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BINDING OF ACTIVATED PROGESTERONE RECEPTOR TO MICROSOMES

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Summary—Specific binding of steroid hormones to microsomes has been reported for several tissues. In the hen oviduct, this receptor appears to be very similar to activated cytosolic receptor. The microsomal receptor is readily solubilized, and resembles the cytosolic receptor in all physico-chemical characteristics: sedimentation coefficient ~4 S, Stokes radius 5.5 nm, slow dissociation rate of the complex, adsorption to polyanions. It is precipitated by an antibody to the cytosolic receptor. Microsomes display saturable binding of cytosolic receptor, with a B_{max} of ~300 fmol/mg protein. This binding is also observed using microsomes from non-target tissues, and is decreased by treatment with RNase. It seems likely that microsomal binding is due to the high affinity of activated cytosolic receptor for RNA.

INTRODUCTION

Steroid hormones are thought to act by binding to a receptor, which interacts with DNA and/or chromatin to alter the pattern of gene expression. In addition to cytoplasmic and nuclear receptor forms, membrane-bound receptors have been described for several steroids. These include: estrogen binding to rat uterine lysosomes [1], plasma membranes [2] and microsomes [3], androgen binding to rat ventral prostate microsomes [4] and progesterone binding to microsomes of rat uterus [5] and liver [6] and of chick oviduct [7].

The microsomal receptor may be envisaged as part of the cytoplasmic-nuclear pathway; it could be either a ribosome-bound precursor of the cytoplasmic receptor or represent recycled or processed receptor which has left the nucleus. On the other hand, the microsomes might be an entirely different locus of hormonal control. In the course of experiments designed to resolve this question, we found that the microsomal receptor of hen oviduct closely resembles activated cytoplasmic progesterone receptor.

EXPERIMENTAL

[1,2,6,7,21-^{3}H]Progesterone (160 Ci/mmol) was obtained from New England Nuclear and purified by LH-20 (Pharmacia) chromatography in a system of benzene–methanol, 85:15 (v/v). Its purity was verified by thin-layer chromatography in a system of benzene–ethyl acetate, 8:2 (v/v). It was stored at −20°C in ethanol.

Laying hens, Rhode Island Reds, were decapitated, and the magnum portion of the oviduct removed and rinsed in saline. It was homogenized (4:1, v/w) in P buffer containing 10 mM potassium phosphate, 1.5 mM EDTA, 12 mM thioglycerol, 10% glycerol, pH 7.8. Phenylmethylsulfonylfluoride, a serine protease inhibitor, was added to a final concentration of 0.3 mM. The homogenate was centrifuged at 20,000 g × 30 min to obtain a postmitochondrial supernatant, which was then centrifuged at 105,000 g × 60 min. The resulting supernatant was cytosol; the microsomal pellet was rinsed and resuspended in the same buffer.

Cytosol was treated with a suspension of charcoal–dextran, 0.5%/00.05% for 5 min at 0°C to remove endogenous steroid, and then the charcoal removed by centrifugation at 2,000 g × 5 min. The receptor was labeled with [^{3}H]progesterone by a 2 h incubation at 25°C, in the presence of 10 nM [^{3}H]progesterone and 1 μM cortisol. A parallel incubation was carried out, including 100-fold excess unlabeled progesterone, in order to calculate nonspecific binding; generally ~10%. Excess steroid was removed by incubation with charcoal/dextran. Immature chicks, used for some experiments, were estrogen-primed by treatment for at least 2 weeks with 1 mg estradiol benzoate/day. Sucrose gradient analysis and dissociation kinetics were carried out as previously described [8].

Bio-Gel A-1.5 m was obtained from Bio-Rad. A 300-ml column was equilibrated in P buffer and calibrated using the following standards for Stokes radius: thyroglobulin, 8.5 nm; β-galactosidase, 6.90 nm; ferritin, 6.14 nm; catalase, 5.20 nm; alcohol dehydrogenase, 4.55 nm; bovine serum albumin, 3.63 nm; ovalbumin, 2.80 nm. The void volume was determined with Blue Dextran and the total volume with potassium dichromate.

DEAE-Sephacel was a product of Pharmacia. It was equilibrated in P buffer. The sample: gel ratio was ~4:1. The sample was applied and the column

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washed until the effluent contained no protein. A linear KCl gradient, 0–0.4 M in P buffer, was then applied, and fractions monitored for radioactivity and protein. ATP-Sepharose was obtained from Sigma, and binding to the resin was carried out as described by Miller and Toft[9]. Protein was determined by the method of Bradford[10] as modified by Seeley *et al.*[11].

Radioactivity was measured using Econofluor (NEN) in a Packard Tricarb scintillation counter with an efficiency of ~40% for ^3H, except when [^{14}C]BSA was used as a marker on sucrose gradients, in which case Aquasol (NEN) was used as a scintillation solvent. EN^{3}HANCE spray was a product of NEN. Silica gel G plates for thin-layer chromatography were from Woelm. X-Ray films and cassettes were from Kodak, and were developed in the Department of Radiology, Magee Womens Hospital. Polyclonal antibodies to the chick oviduct progesterone receptor and pre-immune IgG from the same animal were the generous gift of Dr E.-E. Baulieu[12].

Non-solubilized microsomes were obtained after centrifugation at 105,000 *g* × 60 min, and were resuspended in P buffer. These were labeled with [^3H]progesterone in the same way as cytosol, above. Charcoal treatment did not lead to any loss of protein, as was also noted by Haukkamaa *et al.*[7]. The labeled microsomes were then recentrifuged at 105,000 *g* × 60 min and the resulting supernatant, the "solubilized" microsomes, contained 65–88% of the original progesterone binding of the insoluble microsomes. Facile solubilization of microsomal receptors in the presence of steroid has previously been noted for estrogen receptors of rat uterus and pituitary [13].

Glucose-6-phosphate dehydrogenase [14] and lactate dehydrogenase [15] were assayed as marker enzymes for cytosol, and NADH: cytochrome *c* reductase [16] as a microsomal marker. Measurements were made at three different dilutions of each cell fraction. The microsomal fraction was enriched in NADH: cytochrome *c* activity (10–30-fold increased specific activity over the post-mitochondrial supernatant), and contained < 1% of the glucose-6-phosphate dehydrogenase and lactate dehydrogenase activity found in the post-mitochondrial supernatant.

RESULTS

Characterization of the solubilized microsomal receptor

Progesterone receptors undergo a shift in sedimentation coefficient from ~8 to ~4 S upon activation [8, 17]. When analyzed on sucrose gradients in low salt, the solubilized microsomal receptor displayed a sedimentation coefficient of 3.8 ± 0.2 S. This was the case even when cytosol from the same animals contained both 4 and 8 S receptor forms. (Fig. 1A and B).

The Stokes radius of the microsomal receptor, as determined on Bio-Gel A-1.5 m, was 5.5 nm. (Fig. 2) The two parameters, Stokes radius and sedimentation coefficient, permit calculation of a molecular weight [18] from the microsomal receptor of ~86,000, which is approximately the size of a single subunit of the cytosolic progesterone receptor [19–21].

The profile of elution of the microsomal receptor on DEAE-Sephacel, illustrated in Fig. 3, showed two peaks, at 0.06 M KCl and 0.25 M KCl. These correspond to the positions of elution of the A and B subunits of the cytoplasmic activated receptor [22].

Upon activation, the rate of dissociation of the cytoplasmic non-activated complex decreases [8]. Because the soluble microsomal receptor was considerably more heat-labile than the cytoplasmic receptor, dissociation experiments were done using buffers containing 20% glycerol. Under these conditions, the $t_{1/2}$ of dissociation of both activated cytosol receptor and soluble microsomal receptor was 55 ± 11 min at 25°C.

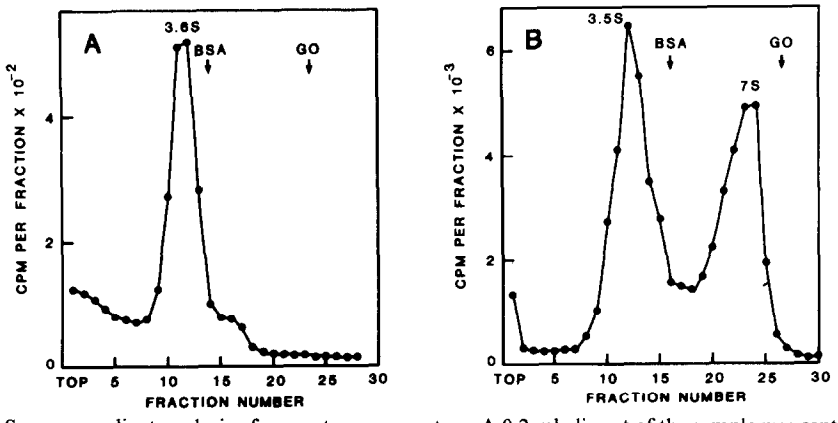

Fig. 1. Sucrose gradient analysis of progesterone receptors. A 0.2-ml aliquot of the sample was centrifuged on linear 5–20% sucrose gradients in P buffer, containing 0.15 M KCl and 50 mM molybdate at 360,000 *g* × 18 h. The internal markers glucose oxidase (G.O.), 7.9 S and BSA, 4.6 S were included. Sedimentation coefficients were calculated according to Martin and Ames[28]. (A) Solubilized microsomal receptor. (B) Cytosolic receptor.

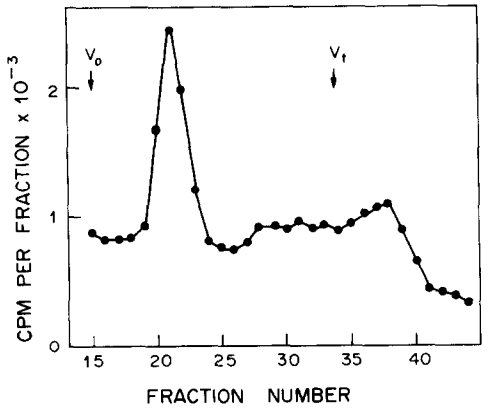

Fig. 2. Gel filtration profile of solubilized microsomal progesterone receptor. A 1-ml sample of the solubilized microsomal receptor was chromatographed on a Bio-Gel A-1.5 m column, eluted with P buffer. Three-ml fractions were collected. See the Experimental section for calibration.

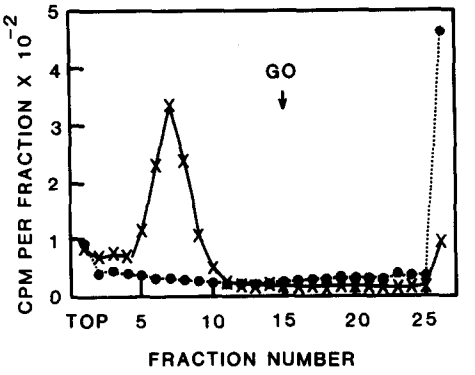

Fig. 4. Reaction of solubilized microsomal progesterone receptor with antibody to the cytosolic receptor. Two hundred μl of microsomal receptor was incubated at 0°C for 5 h with 50 μg of IgG, in a final volume of 350 μl. Aliquots of 200 μl were centrifuged on linear sucrose gradients (see legend to Fig. 1 for details), ●——●, IgG was specific anti-receptor; ×——×, IgG was pre-immune goat antiserum. Control, i.e. buffer in place of IgG, was identical to pre-immune profile. G.O., glucose oxidase.

An antibody has been raised to the non-activated cytosolic progesterone receptor which also reacts with the activated receptor [12]. This antibody was allowed to react with the soluble microsomal progesterone receptor, and then the complex analyzed on sucrose gradients. These are presented in Fig. 4. The ~4 S form of the receptor, present in the absence of antibody, disappeared after reaction with the antiserum, and heavier, aggregated forms were observed on the bottom of the tube. No displacement of radioactivity was observed when pre-immune serum was used. The same pattern was seen for activated cytosolic receptor.

Activated receptor, unlike non-activated, will bind to nuclei and other polyanions [23]. Both activated cytosolic receptor and soluble microsomal receptor bound to ATP-Sepharose. The amount of receptor bound and eluted was 15–21% of that applied, for both the cytosolic and microsomal form. In our laboratory, this is the maximum binding observed for this resin.

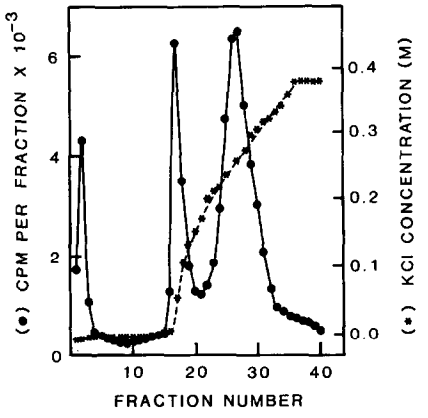

Fig. 3. Ion-exchange chromatography of solubilized microsomal progesterone receptor. A 2-ml DEAE-Sephacel column was equilibrated in P buffer, and 9 ml of solubilized microsomal receptor was applied. Two-ml fractions were collected. A linear KCl-gradient was begun at fraction No. 13. See the Experimental section for further details.

Binding of activated cytosolic receptor to microsomes

Because the microsomal receptor so closely resembled activated cytoplasmic receptor, we attempted to discover whether the cytoplasmic form would bind to microsomes.

Microsomes as isolated bound ~10% of the [^{3}H]progesterone bound by cytosol. The total amount of receptor in the oviduct was dependent on the tissue weight and stage of the laying cycle; in a typical experiment, the cytosol contained 320 fmol of receptor and 940 mg protein, and the microsomal preparation contained 28 fmol of receptor and 34 mg protein.

Microsomes were incubated either with free [^{3}H]progesterone or with [^{3}H]progesterone-labeled cytosolic receptor, at 25°C for 1 h. The amount of radioactivity was equivalent in both incubations. At the end of the incubation period, the microsomes were pelleted by centrifugation at 105,000 g × 60 min, rinsed, and resuspended in buffer. Excess steroid was removed with charcoal/dextran. When microsomes were labeled with cytosolic receptor, the amount of binding observed was 3-fold higher than when microsomes were labeled with free steroid. (Table 1).

This higher degree of microsomal binding could also be accomplished by incubating (unlabeled) chick post-mitochondrial supernatant, with [^{3}H]progesterone at 25°C and then separating cytosol from microsomes and removing free steroid. Under these conditions, the microsomes contained 8–12% of the radioactivity of the cytosol. If incubation took place at 0°C or in the presence of 20 mM molybdate, conditions which prevent activation from occurring [8, 14, 24], however, the microsomes contained only 1–2% of the radioactivity. (Table 1).

This binding was saturable. When increasing

Table 1. Effect of temperature, molybdate and method of labeling on microsomal progesterone binding

Preparation	cpm bound/mg protein 10^{-4}	% Of radioactivity in microsomes
Hen microsomes		
labeled with free steroid	1.81	
labeled with labeled cytosol	6.25	
Chick cytoplasm		
25°C, no $MoO_4^=$	15.3	10
cytosol microsomes	18.1	
0°C, no $MoO_4^=$	12.1	2
cytosol microsomes	4.59	
25°C, 20 mM $MoO_4^=$	25.0	1
cytosol microsomes	3.71	

amounts of cytosolic receptor were incubated with a constant quantity of microsomal protein, a plateau of specific binding was reached at ~300 fmol/mg protein. (Fig. 5).

After incubation, both cytosol and microsomes were extracted with ethyl acetate and the extract chromatographed on silica gel thin-layer plates in a system of benzene–ethyl acetate, 8:2. Authentic progesterone and 5α-pregnane-3,20-dione, the predominant metabolite at 37°C [25], were included as standards. The developed plates were sprayed with EN^{3}HANCE and exposed to X-ray film for 7 days at −70°C. The fluorogram is shown in Fig. 6. Areas of the plate which displayed radioactivity were scraped and counted in Econofluor.

Most of the ^{3}H included in the incubations was still present as progesterone at the end of the incubation period, for cytosol and microsomes. There was no metabolism of progesterone by microsomes. (Table 2).

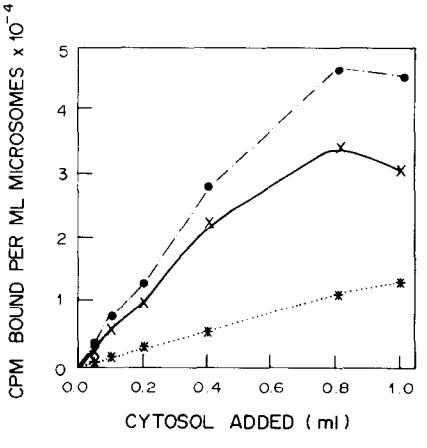

Fig. 5. Saturation of microsomes with cytosolic progesterone receptor Non-solubilized microsomes, 0.5 ml suspended in P buffer, were incubated for 1 h at 25°C with increasing amounts of labeled cytosolic progesterone receptor, in the presence (NS) or absence (T) of excess unlabeled progesterone. The total volume was adjusted to 1 ml with buffer. At the end of the incubation period, the microsomes were pelleted, rinsed, resuspended in buffer and treated with charcoal to remove free steroid. T, total, ●——●; NS, non-specific, *——*; specific binding = T-NS, ×——×.

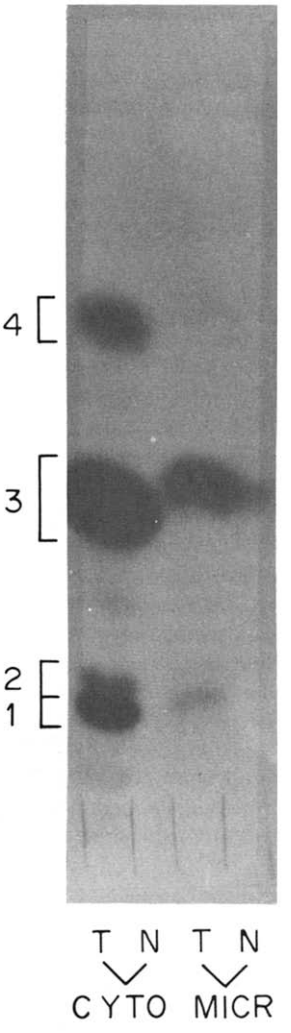

Fig. 6. Fluorogram of thin-layer chromatograph: extract from cytosolic and microsomal progesterone receptor. The ethyl acetate extract was chromatographed on silica gel in benzene–ethyl acetate, 8:2. Fluorography was carried out as described in the Experimental section. Four fractions, corresponding to the areas numbered 1–4, were scraped from each lane and counted. Blanks, of silica gel from non-radioactive lanes, were subtracted. Radioactivity in each fraction is presented in Table 2. T, total; NS, non-specific.

When microsomes from non-target tissues were incubated with free [^{3}H]progesterone, little specific binding was observed. However, when incubated with labeled cytosolic receptor, these microsomes did exhibit binding. Some of them in fact, had a higher specific activity of binding than did oviduct microsomes. (Table 3).

Table 2. Thin-layer chromatography of steroids extracted from progesterone receptors

Area	% Of radioactivity recovered Cytosol		Microsomes	
	T	NS	T	NS
1	6	3	8	6
2	2	2	5	8
3 (progesterone)	84	85	80	75
4 (5α-pregnanedione)	8	10	7	11

Table 3. Progesterone-binding activity of microsomes from various tissues

Tissue	cpm bound/mg protein ($\times 10^{-3}$) Free steroid	Labeled cytosol
Oviduct	4.64	29.2
Heart	0.124	18.9
Lung	0.065	24.2
Liver	1.25	33.5
Intestine	0	86.5

Microsomes were prepared from hen heart, lung, liver and intestine as for oviduct, as described in the text. A constant amount of microsomal protein, 0.42 mg, was incubated for 2 h at 25°C together with free [^{3}H]progesterone or labeled cytosol in a total volume of 1 ml. Binding to microsomes was determined as for oviduct. See text for details.

Binding of receptor to microsomes could be abolished to a large extent by treatment of microsomes with RNase. After such treatment the microsomes bound only 34–50% of their original capacity. Trypsin and phospholipase A had no effect on binding (Table 4).

DISCUSSION

The results presented here suggest that microsomes of hen oviduct bind activated cytosolic progesterone receptor. This binding does not appear to be due to cytosolic contamination: only ~4 S receptor appears in the microsomal fraction, even when cytosol contains 4 and 8 S forms, and cytosolic enzyme markers are present in very low concentration.

The microsomal receptor, both solubilized and non-solubilized, is notably more heat-labile than the cytosolic receptor. Addition of neither exogenous protein nor sodium molybdate helped to stabilize the microsomal receptor. Therefore, the concentration of microsomal sites may be an underestimate. Our estimate is slightly lower than that of Haukkamaa *et al.*[7].

Because we have solubilized only 65–88% of the microsomal receptor, we cannot rule out the possibility that there is yet another microsomal receptor which is different from the cytoplasmic form. The great majority of microsomal receptors, however, appear to be derived from cytosolic receptors.

The finding that microsomes from non-target tissues also bind activated receptors may be taken to mean that the microsomal receptor is merely an artifact of the high affinity of activated receptor for all polyanions, including the RNA of microsomes. However, this general affinity does not exclude the possibility of specific binding sites in the microsomes, just as activated receptors will bind to any heterologous DNA, but exert their influence on transcription of specific gene sequences. Chong and Lippman have found RNA associated with receptors from MCF-7 cells and have suggested that steroid–receptor complexes may bind to specific RNA having appropriate nucleotide sequences [26]. It is of interest in this regard that ribosome-associated estrogen receptors have been shown to affect protein synthesis in the rat uterus [3] and that cGMP can mimic some of progesterone's effects in the chick oviduct [27].

Table 4. Binding of cytosolic progesterone receptor to microsomes after enzyme treatment

Treatment	cpm bound/mg protein ($\times 10^{-4}$)	% Of control
Control	1.22	100
Trypsin	1.38	113
Phospholipase A	1.55	127
RNase	0.61	50

One-ml aliquots of solubilized microsomes were incubated at 37°C for 10 min with one of following: buffer alone (control); RNase A, 1 mg/ml; trypsin, 1 mg/ml; phospholipase A, 0.14 mg/ml. The trypsin and phospholipase incubations also included Ca Cl_2, 20 mM. At the end of the incubation, the microsomes were pelleted, rinsed, resuspended, and incubated with [^{3}H]progesterone labeled cytosol.

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