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Evidence for a Single Steroid-Binding Protein in the Rabbit Progesterone Receptor†

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ABSTRACT: The rabbit uterine progesterone receptor copurifies as two molecular weight (M_r) forms of about 105 000 and 78 000. To investigate whether these are different proteins, we have used protease digestion, reversible denaturation, and photoaffinity labeling in studies on the steroid-binding domain of the receptor. Digestion of the M_r 105 000 and 78 000 forms, photoaffinity labeled with [^{3}H]R5020, with *Staphylococcus aureus* V8 protease revealed identical peptide fragments of M_r 43 000, 39 000, and 27 000–30 000. When receptor in cytosol was denatured, separated by electrophoresis, and then reconstituted, [^{3}H]progesterone bound specifically to a single form at about M_r 105 000. After partial purification, the reversible denaturation procedure revealed both the larger and the smaller progesterone-binding species similar to the photoaffinity-labeled species in this preparation. Receptor in uterine cytosol prepared under mild conditions appeared as a predominant large molecular weight form on photoaffinity labeling with [17α-*methyl*-^{3}H]R5020, [6,7-^{3}H]R5020, or [^{3}H]RU27987. Further purification of this cytosol showed the generation of a smaller labeled species. These results from three different approaches reinforce the view that the rabbit progesterone receptor contains a single steroid-binding protein.

Most steroid receptors consist of a single hormone-binding subunit, which may be present in a homo- or heterodimer or tetramer (Greene et al., 1979; Wilson & French, 1979; Katzenellenbogen et al., 1983; Vedeckis, 1983; Chang et al., 1984; Miesfeld et al., 1984; Monsma et al., 1984; Rowley et al., 1984; Sakai & Gorski, 1984; Sherman & Stevens, 1984; Wrange et al., 1984). The subunit structure of the progesterone receptor, however, is less clear. For the chicken oviduct progesterone receptor, two dissimilar hormone-binding subunits have been identified with molecular weights of 79 000 and 108 000; these subunits exhibit differential binding to DNA and chromatin (Grody et al., 1982). Other workers have

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identified a single chick progesterone receptor subunit of M_r 84 000–87 000 (Miller et al., 1975; Murayama et al., 1980; Renoir et al., 1982) or 110 000 (Renoir et al., 1984). In mammals, the progesterone receptor from T47D cells, a human breast cancer cell line, has been proposed as formed of dissimilar subunits, which resemble those of the chicken progesterone receptor (Lessey et al., 1983; Horwitz & Alexander, 1983). Molecular weights of 40 000–50 000 (Smith et al., 1981; Holmes & Smith, 1983) and 110 000 (Smith et al., 1975) or 108 000 (Manz et al., 1982) have been reported for the human uterine progesterone receptor. Using photoaffinity labeling, heterogeneous forms of the rabbit uterine progesterone receptor with observed molecular weights of 95 000 and 80 000 have been reported by Westphal et al. (1981) and of 100 000, 80 000, and 65 000 by Jänne (1982).

We reported earlier the presence of two molecular weight forms of rabbit uterine progesterone receptor of M_r 102 000 and 78 000, which could not be distinguished on the basis of their ionic properties, their ligand specificity, or their DNA binding (Lamb & Bullock, 1984). At that time we stated that we did not know whether these two forms were different proteins or whether one was derived from the other. Since then, using immunological approaches, Loosfelt et al. (1984) and Logeat et al. (1985) have provided strong evidence that the rabbit receptor contains a single steroid-binding subunit of M_r 110 000. In this paper, we report the use of biochemical techniques that support that view and indicate that the smaller molecular weight form is derived from the larger.

Experimental Procedures

Materials. [1,2-^{3}H]Progesterone (57.5 Ci/mol), [17α-*methyl*-^{3}H]promegesterone (R5020, 89 Ci/mmol), and Aquasol were purchased from New England Nuclear (Boston, MA); DE-52 cellulose was obtained from Whatman, Inc. (Clifton, NJ); charcoal, Bio-Rad protein reagent, Coomassie Brilliant blue R250, acrylamide, BIS, ammonium persulfate, and TEMED were from Bio-Rad Laboratories (Richmond, CA); DNA–cellulose and phosphocellulose were gifts from Dr. W. T. Schrader (Baylor College of Medicine, Houston, TX). The DNA–cellulose was prepared as described by Alberts and Herrick (1971) and Coty et al. (1979). [6,7-^{3}H]Promegesterone (R5020) and [6,7-^{3}H]RU27987 [21(*S*)-hydroxypromegesterone] were generous gifts from Dr. J. P. Raynaud (Roussel-Uclaf) and Drs. P. Bremer and I. A. Eckert (Hoechst).

Preparation of Cytosol. Adult virgin female New Zealand white rabbits were injected subcutaneously for 3–5 days with 50 μg of estradiol benzoate in sesame oil. The animals were anesthetized with sodium pentobarbitone, the uterine vasculature was extensively perfused with saline, and the uteri were excised. The uteri were homogenized in 4 volumes of TGETMo [10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 15 mM monothioglycerol, 10% (v/v) glycerol, 10 mM sodium molybdate] and a 105000*g* cytosol was prepared, as described previously (Lamb & Bullock, 1984).

Cytosol was immediately chromatographed on an 8 × 2 cm phosphocellulose column (Lamb & Bullock, 1984), and the unbound fraction was used for experiments. Receptor was labeled with 10 nM of the indicated ^{3}H steroid for 1 h at 4 °C in the presence of 10 μM cortisol. Free steroid was removed by incubation for 10 min with the pellet from 1.33 volumes of charcoal suspension (0.05% dextran, 0.5% charcoal). The starting cytosol contained approximately 15 pmol/mL [^{3}H]progesterone-binding activity.

Column Chromatography of Progesterone Receptor. Cytosol (30–75 mL) was applied to a DE-52 column (12 × 4 cm) in TGETMo. The protein was washed through the column until the absorbance (A_{280}) of the eluate reached base line. The column was step-eluted with 60 mL of 0.1 M NaCl in TGETMo and then with 60 mL of 0.3 M NaCl in TGET. Receptor in the second eluate was allowed to activate overnight in the salt-containing elution buffer.

The DE-52 0.3 M NaCl eluate was diluted to 100 mM NaCl before application onto a 2-mL DNA–cellulose column. The receptor was washed through in TGET, and the column was eluted with a gradient of 0–0.5 M NaCl in TGET. Fractions of 1.5 mL were collected, and 25-μL portions were removed for counting radioactivity.

Photoaffinity Labeling. Cytosol, after passage over a phosphocellulose column, was labeled for 1 h at 4 °C with 10 nM [17α-*methyl*-^{3}H]R5020, [6,7-^{3}H]R5020, or [6,7-^{3}H]-RU27987 in the presence of 10 μM cortisol. After removal of the unbound ligand by incubation with charcoal as described above, portions of the eluates from each column were irradiated under UV light at 320 nm for 15 min (Dure et al., 1980).

Gel Electrophoresis. Electrophoresis on 7.5% or 10% acrylamide slab gels was carried out as described by Porzio and Pearson (1977). The molecular weight standards were myosin (200 000), β-galactosidase (116 000), phosphorylase B (97 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (34 000), and soybean trypsin inhibitor (21 000). The gels were fixed and stained overnight in 10% glacial acetic acid, 25% 2-propanol, and 0.03% Coomassie blue R250 and were destained with repeated changes of 20% methanol.

To detect photoaffinity-labeled receptor, gel lanes were cut, sliced (2 mm each), and solubilized in 500 μL of NCS–H_2O (9:1) overnight before counting in 5 mL of Aquasol or were enhanced, dried, and exposed to X-ray film at −70 °C. For protease digestion by the method of Cleveland et al. (1977), slices from an adjacent lane corresponding to the peak fractions of [^{3}H]R5020–receptor complex were excised, placed in the gel lanes of a second acrylamide slab gel, and then covered with 60 ng of *S. aureus* V-8 protease in 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA, and 10% glycerol. Electrophoresis proceeded until the bromophenol blue tracking dye approached the bottom of the stacking gel. Enzymatic digestion was allowed to continue in the stacking gel for 30 min, and then current was reapplied to allow migration of the peptide fragments through the separating gel. The gel was enhanced, dried, and exposed to Kodak XAR X-ray film for 6 weeks at −70 °C.

Reversible Denaturation. This procedure was carried out as described by Sakai and Gorski (1984). The receptor preparations were precipitated with 10 volumes of 100% ethanol at −20 °C. The precipitate was dissolved in sample buffer [0.01 M Tris-HCl, pH 6.8 (4 °C), 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol; Laemmli, 1970], and electrophoresis on 3-mm-thick, 10% Porzio and Pearson gels was performed. The gels were frozen at −70 °C and the sample lanes cut into 2- or 4-mm slices. Each slice was homogenized, on a motor-driven Teflon pestle, in 2.5 mL of elution buffer [0.1% SDS, 0.05 M Tris-HCl, pH 7.9 (25 °C), 0.15 M NaCl, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.1 mg/mL bovine serum albumin (fraction V)]. After elution for 1 h or overnight at room temperature, the acrylamide was removed by centrifugation at 800*g* for 5 min. The supernatant was divided into two 1-mL portions, and protein was precipitated with 100% ethanol at −20 °C overnight. The pellet was washed with 80% ethanol in dilution buffer [0.05 M Tris-HCl, pH 7.9 (25 °C), 0.15 M NaCl, 20% glycerol (v/v), 1 mM dithiothreitol, 0.1

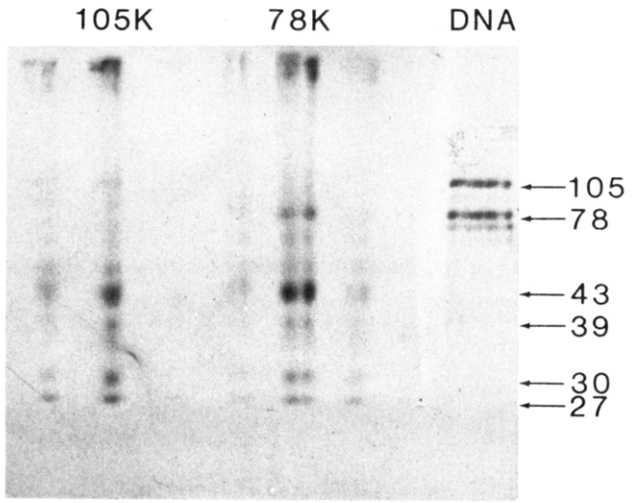

FIGURE 1: Autoradiographic pattern of photoaffinity-labeled receptor fragments after protease digestion. [^{3}H]R5020-labeled receptor was partially purified by DE-52 cellulose and DNA–cellulose chromatography prior to photoaffinity labeling. The receptor eluted from DNA–cellulose was analyzed by 10% polyacrylamide gel electrophoresis, and the M_r 105 000 and 78 000 bands were excised and placed in separate wells of the stacking gel of a 12% polyacrylamide gel. After digestion with 60 ng of *S. aureus* V-8 protease, the photoaffinity-labeled peptide fragments were separated electrophoretically and detected by autoradiography. The starting material is in the lane labeled DNA; lanes on either side of the 105 000 and 78 000 lanes are digests of adjacent slices above and below the bands of interest. The M_r ($\times 10^{-3}$) of the bands are shown at the right.

M EDTA] and dissolved in 50 μL of 6 M guanidine hydrochloride by frequent vortexing for 30 min at room temperature. The samples were then diluted with 1 mL of dilution buffer containing 2 nM [^{3}H]progesterone and 1 μM cortisol, with or without 100-fold excess progesterone to estimate nonspecific binding. Bound radioactivity was determined with hydroxyapatite (Sakai & Gorski, 1984).

RESULTS

Cleveland Digestion of Photoaffinity-Labeled Receptor. Figure 1 shows the autoradiographic pattern of photoaffinity-labeled fragments from the larger and smaller forms after excision from a polyacrylamide gel, digestion with *S. aureus* V-8 protease, and subsequent SDS–polyacrylamide gel electrophoresis. The starting material contains the larger and smaller forms of the receptor, with a small amount of a lower molecular weight species. In the digested lanes, faint bands at 105 000 or 78 000 represent residual undigested starting protein. Similar digestion products are present at M_r 43 000, 39 000, 30 000, and 27 000 for both the 105 000 and 78 000 forms. There is no reason a priori why V-8 protease should generate the 78 000 from the 105 000 form. Thus, the two forms of the progesterone receptor are either identical or structurally similar proteins with respect to their steroid-binding domain.

Reversible Denaturation of the Progesterone Receptor. We used the reversible denaturation procedure described by Sakai and Gorski (1984) to study the receptor forms under conditions where proteolysis should be minimized. Cytosol was prepared, and a portion was immediately denatured by the addition of Laemmli sample buffer. Following SDS–polyacrylamide gel electrophoresis, elution, and renaturation, specific [^{3}H]-progesterone binding was found predominantly in the larger molecular weight form. Figure 2 shows the results of two experiments. In the first (Figure 2A), 10% of the binding activity did not enter the gel and 10% migrated near the dye front. A single peak of binding activity (31%) renatured at

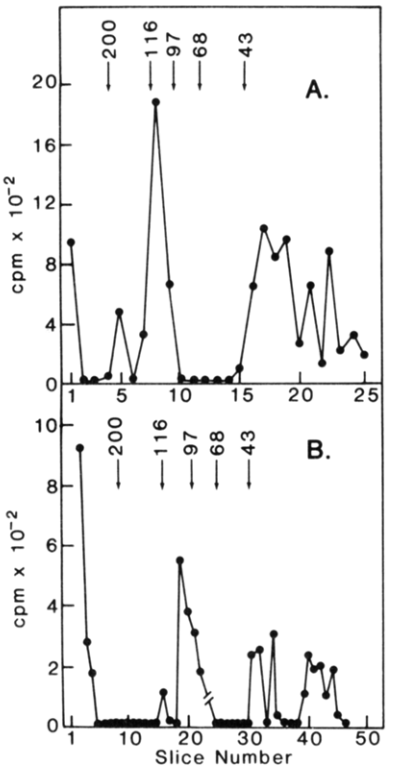

FIGURE 2: Reversible denaturation of progesterone receptor in cytosol. Cytosol was prepared and denatured immediately by ethanol precipitation and the addition of SDS–sample buffer. Following SDS–polyacrylamide gel electrophoresis, the gel was sliced and each slice was subjected to elution and renaturation. The fractions were incubated with 2 nM [^{3}H]progesterone and 1 μM cortisol ± 100-fold excess progesterone. Steroid binding was assessed by the hydroxyapatite assay. The migration of molecular weight standards ($\times 10^{-3}$) is shown at the top. Two experiments are shown, with one gel cut into 4-mm slices (A) and one into 2-mm slices (B).

a position consistent with the larger of the two molecular weight forms, and 38% of the binding activity renatured as smaller peptides between M_r 30 000 and 38 000. In the second experiment (Figure 2B), similar results were obtained. Nineteen percent of the binding activity was found at the top of the gel, and 22% renatured near the dye front. Again, a single peak accounting for 31% of the renatured binding activity migrated to a position consistent with the larger molecular weight form of the receptor, and small peaks were detected in the region of M_r 30 00–40 000. In neither case was there evidence of renaturation of the M_r 78 000 form.

In the second experiment, receptor in the remaining, undenaturated cytosol was partially purified by ion-exchange and DNA–cellulose chromatography. After DNA–cellulose, a portion of the receptor was photoaffinity labeled with [^{3}H]-R5020, and the rest was subjected to reversible denaturation. Figure 3A shows that both the larger and smaller molecular weight forms were renaturated in the partially purified preparation, with smaller peaks at low molecular weight again present. The photoaffinity labeled receptor in this preparation was analyzed on the same gel and revealed both the larger and smaller molecular weight forms (Figure 3B) in positions similar to those of the two dominant renatured species. The lower molecular weight fragments, however, were not detected by photoaffinity labeling.

Photoaffinity Labeling of the Receptor. At each step of the partial purification procedure (cytosol after phosphocellulose, DE-52 cellulose, and DNA–cellulose), portions of the preparation were removed for photoaffinity labeling with three different ligands: [17α-*methyl*-^{3}H]R5020, [6,7-^{3}H]-

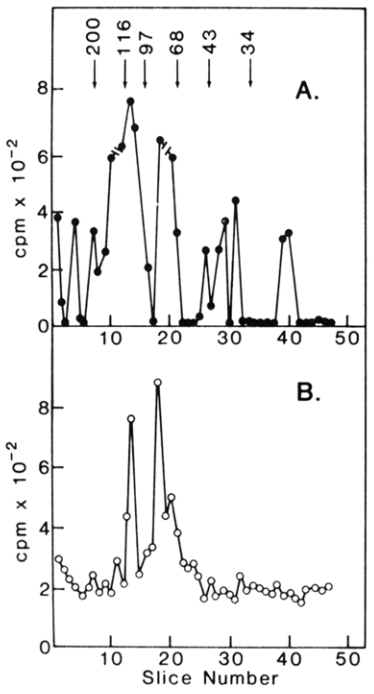

FIGURE 3: Reversible denaturation of the progesterone receptor after partial purification by chromatography on phosphocellulose, DE-52 cellulose, and DNA–cellulose, as described under Experimental Procedures. A portion of the DNA eluate was photoaffinity labeled with [^{3}H]R5020 and analyzed along with the rest of the eluate on a SDS–polyacrylamide gel. Nonlabeled receptor lanes were sliced and carried through the reversible denaturation procedure, while lanes with covalently labeled receptor were sliced and the radioactivity was counted. The specific [^{3}H]progesterone binding after reversible denaturation is shown in panel A, and the migration of covalently labeled receptor is shown in panel B. The migration of molecular weight standards ($\times 10^{-3}$) is shown at the top of the figure.

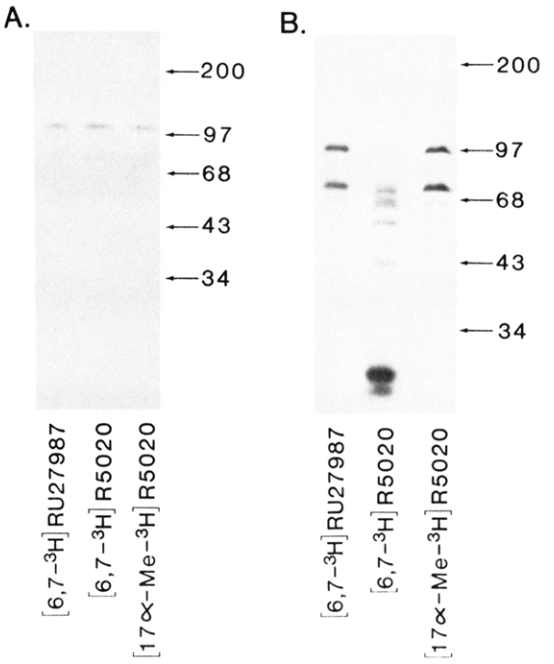

FIGURE 4: Autoradiographic patterns of photoaffinity-labeled receptor after DE-52 cellulose and DNA–cellulose chromatography. Cytosol was passed through a phosphocellulose column and labeled with [17α-*methyl*-^{3}H]R5020, [6,7-^{3}H]R5020, or [6,7-^{3}H]RU27987. After DE-52 cellulose chromatography, part of each 0.3 M NaCl eluate was photoaffinity labeled and analyzed on a 10% SDS gel (A). The remainder of each fraction was applied to a DNA–cellulose column. In each case, all of the receptor bound to the column and was eluted with a NaCl gradient. The receptor eluted from the DNA–cellulose columns was photoaffinity labeled and analyzed on a 10% SDS–polyacrylamide gel (B). Photoaffinity-labeled receptor was detected by autoradiography. The molecular weight ($\times 10^{-3}$) of standard proteins is shown at the right.

R5020, or [6,7-^{3}H]RU27987. The cytosol and DE-52 fractions were analyzed on one gel for fluorography, and the DNA–cellulose fractions were analyzed in duplicate on a second gel, half of which was exposed to X-ray film, while the other half was sliced for counting radioactivity. There was insufficient radioactivity in the cytosol samples to obtain a signal on film after 3 weeks of exposure. The results from the DE-52 fraction, however, are shown in Figure 4A, where the dominant signal is from the larger of the two M_r forms, with little of the smaller form being present. After DNA–cellulose chromatography, when all these preparations bound quantitatively and eluted as single peaks, breakdown of the larger form to the smaller form had occurred (Figure 4B). There was little difference in intensity of labeling between the three ligands, but pronounced degradation of the receptor occurred with [6,7-^{3}H]R5020 compared to the other two steroids. There is no immediate explanation for this discrepancy, which we attribute to an unknown experimental error in handling this sample. In another experiment, this steroid labeled only the two dominant molecular weight forms (Figure 5).

Quantitation of the photoaffinity labeled receptor in the two molecular weight forms, by slicing the gel shown in Figure 4B, solubilizing, and counting, revealed that both forms were now present in approximately equimolar concentrations when labeled with [17α-*methyl*-^{3}H]R5020 or [6,7-^{3}H]RU27987 (Figure 6). A similar result was obtained in a separate experiment where [17α-*methyl*-^{3}H]R5020 was used as the photoaffinity label (Figure 7). At the DE-52 cellulose step, 75% of the receptor was present as the larger molecular weight form (Figure 7A). After further purification, the receptor form shifted so that 49% was represented as the smaller molecular weight species after elution from DNA–cellulose (Figure 7B).

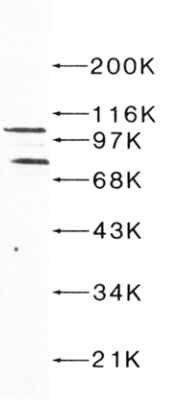

FIGURE 5: Photoaffinity labeling with [6,7-^{3}H]R5020 of progesterone receptor after elution from DNA–cellulose. The conditions are as in Figure 4B. Molecular weight standards are shown at the right.

Discussion

Conflicting reports exist as to the structure of the mammalian progesterone receptor. In the human uterus, Smith's group reported receptor forms of M_r 110 000 (Smith et al., 1975), 45 000 (Holmes & Smith, 1983), and 40 000 (Smith et al., 1981). For the progesterone receptor from rabbit uterus, our studies, using hydrophobic and affinity chromatography, identified a single form of M_r 72 000. This form corresponded with the major affinity-labeled protein in cytosol, although small peaks of label were also observed at higher molecular weights (Lamb et al., 1982). We showed subsequently that

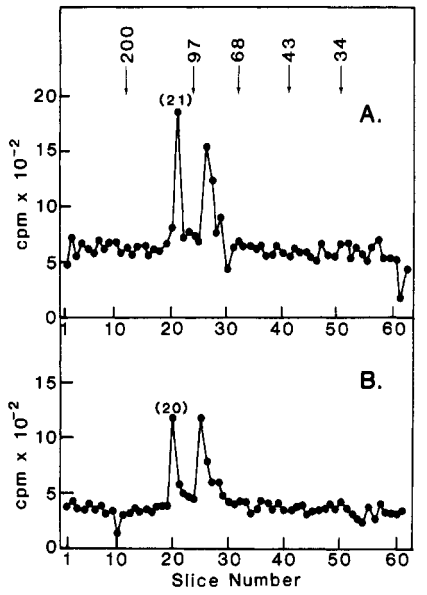

FIGURE 6: Quantitation of photoaffinity labeling of progesterone receptor with [17α-*methyl*-^{3}H]R5020 (A) and [6,7-^{3}H]RU27987 (B). Duplicate lanes from the same gel as in Figure 4B were cut into 2-mm slices and solubilized, and the radioactivity was counted. The migration of molecular weight standards is shown at the the top of the figure.

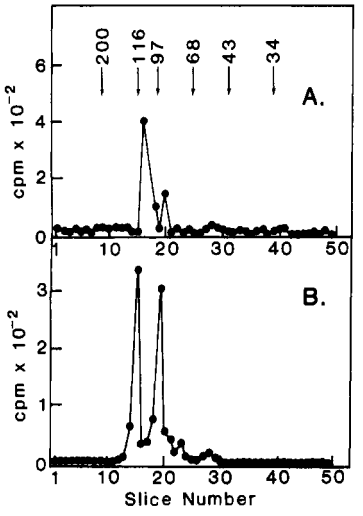

FIGURE 7: Photoaffinity labeling with [17α-*methyl*-^{3}H]R5020 of progesterone receptor after elution from DE-52 cellulose (A) followed by DNA–cellulose (B) in the same preparation. The receptor was analyzed on a SDS–polyacrylamide gel, which was sliced, solubilized, and counted for radioactivity. Migration of molecular weight standards is shown at the top of the figure.

after passage over a phosphocellulose column to diminish endogenous protease activity, the receptor migrated at molecular weights of 102 000 and 78 000 (Lamb & Bullock, 1984).

Horwitz and colleagues (Lessey et al., 1983; Horwitz & Alexander, 1983) found two subunits of human breast cancer (T47D) progesterone receptor of 81 800 and 114 800 and suggested that these two forms exhibited differential DNA-binding characteristics, although photoaffinity labeling of the DNA-bound receptor was not performed. Using an in situ photoaffinity labeling technique, these workers showed that both molecular weight forms were present in the cytosol and both could be translocated to the nucleus, by progestin treatment in vivo, in equimolar concentration, and without a change in molecular weight. Thus, studies on the human and rabbit receptors suggested that, like the progesterone receptor from chicken oviduct, the mammalian progesterone receptor consisted of two hormone-binding subunits of different molecular weights.

To ask whether these two forms of rabbit progesterone receptor were different proteins or whether the 78 000 form was derived from the larger form, we used protease digestion (Cleveland et al., 1977) to study the proteolytic fragments of these two proteins. Since we could not obtain pure M_r 102 000 and 78 000 receptor, we were not able to follow the digestion by examining silver-stained peptide fragments. We used photoaffinity-labeled receptor, therefore, and observed identical digestion patterns for both molecular weight forms. Although this result does not necessarily mean that these proteins are the same, it indicates that they possess similar hormone-binding domains.

The results of the reversible denaturation studies and the photoaffinity labeling experiments prove that the smaller receptor is derived from the larger protein. In the reversible denaturation studies, where proteolysis was limited by immediate denaturation, the cytosol preparation contained only the larger of the two molecular weight forms. After several purification steps the procedure reconstituted both the larger and the smaller species of the progesterone receptor. Recovery with this technique is low and variable (Sakai & Gorski, 1984), yet the low recovery (10–20%) did not preclude detection of more than one form of the receptor. Smaller degradation products, however, were still detected, both in cytosol and in partially purified preparations. Note that all labeling was done in comparison to an excess of progesterone. These degradation products were not detected by photoaffinity labeling (Figure 3B) due to the lower sensitivity of this technique, which covalently links less than 5% of bound steroid, compared to [^{3}H]progesterone binding. Broadening of the major reconstituted receptor peaks in Figures 2 and 3 is due partly to pooling gel slices and partly to heavy protein loading impairing resolution. It is thus difficult to estimate molecular weights accurately. The data serve to indicate that in cytosol the procedure reveals only one of the two dominant forms, whereas after several purification steps both molecular weight forms are present.

Similarly, the photoaffinity-labeling studies demonstrated unequivocally that the 78 000 protein was derived from the larger receptor. Since all the [^{3}H]progesterone–receptor complex from the DE-52 eluate (Figure 4A, one molecular weight form) bound to the DNA–cellulose and eluted as two molecular weight forms (Figure 4B), it cannot be argued that the 78 000 form bound preferentially to DNA, making it appear as though the 78 000 protein was only detected after DNA–cellulose chromatography. The occurrence of the two molecular weight forms in roughly equal amounts, here and earlier (Lamb & Bullock, 1984), is striking. The proteolytic activity appears to copurify with the receptor and may even be a component of the receptor complex that could be involved in biological activity. We compared the efficiency of photoaffinity labeling with [17α-*methyl*-^{3}H]R5020 (on average, about 2.5%) to that of two other steroids, [6,7-^{3}H]R5020 and [6,7-^{3}H]RU27987, but found little difference (Figure 4). The anomalous result with [6,7-^{3}H]R5020 (Figure 4B) is puzzling. We think it is unlikely that the same fragments were present in all three samples and were detected by only one steroid (cf. Figure 5). This result further illustrates the extreme lability of the rabbit progesterone receptor (Lamb et al., 1982; Lamb & Bullock, 1984).

The work of Loosfelt et al. (1984) provided strong evidence for a single form of the rabbit progesterone receptor, based on recognition by several monoclonal antibodies. Electro-

phoretic transfer of rabbit uterine cytosol and immunoblotting with these monoclonal antibodies showed, when proteolysis was minimized, that only one receptor protein at M_r 110000 was identified. The difference in our estimate of the molecular weight may be due to differences in conditions of electrophoresis. In a recent publication, this group (Logeat et al., 1985) also reported copurification, by immunoaffinity chromatography, of two forms at M_r 110000 and 79000. Using peptide analysis of ^{125}I-labeled purified receptor, these authors reached the conclusion that the smaller form is derived from the larger by proteolysis.

The results presented in this paper, based on photoaffinity labeling, protease digestion, and reversible denaturation, confirm this conclusion and support the view that the rabbit progesterone receptor contains a single steroid-binding protein. Several discrepancies, however, remain to be reconciled in the structures of the progesterone receptors from rabbits, humans, and chickens. These questions are unlikely to be resolved until the genes coding for these proteins are cloned.

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