The Nonactivated Progesterone Receptor Is a Nuclear Heterooligomer*

The discovery of the nuclear localization of estradiol and progesterone receptors in the absence of the steroid hormone has led to reconsideration of the model of cytoplasmic to nuclear translocation of these receptors upon exposure to hormone. Unoccupied nonactivated receptors are thought to be weakly bound to nuclei of target cells from which they are leaking during tissue fractionation and thus found in the cytosol fraction of homogenates in a nontransformed heterooligomeric "8-9 S" form, which includes hsp90. However, no direct biochemical evidence has yet been obtained for the presence of such heterooligomers in the target cell nucleus, possibly because it dissociates in high ionic strength conditions used for extraction of the nuclear receptor. We took advantage of the combined stabilizing effects of tungstate ions and antiprogestin RU486 to extract a nuclear non-DNA binding nontransformed, 8.5 S-RU486-progesterone receptor complex from estradiol-treated immature rabbit uterine ex- 

munsibiochemical data demonstrating the nuclear localization of estradiol and progesterone receptors (PR), including Mr 59,000.

Biochemical analyses of steroid hormone receptors have been hampered by extraction conditions. In the hormone-free state, receptors are easily extracted in low salt-containing medium and are recovered in the cytosol fraction of homogenates, a result contrasting with immunohistochemical data demonstrating the nuclear localization of estradiol and progesterone receptors (PR),1 including in the absence of steroid (1-3). These unoccupied cytosol receptors are heterooligomers which include hsp90 (4-8) and, in the case of mammals, another nonhormone binding protein p59 (9). In the presence of hormone, the receptors become difficult to extract in low salt medium, and high ionic strength (≥0.4 M KCl) is then required to recover the major part from nuclei (10), probably because receptors are tightly bound to chromatin components. Unfortunately, ionic strength is well known to also dissociate the large non-DNA binding nontransformed ("8 S") form of steroid receptors found in the cytosol (11, 12). Numerous reports have indicated that transition metal oxanions protect this large receptor form from high ionic strength- or heat-induced dissociation (transformation) and the subsequent ability to bind DNA. Among these agents, molybdate ions have been the most widely used, and tungstate ions have also been described as effective (13, 14). In this work2 we used the higher stabilization effect of tungstate ions, as compared with molybdate ions, against the KCl-induced transformation of the "8 S" rabbit uterus PR, to identify a nuclear form of PR containing hsp90 and p59. This form was also stabilized by the antiprogestin RU486 which has been previously shown to maintain the association between rabbit PR and hsp90 (15).

MATERIALS AND METHODS

The following compounds were used. [3H]R5020, specific activity 1.88 TBq/mmol, and [3H]RU486, specific activity 1.85 TBq/mmol, were a gift from Roussel-Uclaf (Romainville, France). All reagents were analytical grade and purchased from Merck (Darmstadt, Federal Republic of Germany). Other biochemicals were obtained from the following sources. Protein A-Sepharose and DNA-cellulose (1.9 mg of DNA/ml of cellulose) were from Pharmacia (Uppsala, Sweden); acrylamide, N,N,N',N'-tetramethylenediamine was from Kodak; monoclonal antibody raised against nontransformed rabbit PR and reacting against rabbit p59 (16): AC88, mouse monoclonal antibody raised against nontransformed rabbit PR and reacting against rabbit p59 (16); AC88, mouse monoclonal antibody raised against a protein of M, 88,000 of Achlya ambisexualis (17) and reacting with hsp90, was a gift from Dr. D. O. Toft (Mayo Clinic, Rochester MN); mPRRI mouse monoclonal antibody raised against the purified rabbit uterus PR (18) and recognizing the intact receptor as well as the 85,000-dalton proteolytic fragment in immunoblot (Transbio, Paris, France).

Preparation of Cytosol from Rabbit Uterus—Cytosol from the uterus of estrogen-stimulated immature rabbits (New Zealand White) was prepared in 10 mM phosphate, 10% glycerol (v/v), pH 7.8 (PG buffer) containing either 20 mM molybdate (PGM) or 20 mM tungstate (PGW) as described previously (7, 15). The homogenate was supplemented by a mixture of protease inhibitors (Sigma), and 1 µM radioc- inent cortisol was added to the cytosol in order to block transcortin and glucocorticosteroid receptor (GR) binding sites (7, 15). Cytosol was incubated at least 2 h at 0 °C with 20 nM either [3H]RU486 or [3H]R5020 in order to label PR.

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1 The abbreviations used are: PR, progesterone receptor; GR, glu- cocorticosteroid receptor; R5020, 17,21-dimethyl-19-nor-pregna-4,9- dien-3,20-dione; RU486, 17a-(l-propynyl)-estra-4,9-dien-3-one; hsp90, heat shock protein of the Endocrine Society, Seattle, WA, June 21-24, 1989, Abstr. 992.
Preparation of Nuclear Extract from Rabbit Uterus Explants—

Pieces (~1 mm³) of uterus (3 g) from estrogenized immature rabbit were incubated for 1 h at 37 °C in Dubelco's modified Eagle's medium (10 ml) with 40 nM \([1^H]R5020\) or \([1^H]RU486\) in the presence of 1 μM cortisol. After incubation, pieces of tissues were rinsed 2 times with ice-cold 20 mM tunicaste containing phosphate buffer saline followed by centrifugations (700 x g for 10 min). Homogenization was carried out in 2 volumes of PGW buffer and protease inhibitors, as described previously (7), using a glass/grass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 105,000 x g for 1 h, and the pellet was washed once with 50 ml of Triton X-100 (1%) containing buffer (5 mm Tris, 0.33 M sucrose, 3 mM MgCl₂, pH 7.6, at 4 °C) and twice with the same volume of buffer without Triton. The pellet was resuspended in 1 volume of 0.4 M KC1 containing PGW buffer supplemented with protease inhibitors (7), and extraction was carried out for 60 min at 0 °C. Soluble nuclear fraction, referred to as 0.4 M KC1 nuclear extract, was obtained by centrifugation of the nuclear suspension at 105,000 x g for 30 min.

Cross-linking with Dimethylpimelimidate—This reaction was performed as described previously (19) on the cytosol and the nuclear extract.

Sucrose (5-20%) Gradient Ultracentrifugation Analysis—Aliquots to which was added or not antibody EC1 for 4 h at 4 °C were loaded on preformed 5-20% sucrose gradients and centrifuged in a SW 60 rotor (Beckman) for 16 h at 46,000 rpm at 4 °C with enznic inhibitors (glucose oxidase, 7.9 S and peroxidase, 3.6 S).

Immunopurification of Nuclear PR—Anti-p59, EC1, monoclonal antibody, was coupled to Affi-Gel 10 agarose beads as described previously (9), and anti-PR, mPR II monoclonal antibody, was cross-linked to Protein A-Sepharose as described in Logeat et al. (18). Two or 9 ml of nuclear extract containing ~10 pmol of PR/mg of protein were rotated overnight with both of these immunoaffinity matrices (0.3-0.5 ml containing 0.05-0.2 mg of antibody/ml) equilibrated in PGW. The gels were then rinsed with 10 ml of PGW buffer and eluted 2 times with 0.3 ml of 50 mM sodium lauryldimethylamine, 20 mM tunicaste, pH 10.4, for 30 min. After immediate neutralization, the two eluates were mixed and referred to as Affi-Gel 10-EC1, or Protein A-mPR II eluate, respectively. Control experiments were carried out by loading the same amount of 0.4 M KC1 nuclear extract onto either Affi-Gel 10 or Protein A-Sepharose nonimmune mouse IgGs; these immunoabsorbsents were treated as described above. Their eluates were referred to as Affi-Gel 10 and Protein A-nonimmune eluates.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting Analyses—Electrophoresis on 7.5% denaturing polyacrylamide gels (20) was conducted at 35 mA/gel for 4 h, with rainbow markers (Amersham Corp.) as molecular weight standards. Transfer of proteins to nitrocellulose (0.45 μm, Schleicher & Schuell) was carried out with a horizontal X Blot apparatus (Cera Labo, France) for 90 min. Transfer of proteins to nitrocellulose (0.45 μm, Schleicher & Schuell) was carried out with a horizontal X Blot apparatus (Cera Labo, France) for 90 min. Markers (Amersham Corp.) as molecular weight standards. Transfer of proteins to nitrocellulose (0.45 μm, Schleicher & Schuell) was carried out with a horizontal X Blot apparatus (Cera Labo, France) for 90 min. The gels were then rinsed with 10 ml of PGW buffer and eluted 2 times with 0.3 ml of 50 mM sodium lauryl maltoside, 20 mM tunicaste, pH 10.4, for 30 min. After immediate neutralization, the two eluates were mixed and referred to as Affi-Gel 10-EC1, or Protein A-mPR II eluate, respectively. Control experiments were carried out by loading the same amount of 0.4 M KC1 nuclear extract onto either Affi-Gel 10 or Protein A-Sepharose nonimmune mouse IgGs; these immunoabsorbsents were treated as described above. Their eluates were referred to as Affi-Gel 10 and Protein A-nonimmune eluates.

Protein Measurements and Radioactivity Counting—Proteins were measured by the method described by Schaffner and Weissmann (21), and radioactivity was measured in a Scintillation Minaxi counter (Packard Instruments) with 55% efficiency.

RESULTS AND DISCUSSION

First, we tested the effect of tunicaste ions on the stabilization of rabbit uterus cytosol PR. Experiments depicted in Fig. 1 establish that, once stabilized by tunicaste ions during (or soon after) homogenization in low salt medium (PG buffer), the rabbit uterine cytosol PR, labeled with the synthetic progestin [1^H]R5020 or the antagonist [1^H]RU486, sediments as a 9.5 S entity on density gradient prepared in PG buffer (Fig. 1a) and as 8.5 S when the PG buffer contains 0.4 M KC1 (Fig. 1b). In contrast to tunicaste ions, molybdate ions were unable to prevent the KC1-induced decrease to 4.5 S of the 9.5 S-PR complexes (Fig. 1b). The 9.5 S PR form, stabilized or not by oxyanions, includes in addition to the hormone binding unit, two nonhormone binding proteins, hsp90 (4, 7, 9) and p59 (9). Upon incubation with EC1, the 8.5 S-PR observed in the 0.4 M KC1-containing gradients. Identical patterns were obtained in the presence of oxyanions and after incubation with EC1, c, samples containing ~1.2 pmol of cross-linked PR were incubated with 20 μg of mPR (O---O) or not (A---A) and analyzed as in b. Positions of internal standards are indicated by arrows. GO, glucose oxidase; PO, peroxidase.

FIG. 1. In vitro KC1 activation of the cytosol rabbit PR stabilized by molybdate or tunicaste ions. a, samples containing ~1.2 pmol of [1^H]R5020-PR were incubated with 30 μg of EC1 (O---O) or not (A---A) and then loaded on a 5-20% sucrose gradient in PG buffer. Superimposable profiles were obtained for tunicaste or molybdate-stabilized [1^H]R5020-PR analyzed in PGW or PGM, respectively, and also for [1^H]RU486-labeled PR (not shown); b, samples containing ~1.2 pmol of tunicaste (A---A) or molybdate (C---C) stabilized [1^H]R5020-PR were run in 0.4 M KC1-containing gradients. Identical patterns were obtained in the presence of oxyanions and after incubation with EC1, c, samples containing ~1.2 pmol of cross-linked PR were incubated with 20 μg of EC1 (O---O) or not (A---A) and analyzed as in b. Positions of internal standards are indicated by arrows. GO, glucose oxidase; PO, peroxidase.
continuous presence of tungstate ions. In both RU486 and R5020 experiments, ~30% of radioactivity was found in the nuclear fraction after 60-90 min of incubation. Western blotting of nuclear extracts with mPR II revealed a major band corresponding to progesterone binding, PR B unit (~120 kDa), and two minor bands, at ~85 kDa (likely subunit A) and ~75 kDa. In addition, positive signals were observed with AC88 and EC, at M₉, 90,000 and 59,000, respectively. AC88 was described to recognize hsp90 in a large variety of species (17), and we showed recently that EC, recognizes the M₉, 59,000 protein in several mammals (22). These findings indicate that hsp90 and p59, respectively, were also present in the nuclear extracts (Fig. 2, a and b, 1). To assess if the three proteins were associated to form the nuclear heterooligomeric component in a multimeric structure. In contrast, in the R5020 experiment (Fig. 2b, 2), only PR subunits (~120 and 85 kDa) were found, suggesting that dissociation of the preexisting heterooligomer occurred upon agonist binding and, at the same time, indicating the lack of nonspecific interactions during these experimental procedures.

In parallel experiments, we studied the nuclear PR by sucrose gradient ultracentrifugation analysis. In the RU486 incubation experiment, only a ~8.5 S peak, nondisplicable by EC, was observed (not shown), suggesting release of p59 and thus apparently in contradiction with the results obtained after immunopurification. However, if the nuclear extract was treated with dimethylpimelimidate immediately after extraction, then part of 8.5 S PR analyzed after cross-linking in high salt gradient was displaced to ~10 S after incubation with EC (Fig. 3a). The complete dissociation of p59 in the absence of cross-linking is best explained by the prolonged incubation of the p59-containing heterooligomer in the 0.4 M KC1 extract with the antibody. The partial dissociation of p59 in the cross-linking experiment is also probably due to the 1 h exposure to high salt during extraction. It is likely that the 0.4 M KC1 nuclear extract contains a heterogeneous population of RU486-PR complexes, some including p59 and others lacking p59 that has dissociated in the presence of high salt. Conversely, dissociation does not seem to occur when immunoadsorption is immediately performed (Fig. 2a, 2). Therefore, immunopurification of the non-cross-linked nuclear PR.
on Affi-Gel 10-EC, affinity resin was also performed. Only part of the RU486 PR complexes was bound to the column; the eluted PR migrated as a sharp ~9 S peak in density gradient and was completely displaced to ~10.5 S by EC1 (Fig. 3b). This result demonstrates that it is possible to isolate a homogeneous population of nuclear heterooligomeric PR containing hsp90 and p59. Unfortunately, similar experiments of immunoadsorption and sucrose gradient analysis are not possible with any anti-hsp90 antibody that we tested, namely AC86 which does not interact with hsp90 included in the 8.5 S nontransformed form of PR (17). While these results were obtained with RU486 as ligand, in the R5020 experiment and as expected from data of Fig. 2b, 2, no significant 8-9 S large PR form was found in the nuclear extract. Only a ~4.5 S PR, neither displaced by EC1 (Fig. 3c) nor retained on Affi-Gel 10-EC, affinity resin (not shown), was found. This form, unlike the RU486-bound one, was apparently unstable and rapidly released free radioactive R5020. According to the immunofluorescence data reported above (Fig. 2b, 2), it is likely that this 4.5 S PR did not appear during incubation and ultracentrifugation but rather was initially present in the nuclear extract of agonist-treated explants.

Control experiments demonstrated that always cytosol contamination can be ruled out, since lactate dehydrogenase was never found in nuclear extracts, while normal lactate dehydrogenase levels were measured in cytosol supplemented or not with 0.4 M KCl. From these results, we conclude that hsp90 as well as p59 are originally associated to the PR in the nuclei of immature rabbit uterine cells. Tungstate ions do not induce artificial formation of heterooligomer during homogenization and extraction, as indicated by results with R5020 (Fig. 3c); these ions appear only required to maintain hsp90 bound to the receptor exposed to high salt in the absence of chemical cross-linking. The latter is still necessary to prevent the release of p59.

We also measured the DNA cellulose binding of the nuclear receptor, a criterion for in vitro activation. After 60 min of incubation with the explants with agonist or antagonist, the DNA cellulose binding of nuclear 8.5 S [3H]RU486-PR, as expected for a nontransformed receptor form, was much lower than that of the 4.5 S [3H]R5020-PR (5 versus 46%).

How does the observation of a nuclear stabilized RU486-8 S receptor form fit with data related to receptor function and RU486 mechanism? Altogether, our results are consistent with the decrease of RU486-PR and -GR complexes tight binding to nuclei, as compared with that of agonist-receptors complexes (26, 27), and the lack of down-regulation observed after 30 min of hormone binding (28) or chronomastiguin but in contrast to the effect of progestin (30). The subcellular distribution of GR is still a controversial matter, but there is definitively nuclear GR in the absence of hormone and agonist-induced increased binding to nuclei (31). Stabilization of 8 S GR may also explain that experimentally the chemical methylation of hormone response element of the glucocorticosteroid-regulated tyrosine aminotransferase gene is possible, in intact cells, in the presence of RU486, contrary to dexamethasone (32). Differently, other observations are not in favor of the hsp90 stabilization hypothesis and its consequence on RU486-induced lack of DNA binding of the receptor. For instance, in vitro binding of RU486-PR and -GR complexes to corresponding HREs (23, 33) was reported, but the receptors which were used were dissociated from hsp90, and this experimental situation may be artificial. Our data may also appear to contradict reports of competition by RU486-GAL-GR chimera (34) or RU486-PR (36) complexes for the transcriptional activity of GAL4 or constitutively active truncated PR, respectively, suggesting total or partial release of hsp90 from receptors upon binding of RU486 and subsequent binding of RU486-receptor complexes to HRE. Antihormone activity would then be due to the abortive nature of the complexes at the DNA binding level (imperfect interaction) or after it (e.g. steric hindrance precluding binding of transcription factor(s) to the DNA or the receptor itself).

In fact, there is no direct evidence for DNA binding activity of RU486-receptor complexes in intact cells, and the competitive effect in the transcription experiments may simply be due to physically impeded access to DNA of active GAL4 or truncated receptor by the nuclear stabilized RU486-8 S receptor. In any case, it is presently impossible to reason in terms of “simple” receptor-DNA interaction with models not taking into account transcription factors, probably other chromatin proteins, phases of nucleosomes (as demonstrated in the promoter region of the glucocorticosteroid-inducible mouse mammary tumor virus (37)), and yet undefined features of chromatin structure.

Presently no data contradict the hsp90-stabilizing hypothesis, but it may well be that the antihormone effect depends on several, possibly sequential, molecular mechanisms. For instance, there could be a reordering effect on activation, and secondarily, formation of abortive RU486-receptor complexes as described above. In fact, we have observed an increase of DNA cellulose binding (23%) of [3H]RU486-PR complexes isolated from nuclei of explants exposed for 90 min to the antihormone. In parallel, we noted the appearance of a form sedimenting at ~6 S with a concomitant decrease of 8.5 S RU486-PR (not shown). With regard to previous reports on both the estrogen (8) or the glucocorticosteroid (38) receptors, the 6 S form may be a dimeric form of PR, as recently demonstrated (39).

It is clear that more work is still needed to elucidate the RU486 mechanism in detail. It remains that the present report strongly suggests that, originally the absence of hormone as after stabilization by RU486, the nuclear receptor is present in a heterooligomeric non-DNA binding form, presumably inactive. Even if a role of nuclear p59 in the heterooligomeric form of steroid receptor has not yet been established, we predict that it may undergo the hsp90-receptor complex within the nucleus until hormone binds to receptor which is then released and can initiate the response.

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3 Recently, the dioxin receptor (40) was reported to associate with hsp90 in a nuclear 8 S species.
