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Progesterone Receptor Does Not Form Oligomeric (8S), Non-DNA-Binding Complex in Intact Cell Nuclei

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Abstract We raised a polyclonal antibody, αD, against a synthetic peptide (amino acids 522–535) of chicken progesterone receptor (PR). The sequence is located between the DNA-binding domain and the hormone-binding domain in the region within the sequences required for stability of the oligomeric form of PR. In the immunoblot, αD reacted with both A and B forms of PR. In the sucrose gradient and dot-blot the antibody did not recognize the so-called 8S form of PR, which is an oligomeric complex of PR and other proteins. When the oligomeric complex was dissociated by salt treatment, the antibody recognized the resulting 4S form of PR. This would suggest that the epitope is masked in the 8S form of PR and exposed in the 4S form. To study whether a similar complex exists in vivo, we used the antibody for immunohistochemistry. Two different fixation techniques were employed, freeze-drying-vapor fixation and liquid fixation. In the animals not treated with progesterone, intensive nuclear staining was detected independent of the fixation technique. When receptor from similarly treated animals was analyzed by sucrose gradient, all of the receptor molecules were in the oligomeric complex (8S). Ligand binding is known to promote a dissociation of this complex. Thus progesterone treatment should lead to an increased immunodetection of the epitope; however, progesterone treatment decreased the intensity of PR immunostaining. These results suggest that the oligomeric complex (8S), present in tissue extracts, does not exist in intact cell nuclei. They also call into question the proposed role of hsp90 in regulating progesterone receptor function.

Key words: steroid receptor, hsp90, gene regulation, molecular composition, immunohistochemistry

Steroid receptors are transcription factors whose activity is regulated by hormone binding [Gronemeyer, 1992; Carson-Jurica et al., 1990]. The mechanism whereby steroids regulate the activity of the receptors is unknown. Steroid binding might change receptor structure to ensure DNA binding, dimerization and interaction with other transcription factors [Gronemeyer, 1991]. Steroid binding is known to increase phosphorylation of the receptor [Auricchio, 1989], which may be an important early regulatory step. A widely accepted hypothesis is that nonliganded receptor is nonactive due to binding of inhibitory factors. Upon ligand binding, the inhibitory factors dissociate and receptors become active [Renoir and Mester, 1984]. Evidence for these inhibitory factors comes from early steroid receptor studies in which nonliganded receptors were seen to form oligomeric complexes, which sediment as 8S in sucrose gradient centrifugation and which do not bind to DNA in vitro. Elevated salt conditions and steroid administration promote the dissociation of the complex and result in a receptor form which sediments as 4S in sucrose gradient and is able to bind to DNA in vitro [Dougherty et al., 1984].

Heat shock protein 90 (hsp90) was the first protein shown to interact with the steroid receptors in the oligomeric complex. When nonactivated chicken progesterone receptor was purified, hsp90 was found to be associated with the receptor [Catelli et al., 1985]. hsp90 was found to be associated also with other steroid receptors [Joab et al., 1984]. hsp90 interacts with the hormone binding domain and that which is between the DNA binding and hormone binding domains, and it is thought to inactivate steroid receptors by steric interference with DNA binding [Carson-Jurica et al., 1989]. There are also other proteins that copurify with nonactivated steroid receptors, but it is not known how they interact with the receptors [Kost et al., 1989; Smith et al., 1990; Smith and Toft, 1992].

In spite of a vast on literature the structure and dissociation of the oligomeric (8S) form of

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the steroid receptors in vitro [Carson-Jurica et al., 1990; Baulieu, 1987; Pratt, 1990], there is no evidence as to whether nuclear steroid receptors (estrogen receptor and progesterone receptor) form such an oligomeric complex in vivo. We have recently presented evidence that the hsp90 is not associated with nuclear progesterone receptor in vivo in chicken tissues [Pekki, 1991; Tuohimaa et al., 1993]. To further study whether the oligomeric complex exists in vivo, we raised an antibody against an epitope of PR that is hidden in the oligomeric form and that becomes accessible when the oligomeric form dissociates. It emerged that the epitope is fully detectable by immunohistochemistry, even though all the receptors extracted from these tissues are in oligomeric form.

MATERIALS AND METHODS

Animals and Treatments

Immature 2-week-old white Leghorn chicks were treated with β-estradiol-benzoate (Sigma Chemical Co., St. Louis, MO) (1 mg/animal/day) for 1 week, followed by a withdrawal period of 1 week. Thereafter, they received a single dose of progesterone (Merck, Darmstadt, Germany) (20 mg/kg body weight) or sesame oil (0.05 ml/animal). The chicks were killed 1, 6, 18, or 48 h after injection. Oviduct pieces (2–4 mm in diameter) were dissected for use in immunohistochemical studies.

Cytosol Preparation

Tissue samples were homogenized in 4–6 vol of TE-buffer (10 mM Tris, 12 of Tris-buffer (10 mM Tris, 12 mM monothioglycerol, 1.5 mM EDTA and 10% glycerol, pH 7.4) at 0°C. The protease inhibitor phenylmethyl sulfonyl fluoride was added to the buffer in a final concentration of 0.9 nM. For immunoblotting leupeptin (0.1 mM) and aprotinin (77 μg/ml) were included as protease inhibitors during the homogenization. A supernatant fraction obtained by centrifugation (100,000*g* for 1 h at +4°C) was used as cytosol. For some experiments, sodium molybdate, Na_2MoO_4 (final concentration 50 mM), or KCl (final concentration 0.3 M) was added and incubated at 0°C for 1 h after centrifugation.

PR Antibodies

The peptide (Leu-Thr-Gln-Arg-Leu-Ser-Phe-Ser-Pro-Asn-Gln-Glu-Ile-Pro-Lys) corresponding to residues 522–535 of chicken PR-B [Gronemeyer et al., 1987; Conneely et al., 1987] was conjugated to thyroglobulin (TG) through the lysine. Male California rabbits were injected with 100 μg of the peptide in 0.5 ml of Freund's complete adjuvant. Antigen injections were repeated five times in Freund's incomplete adjuvant. Sera were precipitated by dropwise addition of saturated $(NH_4)_2SO_4$ to a final concentration of 40% (v/v). IgG(αD) was tested by direct immunoassay against the corresponding ovalbumin-conjugated peptide (2 μg/ml) immobilized on solid phase (Immunoplate, Nunc, Roskilde, Denmark). We also used polyclonal rabbit antibody 907 [Weigel et al., 1989], similar to αD, and the mouse monoclonal antibody PR22, which recognizes both A and B components of PR [Sullivan et al., 1986; Scheiner et al., 1988].

Immunoblotting

The PR was precipitated with ammonium sulfate (a final concentration of 35%) and centrifuged. The pellet was taken in 4 vol of sample buffer (62.5 mM Tris–HCl, 10% glycerol, 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 0.05% bromophenol blue) and boiled for 5 min. The proteins were resolved in 12% polyacrylamide slab gels containing 0.1% SDS [Laemmli, 1970] and transferred to nitrocellulose membrane with an electrophoretic transfer apparatus (Mini Trans-blot, BioRad, Richmond, CA). The membranes were saturated with 5% bovine serum albumin (BSA) in TBS (50 mM Tris, 0.9% NaCl, pH 8.0) and then incubated with the primary antibody, αD (5 μg/ml) or PR22 (0.5 μg/ml), at 4°C overnight. After washing, the membranes were incubated with peroxidase-conjugated goat antirabbit IgG (Cappel, West Chester, PA) diluted 1:10,000 in TBS containing 1% BSA. The peroxidase was visualized using the ECL method according to the instructions of the manufacturer (Amersham, UK). The molecular-weight standards were phosphorylase b M_r 97,000, BSA M_r 66,000, ovalbumin M_r 45,000, carbonic anhydrase M_r 31,000, soybean trypsin inhibitor M_r 21,000, and lysozyme M_r 14,000 (BioRad, Richmond, CA).

Sucrose Gradient Centrifugation

Cytosol was labeled with ^{3}H-Org 2058 (Amersham, England) (final concentration of 10 nM) at 0°C for 2 h. The unbound ligand was removed with dextran-coated charcoal. The cytosol was then incubated with PR22 or αD antibody at a

dilution of 20 μg/ml at 0°C for 4 h. A sucrose gradient (5–20%) was prepared in 10 mM Tris, 12 mM monothioglycerol, 1.5 mM EDTA, 10% glycerol, 50 mM Na_2MoO_4, 0.15 M KCl, at pH 7.5. The tubes were centrifuged at 200,000*g* for 16 hs. Fractions (2 drops) were collected by piercing the bottom of the tube and counted for radioactivity in a liquid scintillation counter (Ultrabeta 1210, LKB, Wallac). Glucose oxidase (7.9 S) and horseradish peroxidase (HPO) (3.6 S) were used as internal standards.

Immunoprecipitation of PR

The experiment was carried out as described by Kost et al. [1989]. The PR was immunoprecipitated from KCl-treated cytosol. A part of cytosol was incubated with 10 nM progesterone for 2 h at 4°C. The antibody αD was used at concentration 30 μl/ml. The immune complexes were resolved by using 7.5% SDS–PAGE. The immunoblotting was made as described above. PR22 was used as a primary antibody in immunoblotting.

Immunohistochemistry

Oviduct samples were processed for immunohistochemistry as previously described [Pekki and Tuohimaa, 1989]. Briefly, the freeze-dried samples were fixed in *p*-benzoquinone vapor for 3 h and then embedded in paraffin. Some samples were immersion-fixed in Baker's fluid (4% paraformaldehyde and 1% $CaCl_2$ in distilled water, pH 6.7) for 1.5 h at 0°C. Specimens were then dehydrated and embedded in paraffin. All sections (5 μm) were stained using the avidin–biotin and immunoperoxidase technique (Vectastain Reagents, Vector Laboratories, Burlingame, CA). The concentration of the primary antibodies was 4 μg/ml.

Histochemical Controls

In order to verify the specificity of the staining of PR, primary antibody was substituted with phosphate buffer or an equal concentration of a nonspecific antibody (monoclonal antihuman-LH, Medix Biochemica, Finland). The presaturation controls were made with affinity-purified B subunit of PR and ovalbumin-conjugated synthetic peptide by incubating them with the primary antibody at 0°C for 4 h.

Presaturation experiments were also carried out with a chicken oviduct cytosol prepared in molybdate-containing buffer, where PR is mainly in the 8S form, and with a cytosol treated with 0.3 M KCl for 2 h, where PR is in 4S form. The cytosols were incubated with the primary antibody at 0°C for 2 h.

A further presaturation experiment was performed with cytosol prepared from COS cells (monkey kidney cells) transfected with chicken PR cDNA. The COS cells were grown in Dulbecco's modified medium supplemented with 5% fetal calf serum (FCS), then plated at 20–30% confluence in Petri dishes. Whole-length chicken PR cDNA [Jeltsch et al., 1986], subcloned into the eukaryotic expression vector pSG5 [Green et al., 1988], was transfected into the cells using the calcium-phosphate technique [Wigler et al., 1978]. The calcium–phosphate precipitate was removed 18–22 h later, and the cells were further incubated for 24 h, harvested into phosphate-buffered saline (PBS), and placed in extraction buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 0.4 M NaCl and 10% glycerol). The transfected COS cells and an identical sample of untreated cells were broken by three cycles of freezing in liquid nitrogen and thawing on ice. To obtain the cytosol, the homogenate was centrifuged for 15 min at 16,000*g*.

Immunodot Assay

The molybdate (8S) and salt-treated (4S) cytosols were diluted 1:10 with corresponding buffers. 250 μl of cytosol was applied on nitrocellulose membrane using a dot-blot apparatus at 4°C. The membranes were fixed in Baker's fixative (4% paraformaldehyde and 1% $CaCl_2$ in distilled water, pH 6.7) for 2 h at 4°C and washed with PBS (3 × 10 min). The membranes were incubated with the blocking buffer (5% BSA, 0.05% Tween in PBS) at 37°C for 1 h and thereafter with the primary antibodies diluted in dilution buffer (1% BSA and 0.05% Tween in PBS). The final concentrations of antibodies were 0.5 μg/ml (PR22) and 5 μg/ml (αD). They were then washed with the dilution buffer and incubated with secondary antibody (peroxidase-conjugated antimouse or antirabbit (Cappel, West Chester, PA) diluted in 1:10,000) at room temperature (RT) for 1 hr. After washing, the peroxidase was visualized by the enhanced chemiluminescence (ECL) method according to manufacturer's (Amersham, UK) instructions. The specificity of immunostaining was verified using the antibody αD presaturated with the peptide used for immunization.

RESULTS

The antigen used for immunization was a synthetic peptide deduced from the chicken PR sequence (amino acids 522–535 of the B form of the cPR). The sequence is located between the DNA-binding domain and the hormone-binding domain [Gronemeyer et al., 1987; Conneely et al., 1987] in the region which is required for stability of the oligomeric form of PR [Carson-Jurica et al., 1989; Schowalter et al., 1991]. Hydropathicity analysis revealed that the region is relatively hydrophilic, suggesting that the sequence is exposed on the surface of the protein. The region showed no homology with other steroid receptors. Rabbits were immunized with the peptide conjugated with thyroglobulin. The IgG fraction of resulting antiserum (αD) was precipitated with ammonium sulfate and was shown to recognize the peptide specifically in a direct immunoassay. When chick oviduct cytosol was immunoblotted with the αD antibody, both A and B forms of PR were detected (Fig. 1, lane 2). When the antibody was saturated with the peptide, no staining was seen (Fig. 1, lane 3). A similar immunoblot was obtained with monoclonal anti-PR antibody PR22 (Fig. 1, lane 1). PR22 recognized the antigen more efficiently, which is not unusual when comparing monoclonal with polyclonal antibodies. Both antibodies also recognized smaller receptor fragments, which are probably degradation products of PR (Fig. 1, lanes 1 and 2).

To study the recognition of the native (nondenatured) form of PR with the αD antibody, oviduct cytosol was incubated with the antibody and analyzed by a sucrose gradient centrifugation. When the 4S form of PR (cytosol treated with 0.3M KCl) was incubated with the αD, the 4S peak disappeared in the sucrose gradient and receptor sedimented as multiple-sized receptor–antibody complexes (Fig. 2A). When the oligomeric form (8S) of the receptor (cytosol prepared in hypotonic buffer with molybdate) was analyzed, αD did not change the sedimentation pattern of PR (Fig 2B). The monoclonal antibody PR22 shifted both the 8S and 4S receptors. The result was similar to that shown by Weigel et al. [1989]. These results indicate that the antibody directed against the AB domain (PR22) of PR recognizes both the oligomeric and the dissociated form, whereas the antibody directed against the D domain (αD) recognizes only the dissociated form.

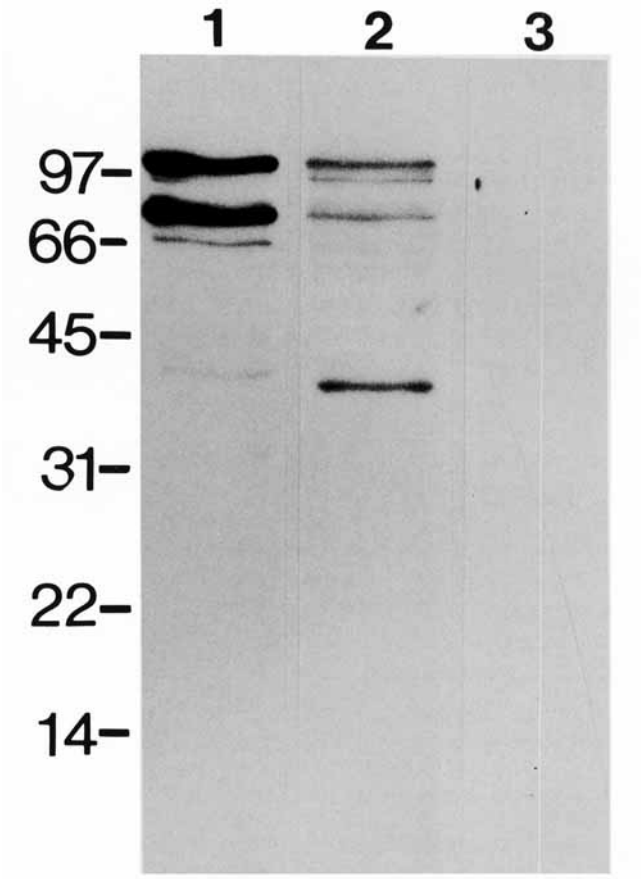

Fig. 1. Immunoblot analysis of oviduct cytosol with PR22 (lane 1) and αD (lane 2) antibodies. Cytosols were precipitated with ammonium sulphate, subjected to SDS-gel electrophoresis and transferred to nitrocellulose. Membranes were incubated with PR22 (lane 1), αD (lane 2), and αD presaturated with the peptide used for immunization (lane 3). Membranes were subsequently incubated with peroxidase-conjugated secondary antibodies, and peroxidase was detected by an enhanced chemiluminescence (ECL) method.

To study whether the antibody can recognize both liganded and nonliganded 4S-PR, we immunoprecipitated progesterone receptor with αD and immunoblotted the precipitate with PR22. Both the A and B forms of the receptor were precipitated. The amount of precipitated PR was not changed by ligand binding, indicating that the antibody recognizes both liganded and nonliganded receptor with the same affinity (Fig. 3).

In addition to the αD antibody, we have used another antibody (907) which is also directed against the same sequence of the D domain [Weigel et al., 1989]. It shows characteristics similar to those of the αD antibody. It is specific for PR, it recognizes only the 4S form of PR and it recognizes both the A and B forms of PR

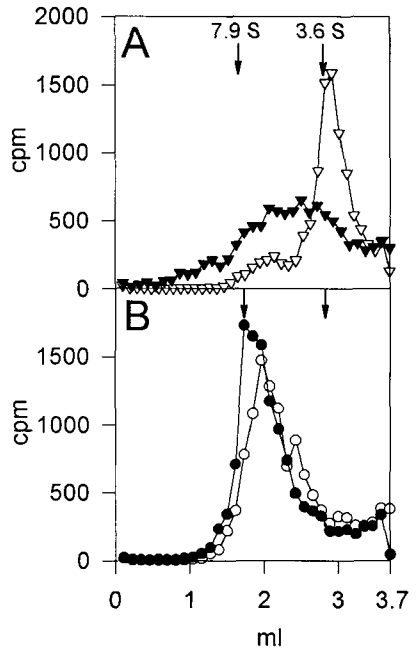

Fig. 2. Detection of the 4S form (**A**) and the 8S form (**B**) of progesterone receptor with the αD antibody. Cytosol was prepared in TE buffer (see Materials and Methods). To prepare 4S cytosol, KCl was added to the cytosol and to prepare 8S cytosol, sodium molybdate was added instead. **A:** 4S cytosol was incubated with the antibody and then the receptor was analyzed by sucrose gradient centrifugation. Closed triangles represent cytosol incubated with the antibody and open triangles cytosol from which the antibody was omitted. **B:** 8S cytosol was labelled with ^{3}H-Org2058, stripped with charcoal and incubated with the αD antibody. The receptor was analyzed as in A. Closed circles represent cytosol incubated with the antibody αD, and open circles cytosol not incubated with the antibody. Two molecular-weight markers were used in sucrose gradient centrifugation: glucose oxidase (7.9 S) and horseradish peroxidase (3.6S) indicated with arrows.

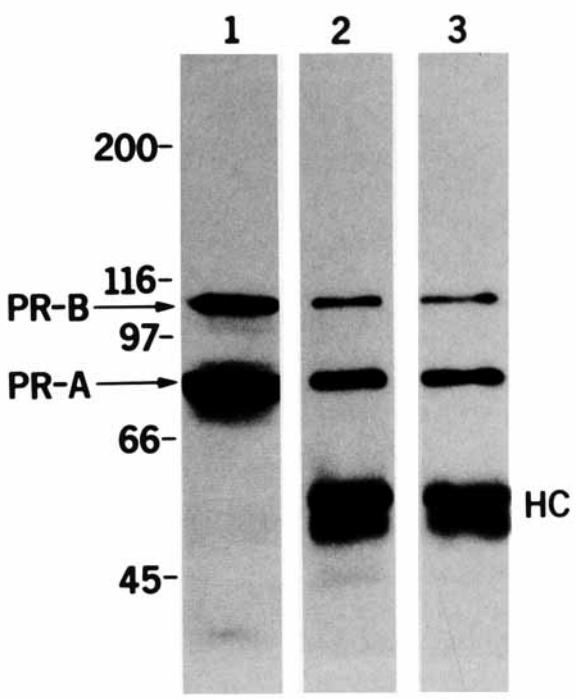

Fig. 3. Immunoprecipitation of the nonliganded and liganded PR using a polyclonal αD antibody. Lane 1, Chicken oviduct cytosol before immunoprecipitation. The different forms of PR are indicated. Lane 2, Immunoprecipitation of the nonliganded PR with αD. Lane 3, Immunoprecipitation of the liganded PR with αD. A position of antibody heavy chain (HC) is indicated on the right. PR22 was used as a primary antibody in the immunoblotting. The molecular-weight standards were myosin M_r 200,000, β-galactosidase 116,000, phosphorylase b M_r 97,000, BSA Mr 66,000 and ovalbumin M_r 45,000 (BioRad, Richmond, CA).

[Weigel et al., 1989]. Weigel et al. [1989] have further characterized the antibody and shown that it recognizes the liganded and nonliganded receptors with the same affinity.

Immunohistochemistry of Nonliganded PR

By means of these antibodies, which can distinguish between the oligomeric and dissociated forms of PR, we studied the distribution of the two forms in the chick oviduct. In our previous works, we studied the distribution of PR in the chicken oviduct with antibodies which detect the 8S and 4S forms with equal sensitivity. In the estrogen-primed oviduct, all cell types were found to contain progesterone receptors. The staining was the most intense in the epithelial cells and in muscle cells, and lowest in stromal cells [Joensuu, 1990]. In all tissue sections αD and 907 antibodies gave similar staining, which was indistinguisable from that seen with the other PR antibodies (Fig. 4A,C). There were no cells or cell types devoid of staining, and the relative staining intensities between different cell types were similar to those with the other antibodies. This was a surprising result, since in sucrose gradient analysis of similarly prepared tissue samples all of the receptors sedimented as 8S and were not detected by αD (Fig. 2A) nor by 907 [Weigel et al., 1989].

Since it is possible that fixation might alter protein–protein interaction, we decided to study whether fixation could explain the conflicting results. Since liquid fixation with paraformaldehyde is known to be a slow process and prone to produce diffusion artifacts, we chose freeze-drying-vapour fixation [Pekki and Tuohimaa, 1989]. In this technique, tissue sections are frozen in liquid nitrogen immediately after dissection and subsequently freeze-dried. After dry-

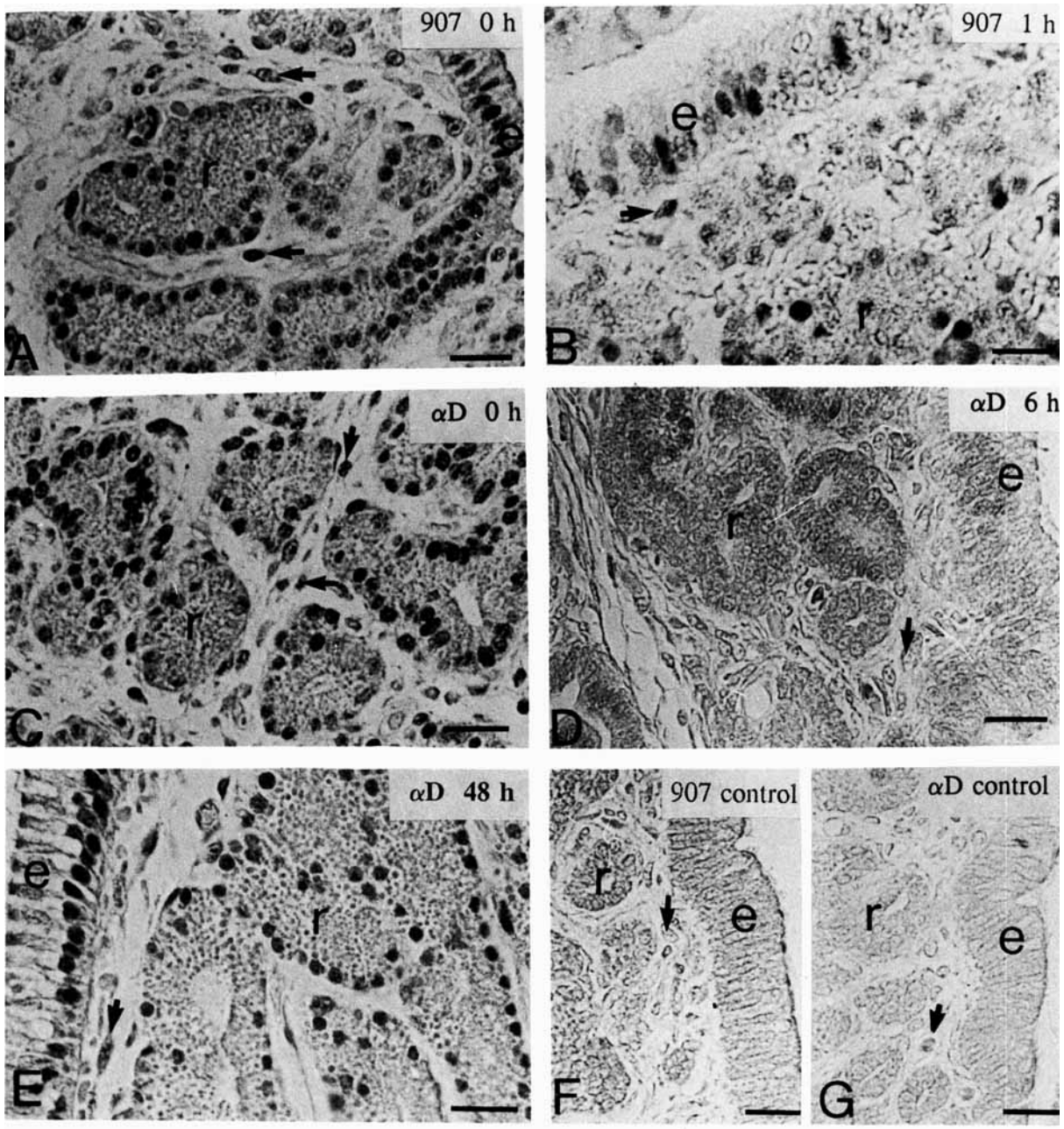

Fig. 4. Immunohistochemical analysis of progesterone receptor in chick oviduct before **(A,C)** and after **(B,D,E)** progesterone treatment. Two different antibodies (αD and 907) and two different fixation methods (liquid fixation and freeze-dried-vapor fixation) were used. In the freeze-dried-vapor-fixed sections **(A,B)**, intense nuclear staining was seen with 907 in chicks not treated with progesterone (A), while staining intensity was markedly diminished 1 h after progesterone treatment (B). In liquid-fixed sections **(C–E)**, similar intense nuclear staining was seen with antibody αD in chicks not treated with progesterone (C). The staining was abolished 6 h after progesterone treatment (D) and returned to original level in 48 h (E). Specificity of the immunostaining was studied by carrying out histochemical staining with antibodies presaturated either with purified PR **(F)** or with a cytosol from COS cells transfected with PR cDNA **(G)**. The section in F has been fixed by the freeze-drying-vapor method and in G by liquid fixation. e, epithelium; r, gland. arrows, mesenchymal cells. Bar = 20 μm.

ing, the sections are fixed with *p*-benzoquinone vapour. When the sections were stained with αD or 907 (Fig. 4A), the results were similar to those obtained with liquid-fixed sections (Fig. 4C).

To study further the effect of fixation on the structure of the oligomeric complex, we applied the 8S and 4S cytosols on nitrocellulose and fixed the membrane in Baker's fixative for 2 h. PR was then detected with PR22 and αD antibod-

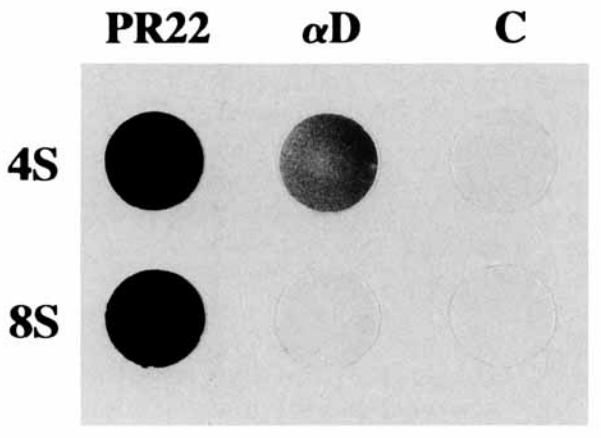

Fig. 5. Reconstitution of fixation and immunodetection process for cytosolic 4S and 8S receptors. The 4S and 8S cytosols were prepared as indicated in Figure 2 and in Materials and Methods; 250 μl of the diluted cytosol was dotted on nitrocellulose membrane and fixed in Baker's fixative for 2 h at 4°C. After washing, the membrane was immunostained with PR22, αD and αD presaturated with the peptide used as antigen (C).

ies. PR22 recognized both forms of PR, whereas D recognized only the 4S form. This implies that fixation is not detrimental to the stability of the oligomeric form but stabilizes the complex (Fig. 5). Both results suggest that nonliganded PR is in the nucleus in dissociated and not in oligomeric form as earlier thought. Such an assumption is further supported by the observation that αD staining is presaturable with 4S receptor but not with 8S receptor (Fig. 6).

Effect of Ligand on PR Staining

If unliganded PR formed an oligomeric complex whose stability is decreased by ligand binding in vivo as well as in vitro, hormone administration should increase the immunostaining with αD and 907. When we stained oviduct sections taken from progesterone-treated chicken with αD and 907, the staining intensity decreased (Fig. 4B,D). The most pronounced decrease was found 6 hours after a single injection of progesterone (Fig. 4D). The staining intensity was still low 18h after injection and was restored to the original level in two days (Fig. 4E). Similar downregulation was seen when the sections were stained with PR22.

The specificity of the immunostaining was studied by incubating the antibodies with purified PR, peptide used for immunization or cytosol from the COS cells transfected with chicken PR cDNA. The immunostaining was saturable with the peptide, purified PR (Fig. 4F) and with the cytosol (Fig. 4G). Cytosol from untransfected cells did not affect immunostaining. When the primary antibodies were substituted with buffer or nonspecific antibodies (hLH), no staining was seen.

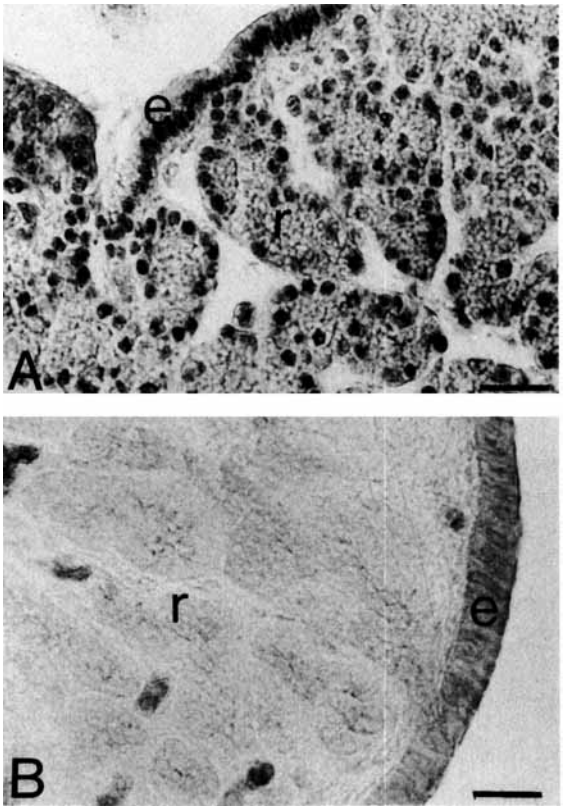

Fig. 6. Immunohistochemistry of PR with the αD antibody presaturated with 8S cytosol (**A**) or with 4S cytosol (**B**). The estrogen withdrawn chick oviduct sections were fixed by freeze-drying-vapor technique and immunostaining was carried out as in Figure 3. e, epithelium; r, gland. Bar = 20 μm.

DISCUSSION

Nonliganded steroid receptors form oligomeric complexes when extracted in hypotonic buffer. The oligomeric form is regarded as an nonactivated from, which needs to be activated before it can interact with DNA and regulate transcription [Gronemeyer, 1992; Carson-Jurica et al., 1990]. This activation implies transformation of the 8S into the 4S form and is thought to be triggered by ligand binding [Renoir and Mester, 1984]. The exact composition of the 8S form of receptor is not known. When the nonactivated form of chicken PR was purified by affinity chromatography, hsp90 copurified with PR in a ratio of two molecules of hsp90 to one molecule of PR [Renoir and Mester, 1984; Dougherty et al., 1984; Catelli et al., 1985].

Later several other proteins (hsp70, p60, p54, p50, p23) were shown to be associated with the nonactivated form of chicken PR [Kost et al., 1989; Smith et al., 1990; Smith and Toft, 1992]. Of these proteins, only hsp70 also copurifies with the activated form (4S) [Kost et al., 1989]. The amino acids 495–632 of cPR have been shown to be important for the formation of the 8S complex [Carson-Jurica et al., 1989]. When this region is deleted, the mutated receptor is unable to form the oligomeric complex. Other mutations have no effect on 8S formation [Carson-Jurica et al., 1989].

To study the different forms of PR on tissue sections we raised an antibody which can distinguish between the 4S and 8S forms. The epitope for the antibody is located in the D domain of the chicken progesterone receptor in the region required for the formation of the oligomeric complex. The antibody can be considered specific for PR since it recognized both A and B forms of PR in immunoblotting, its immunostaining was presaturable with a cytosol from COS cells transfected with a cPR cDNA, it recognized PR in sucrose gradient centrifugation and can precipitate the A and B forms of PR from cytosol. Sucrose gradient analysis further showed that the antibody recognized only the 4S form of the receptor, whereas the monoclonal antibody directed against the AB domain recognized both 4S and 8S forms. We also used another antibody (907) that can distinguish between the two forms of PR [Weigel et al., 1989]. The antibodies αD and 907 are thus directed against the region, which is directly involved in the formation of the oligomeric complex and which is masked in the oligomeric form. This masking may be due to steric interference or to alteration of the structure of PR. Since this region is very close to the DNA-binding domain, the same mechanism probably explains the inability of the 8S form to bind to DNA. The antibodies αD and 907 can thus be used to study conversion of the non-DNA-binding form (8S) of PR into the DNA-binding form (4S).

We obtained conflicting results when we analyzed the receptor in hypotonic cell extract (cytosol) and on tissue section. When nonliganded PR was extracted with hypotonic buffer all of the receptors sedimented in oligomeric form, which was not recognized by these antibodies. When the same tissue samples were analyzed with immunohistochemistry both αD and 907 showed an intense nuclear staining which was comparable with the results obtained with other PR antibodies. These results raise the question, which one of these techniques better reflects the in vivo situation.

Since tissue fixation might alter protein–protein interaction we tested different methods of fixing tissues. We also reconstituted a fixation process for the cytosolic receptor by fixing the 8S and 4S receptors on a nitrocellulose membrane. We found no indication that fixation conditions might so alter the structure of the oligomeric form that the epitope for αD and 907 antibodies became exposed. It might rather be conceived that when extracted in a hypotonic milieu charged molecules like steroid receptors form complexes with opposite-charged molecules (like hsp90) and that these dissociate in elevated salt conditions. In fact, we have shown that the 8S form of PR can be reconstituted when the unliganded 4S form of the receptor is homogenized with hsp90-containing tissue. The reconstitution did not take place when liganded receptor was used instead of nonliganded [Tuohimaa et al., 1993]. This might be due to alteration of receptor structure upon ligand binding, which decreases the affinity of PR for hsp90 and other components of the oligomeric form. Thus ligand binding rather inhibits the formation of the PR–hsp90 complex during tissue processing than causes dissociation of the putative preexisting complex.

When we studied tissue sections from animals treated with progesterone we found that the staining intensity was decreased. A decrease in progesterone receptor concentration after progesterone administration has also been seen with immunohistochemistry using antibodies directed against different epitopes [Pekki and Tuohimaa, 1989], with immunoblot [Sullivan et al., 1988] and with ligand binding assays [Mester and Baulieu 1977]. Thus the decrease in αD and 907 staining after progesterone treatment does not reflect changes in the putative receptor oligomerization, but receptor downregulation. Similar autoregulation of steroid receptors is seen with other steroids [Burnstein et al., 1990]. The mechanism by which the receptors are downregulated so rapidly is unknown.

The results discussed here are in concert with our previous reports suggesting that PR does not exist in a significant quantity as an oligo-

meric, non-DNA-binding, form in nuclei. First, we have shown, using an advanced immunohistochemical technique for soluble proteins with antibodies of equal sensitivity to PR and hsp90, that hsp90 and PR are located in different subcellular compartments, hsp90 in the cytoplasm and PR in the nucleus [Tuohimaa et al., 1993]. Second, by a sensitive immunoelectron microscopic technique, we found no significant colocation of hsp90 and PR [Pekki, 1991]. Third, when HeLa cells were transfected with chicken hsp90 and PR, was hsp90 located in the cytoplasm and PR in the nucleus [Tuohimaa et al., 1993]. Recently, it has been shown that nuclear localization deficient PR can be rendered, at least partially, nuclear by cotransfecting it with a hsp90 carrying a nuclear localization signal [Kang et al., 1994]. This suggests that this artificial hsp molecule can interact with a mutated PR at least in the cytoplasm. In contrast, wild type PR is unable to render wild type hsp90 nuclear when these are cotransfected [Tuohimaa et al., 1993].

Taken together, our results suggest that unliganded PR is in the nuclei predominantly in 4S form and that ligand-induced PR activation does not imply dissociation of the oligomeric, non-DNA-binding form. The oligomeric complex found in hypotonic tissue extracts is possibly formed during tissue processing. It is, however, possible that hsp90 and other components of the oligomeric form might function as a chaperon during receptor synthesis [Wiech et al., 1992], thus being important for the proper structure and stability of the receptor [Nemoto et al., 1990].

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REFERENCES

Auricchio F (1989): Phosphorylation of steroid receptors. J Steroid Biochem 32:613–622.

Baulieu E-E (1987): Steroid hormone antagonists at the receptor level: A role for the heat shock protein MW 90,000 (hsp90). J Cell Biochem 35:161–174.

Burnstein KL, Jewell CM, Cidlowski JA (1990): Human glucocorticoid receptor cDNA contains sequences for receptor down-regulation. J Biol Chem 265:7284–7291.

Carson-Jurica MA, Lee AT, Dobson AW, Conneely OM, Schrader WT, O'Malley BW (1989): Interaction of the chicken progesterone receptor with heat shock protein (hsp)90. J Steroid Biochem 34:1–9.

Carson-Jurica MA, Schrader WT, O'Malley BW (1990): Steroid receptor family: Structure and functions. Endocr Rev 11:201–220.

Catelli MG, Binart N, Jung-Testas I, Renoir J-M, Baulieu E-E, Feramisco JR, Welch WJ (1985): The common 90-kd protein component of non-transformed "8S" steroid receptors is a heat-shock protein. EMBO J 4:3131–3135.

Conneely OM, Dobson ADW, Tsai MJ, Beattie WG, Toft DO, Huckaby CS, Zarucki T, Schrader WT, O'Malley BW (1987) Sequence and expression of a functional chicken progesterone receptor. Mol Endocrinol 1:517–525.

Dougherty JJ, Puri RK, Toft DO (1984): Polypeptide components of two 8S forms of chicken oviduct progesterone receptor. J Biol Chem 259:8004–8009.

Green S, Issemann I, Sheer E (1988): A versatile in vivo and in vitro eukaryotic expression vector for protein engineering. Nucleic Acid Res 16:369.

Gronemeyer H (1991): Transcription activation by estrogen and progesterone receptors. Annu Rev Genet 25:89–123.

Gronemeyer H (1992): Control of transcription activation by steroid hormone receptor. FASEB J 6:2524–2529.

Gronemeyer H, Turcotte B, Quirin-Stricker C, Bocquel MT, Meyer ME, Krozowski Z, Jeltsch JM, Lerouge T, Garnier JM, Chambon P (1987) The chicken progesterone receptor: sequence, expression and functional analysis. EMBO J 6:3985–3994.

Jeltsch JM, Krozowski Z, Quirin-Stricker C, Gronemeyer H, Simpson RJ, Garnier JM, Krust A, Jacob F, Chambon P (1986): Cloning of the chicken progesterone receptor. Proc Natl Acad Sci USA 83:5424–5428.

Joab I, Radanyi C, Renoir J-M, Bouchou T, Catelli MG, Binart N, Mester J, Baulieu E-E (1984): Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid hormones. Nature 308: 850–853.

Joensuu TK (1990): Chick oviduct differentiation. The effect of estrogen and progesterone on the expression of progesterone receptor. Cell Diff Dev 30:207–218.

Kang KI, Devin J, Cadepond F, Jibard N, Guiochon-Mantel A, Baulieu E-E, Catelli MG (1994): In vivo functional protein–protein interaction: Nuclear targeted hsp90 shifts cytoplasmic steroid receptor mutants into the nucleus. Proc Natl Acad Sci USA 91:340–344.

Kost SL, Smith DF, Sullivan WP, Welch WJ, Toft DO (1989): Binding of heat shock proteins to the avian progesterone receptor. Mol Cell Biol 9:3829–3838.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685.

Mester J, Baulieu E-E (1977): Progesterone receptor in the chick oviduct. Determination of total concentration of binding sites in the cytosol and nuclear fraction and effect of progesterone on their distribution. Eur J Biochem 72:405–414.

Nemoto T, Ohara-Nemoto Y, Denis M, Gustafsson J-Å (1990): The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. Biochemistry 29:1880–1886.

Pekki A (1991): Different immunoelectron-microscopic locations of progesterone receptor and hsp90 in chick oviduct epithelial cells. J Histochem Cytochem 39:1095–1101.

Pekki A, Tuohimaa P (1989): New freeze-dry and vapor fixation method for immunohistochemistry of soluble proteins. Subcellular location of the progesterone receptor. J Histochem Cytochem 37:1207–1213.

Pratt WB (1990): Interaction of hsp90 with steroid receptors: organizing some diverse observations and presenting the newest concepts. Mol Cell Endocrinol 74:C69–C76.

Renoir J-M, Mester J (1984): Chick oviduct progesterone receptor, structure, immunology, function. Mol Cell Endocrinol 37:1–13.

Scheiner W, Toft DO, Sullivan W, Shyamala G (1988): Interaction of murine progesterone receptors with specific monoclonal antibodies to the avian progesterone receptor. J Steroid Biochem 29:297–306.

Schowalter DB, Sullivan WP, Maihle NJ, Dopson ADW, Conneely OM, O'Malley BW, Toft DO (1991): Characterization of progesterone receptor binding to the 90- and 70-kDa heat shock proteins. J Biol Chem 266:21165–21173.

Smith DF, Faber LE, Toft DO (1990): Purification of unactivated progesterone receptor and identification of novel receptor-associated proteins. J Biol Chem 265:3996–4003.

Smith DF, Toft DO (1992): Composition, assembly and activation of the avian progesterone receptor. J Steroid Biochem Mol Biol 41:201–220.

Sullivan W, Beito T, Proper J, Krco C, Toft DO (1986): Preparation of monoclonal antibodies to the avian progesterone receptor. Endocrinology 119:1549–1557.

Sullivan WP, Smith DF, Beito TG, Krco CJ, Toft DO (1988): Hormone-dependent processing of the avian progesterone receptor. J Cell Biochem 36:103–119.

Tuohimaa P, Pekki A, Bläuer M, Joensuu T, Vilja P, Ylikomi T (1993): Nuclear progesterone receptor is mainly hsp90-free in vivo. Proc Natl Acad Sci USA 90:5848–5852.

Weigel NL, Schrader WT, O'Malley BW (1989) Antibodies to progesterone receptor peptide 523–536 recognize a site exposed in receptor–deoxyribonucleic acid complexes but not in receptor-heat shock protein-90 complexes. Endocrinology 125:2494–2501.

Wiech H, Buchner J, Zimmermann R, Jakob U (1992): Hsp90 chaperones protein folding in vitro. Nature 358: 169–170.

Wigler M, Pellicer A, Silverstein S, Axel R (1978): Biochemical transfer of single copy eukaryotic genes using total cellular DNA as donor. Cell 14:725–731.