Involvement of a Non-Hormone-Binding 90-Kilodalton Protein in the Nontransformed 8S Form of the Rabbit Uterus Progesterone Receptor†

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ABSTRACT: Nontransformed 8S progesterone receptor (8S-PR) was purified by hormone-specific affinity chromatography from rabbit uterine low-salt cytosol containing 20 mM molybdate. In the eluate obtained with radioactive progestin, sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) showed the presence of several bands, including three that corresponded to ~90-, ~120-, and ~85-kDa proteins. None of these three proteins was found in the eluate of the affinity column when the molybdate-containing cytosol was chromatographed in the presence of nonradioactive progesterone ("mock purification"). Subsequent purification of the affinity eluate by DEAE-Sephacel chromatography gave a single radioactive receptor peak at 0.15 M KCl (~20% yield, 19% purity on the basis of one binding site per ~100 kDa) with a sedimentation coefficient of 8.5 S. Silver staining after SDS–PAGE revealed that this purified 8S-PR fraction contained mainly the 120-, 90-, and 85-kDa proteins. [^{3}H]R5020-labeled 8S-PR purified by DEAE-Sephacel column chromatography was UV irradiated, and after SDS–PAGE the 120- and 85-kDa proteins were revealed, but the 90-kDa protein was not. Further evidence for the presence of the 90-kDa non-hormone-binding protein in the purified molybdate-stabilized nontransformed 8S-PR structure was demonstrated by (1) the presence of the three proteins in the 8.5S radioactive peak in a density gradient loaded with purified [^{3}H]R5020-labeled PR, as in the 7.7-nm radioactive peak of this form analyzed by HPLC, (2) the shift of sedimentation coefficient of the purified 8S-PR in density gradient analysis, occurring after incubation with a specific antibody against a non-hormone-binding, calf uterine 90-kDa protein, and (3) the presence in this shifted peak of both 90-kDa protein and 120- and 85-kDa receptor proteins. In the course of this work, it was verified that 0.3 M KCl added in cytosol in the absence of molybdate dissociated the 8S-PR complex, and purified 120- and 85-kDa progestin binding proteins were obtained by hormone-specific affinity chromatography of the salt-treated cytosol. In addition, despite the stabilization effect of molybdate ions, partial dissociation occurred during chromatography, and "4S-PR" was found in the flow-through of the DEAE-Sephacel column; after further purification by DNA–cellulose, it yielded the 120- and 85-kDa progestin binding units with 60% purity. In summary, the results demonstrated that, as for the nontransformed avian 8S-PR [Renoir, J. M., Buchou, T., Mester, J., Radanyi, C., & Baulieu, E. E. (1984) *Biochemistry 23*, 6016–6023], the nontransformed 8S form of the rabbit uterus PR includes a non-hormone-binding 90-kDa protein.

The nontransformed molybdate-stabilized 8S form of the chick oviduct progesterone receptor (8S-cOvPR) has been recently obtained ~30% pure (on the basis of one binding site of hormone per 100 kDa) in two different laboratories (Puri et al., 1982; Renoir et al., 1982a, 1984a; Dougherty et al., 1984) by the use of affinity and ion-exchange chromatographies. Two molecules of the 90-kDa[1] protein (Dougherty et al., 1984; Renoir et al., 1984a), which does not bind the hormone (Renoir et al., 1984a,b; Joab et al., 1984), are, in the 8S-PR, associated either with one A (~80 kDa) or one B (~110 kDa) progestin binding subunit previously described by Schrader et al. (1981). It has been suggested that the 90-kDa protein is a contaminant of purification (Birnbaumer et al., 1984) and/or that its association with the A or B subunits may be due to the use of molybdate. In fact, purification in the presence of molybdate and radioinert progesterone containing cytosol showed that neither the hormone binding subunits nor the 90-kDa protein were retained by the affinity gel (Renoir et al., 1984a), excluding a purification artifact. Immunological studies with the monoclonal anti-90-kDa protein antibody BF_4 (Radanyi et al., 1983) indicated that molybdate does not provoke the formation of the 8S structure but that it only stabilizes it (Joab et al., 1984). The non-hormone-binding 90-kDa subunit has been detected in the nontransformed forms of all chick oviduct steroid hormone receptors tested so far (Joab et al., 1984) and identified as heat-shock protein hsp 90 (Catelli et al., 1985). It has been known for a long time that all mammalian steroid receptors can be obtained in their nontransformed "8S form" (Gorski et al., 1968; Baulieu et al., 1971). In order to examine if these nontransformed 8S-PR of mammals also contained in their structure a non-hormone-binding component, we have purified the molybdate-stabilized (8S) and the transformed (4S) forms from the cytosoluble fraction of the rabbit uterus by successive affinity and ion-exchange chromatographies. After analysis by silver staining of SDS–PAGE, and by using the synthetic progestin [^{3}H]R5020 to label the hormone binding macromolecule(s) covalently after UV irradiation, we have identified, in the structure of the nontransformed 8S-PR form of the rabbit uterus (8S-rUtPR), a 90-kDa non-hormone-binding

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[1] Abbreviations: PR, progesterone receptor; GR, glucocorticosteroid receptor; rUtPR, rabbit uterus PR; cOvPR, chick oviduct PR; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NA-DAc–Sepharose, *N*-(12-aminododecyl)-3-oxo-4-androstene-17β-carboxamide–Sepharose CL-4B, 2–3 μmol ligand^{-1} (mL of gel)$^{-1}$ (Renoir et al., 1982); E, estradiol; TCA, trichloroacetic acid; kDa, kilodalton; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; IgG, immunoglobulin G.

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protein associated with the progestin binding units.

Materials and Methods

Chemicals. [2,4,6,7-^{3}H]Progesterone ([^{3}H]P, sp act. 96–110 Ci/mmol) was from the Radiochemical Centre (Amersham, Bucks, UK), and the synthetic progestin 17,21-[17-*methyl*-^{3}H]dimethylpregna-4,9(10)-diene-3,20-dione ([^{3}H]R5020, sp act. ~50 Ci/mmol) was a gift from Roussel-Uclaf (Romainville, France). Nonradioactive cortisol and progesterone (>95% pure) were obtained from Roussel-Uclaf. DEAE-Sephacel was from Pharmacia (Uppsala, Sweden) and DNA cellulose from P-L Biochemicals, Inc. (Milwaukee, WI). All other reactants were from Merck (Darmstadt, West Germany).

Buffers. Buffer A included 10 mM potassium phosphate, 1.5 mM EDTA, 10% (v/v) glycerol, and 12 mM 1-thioglycerol, pH 7.4, at 25 °C. Na_2MoO_4 (20 mM) was added to buffer A to make buffer B.

Rabbit Uterus Cytosol. Immature New Zealand rabbits injected for 10 days with 10 μg of diethylstilbestrol (DES)/day were used. Uteri were excised and placed immediately at 0 °C, minced, and homogenized in buffer B, as described in Renoir et al. (1984a,b) for chicken oviducts, in order to purify the nontransformed PR form. For purification of transformed PR, cytosol was prepared in buffer A. The following protease inhibitors were added immediately after homogenization of the uteri: (a) PMSF, phenylmethanesulfonyl fluoride (Calbiochem; 0.3 mM final concentration), (b) leupeptin (Sigma; 20 μM), (c) aprotinin from bovine lung (Sigma; 0.5 TIU/mL), (d) pepstatin A (Sigma; 0.25 μg/mL), (e) bacitracin (Sigma; 10 μg/mL), and (f) benzamidine (Sigma; 25 mM). Nonradioactive cortisol (1 μM) was added to all cytosol in order to prevent affinity column binding to either transcortin or glucocorticosteroid receptor.

Affinity Chromatography. About 30 uteri were routinely used for one purification. Cytosol (150–200 mL) was loaded overnight at 0–4 °C on two columns of 25–30 mL of packed NADAc affinity gel (Renoir et al., 1982a), at a flow rate of 5 mL/h. The gel was then washed (flow rate ~300 mL/h) successively with buffer B containing 0.3 M KCl (3 column volumes), buffer B alone (10 column volumes), buffer B containing 2.25 M urea (3 column volumes), and buffer B alone (10 column volumes). The washing step of the affinity columns did not last more than 2.5 h.

For purification of the transformed PR, the cytosol prepared in buffer A was incubated for >2 h at 4 °C in presence of 0.3 M KCl before loading on the affinity gel. The washing procedure was the same as described above, but buffer A was used instead of buffer B.

Specific elution of the receptor was carried out in 1 column volume of the appropriate buffer, by overnight exchange at 4 °C with 1 μM final of either [^{3}H]P, sp act. 9 Ci/mmol, or 0.5 μM [^{3}H]R5020, sp act. 18 Ci/mmol. The ^{3}H-liganded PR was recovered as described in Renoir et al. (1984a). Each affinity eluate volume was ~60 mL/column.

DEAE-Sephacel Chromatography. The eluted PR from the affinity gels was loaded (flow rate ~30 mL/h) on top of a 1-mL DEAE-Sephacel column equilibrated with the appropriate buffer. The gel was then washed with 10 mL of buffer in order to remove the free steroid, and ^{3}H-liganded receptor complexes were eluted by a 0–0.5 M KCl linear gradient in either buffer A or B.

DNA–Cellulose Chromatography. Fractions containing ^{3}H steroid–PR complexes were loaded on top of DNA–cellulose (~25 mL) packed in a 2.5-cm diameter IBF column (Genevilliers, France). After the fractions were loaded, the gel was washed with 5 column volumes of buffer A and the PR was eluted by a linear 0–0.5 M KCl gradient in buffer A.

Progesterone Receptor Binding Assay and Ultracentrifugation Analysis. Steroid binding activity was measured as in Renoir et al. (1984a). For the initial cytosol, total binding was determined by incubation with 20 nM [^{3}H]P. Nonspecific binding was measured after incubation he same conditions plus a 500-fold excess of unlabeled P. Dextran (0.025%)–charcoal (0.25%) adsorption was used to discriminate between free and protein-bound steroid. The amount of affinity-eluted receptor-bound ^{3}H steroid was measured after using a similar dextran–charcoal suspension containing 2 mg of gelatin/mL. The binding activity of the purified receptor after ion-exchange chromatography was determined by direct counting of 0.05-mL aliquots. Sedimentation coefficients were determined by 10–35% glycerol gradient ultracentrifugation analysis as in Renoir et al. (1982b) after 16 h at 200000*g* at 2 °C in a SW 60 Beckman rotor. Glucose oxidase, $s_{20,w}$ = 7.9 S, and peroxidase, $s_{20,w}$ with 3.6 S, were used as internal markers. The affinity eluate samples were treated with a dextran–charcoal (0.5–0.05% w/v) suspension, for 10 min at 0–4 °C, prior to loading on top of the performed gradients.

Affinity Labeling of [^{3}H]R5020–PR Complexes. Affinity labeling of [^{3}H]R5020–PR complexes was obtained by UV irradiation during 2.5 min at 0 °C, as described in Renoir et al. (1984a). Affinity-labeled PR samples were then precipitated by 50% ammonium sulfate, and the pellets were redissolved in 0.1% SDS electrophoresis buffer before analysis by SDS–disc tube electrophoresis (7.5% final acrylamide concentration, 11.5-cm length) according to the method of Laemmli (1970). After migration, each gel was cooled at 0 °C and cut into ~1-mm slices. Each slice was counted in 10 mL of Ready-Solv solution (Beckman) after overnight digestion with 0.5 mL of NCS (Amersham) at 37 °C.

High-Performance Liquid Chromatography (HPLC) Analysis. Samples (100 μL) of crude or purified PR were chromatographed on the TSKG-3000 S_{10} column (30 cm; LKB, Uppsala, Sweden), equilibrated in buffer B. Fractions of 0.5 mL were collected at a flow rate of 1 mL/min at 4 °C; each total fraction was counted as described below. The column was calibrated with the following standard proteins: (1) bovine thyroglobulin, R_s 8.6 nm; (2) crude 8S-PR from chick oviduct cytosol, R_s 7.7 nm; (3) β-galactosidase from *Escherichia coli*, R_s 6.9 nm; (4) pure "B" subunit of the chick oviduct PR, R_s 6.1 nm; (5) catalase from bovine liver, R_s 5.22 nm; (6) human transferrin, R_s 3.6 nm [for references of R_s, see Renoir et al. (1982a, 1984a,b)]. K_D values were calculated as $[(V_e - V_0)/(V_t - V_0)]$ where V_t is the total volume of the column measured with tryptophan, V_0 the excluded volume measured with dextran blue, and V_e the elution volume of the protein.

SDS Slab Gel Electrophoresis and Staining. Analytical denaturing slab gel electrophoreses were generally performed in 7.5–15% polyacrylamide gradient (except when noted) according to the method of Laemmli (1970). Samples (0.3–1 μg) of purified PR fractions or of molecular weight markers (MW-SDS-200 kit; Sigma, St. Louis, MO) were electrophoresed, and silver staining was performed according to the method of Wray et al. (1981). Molecular weight markers ($\times 10^{-3}$) [for references, see Renoir et al. (1984a,b)] are as follows: myosin, 212; β-galactosidase, 130; phosphorylase *b*, 97.4; BSA, 66; ovalbumin, 45; carbonic anhydrase, 29.

Immunological Studies. We used a rabbit polyclonal antibody against the 90-kDa protein detected in the nontransformed form of the calf estradiol receptor (Redeuilh et al.,

Table I: Purification of Rabbit Uterus 8S- and 4S-PR, a Representative Experiment

	vol (mL)	binding act.[a] (pmol/mL) (*a*)	proteins (mg/mL) (*b*)	sp act. (*a*/*b*)	purifcn (*x*-fold)	yield (%)
cytosol	220	17.3	11	1.57		
affinity chromatography[b]	120	11.1	0.012	920	590	35
DEAE-Sephacel (8S-PR)	9.9	74.7	0.043	1740	1110	19.3
DNA-cellulose[c] (4S-PR)	14.3	19.5	0.0032[d]	6100	3879	7

[a] The binding activity was measured as described under Materials and Methods, and is expressed in picomoles of bound [^{3}H]P, taking into account the isotopic dilution of the ligand used at the affinity chromatography elution step, on the basis of ~100 Ci/mmol. [b] Sixty milliliters of a batch of NADAc gel containing 0.38 μmol of deoxycorticosterone grafted/mL of gel. [c] This column was loaded with the flow-through fractions of the DEAE-Sephacel column, and chromatography was performed as described under Materials and Methods. [d] Due to the low protein concentration at this level of purification, the calculated purity of 4S-PR is approximate. Proteins at each purification step were analyzed by SDS–PAGE (Figures 2b and 4b).

1985) and cut out from an SDS–PAGE. Western blotting technique (Bowen et al., 1980) was used after SDS–PAGE as described in Renoir et al. (1984b). The anti-90-kDa antiserum was used at a dilution of $^1/_{1000}$ and revealed with 5 × 10^5 cpm/mL of a second ^{125}I-labeled anti-rabbit–goat polyclonal antibody. Nitrocellulose filters (Schleicher and Schüll; 0.45-μm pore size) and X-Omat (XAR-5) films (Kodak) were used for transfer and fluorography, respectively.

Protein Determination. Quantification of proteins was performed according to the method of Schaffner and Weissmann (1973), modified as in Renoir et al. (1984a). Duplicate samples were assayed in which at least 4 μg of proteins were measured.

Radioactivity Counting. Samples were mixed with 7 mL of Scintimix (0.4% in toluene) and counted in a Packard liquid scintillation spectrometer (45% efficiency).

Results

Affinity Chromatography

Nontransformed 8S-PR. Affinity chromatography of molybdate-containing cytosol from rabbit uterus gave a purification factor of 450 ± 150 (mean ± SD, n = 8) with always more than 20% yield (see an example in Table I). The capacity of the NADAc–Sepharose affinity gel was calculated to be ~40 pmol of 8S-PR/mL of gel. The gel was washed as described under Materials and Methods. It was verified previously that the nontransformed PR from rabbit uterus cytosol remained predominantly (>60%) in its 8S form after a 1-h exposure to 2.25 M urea at 0 °C in buffer B (T. Buchou, unpublished results). The elution yield was ~60%. Ultracentrifugation analysis performed in the presence of 20 mM molybdate showed a peak sedimenting at the 8.5S position (Figure 1a); even after charcoal treatment, the largest amount of radioactivity migrated as a broad peak in the 2–5S region. It contains some free steroid dissociating from ^{3}H hormone–receptor complexes during the centrifugation in such diluted and purified preparations and also 20% of 4S transformed PR (as measured by charcoal adsorption), either already present in the affinity eluate or formed during the centrifugation period.

Transformed PR. Affinity chromatography of cytosol containing 0.3 M KCl (and no molybdate) was performed in order to purify transformed PR complexes and to compare them with the nontransformed PR. The purification factor and the yield were similar (~600-fold and ~35%, Table I and data not shown). Density gradient experiments demonstrated a broad 3–5S radioactive peak, very unstable in term of binding activity, and no radioactivity in the ~8S region (not shown).

Protein Content of Affinity Chromatography Eluates. Analysis by SDS–PAGE of the proteins present in the affinity eluates revealed a major silver-stained band at ~72 kDa (Figure 2a, lanes 1–3). This band and other bands at ~212 and 160 kDa and between 66 and 45 kDa were also present

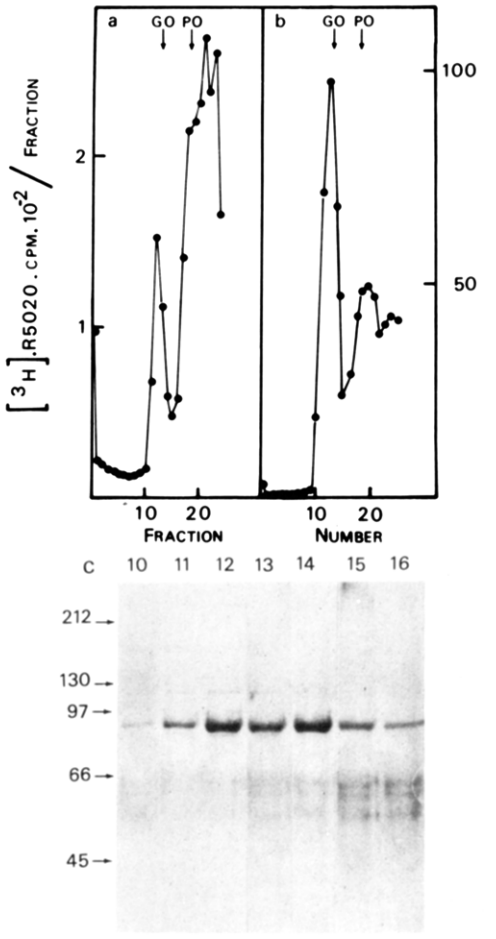

FIGURE 1: Glycerol (10–35%) gradient ultracentrifugation of purified 8S-PR. (a) An aliquot (50 μL) of affinity chromatography eluate in buffer B, from which the excess of steroid was removed by dextran–charcoal adsorption, was loaded on top of a preformed 10–35% glycerol gradient and run as described under Materials and Methods. GO, glucose oxidase; PO, peroxidase. (b) A sample (50 μL) of the 0.15 M KCl radioactive peak from the DEAE-Sephacel column was ultracentrifuged in glycerol gradient made in 0.15 M KCl containing buffer B. Note the difference of scale from (a). (c) A sample (300 μL) of the 0.15 M KCl radioactive peak from the DEAE-Sephacel column was immediately ultracentrifuged as above. After detection of the 8.5S radioactive peak in 10-μL aliquots, the remaining part of each fraction was boiled in 1% SDS sample buffer and analyzed in 7.5% SDS–PAGE. Analyzed fractions are numbered at the top of the gel, and molecular weights of markers (×10^{-3}) are on the left.

in the affinity eluates of the "mock purification"—that is, after chromatography of molybdate-containing cytosol supplemented with 1 μM cold progesterone in order to prevent binding of the PR to the affinity column (Figure 2a, lane 3, and Figure 2, lane 2). Therefore they may be considered as contaminants unrelated to PR since they were present in the eluates of the affinity columns in similar amounts, whether the PR was

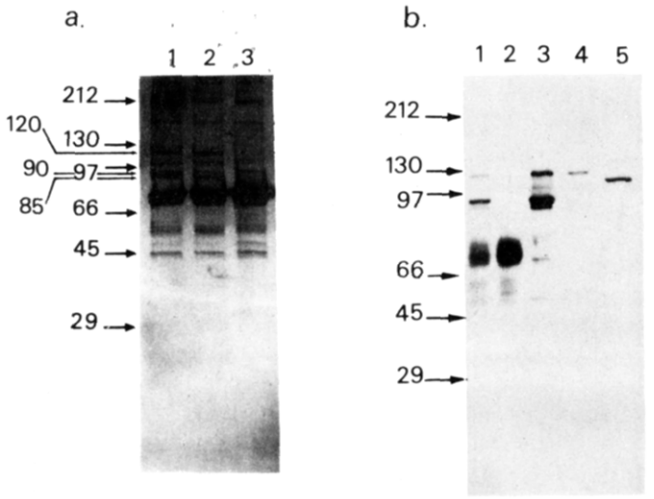

FIGURE 2: SDS–polyacrylamide gel electrophoresis of purified rabbit uterus PR. The gels were performed as mentioned under Materials and Methods and molecular weights of markers ($\times 10^{-3}$) are indicated by arrows. (a) Analysis of affinity chromatography eluates. The cytosol of rabbit uteri was prepared in the presence of protease inhibitors a, b, and c and divided into three parts. Each part was supplemented with (1) 20 mM molybdate, (2) 0.3 M KCl followed by a 3-h incubation at 0–4 °C, and (3) 20 mM molybdate plus 1 μM radioinert progesterone ("mock purification"). Each sample was loaded on top of an affinity gel column in an identical cytosol-to-gel ratio and washed as described under Materials and Methods. After biospecific elution, samples of eluates were boiled in SDS buffer and loaded at the same protein concentration (1 μg) on top of the gel. Lane 1, purification of 8S-PR; lane 2, purification of 4S-PR; lane 3, "mock purification" of 8S-PR. (b) SDS gel analysis of PR samples obtained at each purification step. Uterine cytosol was prepared in the presence of molybdate and of protease inhibitors a–f; after affinity chromatography and biospecific elution, the eluted 8S-PR was rechromatographed on DEAE-Sephacel and the flow-through was loaded on DNA–cellulose as described under Materials and Methods. An aliquot of each eluted PR fraction was boiled in SDS and loaded on top of the gel. Lane 1, 8S-PR (0.9 μg of protein) eluted from the affinity gel; lane 2, eluate from a parallel "mock purification" (0.9 μg); lane 3, pooled 8S-PR eluted from the DEAE-Sephacel column (1 μg); lane 4, pooled 4S-PR eluted from the DNA–cellulose column (0.4 μg); lane 5, B subunit of the chick oviduct PR (1 μg) (Renoir et al., 1984a). Lanes correspond to the fractions described in Table I.

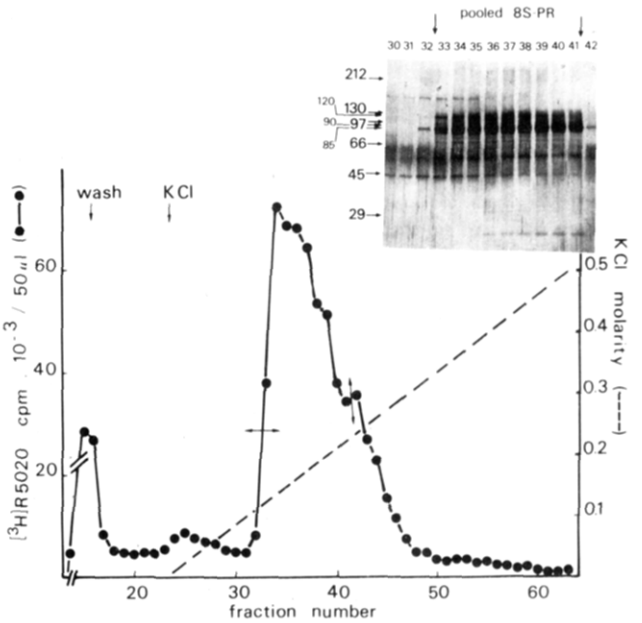

FIGURE 3: DEAE-Sephacel chromatography of affinity-purified 8S-PR. Elution profile of the 8S-PR. Uterine cytosol was prepared as mentioned in Figure 2b and purified by affinity chromatography. After exchange elution by [^{3}H]R5020, the affinity-eluted 8S-PR (120 mL) was loaded on top of the 1-mL ion-exchange gel, and chromatography was performed as described under Materials and Methods. Nine-milliliter fractions from fraction number 1 to fraction number 15, and then 1.1-mL fractions, were collected, respectively. A 50-μL portion of each fraction was counted for radioactivity (●). KCl concentration was determined by measuring the conductivity of each fraction (---). Fractions between the double-headed arrows were pooled. (Inset) SDS gel electrophoresis of individual fractions corresponding to pooled 8S-PR. Portions (100 μL) of fractions of the DEAE-Sephacel column reported in (a) were boiled in SDS and electrophoresed according to the method of Laemmli (1970). Arrows on the left indicate the molecular weights of markers ($\times 10^{-3}$).

retained (and further eluted) or not.

In the "mock purification", an insignificant amount of radioactivity (corresponding to ~2% of the 8S-PR eluted in parallel real affinity chromatography) was found to be charcoal resistant. Interestingly, while bands of 120, 90, and 85 kDa were detected in the 8S-PR-containing eluate (Figure 2a, lane 1, and Figure 2b, lane 1), they were very weakly present or absent in the corresponding fraction after "mock purification" (Figure 2a, lanes 1 and 3, and Figure 2b, lanes 1 and 2). In addition, in the 4S-PR-containing eluates of affinity chromatography of KCl-treated cytosol, only a very faint band at 90 kDa was silver stained, while the 120- and 85-kDa bands were observed (Figure 2a, lane 2). It was then concluded that the hormone-binding units have M_r ~120000 and 85000 and that the 90-kDa protein does not bind hormone but is part of the 8S-PR with which it is retained during affinity chromatography of molybdate-containing cytosol.

DEAE-Sephacel Chromatography

Nontransformed PR. The affinity eluate obtained in the presence of 20 mM molybdate was loaded on the ion-exchange column; a single radioactive peak was eluted at ~0.15 M KCl during the linear salt gradient (Figure 3). [^{3}H]PR complexes sedimented mostly as in a 8.5S position after ultracentrifugation analysis (Figure 1b), while a small 4S peak and some free steroid were also observed. After this second purification step, the 8S-PR was 19% pure (see Table I), based on specific activity (calculated according to one hormone binding site per 100 kDa). The purification was 800 ± 150-fold (mean ± SD, n = 8), and the yield was 20%. As observed in Figure 3, a large amount of radioactivity was found in the flow-through fractions of the DEAE-Sephacel column. It corresponded to the excess of free steroid used at the elution step of the affinity gel and also to some [^{3}H]PR complexes, as reflected by dextran–charcoal measurements (data not shown).

Transformed PR. The 4S-transformed PR eluted from the NADAc affinity gel was not retained on the ion-exchange chromatography column, all specific binding being recovered in the flow-through fractions (not shown).

Protein Content of DEAE-Sephacel Purified 8S-PR. Analytical denaturing electrophoresis of the 0.15 M peak eluted from DEAE-Sephacel revealed that the 90-kDa protein was the most abundant protein as compared to 120- and 85-kDa ones (Figure 2b, lane 3) and that other silver-stained (probably protein contaminants) bands detected after affinity chromatography had decreased in staining intensity (compare lanes 1 and 3 in Figure 2b). Each fraction of the DEAE-Sephacel chromatography was analyzed in SDS gel. As shown in Figure 3, only the proteins at 120, 90, 85, and 60 kDa followed the radioactive peak. The 60-kDa silver-stained protein may correspond to that reported by Tai and Faber (1984a,b).

Individual fractions collected after sedimentation gradient analysis of the 0.15 M KCl peak of the ion-exchange column were analyzed by SDS gel electrophoresis. Comigration of

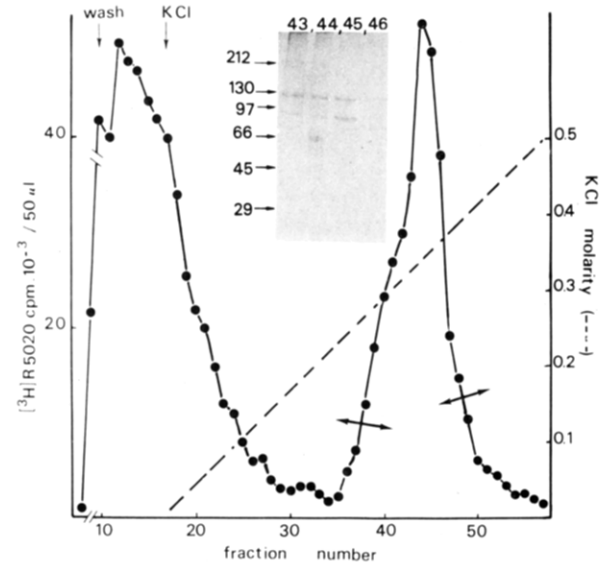

FIGURE 4: DNA–cellulose chromatography of the flow-through of the DEAE-Sephacel column. Elution profile of the purified 4S-PR. The flow-through fraction of the DEAE-Sephacel column was loaded on top of the DNA–cellulose column equilibrated in buffer A; the chromatography was performed as described under Materials and Methods. Nine-milliliter fractions from fraction number 1 to fraction number 19, and then 1.1-mL fractions, were collected, respectively. A 50-µL portion of each fraction was counted for radioactivity (●) and the KCl concentration (---) was measured as in Figure 4a. The fractions between the double-headed arrows were pooled and referred to as pooled 4S-PR. (Inset) SDS electrophoresis analysis of 4S-PR shown above. Aliquots (130 µL) of fractions 43–46 of the top of the radioactive peak were electrophoresed after boiling in SDS buffer as in Figure 3.

the 90-kDa protein with the 120- and 85-kDa proteins was detected in the 8.5S radioactive peak (Figure 1c), suggesting that they were associated in a heteromeric structure. Some 90-kDa protein was also present in fractions following the 8.5S radioactive peak (~6.5S, suggested to be a homodimer, C. Radanyi, unpublished results). This might be explained by (a) a transformation process having occurred during affinity and/or ion-exchange chromatography (see later the recovery of transformed receptor in the flow-through of the DEAE-Sephacel) or (b) some dissociation from the 8S-structure occurring during ultracentrifugation.

Purification of 4S-PR by DNA–Cellulose Chromatography. DNA–cellulose chromatography was used to purify the transformed 4S-PR found in the flow-through fraction of the DEAE-Sephacel chromatography of 8S-PR column (see Figure 1). A radioactive peak was eluted at ~0.35 M KCl of a linear KCl gradient (Figure 4). This procedure allowed obtaining of purified transformed PR of ~60% purity on the basis of a molecular weight of 100000 (Table I), with recovery of 5–7% of the original cytosol PR. Glycerol gradient analysis demonstrated a broad 4–6S peak (not shown), with a large amount of free (dissociated) steroid, due to the unstability of hormone binding of this highly purified and diluted 4S-PR form, as already observed for the 4S-PR eluted from the affinity column. Denaturing gel electrophoresis analysis (Figure 4) revealed two silver-stained bands at 120 and 85 kDa, the former being more abundant than the latter (Figure 2b, lane 4), coinciding with the radioactive peak. They likely correspond to the B and A subunits that have been identified and purified from chick oviduct (Schrader & O'Malley, 1972; Sherman et al., 1976; Schrader et al., 1981; Renoir et al., 1984b), rabbit uterus (Lamb & Bullock, 1984; Logeat et al., 1985) and human MCF7 breast cancer cells (Lessey et al., 1983). The 72- and 66-kDa proteins, which were present in the affinity chromatography eluate and in the flow-through of the DEAE-Sephacel column, were eliminated during the loading and washing of the DNA–cellulose column. Contrary to the transformed cOvPR, which, on such a column, is resolved into two B and A peaks [according to their order of elution by ionic strength, Schrader et al. (1972)], the transformed rUtPR was eluted as a single peak containing 85- and 120-kDa proteins. There was no 90-kDa protein in any fraction of DNA–cellulose chromatography. Some 90-kDa protein nonassociated to 8S-PR is often present in affinity chromatography eluate, due to secondary partial transformation of 8S-PR, and therefore its absence after DNA–cellulose column chromatography suggested that it was retained by the DEAE-Sephacel column (behaving similarly to the 8S-PR itself), and then absent of its flow-through. As compared to the molecular weight of the purified B subunit of the cOvPR (~110000; Renoir et al., 1984b), the molecular weight of the B-like subunit of the rUtPR appeared larger (~120000 ± 7000, n = 12; Figure 2b), in accord with the value reported by Logeat et al. (1985).

Affinity Labeling. In crude cytosol of rabbit uterus prepared either in the presence or in the absence of molybdate and in the presence of protease inhibitors, two radioactive peaks at positions at 118 ± 12 kDa (n = 6) and 85 ± 9 kDa (n = 6) were photolabeled with [^{3}H]R5020 (Figure 5a). These peaks were almost completely abolished by addition of 2 µM radioinert progesterone and by 24-h exchange at 4 °C before irradiation, indicating specific binding of the synthetic progestin to the 120- and 85-kDa proteins. The 85-kDa peak was variable in intensity but always present and at least half as intense as the 120-kDa peak, in spite of the use of protease inhibitors. The same results were obtained in the DEAE-Sephacel [^{3}H]R5020–8S-PR eluate (Figure 5b) and in the DNA–cellulose 4S-PR eluate (Figure 5c). The extent of the competition for [^{3}H]progestin by nonradioactive progesterone was more pronounced when performed on the 8S-PR eluted from the DEAE-Sephacel column than on the 4S-PR eluted from the DNA–cellulose column. This is in agreement with the higher dissociation rate of the hormone from labeled nontransformed 8S-PR complexes than that from labeled transformed 4S-PR complexes (Seeley & Costas, 1983).

HPLC Analysis. As illustrated in Figure 6, the purified molybdate-stabilized 8S-PR eluted as a sharp radioactive peak with a R_s of 7.7–7.8 nm, not significantly different of that measured for crude 8S-PR. On the basis of the formula of Siegel and Monty (1966), the molecular weight of the rabbit uterus 8S-PR was calculated to be ~280000. The instability of the binding activity of the purified transformed rabbit uterus 4S-PR precluded its analysis on the HPLC column, and a smaller and broad radioactive peak always was obtained (not shown). The 8S-PR was more stable, and 50% of the PR remained intact. Electrophoretic analysis of the 8S-PR radioactive peak of the HPLC column showed that, as for the 8S-PR eluted from the DEAE-Sephacel column, the 90-kDa protein comigrated with the hormone binding 120- and 85-kDa proteins. As in density gradient experiments, another population of the 90-kDa protein was also detected, not associated with the 7.7-nm radioactive peak. This result is likely due to the same reasons as those evoked above.

Immunological Recognition of a 90-kDa Protein in 8S-rUtPR. With the use of a polyclonal antibody anti-90-kDa protein of the calf uterus (Redeuilh et al., 1985), Western blots showed that this antibody recognized the 90-kDa protein of the tested mammalian cytosol uteri and of chick oviduct (Figure 7a). In purified 8S steroid hormone receptor

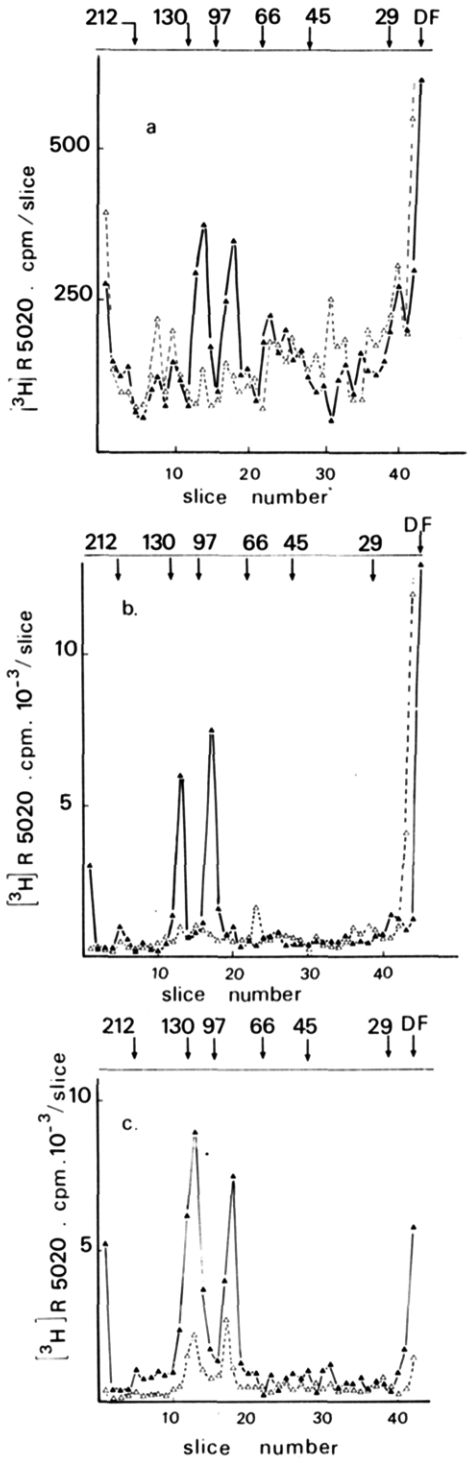

FIGURE 5: Photoaffinity labeling of 8S- and 4S-PR at different purification steps. (a) UV irradiation of initial cytosol. Uterine cytosol was prepared with protease inhibitors a–f or with only PMSF, in the presence or in the absence of molybdate. Aliquots of cytosol were then labeled with (1) (▲) 20 nM [^{3}H]R5020 or (2) (△) 20 nM [^{3}H]R5020 plus 2 μM radioinert progesterone during 3 h at 0 °C. Portions of each incubation (100 μL) were diluted to 1 mL and UV irradiated as previously described (Renoir et al., 1984b). After concentration by ammonium sulfate precipitation, irradiated samples were electrophoresed in 7.5% acrylamide disc tubes (see Materials and Methods). (b) and (c) Irradiation of purified 8S- and 4S-PR bioaffinity eluted with [^{3}H]R5020. Samples (1 mL) of the 8S-PR obtained from the DEAE-Sephacel column and of the 4S-PR obtained from the DNA–cellulose column (top of the radioactive peaks) (c) were UV irradiated and analyzed as in (▲). Identical samples containing the same amount of bound [^{3}H]R5020 were incubated for 24 h at 0 °C with 2 μM of cold P and subsequently irradiated (△). Two specific binding peaks were observed. Molecular weights of markers ($\times 10^{-3}$) are indicated at the top of each panel. DF, dye front.

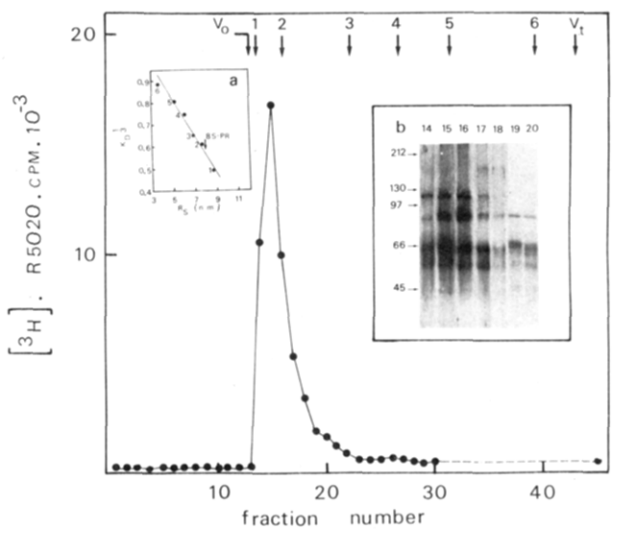

FIGURE 6: HPLC chromatography of purified 8S-PR. Aliquots of either crude or DEAE-Sephacel purified 8S-PR, labeled with tritiated ligand, were injected on top of the TSKG column and chromatographed as described in the text. Each collected fraction (500 μL) was counted for radioactivity, and the Stokes radius of PR samples was calculated by reference to the standard curve (inset a) obtained with proteins of known R_s. The two samples gave exactly the same profile. (Inset b) Fractions 14–20 of four successive runs of [^{3}H]-R5020-labeled DEAE-Sephacel purified 8S-PR were pooled and precipitated by 7% final TCA, pelleted by centrifugation, and boiled for SDS–PAGE (7.5% acrylamide). Molecular weights of markers ($\times 10^{-3}$) are indicated to the left. The diffuse bands at ~66 and ~50 are silver-stained artifacts.

preparations, a signal was observed at a 90-kDa position with the 8S-rUtPR, the 8S-cOvPR, and the nontransformed form of the calf estradiol receptor (G. Redeuilh, unpublished results).

In density gradient analysis (Figure 7b), a shift of the purified 8S-rUtPR from 8.5S to ~11S was observed, after incubation with IgGs of the anti-90-kDa protein of the calf uterus. Some radioactivity was also found in the bottom of the tube (possibly larger anti-90-kDa antibody-8S-rUtPR complexes). SDS–PAGE analysis of the top fraction of the 11S peak revealed the presence of the 90-kDa protein and that of both 120- and 85-kDa binders. No shift occurred after incubation with the same amount of nonimmune rabbit IgGs; in this latter case, some transformation of 8S-rUtPR into a 4S form was observed, as we previously observed when working with partially purified nonimmune IgGs whatever the species used (Renoir et al., 1982b; Joab et al., 1984; Groyer et al., 1985). This was not the case when working with anti-90-kDa specific IgGs. Interaction with anti-90-kDa antibodies seems to stabilize the nontransformed structure of the receptor, as already observed in the case of the 8S-cOvPR (Renoir et al., 1982b; Joab et al., 1984). The fact that a large amount of anti-90-kDa protein antibody was necessary to shift the 8.5S peak could be explained by (1) low affinity of antibodies, (2) difference of antigenic sites in the denatured protein (against which was generated the antibody) and in the native protein assayed here, and (3) masking of antigenic sites when the 90-kDa protein is included in the nontransformed 8S-PR structure.

Activation of Purified 8S-rUtPR. Purified [^{3}H]R5020–nontransformed 8S-rUtPR was chromatographed on DNA–cellulose just after its elution from the DEAE-Sephacel column. As shown in Figure 8a, no binding to DNA–cellulose occurred, as shown by recovery of all radioactivity in the flow-through. An identical [^{3}H]R5020-labeled, nontransformed 8S-rUtPR preparation was treated with 3 M KCl

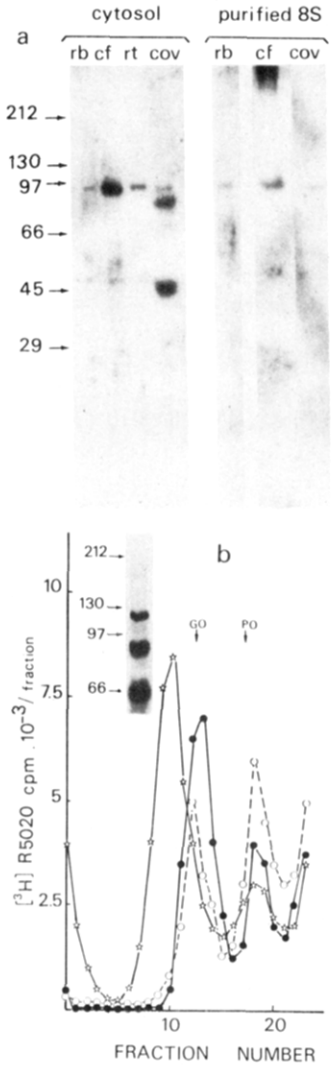

FIGURE 7: Immunological recognition of the 90-kDa protein in 8S-PR. (a) Western blots of crude cytosols with polyclonal anti-90-kDa antibody. Cytosols from rabbit (rb) and rat uteri (rt) were prepared as described under Material and Methods, cytosol from chick oviduct (cOv) was prepared as in Renoir et al. (1984a), and cytosol from calf uteri (cf) as in Bucourt et al. (1978). Two microliters of nondiluted cytosols, except 10 μL with cOv, were loaded on 7.5–15% SDS–PAGE. Samples (100 μL) of purified 8S-rUtPR [total protein: 0.4 μg in rb, 0.6 μg of purified calf estradiol receptor (Redeuilh et al., 1985), and 1 μg in purified 8S-cOvPR (Renoir et al., 1984a)] were loaded, electrophoresed, and treated as described under Materials and Methods. Parallel blots performed with nonimmune rabbit IgGs (not shown) revealed no signal at the 90-kDa position with any sample. Two spots corresponding to the abundant ovalbumin (45 kDa) and conalbumin (80 kDa) were observed in cOv cytosol preparations. (b) Glycerol (10–35%) density gradient experiments. Freshly purified 8S-rUtPR (0.20 pmol) after DEAE-Sephacel chromatography, labeled with nonisotopically diluted [^{3}H]R5020 (sp act. = 87 Ci/mmol) was incubated or not (●) with 1.2 mg of partially purified polyclonal anti-90-kDa IgGs (☆) or 1.2 mg of partially purified nonimmune rabbit IgGs (○) during 4 h at 4 °C in buffer B. The incubates were loaded on top of preformed and cooled (4 °C) gradients; centrifugation ran during 17 h at 200000g as described earlier (Renoir et al., 1982b), with external glucose oxidase (7.9S) and peroxidase (3.6S) markers. After 20 μL of each collected fraction was counted, the remaining part of fractions 8–11 was pooled, TCA precipitated, and boiled for SDS–PAGE (7.5% acrylamide). Only the upper part of the gel was silver stained to avoid staining of the ~53-kDa heavy chains of the large amount of used IgGs.

during 1 h at 4 °C, then diluted in buffer A to an ionic strength of ~0.05 M KCl, and loaded on top of a parallel DNA–cellulose column; in this case a radioactive peak was eluted at ~0.3 M KCl during the linear 0–0.5 M KCl gradient.

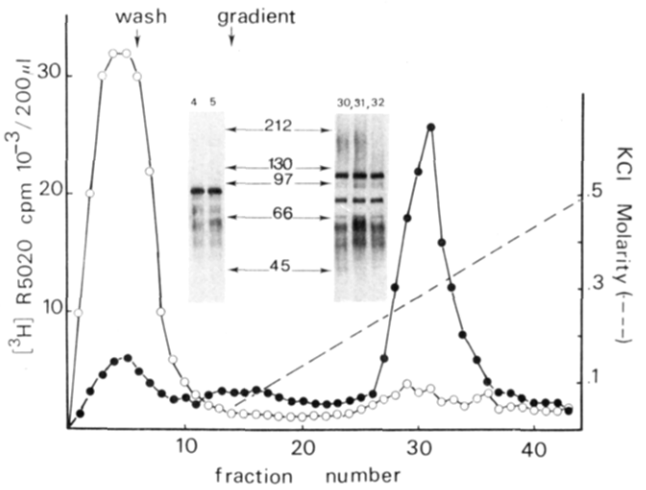

FIGURE 8: Activation of purified 8S-PR. An aliquot (5 mL, ~0.4 nmol) of [^{3}H]R5020-labeled DEAE-Sephacel purified 8S-PR was loaded on top of 10 mL of DNA–cellulose preequilibrated in buffer A (○). In a parallel experiment, 5 mL of the same 8S-PR preparation was incubated for 1 h at 0 °C with 0.3 M KCl, diluted 7 times in buffer A, and chromatographed on an identical column (●). After washing with 50 mL of buffer A, a 0–0.5 M KCl gradient (50 mL) was established as in Figure 4. Fractions of 2 and 5 mL were collected between fractions 1 and 6 (○ and ●, respectively), 10 mL between fractions 7 and 15 for both columns, and 1 mL during the gradient. Aliquots of 200 μL were assayed for radioactivity. (Inset) SDS–PAGE analysis (7.5% acrylamide) of proteins eluted or not from DNA–cellulose chromatographies. Aliquots (150 μL) of fractions 1–9 and 28–36 of the DNA–cellulose columns loaded with the KCl-treated purified 8S-PR (○) were boiled in SDS, electrophoresed (7.5% acrylamide), and silver stained as described under Materials and Methods. Only representative protein profiles of fractions 4, 5, and 30–32 are shown. Molecular weights of markers ($\times 10^{-3}$) are indicated on both sides of gels.

SDS–PAGE (Figure 8) showed that this peak contained the 120- and 85-kDa proteins, while the 90-kDa protein present in the original 8S-PR was found in the flow-through. This suggested that (1) purified 8S-rUtPR can be in vitro transformed into a form that can bind to DNA–cellulose as the activated receptor should, (2) the 90-kDa protein association prevents DNA binding of the PR, and (3) the 90-kDa protein does not bind to DNA–cellulose. The same conclusions have recently been obtained with purified 8S-cOvPR (Renoir & Baulieu, 1985).

This highly purified 90-kDa protein was analyzed by density gradient ultracentrifugation, HPLC, and SDS–PAGE (not shown). A sedimentation coefficient of ~6.5 S and Stokes radius of ~7.0 nm were found, suggesting a dimeric structure (calculated M_r ~190000). This is significantly different from what was found for the purified [^{3}H]progestin-labeled 8.5S-rUtPR (8.5 S and 7.75 nm).

Discussion

The structure of the progesterone receptors has been extensively studied. From the hen or chick oviduct cOvPR, two hormone binding proteins of different molecular weights, called subunits A and B, have been described, and their purification was reported (Schrader & O'Malley, 1972; Sherman et al., 1976; Kuhn et al., 1977; Coty et al., 1979; Renoir et al., 1984a,b). It was suggested by Schrader et al. (1981) that the structure of the native receptor has a sedimentation coefficient of 6 S and is a dimer of A and B subunits. On the other hand, with molybdate as a stabilizing agent for the nontransformed "form" of the chicken PR (Wolfson et al., 1980), an 8S form was purified (Renoir et al., 1982a, 1984a; Puri et al., 1982; Dougherty et al., 1984), and Renoir et al. (1984a) proposed that the nontransformed 8S form of cOvPR, of M_r ~260000,

includes one molecule of the binding proteins A (79 kDa) or B (110 kDa) and two molecules of a non-hormone-binding 90-kDa protein [see review in Renoir and Mester, (1984)].

Several molecular weight values have been reported for the progesterone binding proteins of the rUtPR (Westphal et al., 1981; Jänne, 1982; Lamb & Bullock, 1984; Tai & Faber, 1984; Logeat et al., 1985) and for the human PR (Smith et al., 1981; Lessey et al., 1983). Besides early report indicating that mammalian PRs have a sedimentation coefficient ~8 S (Baulieu et al., 1971; Sherman et al., 1983), no report has yet appeared dealing with the purification and structure of mammalian nontransformed 8S-PRs.

In this work, we have purified the nontransformed 8S-rUtPR in the presence of molybdate and by successive affinity and DEAE-Sephacel chromatographies. The purified preparations contain a 120- and an 85-kD progestin binding protein, which correspond to the subunits B and A of the cOvPR of 110 and 79 kDa, respectively. In addition, there is a 90-kDa protein present in the 8S-PR structure, which does not bind progestin. We have reported comparatively the protein composition of the affinity eluates containing the 8S-PR, the 4S-PR, and that obtained after "mock purification". The results are crucial to the concept that the non-hormone-binding 90-kDa protein is purified correlatively to the progesterone binding 120- and 85-kDa units only in the 8S-PR purification experiments. It has been difficult to demonstrate that the 90-kDa protein in purified 8S-rUtPR preparations comigrated with the hormone binding proteins either in ultracentrifugation or in HPLC analysis. This was mainly due to the great instability of the nontransformed structure of the rabbit 8S-PR, which, even in presence of molybdate, always transformed, producing dissociation of the 90-kDa protein from the hormone binding proteins. However, when analyzing fresh 8S-PR preparations in density gradients or HPLC just after elution from the DEAE-Sephacel column, SDS–PAGE revealed the comigration of the 90- with the 120- and 85-kDa proteins. The use of a specific anti-90-kDa mammalian protein antibody gave evidence for the presence of the 90-kDa protein in the 8S-rUtPR, since a shift of the radiolabeled 8S-rUtPR, after incubation of the [^{3}H]R5020–8S-rUtPR with this antibody, was observed in density gradient experiments. SDS–PAGE here, too, revealed the presence in the shifted radioactive peak of the 90-kDa protein with the hormone binding units.

We have previously reported that a 90-kDa protein that does not bind steroid hormones is involved in the structure of the nontransformed progesterone, androgen, estrogen, and glucocorticosteroid receptors found in the chick oviduct cytosol (Joab et al., 1984; Groyer et al., 1985). This 90-kDa protein has now been identified in chick as heat-shock protein hsp 90 (Catelli et al., 1985). The present results with the rUtPR, as the preliminary findings reported for the calf estradiol receptor (Redeuilh et al., 1985), also suggest that a 90-kDa protein is associated with steroid-binding units in the 8S structure of mammalian steroid receptors. Very recently, the molybdate-stabilized glucocorticosteroid receptor from either rat liver of untransformed L-cell cytosol was shown to be a heteromer (Housley et al., 1985; Okret et al., 1985), which includes a 90-kDa heat-shock protein associated with a 94–98-kDa hormone binding molecule (Sanchez et al., 1985). As in the chick oviduct (Gasc et al., 1983, 1984), there is evidence that the rUtPR is nuclear, even in the absence of hormone (Perrot-Applanat et al., 1985), and the possible role of the 90-kDa protein as a regulatory component of receptor activity is investigated in our laboratory. One cannot exclude a possible artifactual association of receptors with hsp 90 occurring during cell fractionation. One cannot reject also that such a complex does accumulate inside the nucleus, since the 90-kDa protein has been detected at this level in the chick oviduct system (Gasc et al., 1984). This association could maintain the receptor in a form unable to bind to DNA.

Several proteins other than 120, 90, and 85 kDa were detected in the purified preparation of 8S-rUtPR (see Figures 3 and 4). Among those, proteins at 210, 160, and 72 kDa and between 60 and 45 kDa had to be considered as contaminants, since they were still present approximately in the same amount in the corresponding eluates of regular and mock purifications (Figure 2). A 60-kDa protein has been described as involved in the nontransformed rUtPR (Tai & Faber, 1984a,b). However, we could not confirm this result since we failed to displace the [^{3}H]P–8S-rUtPR with the anti-60-kDa monoclonal antibody, generously given to us by Dr. Faber, in density gradient experiments and did not reveal a protein by immunoblotting of purified 8S-rUtPR.

The molecular weight values of the progesterone binding proteins of rUtPR were in agreement with those found for other mammalian PRs (Westphal et al., 1981; Lessey et al., 1983; Lamb & Bullock, 1984; Tai & Faber, 1984; Logeat et al., 1985). On the basis of a strong argument for a single ~120-kDa protein proposed by Logeat et al. (1985) and using antiproteolytic compounds, we tried and failed to avoid the formation of the 85-kDa unit in case it is a proteolysis product of the 120-kDa subunit. However, we never found proteolytic fragments as those detected by Sherman et al. (1983) and Lamb and Bullock (1984). We measured ~120 kDa for the molecular weight of the largest progesterone binding unit of the rUtPR, that is, slightly larger than that of the chicken (~110 kDa); small phylogenic differences may also explain that anti-rUtPR monoclonal antibodies do not cross-react with cOvPR (Feil, 1983; Logeat et al., 1983) and that conversely polyclonal antibodies against cOvPR-B recognize poorly mammalian PRs (Tuohimaa et al., 1984).

Finally, the instability of the 8S-rUtPR has created the opportunity to describe a new efficient method for purifying the 4S progesterone binding units, by further chromatography on DNA–cellulose of the flow-through fraction of the DEAE-Sephacel column used to isolate the untransformed receptor. The main result of this work remains, however, the involvement of the non-hormone-binding 90-kDa protein in the nontransformed form of the progesterone receptor of the rabbit uterus. The transformation process of cytosoluble steroid hormone receptor includes the dissociation of non-hormone binding from hormone-binding proteins; this is accompanied by a change in sedimentation coefficient (from 8–9 S to 4 S), and probably by a conformational change of the released hormone binding unit, that apparently we have been so far unable to revert in recombination experiments (by addition of hsp90).

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