., Iwamoto, K., Taguchi, T., & Sunamoto, J. (1985) *Biochim. Biophys. Acta 817*, 95.
Papahadjopoulos, D., Vail, W. J., Jacobson, K., & Poste, G. (1975) *Biochim. Biophys. Acta 394*, 483.
Saito, H., Sugimoto, Y., Tabeta, R., Suzuki, S., Izumi, G., Kodama, M., Toyoshima, S., & Nagata, Ch. (1983) *J. Biochem. (Tokyo) 94*, 1877.
Schubert, R., Jaroni, H., Schölmerich, J., & Schmidt, K.-H. (1983) *Digestion 28*, 181.
Shinitzky, M., & Barenholz, Y. (1974) *J. Biol. Chem. 249*, 2652.
Small, D. M. (1967) *Gastroenterology 52*, 607.
Ulmius, J., Lindblom, G., Wennerström, H., Johansson, L. B.-A., Fontell, K., Söderman, O., & Arvidson, G. (1982) *Biochemistry 21*, 1553.
Van Blitterswijk, W. J., van Hoeven, R. P., & van der Meer, B. W. (1981) *Biochim. Biophys. Acta 644*, 323.

A 59-Kilodalton Protein Associated with Progestin, Estrogen, Androgen, and Glucocorticoid Receptors†

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ABSTRACT: Previous studies of the anti 8.5S progestin receptor monoclonal antibody KN 382/EC1 showed that it was specific for nontransformed progestin receptors. However, with different methods of tissue disruption and the use of protease inhibitors, we found that other nontransformed steroid receptors formed immune complexes with KN 382/EC1. Binding of the antibody to rabbit uterine estrogen, progestin, and androgen and liver glucocorticoid receptor systems was characterized by sucrose density gradient centrifugation, high-pressure liquid chromatography (HPLC), immunoadsorption, and immunoblotting. Immobilized KN 382/EC1 adsorbed both M_r 59 000 and M_r 92 000 proteins. The M_r 92 000 protein appeared to be bound to the antigenic M_r 59 000 protein, and the two proteins were present in apparently the same stoichiometric relationship in several tissues. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoadsorbed material revealed appreciable amounts of both proteins in testis, stomach, lung, liver, uterus, and kidney. Only trace amounts were found in skeletal or heart muscle, and none was found in blood serum. Cleveland digestion of isolated M_r 59 000 and 92 000 proteins revealed dissimilar peptide constituents. Immunoblots of material from uterus and liver resulted in staining of the M_r 59 000 protein but not the M_r 92 000 protein. We conclude that similar antigenic determinants reside in components of several nontransformed steroid receptors and they reside on an M_r 59 000 protein. It is likely, therefore, that there are common components present in nontransformed steroid receptors.

Most steroid receptors exhibit sedimentation coefficients (Faber, 1980) that can be divided into two groups. Larger complexes (7 S and greater) are referred to as nontransformed (Puri et al., 1982), untransformed, (Reker et al., 1985), or native (Baulieu, et al., 1983) receptors. Smaller receptor forms (5.5 S or less), commonly designated activated or transformed receptors, have the well-known characteristics of binding to nuclei, DNA, chromatin, and specific isolated gene sequences (Saffran et al., 1976; Baulieu et al., 1983; Renoir & Mester, 1984; von der Ahe et al., 1985). Earlier studies suggested that the conversion of the larger to the smaller forms accompanying transformation resulted from subunit dissociation (Stancel et

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al., 1973; Saffran et al., 1976; Vedeckis, 1983). However, dissociation of nontransformed receptors is poorly understood because the proteins involved have not been identified. Included among proposals for the subunit composition of nontransformed receptors (Vedeckis, 1983; Sherman et al., 1983) is the suggestion of nonsteroid binding components (Jensen & DeSombre, 1972; Vedeckis, 1983; Murayama, 1985). However, the evidence for nonsteroid subunits has been based on indirect data, because the study of nontransformed receptors has been limited to measurement of radioactive steroids bound to receptors. Within the last several years, however, monoclonal antibodies directed against nontransformed receptors have been available (Radanyi et al., 1983; Sullivan et al., 1985; Nakao et al., 1985) which allow identification of nontransformed receptor constituents.

Two laboratories have shown the existence of a M_r[1] 90000 nonsteroid binding protein component of the nontransformed chick oviduct receptor (Dougherty et al., 1984; Renoir et al., 1984). Joab et al. (1984) and Sullivan et al. (1985) described the ubiquitous nature of this protein, and Sanchez et al. (1985) and Schuh et al. (1985) established that, in mouse fibroblasts and chick oviduct, the M_r 90000 protein was indistinguishable from a major heat shock protein.

In light of the ubiqutous nature of M_r 90000 nonsteroid binding subunit, we have assessed the distribution of the antigenic component of the nontransformed progestin receptor bound by KN 382/EC1, a monoclonal antibody developed in our laboratory (Nakao et al., 1985). Immunoprecipitation of receptor complexes with this antibody demonstrated four protein components of the nontransformed rabbit uterine progestin receptor (Tai & Faber, 1985). Two were steroid binders (M_r 116000 and 90000), and two did not bind steroid (M_r 92000 and 59000). Furthermore, KN 382/EC1 appeared to recognize the M_r 59000 protein.

This report presents evidence showing that the epitopes of the M_r 59000 receptor-associated protein are present in nontransformed estrogen, androgen, progestin, and glucocorticoid receptors. The M_r 59000 protein was also found to be present in a number of organs.

Immunoblots and peptide mapping of purified M_r 59000 protein supported our suggestion that the KN 382/EC1 antibody preferentially binds the M_r 59000 receptor-associated protein. This data also provides strong evidence that the M_r 59000 protein is not derived from the M_r 92000 protein. Thus we conclude that the M_r 59000 receptor-associated protein is a unique and common component of nontransformed rabbit receptors.

Experimental Procedures

Steroids. [*17α-methyl*-^{3}H]Methyltrienolone ([*17α-methyl*-^{3}H]R1881) (87 Ci/mmol), [6,7-^{3}H(N)]dexamethasone (44 Ci/mmol), 17β-[1,2,6,7-^{3}H]estradiol (114 Ci/mmol), and [*17α-methyl*-^{3}H]promegestone ([*17α-methyl*-^{3}H]R5020) (87 Ci/mmol) in addition to unlabeled methyltrienolone and promegestone were purchased from New England Nuclear. Unlabeled dexamethasone, 17β-estradiol, progesterone, triamcinolone acetonide, and hydrocortisone were obtained from Sigma. All steroids were stored in absolute ethanol at −20 °C.

Protease Inhibitors. Leupeptin, pepstatin A, phenylmethanesulfonyl fluoride (PMSF), and benzamidine were purchased from Sigma.

Buffers. The following buffers were used: 5 mM PGTA (5 mM potassium phosphate, 10% glycerol (v/v), 10 mM monothioglycerol, and 0.02% sodium azide, pH 7.4); 5 mM PGTMA (5 mM PGTA plus 10 mM sodium molybdate); 5 mM PGTAP (5 mM PGTA plus 0.5 mM PMSF, 20 mM benzamidine, 2 μg/mL pepstatin A, and 10 μg/mL leupeptin); 5 mM PGTMAP (5 mM PGTAP plus 10 mM sodium molybdate); 100 mM PGTA (100 mM potassium phosphate, 10% glycerol (v/v), 10 mM monothioglycerol, and 0.02% sodium azide, pH 7.4); 100 mM PGTMA (100 mM PGTA plus 10 mM sodium molybdate); Tris buffer (20 mM Tris-HCl and 0.02% sodium azide, pH 7.2); SDS sample preparation buffer (3% SDS, 3% mercaptoethanol, 33% glycerol (v/v), and 0.167 M Tris-HCl, pH 6.8).

Animals and Cytosol Preparation. For most studies New Zealand white rabbits, purchased from Turtle Creek Rabbitry (Milbury, OH), received a single injection (250 μg per animal) of Depoestradiol (Upjohn) 6 days prior to killing. Their livers were perfused through the portal vein with ice-cold saline. Excised livers and uteri were sliced and minced prior to homogenization. In some instances frozen testis and uteri were purchased from Pel-Freeze (Rogers, AR). Frozen organs were pulverized on dry ice with a mortar and pestle (placed on dry ice). Minces or frozen powder were homogenized in the presence of protease inhibitors (5 mM PGTAP) with a Polytron PT-10 as described (Tai & Faber, 1985). Homogenates were centrifuged at 800*g* for 10 min. After the pellet was discarded, supernatants were recentrifuged at 100000*g* for 1.0 h, with a Beckman type 65 rotor yielding the cytosol fractions. In the tissue distribution studies samples of liver, uterus, kidney, lung, stomach, heart, and skeletal muscle (from tissue surrounding the femur) were excised from untreated rabbits. They were frozen, powdered, and homogenized as described in 5 mM PGTAP buffer.

Cytosol Labeling. Androgen receptors were prepared by preincubating uterine cytosol with 5 μM triamcinolone acetonide for 20 min, followed by a 1-h incubation with 10 mM [*17α-methyl*-^{3}H]R1881 ± 2 μM unlabeled R1881. Estrogen receptors were labeled with 10 mM 17β-[1,2,6,7-^{3}H]estradiol ±2 μM unlabeled estradiol for 1 h at 4 °C. In this particular instance the cytosols were preincubated with 2 μM hydrocortisone and 2 μM progesterone. Liver cytosol was incubated with 10 nM [6,7-^{3}H(N)]dexamethasone to label the glucocorticoid receptor system. Progestin receptors were labeled with 80 nM [*17α-methyl*-^{3}H]R5020 ± 8 μM nonradioactive R5020, following a 20-min preincubation with 2 μM hydrocortisone.

Preparation of Immunoglobulins. Immune IgG_1 (IgG_I) derived from pooled ascites fluid produced by cell line KN 382/EC1 (Nakao et al., 1985) was purified by DEAE Affi-Gel Blue (Bio-Rad) chromatography as described (Tai & Faber, 1985). Myeloma IgG_1 (IgG_N) (MOPC 21, GI) (IgFK) was purchased from Bionetics.

Analysis by Sucrose Density Gradient Centrifugation. Aliquots (200 μL) of labeled cytosol were incubated with 10 μg of KN 382/EC1 IgG_1 or 10 μg of myeloma IgG_1 at 4 °C for 3 h. For those experiments requiring addition of Na_2MoO_4, 1 M Na_2MoO_4 was added to the mixture to yield a final concentration of 10 mM Na_2MoO_4. Unbound steroid was removed by dextran-coated charcoal (1% Mallinckrodt

[1] Abbreviations: IgG_1, immunoglobulin G_1; IgG_I, KN 382/EC1 monoclonal antibody; IgG_N, Bionetics myeloma monoclonal antibody; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular weight; R5020, promegestone; R1881, methyltrienolone; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

charcoal and 0.1% dexatran T-70, Pharmacia). The sample was layered onto 5–20% sucrose gradients (prepared in the designated buffers) and centrifuged at 50 000 rpm for 16–17 h in a Beckman L5-65 ultracentrifuge (SW 60 Ti rotor). The gradients contained ^{14}C-labeled internal standards (ovalbumin, 3.6 S, and γ-globulin, 7.0 S) purchased from New England Nuclear. They were fractionated with an ISCO Model 185 density gradient fractionator (3 drops/fraction) into minivials. Three milliliters of Beckman Ready-Solv HP scintillation media was added to each vial. They were counted in a Searle Isocap/300 at an efficiency of approximately 25% for ^3H and 10% for ^{14}C.

High-Performance Liquid Chromatography (HPLC) of Antibody–Receptor Complexes. Cytosol was labeled, incubated with IgG_I or IgG_N, and treated with dextran-coated charcoal, as described earlier. Aliquots (250 μL) were chromatography by using a Beckman Model 330 isocratic HPLC and a Toyosoda TSK-4000 column (7.5 mm × 30 cm). Elution was carried out with 100 mM PGTA 10 mM Na_2MoO_4 at a flow rate of 0.4 mL/min. Fractions of 0.25 mL were collected with an ISCO Model 560 fraction collector. Molecular weight standards (Pharmacia) for column calibration were as follows: blue dextran, M_r 2 000 000; thyroglobulin, M_r 669 000; ferritin, M_r 440 000; catalase, M_r 232 000; ovalbumin, M_r 43 000; chymotrypsin, M_r 21 000.

Immunoadsorption of Rabbit Steroid Hormone Receptor Systems. Purified KN 382/EC1 (immune) IgG_1 (1 mg) or Bionetics myeloma (control) IgG_1 (1 mg) was coupled to 1 mL of Affi-Gel 10 (Bio-Rad) in 0.1 M $NaHCO_3$ (pH 8.0) according to the manufacturer's instructions. Unreacted sites were blocked with 2 mL of 0.1 M ethanolamine (pH 8). Bradford protein determination (Bradford, 1976) indicated that 80% of the immunoglobulins were coupled to the gel (800 μg/1000 μL of gel). After being washed extensively with coupling buffer and distilled water, the gels were equilibrated with 5 mM PGTA ± 10 mM Na_2MoO_4.

Aliquots of 1 mL labeled cytosol in 5 mM PGTAP ± 10 mM Na_2MoO_4 were incubated with 200 μL of either immune or control (myeloma) adsorbents at 4 °C overnight. After the gel settled, the supernatants were saved for receptor measurement. The gels were washed with 4 × 1 mL of 5 mM PGTA ± 10 mM Na_2MoO_4 and 3 × 1 mL of 100 mM PGTA ± 10 mM Na_2MoO_4. Immune and control adsorbents were then suspended in 3 mL of Beckman Ready-Solve HP scintillation media and counted for immunoadsorbed radioactivity. The distribution of radioactivity in the supernatant or associated with the adsorbent was expressed as a percentage of the total specific binding of steroid by the receptors. Protein was eluted with 1 mL of sample preparation buffer for SDS–PAGE separation (Tai & Faber, 1985).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–polyacrylamide gel electrophoresis with 7.5% acrylamide was carried out according to Laemmli (1970). Protein bands were visualized by 0.1% Coomassie blue R-250 in 50% methanol and 7.5% acetic acid. Molecular weight standards, purchased from Bio-Rad, included myosin (M_r 200 000), β-galactosidase (M_r 116 000), phosphorylase B (M_r 97 400), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400).

Peptide Mapping. Three milliliters of cytosol, prepared in 5 mM PGTAP from Pel-Freeze frozen rabbit uteri, were immunoadsorbed by 1 mL of KN 382/EC1 coupled Affi-Gel 10 and subjected to SDS–PAGE (7.5% acrylamide) as described above. The gels were stained with Cleveland's staining solution (Cleveland et al., 1977) for 20 min and destained for 50 min. The M_r 59 000 and 92 000 proteins were cut out of the gel and soaked 30 min in solution A (0.125 M Tris-HCl, pH 6.8, 0.1% SDS, and 10 mM EDTA). They were placed in the wells of the second gel (15% acrylamide) containing protease V8 (Sigma, 500 ng/well) according to Cleveland et al. (1977). After electrophoresis peptides in the second gel were visualized by silver staining (Bio-Rad kit).

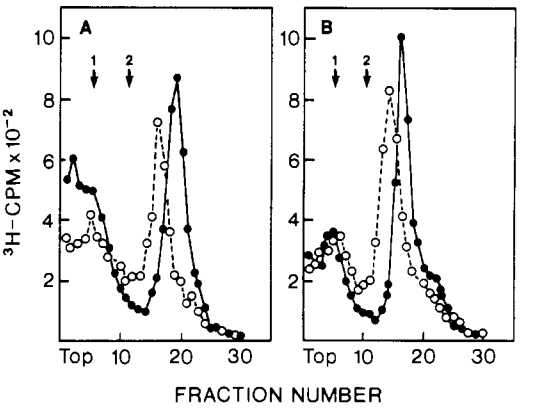

FIGURE 1: Sucrose density gradient analysis of the binding of KN 382/EC1 to various forms of the nontransformed rabbit liver glucocorticoid receptor. Cytosol (5 mM PGTMAP) was prepared as described and incubated with 10 nM [6,7-^3H]dexamethasone. Aliquots (200 μL) were incubated with 10 μg of KN 382/EC1 IgG_1 (●) or 10 μg of the Bionetics myeloma IgG_1 (○) at 4 °C for 3 h. Free steroid was removed by dextran-coated charcoal. (Panel A) 5 mM PGTMAP cytosol; 5 mM PGTMA gradient. (Panel B) 5 mM PGTMAP cytosol; 100 mM PGTMA gradient. All gradients received [^{14}C]ovalbumin (1) and [^{14}C]-γ-globulin (2) internal standards. Centrifugation was for 16 h at 50 000 rpm in a Beckman SW 60 Ti swinging bucket rotor.

Immunoblotting. Protein bands were transferred to nitrocellulose paper (Bio-Rad, 0.4 μm) with a Hoeffer Transblotting apparatus (0.2 A, for 16 h with cooling). Transfer buffer was composed of 25 mM Tris-HCl, 192 mM glycine, and 20% methanol (v/v). After removal of the paper, nonspecific binding sites were blocked with 10% horse serum in Tris-buffered saline (20 mM Tris-HCl and 500 mM NaCl, pH 7.2), incubated for 30 min at room temperature. Nitrocellulose papers were incubated overnight at 4 °C with a 30-mL solution of the first (immunoreactive) antibody (5 μg/mL). Subsequently, the papers were washed twice with Tris-buffered saline (120 mL, 10 min/wash) prior to a 2-h incubation (room temperature) with peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). The second antibody was diluted 1000-fold with the horse serum blocking solution. Nitrocellulose papers are washed 3 times with Tris-Buffered saline (10 min/wash). Antigen bands are visualized by a mixture of 60 mg of 4-chloronaphthol in 20 mL of ice-cold methanol added to 60 μL of H_2O_2 in 100 mL of Tris-buffered saline.

RESULTS

Sucrose density gradient centrifugation has been routinely employed to establish the binding of antireceptor antibodies. Increases in the sedimentation coefficient of the receptor are considered to reflect antigen–antibody interaction. Addition of KN 382/EC1 to cytosols equilibrated with tritiated steroids resulted in shifts to large forms of nontransformed estrogen, androgen, and glucocorticoid receptors (Figure 1, Table I). The presence of sodium molybdate was mandatory for an antibody effect, either in hypotonic (5 mM PGTMA) or hypertonic (100 mM PGTMA) buffers.

The formation of antibody–antigen complexes was also demonstrated by HPLC. Unlike the sucrose gradient results,

Table I: Sedimentation Coefficients of Various Nontransformed Steroid Receptor Systems after Incubation with KN 382/EC1 or Bionetic Myeloma Monoclonal Antibodies[a]

		sucrose gradient buffer					
		5 mM PGTMA[b]		5 mM PGTA[c]		100 mM PGTMA[d]	
receptor system	organ	myeloma	KN 382/EC1	myeloma	KN 382/EC1	myeloma	KN 382/EC1
progestin	uterus	9.25 ± 0.49[e] (n = 4)[f]	11.38 ± 0.49 (n = 4)	8.65 ± 0.48 (n = 2)	8.65 ± 0.83 (n = 2)	8.20 ± 0.54 (n = 2)	10.3 ± 0.37 (n = 3)
estrogen	uterus	8.79 ± 0.66 (n = 6)	11.05 ± 0.48 (n = 6)	9.30 ± 0.10 (n = 3)	9.30 ± 0.10 (n = 3)	8.63 ± 0.29 (n = 6)	9.50 ± 0.30 (n = 6)
androgen	uterus	9.23 ± 0.33 (n = 3)	11.27 ± 0.19 (n = 3)	8.90 ± 0.1 (n = 2)	8.90 ± 0.10 (n = 2)	8.40 ± 0.22 (n = 3)	10.2 ± 0.22 (n = 3)
glucocorticoid	liver	9.53 ± 0.39 (n = 6)	11.37 ± 0.43 (n = 4)	9.30 ± 0.10 (n = 2)	9.3 ± 0.10 (n = 2)	8.40 ± 0.42 (n = 4)	10.5 ± 0.39 (n = 4)

[a] Uterine and liver cytosol was prepared from estrogen-treated rabbits or Pel-Freeze uteri after freezing and pulverization. Powders were homogenized in the presence of protease inhibitors (5 mM PGTAP buffer), and sodium molybdate was added when necessary. Aliquots of cytosol (200 μL) were incubated with 10 μg of KN 382/EC1 or Bionetics myeloma IgG_1 at 4 °C for 3 h. Centrifugation was for 16 h at 50000 rpm in a Beckman SW 60 Ti swinging bucket rotor as described under Experimental Procedures. 5 mM PGTAP = 5 mM sodium phosphate, 10% glycerol (v/v), 10 mM monothioglycerol, 0.02% sodium azide, 5 mM PMSF, 2 mM benzamidine, 2 μg/mL pepstatin A, and 10 μg/mL leupeptin, pH 7.4. [b] 5 mM PGTMA = 5 mM sodium phosphate, 10% glycerol (v/v), 10 mM monothioglycerol, 10 mM Na_2MoO_4, and 0.02% sodium azide, pH 7.4. [c] 5 mM PGTA = 5 mM sodium phosphate, 10% glycerol (v/v), 10 mM monothioglycerol, and 0.02% sodium azide, pH 7.4. [d] 100 mM PGTMA = 100 mM sodium phosphate, 10% glycerol (v/v), 10 mM monothioglycerol, 10 mM Na_2MoO_4, and 0.02% sodium azide, pH 7.4. [e] Standard error of the mean. [f] Number of trials.

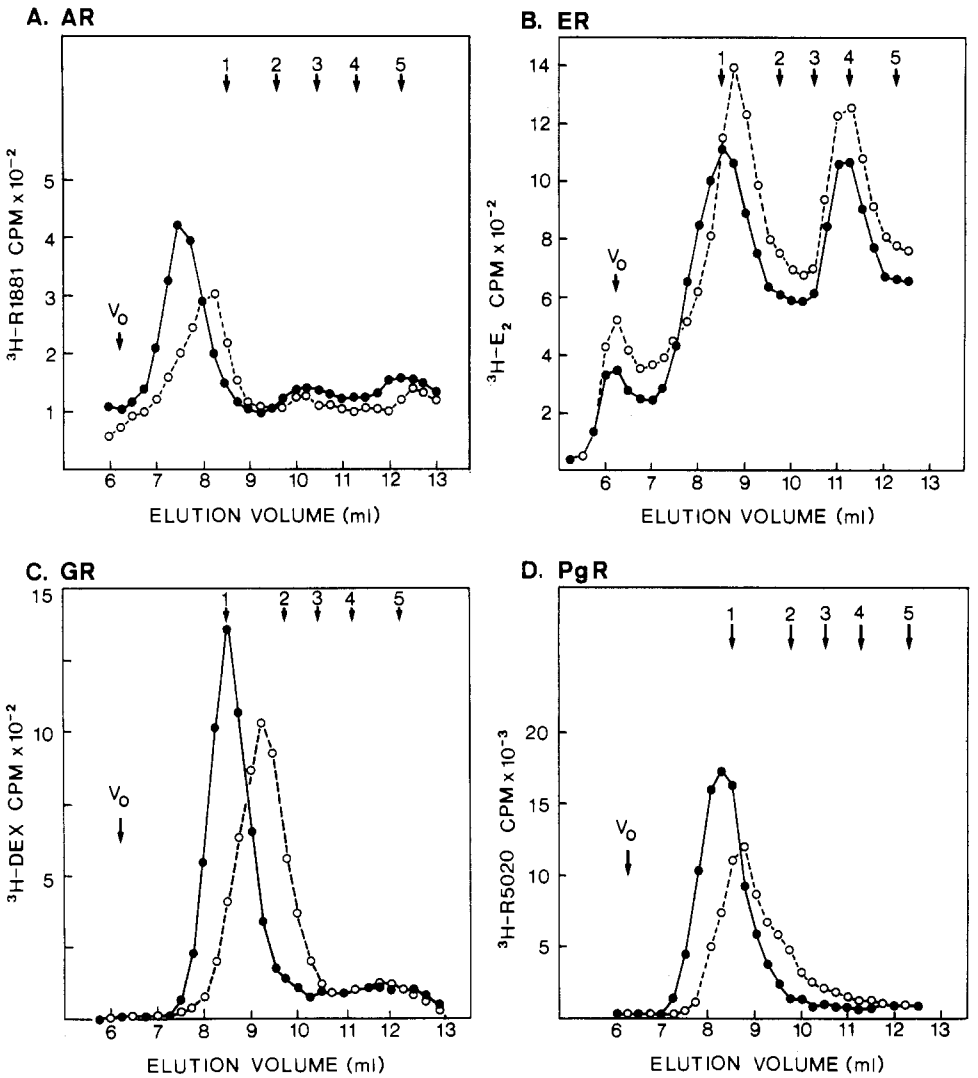

FIGURE 2: High-performance liquid chromatography (HPLC) analysis of the interaction of KN 382/EC1 with various nontransformed steroid receptors. Cytosols were prepared from uterus and liver in 5 mM PGTAP. Samples were labeled with [*17α-methyl*-^{3}H]R1881 to label the androgen receptor (panel A, AR), 17β-[1,2,6,7-^{3}H]estradiol to label the estrogen receptor (panel B, ER), [6,7-^{3}H]dexamethasone to label the glucocorticoid receptor (panel C, GR), or [*17α-methyl*-^{3}H]R5020 to label the progestin receptor (panel D, PgR) as described under Experimental Procedures. After incubation with KN 382/EC1 IgG_1 (●) or Bionetics myeloma IgG_1 (○), 250-μL aliquots were chromatographed with a Beckman Model 330 isocratic HPLC and a Toyosoda TSK 4000 column. The elution buffer was 100 mM PGTA. Standards included blue dextran (V_0), thyroglobulin (1), ferritin (2), catalase (3), ovalbumin (4), and chymotrypsin (5).

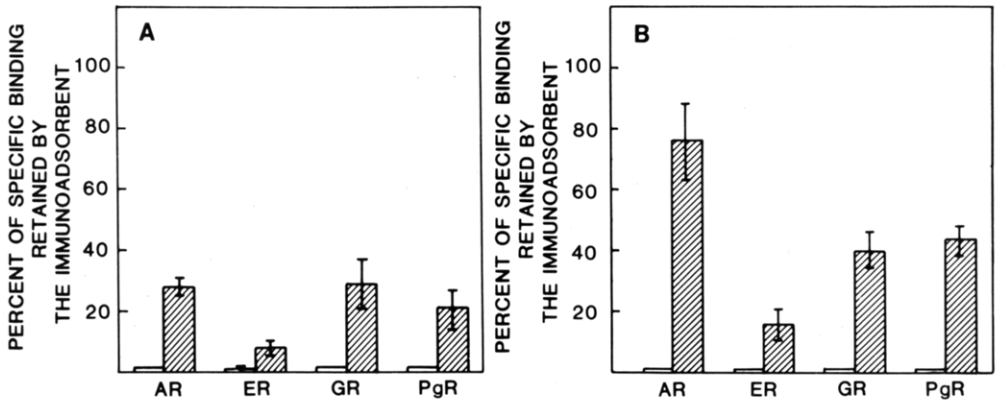

FIGURE 3: Immunoadsorption of various steroid receptors in the presence or absence of sodium molybdate. Rabbit uterine or liver cytosol (1 mL), prepared in 5 mM PGTAP (panel A) or 5 mM PGTMAP (panel B), was labeled with [*17α-methyl*-^{3}H]R1881 (AR), 17β-[1,2,6,7-^{3}H]estradiol (ER), [6,7-^{3}H]dexamethasone (GR), or [*17α-methyl*-^{3}H]R5020 (PgR) and the appropriate nonlabeled steroids. Samples were incubated overnight at 4 °C with 200 μL of Affi-Gel 10 coupled with KN 382/EC1 (crosshatched bars) or Bionetics myeloma IgG_1 (open bars). After settling, pellets were washed 4 times with 1 mL of 5 mM PGTA or 5 mM PGTMA and 3 times with 1 mL of 100 mM PGTA or 100 mM PGTMA prior to counting. Distribution of radioactivity is expressed as percentage of total radioactivity of the original cytosol sample ± the standard error of the mean for three trials.

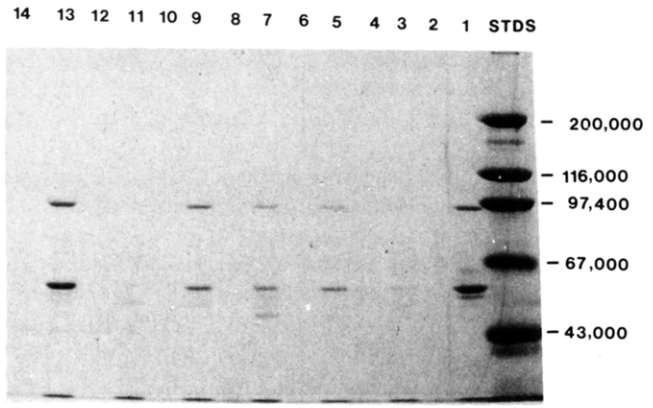

FIGURE 4: SDS–PAGE analyses of material adsorbed by Affi-Gel 10 coupled KN 382/EC1 antibody. Cytosols were prepared from frozen powders in 5 mM PGTAP buffer. Samples of cytosol (1 mL) were incubated with 200 μL of KN 382/EC1 Affi-Gel 10 (odd-numbered lanes) or 200 μL of Bionetic myeloma Affi-Gel 10 (even-numbered lanes) as described under Experimental Procedures. After washing (4 × 1 mL of 5 mM PGTMA followed by 3 × 1 mL of 100 mM PGTMA), bound protein was eluted with SDS sample preparation buffer and subjected to electrophoresis. STDS = standards. Lanes: (1 and 2) testis; (3 and 4) heart; (5 and 6) stomach; (7 and 8) kidney; (9 and 10) lung; (11 and 12) skeletal muscle; (13 and 14) liver. The gel was stained with Coomassie blue R-250.

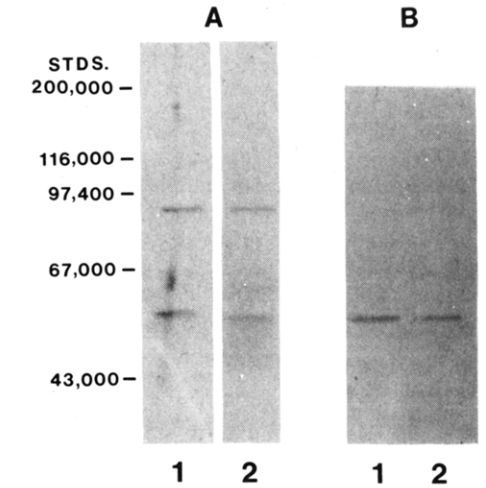

FIGURE 5: Immunoblot of rabbit uterine and liver M_r 59 000 peptide. Cytosols (5 mM PGTMAP) were incubated with Affi-Gel 10 coupled KN 382/EC1 as described under Experimental Procedures. Adsorbed material was washed 4 times with 5 mM PGTMA and 3 times with 100 mM PGTMA prior to elution with SDS sample preparation buffer. Eluted proteins were subjected to SDS–PAGE prior to immunoblotting. Proteins were transferred to nitrocellulose paper (0.2 A for 3 h) and incubated with KN 382/EC1 antibody (5 μg/mL for 16 h at 4 °C). Bands were visualized by sequential incubation with peroxidase-conjugated goat anti-mouse IgG_1 and 4-chloronaphthol in methanol–H_2O_2. (Panel A) SDS–PAGE of material from uterine cytosol (lane 1) and liver cytosol (lane 2). (Panel B) Immunoblot cytosol of material from panel A. STDS = standards. The SDS–PAGE gel was stained with Coomassie blue R-250.

the presence of Na_2MoO_4 was not an absolute requirement for antibody binding. KN 382/EC1 bound to nontransformed progestin, androgen, and glucocorticoid receptors in the absence of Na_2MoO_4 (Figure 2). Almost identical data were obtained when the elution media contained 10 mM Na_2MoO_4 (data not shown). Parenthetically, the antibody did not bind the estrogen receptor system as well, in either molybdate-containing or molybdate-free media.

Confirmation of the binding of KN 382/EC1 to nontransformed receptors in the absence of Na_2MoO_4 was also demonstrated by immunoadsorbtion (Figure 3). In the absence of Na_2MoO_4, KN 382/EC1 coupled Affi-Gel 10 adsorbed 25–30% of cytosolic androgen, glucocorticoid, and progestin receptors (Figure 3). Less binding of KN 382/EC1 to the estrogen receptor, noted in the HPLC experiments (Figure 2), was reflected in the inability of the immunoaffinity gel to adsorb more than 10% of the estrogen receptor (Figure 3A). Addition of 10 mM Na_2MoO_4 to the buffers almost doubled the amount of each receptor that was adsorbed to the matrix (Figure 3B).

Although the above experiments indicated the presence of common epitopes in each of the nontransformed receptors, SDS–PAGE analysis of immunoprecipitated material was necessary to determine if the antigenic sites were on the same or different proteins. As shown by electrophoresis (Figure 4), appreciable quantities of M_r 59 000 and 92 000 proteins were extracted from rabbit testis, stomach, uterus, liver, and kidney cytosol. Only trace amounts were isolated from heart and striated muscle. None was found in blood serum. In control experiments with myeloma-coupled Affi-Gel 10, there was no M_r 59 000 protein adsorbed to the matrix (Figure 4, even-numbered lanes). As was noted earlier (Tai & Faber, 1985), bands corresponding to receptors were not evident. Presumably, the two receptor-associated proteins are in excess, and the amount of receptor is below the sensitivity (~1 μg) of the

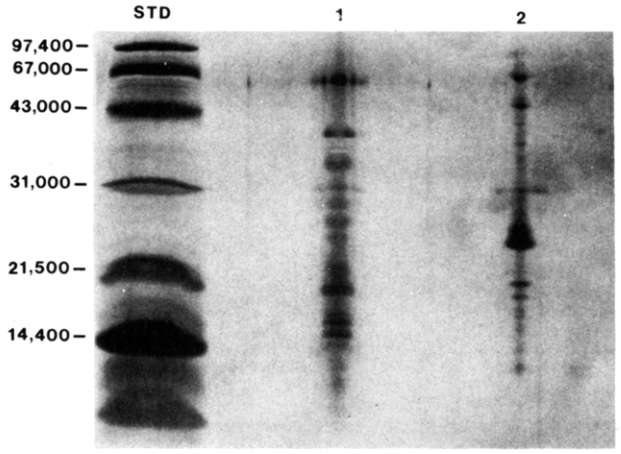

FIGURE 6: Peptide mapping of the M_r 92 000 and 59 000 proteins. Rabbit uterine cytosol (3 mL) was immunoadsorbed by 1 mL of KN 382/EC1 coupled Affi-Gel 10. Protein was eluted and separated by SDS–PAGE (7.5% acrylamide) as described under Experimental Procedures. After the gel was stained with Cleveland's solution (Cleveland et al., 1977), protein bands (M_r 92 000 and 59 000) were cut out, soaked in solution A, and placed in the wells of the second gel (15% acrylamide) containing 500 ng of protease V8. After electrophoresis the gel was stained with silver stain (Bio-Rad kit). STD = standards. Lanes: (1) isolated M_r 59 000 peptide; (2) isolated M_r 92 000 peptide.

Coomassie blue R-250 staining technique.

Coadsorption of the M_r 92 000 and 59 000 proteins suggested that they might share similar epitopes. It was also possible that the M_r 59 000 protein was a proteolytic cleavage product of the M_r 92 000 protein. This does not seem to be the case, for immunoblotting experiments (Figure 5) revealed no binding KN 382/EC1 to the M_r 92 000 protein. Blots of material precipitated by nonimmune myeloma coupled Affi-Gel 10 disclosed a lack of binding to any protein (data not shown).

The inability of KN 382/EC1 to bind to the M_r 92 000 protein was strong, but not conclusive, evidence for the nonidentity of the two receptor-associated proteins. It was possible that common epitopes might be hidden in the M_r 92 000 protein by some perfidious folding of the protein. Conclusive evidence for the absence of a precursor–product relationship was obtained from V8 protease digestion of the two proteins. SDS–PAGE of the resulting peptides shown that the two proteins had no overlapping primary structure (Figure 6).

Discussion

In this report, four separate methodologies, namely, sucrose gradient centrifugation, high-performance liquid chromatography, immunoadsorption, and immunoblotting, were used to demonstrate the identity of the M_r 59 000 protein as a common component of nontransformed estrogen, androgen, progestin, and glucocorticoid receptors. Furthermore, the protein was found to be present in a number of tissues that respond to one steroid or another. Whereas a number of laboratories have shown the presence of a protein of about M_r 90 000 in association with a number of receptor systems, ours is the only one to demonstrate an additional M_r 59 000 protein. It seems logical that the M_r 59 000 protein could be derived from the M_r 92 000 form. Our findings, however, indicate that the two are distinct from each other. This was demonstrated by both immunoblotting and V8 proteolytic cleavage. Additional evidence for the distinct identities of the two proteins is derived from our finding that exhaustive washing of KN 382/EC1 immunoadsorbed material resulted in sequential elution of the M_r 92 000 protein prior to the M_r 59 000 protein (Tai & Faber, 1985). In addition, preliminary studies have shown that anti-heat shock protein antibodies (Riehl et al., 1985; Schuh et al., 1985) did not bind to the M_r 59 000 protein but bound the M_r 92 000 protein.

In our earlier study (Nakao et al., 1985) we were unable to show that KN 382/EC1 reacted to receptors other than progestin receptors. However, in the present study we employed more gentle methods of cytosol preparation (i.e., freezing the tissue and addition of protease inhibitors) than we had used previously. We conclude that protease inhibitors and Na_2MoO_4 not only stabilize the steroid-binding proteins (receptors) but yield more stable preparations of nontransformed receptors. Thus our work concurs with that of Hubbard and Kalimi (1985) that protease inhibitors block dissociation of receptors.

One vexing observation involves the inability to detect antibody binding by sucrose gradient analysis in the absence of Na_2MoO_4 (Table I). The sedimentation coefficient was 8.5–9.3 S, suggesting that we should have seen an antibody effect. An answer for this lies in the work Gregory and Notides (1982) on the role of Na_2MoO_4 in blocking limited proteolysis of the estrogen receptor. They concluded that nonspecific uterine proteases caused modification of the sedimentation and ionic properties of the receptor. We would suggest that protease inhibitors, in concert with Na_2MoO_4, have an additive effect in blocking proteolysis and/or subsequent dissociation of subunits. Furthermore, we believe that Na_2MoO_4 and protease inhibitors prevent dissociation of the M_r 59 000 protein (and perhaps other receptor-associated proteins) from the receptor during ultracentrifugation. An example of this may be seen in the binding of KN 382/EC1 to the molybdate-stabilized 8.5S and not to the molybdate-free 7S rabbit uterine progestin receptor (Tai & Faber, 1985; Faber et al., 1985).

We were intrigued by the observation that Na_2MoO_4 is not an absolute requirement for binding of KN 382/EC1 to nontransformed receptors. This is borne out by both the HPLC (Figure 2) and immunoadsorption experiments (Figure 3). Presumably, the protease inhibitors provided sufficient stability for analysis. This suggests that association of subunits in the presence of Na_2MoO_4 is not a property peculiar to Na_2MoO_4 and that the larger complexes are not Na_2MoO_4-dependent artificial aggregates. Of further interest is the elution volume of the nontransformed receptors (Figure 2). Each eluted between ferritin (M_r 440 000) and thyroglobulin (M_r 669 000), suggesting that these are large complexes. Because of limitations of the methods of analysis (i.e., the possibile metabolism of components and shedding of subunits) we do not emphasize the absolute value of either the molecular weight or Stokes radius of nontransformed receptors. We only wish to point out that either in the presence or in the absence of Na_2MoO_4 the antibody binds to the larger receptor complexes, implying the presence of the M_r 59 000 protein.

Although the data discussed above suggested that the epitopes were common to the nontransformed receptors, it was not clear that the M_r 59 000 protein was a component of each complex. This was partially resolved by the SDS–PAGE analyses of material immunoprecipitated by KN 382/EC1 coupled Affi-Gel 10. For most of the tissues we tested, M_r 59 000 and 92 000 proteins were evident (Figure 4). Detection of the M_r 59 000 protein by electrophoretic means, taken in concert with the sucrose gradient studies and gel permeation (HPLC) analyses, suggests not only that the M_r 59 000 protein is common to receptors but also that it is a ubiquitous cellular protein.

Although the M_r 59 000 and 92 000 proteins are distinct from each other, the two appear to be found together in the

tissues we examined. This may be a reflection of the association of the two as shown by their cofractionation by KN 382/EC1 coupled with Affi-Gel 10. We surmise that the M_r 92 000 protein is bound to the M_r 59 000 protein because the former can be dissociated by reduction in pH (Tai & Faber, 1985).

In summary, there are four major points of this study. First, the M_r 59 000 protein is common to several nontransformed receptors. Second, the M_r 59 000 protein appears to be ubiquitous. Third, the M_r 59 000 protein is in close association with another ubiquitous M_r 92 000 protein. Fourth, the M_r 59 000 and 92 000 proteins are separate and distinct proteins.

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REFERENCES

Baulieu, E.-E., Binart, N., Buchou, T., Catelli, M. G., Garcia, T., Gasc, J. M., Groyer, A., Joab, I., Moncharmont, B., Radanyi, C., Renoir, M., Tuohimaa, P., & Mester, J. (1983) in *Steroid Hormone Receptors: Structure and Function* (Eriksson, H., & Gustafsson, J. A., Eds.) pp 45–72, Elsevier, Amsterdam.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cleveland, D. W., Fisher, S. G., Kirshner, M., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Dougherty, J. J., Puri, R. K., & Toft, D. O. (1984) *J. Biol. Chem. 259*, 8004–8009.

Faber, L. E. (1980) in *Steroid Induced Uterine Proteins* (Beato, M., Ed.) pp 197–216, Elsevier/North-Holland, Amsterdam.

Faber, L. E., Tai, P. K.-K., Maeda, Y., Myers, J. E., & Nakao, K. (1985) *Molecular Mechanism of Steroid Hormone Action* (Moudgil, V. K., Ed.) pp 61–84, de Gruyter, Berlin.

Gregory, M. R., & Notides, A. C. (1982) *Biochemistry 21*, 6452–6458.

Hubbard, J. R., & Kalimi, M. (1985) *Mol. Cell. Biochem. 66*, 101–109.

Jensen, E. V., & DeSombre, E. R. (1972) *Annu. Rev. Biochem. 41*, 203–230.

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

Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.

Murayama, A. (1985) in *Molecular Mechanism of Steroid Hormone Action* (Moudgil, V. K., Ed.) pp 1–30, de Gruyter, Berlin.

Nakao, K., Myers, J. E., & Faber, L. E. (1985) *Can. J. Biochem. Cell Biol. 63*, 33–40.

Puri, R. K., Grandics, P., Dougherty, J. J., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 10831–10837.

Radanyi, C., Joab, I., Renoir, J. M., Richard-Foy, H., & Baulieu, E.-E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2854–2858.

Reker, C. E., Kovacic-Milivojevic, B., Eastman-Reks, S. B., & Vedeckis, W. V. (1985) *Biochemistry 24*, 196–204.

Renoir, J. M., & Mester, J. (1984) *Mol. Cell. Endocrinol. 37*, 1–13.

Renoir, J. M., Buchou, T., Mester, J., Radanyi, C., & Baulieu, E.-E. (1984) *Biochemistry 23*, 6016–6023.

Riehl, R. M., Sullivan, W. P., Vroman, B. T., Bauer, V. J., Pearson, G. R., & Toft, D. O. (1985) *Biochemistry 24*, 6589–6591.

Saffran, J., Loeser, B. K., Bohnett, S. A., & Faber, L. E. (1976) *J. Biol. Chem. 251*, 5607–5613.

Sanchez, E. R., Toft, D. O., Schlesinger, M. J., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 12398–12401.

Schuh, S., Yonemoto, W., Brugge, J., Bauer, V. J., Riehl, R. M., Sullivan, W. P., & Toft, D. O. (1985) *J. Biol. Chem. 260*, 14292–14296.

Sherman, M. R., Moran, M. C., Tuazon, F. B., & Stevens, Y. W. (1983) *J. Biol. Chem. 258*, 10366–10377.

Stancel, G. M., Leung, K. M. T., & Gorski, J. (1973) *Biochemistry 12*, 2137–2141.

Sullivan, W. P., Vroman, B. T., Bauer, V. J., Puri, R. K., Riehl, R. M., Pearson, G. R., & Toft, D. O. (1985) *Biochemistry 24*, 4214–4222.

Tai, P. K.-K., & Faber, L. E. (1985) *Can. J. Biochem. Cell Biol. 63*, 41–49.

Vedeckis, W. V. (1983) *Biochemistry 22*, 1983–1989.

von der Ahe, D., Janich, S., Scheidereit, C., Renkawitz, R., Schutz, G., & Beato, M. (1985) *Nature (London) 313*, 706–709.