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Progesterone Receptor and hsp90 are not Complexed in Intact Nuclei

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In hypotonic cell extract (cytosol), unliganded progesterone receptor (PR) is known to form an oligomeric complex with heat shock protein 90 (hsp90), and this complex does not bind to DNA. Since ligand binding has been shown to render the complex less stable *in vitro*, it has been proposed that ligand binding regulates DNA binding and receptor activity in vivo by altering the stability of the oligomeric complex. However, there is no direct evidence as to whether this oligomeric complex is present *in vivo*. The present study addressed this problem. First, we used an immunoelectron-microscopic technique and monoclonal antibodies to ascertain the location of PR and hsp90 in chick oviduct cells. Hsp90 was found in the cytoplasm and PR in the nucleus. To study the relative affinities of the PR and hsp90 antibodies, we then constructed a chimeric protein (PR–hsp90), which was expressed in the HeLa cells. Both hsp90 and PR antigens of the chimera were detected in the nuclei with the same intensity, which indicates that the antibodies have equal sensitivities in detecting their antigens. This suggests that if significant amounts of nuclear hsp90 were present in intact cells, it should have been detected by our method. Our results indicate that the PR does not exist *in vivo* as an oligomeric, nonDNA-binding form in the cell nuclei and that the oligomeric form found in tissue extracts is possibly formed during tissue processing.

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INTRODUCTION

The chick oviduct progesterone receptor (PR) exists in two forms, A ($M_r = 72,000$) and B ($M_r = 86,000$) [1, 2]. In hypotonic cell extract, unliganded PR is known to form an oligomeric complex, which sediments as 8S in sucrose gradient centrifugation and which does not bind to DNA *in vitro* [3–5]. This complex is composed of one molecule of PR (either A or B form) and two molecules of 90-kDa heat shock protein (hsp90) [6]. Elevated salt conditions and progesterone are able to promote dissociation of the oligomeric complex and the resulting complex sediments as 4S in sucrose gradient and can bind to DNA *in vitro* [5]. There are also other proteins (hsp70, p60, p54, p50 and p23) which copurify with unliganded steroid receptors, but it is not known how these interact with the receptors [7–9].

Immunohistochemical studies revealed that chicken PR is located in the nucleus independently of the presence of the ligand [10–14]. Therefore a nuclear location of hsp90 would be expected if hsp90 were bound to PR in the nucleus *in vivo*. Hsp90 has however been shown to be located in the cytoplasm [10, 15–17] or in both cytoplasm and nucleus [18]. We have recently shown, using a new immunohistochemical technique which minimizes the possibilities of redistribution of soluble proteins, that PR is located exclusively in the nucleus and hsp90 in the cytoplasm [19]. This would suggest that PR is not associated with hsp90 in intact cell nuclei. In the present work we have studied the subcellular location of hsp90 and PR at the electron-microscopic level and compared the sensitivity of the antibodies against these respective components.

EXPERIMENTAL

Animals and treatments

Immature chicks received daily injections of estradiol-17β (1 mg/0.1ml ethanol–sesame oil) for 1 week. After a withdrawal period of 1 week, the chicks were killed and oviduct samples immediately removed.

Antibodies

Mouse monoclonal antibody, PR22, made against progesterone receptor was used. PR22 recognizes both

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the A and B forms of PR [20, 21]. The antibody against hsp90 was D7a [22], which recognizes hsp90 free and bound to PR [22, 23].

Fixation

The specimens were fixed in ice-cold Faglu (4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The fixation time was 4 h at 0°C. Fixed tissues were washed and stored in phosphate buffer containing 0.05% sodium azide at + 4°C. Samples were mounted on a tissue holder and immersed in agarose gel (FMC Corporation Rockland, ME, U.S.A.) before cutting into 20 µm-thick sections by vibratome (Lancer, U.S.A.).

Electron microscopy

Using the immunoperoxidase technique the vibratome sections were processed with the primary antibodies diluted to a concentration of 1 μg/ml, a biotinylated secondary antibody and avidin–biotin complex (Vectastain Reagents, Vector Labs, Burlingame, CA, U.S.A.). The details have been described by Pekki and Tuohimaa [14].

When the immunogold-silver staining technique was used, the vibratome sections were processed using the primary antibody (D7a) diluted to a concentration of 1 µg/ml, the biotinylated secondary antibody, 5nm gold particles (Janssen Pharmaceuticals, Beerse, Belgium) coupled to streptavidin (450µg/ml), and silver intensification.

After immunostaining, the samples were processed for electron microscopy as described by Pekki [17]. Briefly, they were postfixed in glutaraldehyde and osmium tetroxide, embedded in Epon and cut with an ultratome (LKB Wallac, Sweden).

To verify the specificity of immunostaining we made presaturation experiments with an affinity-purified B subunit of PR and immunopurified chicken hsp90 kindly provided by Dr David Toft (Dept. of Biochemistry, Rochester, MN, U.S.A.).

Recombinants

Chicken hsp90 cDNA (a generous gift from Drs M. Catelli and E.E. Baulieu, Kremlin-Bicetre, France) was subcloned into the eucaryotic expression vector, pSG5 [24]. A fusion protein between hsp90 and chicken PR (cPR–hsp2) was constructed by amplifying the 990 N-terminal nucleotide of chicken hsp90 cDNA by PCR with XhoI restriction enzyme-cutting sites at both ends. The PCR product was cut with XhoI and ligated into the XhoI site of cPR21 (a generous gift from Dr H. Gronemeyer, Strasbourg, France). cPR21 is a pSG5 expression vector containing the whole chicken PR coding region. The XhoI site is located immediately before the first ATG [25].

Transfections and immunohistochemical analysis of transfected cells

The cDNAs were transiently expressed in HeLa cells by means of a standard calcium phosphate precipitation technique. After transfection, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Thereafter, they were permeabilized by incubation for 40 min in 0.5% Triton X-100. The cells were then incubated with 3% normal goat serum followed by antibodies PR22 or D7a. Biotinylated secondary antibodies and an avidin–biotin–peroxidase complex were used as a detection system.

RESULTS

PR is a nuclear protein

The intracellular location of nonliganded PR by the peroxidase technique is illustrated in Fig. 1. Using monoclonal antibody PR22, only nuclear staining of unliganded PR was detected in epithelial cells of the chick oviduct. Some of the apical nuclei of the epithelial cells were PR-negative [Fig. 1(A)]. Although the immunoreaction was intranuclear, there was no staining

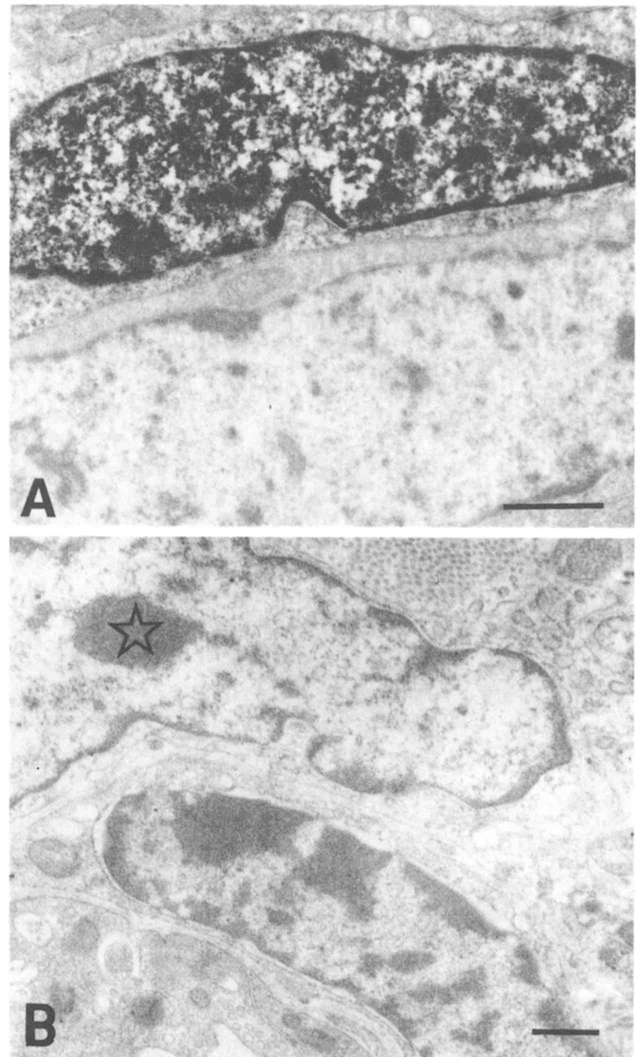

Fig. 1. Immunoperoxidase electron microscopy of unliganded PR stained with monoclonal antibody PR22 in chick oviduct epithelial cells. (A) PR is intranuclear. PR-positive and -negative cells are seen in the epithelium. Bar = 1 μm. (B) Presaturation control for PR22 staining with purified B-subunit of PR. No staining is seen. Nucleoli (sd). Bar = 500 nm.

inside the nucleoli. The specificity of the immunoreaction was verified by presaturation of the primary antibody with purified PR-B. No staining was seen [Fig. 1(B)].

Hsp90 is a cytoplasmic protein

Using the immunogold technique with antibody D7a, hsp90 staining was observed in the cytoplasm of the chick oviduct epithelial cells [Fig. 2(A)]. The nuclei of the epithelial cells were hsp90-negative. In the control staining using D7a presaturated with affinity-purified 90K, no staining was observed [Fig. 2(B)].

Hsp90 and PR antibodies are equally sensitive in detecting their antigens

To demonstrate the relative sensitivity of the immunohistochemical detection of hsp90 and PR, we constructed a chimeric molecule between PR and chicken hsp90 (cPR–hsp2). This chimera expresses an equal number of epitopes for the monoclonal antibodies PR22 (recognizing PR) and D7a (recognizing chicken but not human hsp90 [23]). When HeLa cells were transfected with cPR–hsp2 and stained with either PR22 or D7a at an antibody concentration of 1 μg/ml, nuclear staining of equal intensity was detected with both antibodies [Fig. 3 (A and B)]. When the antibody concentration was reduced to 25 ng/ml, the staining intensity was equally diminished with both antibodies [Fig. 3 (C and D)]. An equal decrease in staining intensity was observed when 20 μ/ml was used instead of 100 μ/ml of peroxidase substrate. Both the PR and the hsp90 epitopes were undetectable when the antibody concentration was below 25 ng/ml or the substrate concentration below 5 μg/ml. Immunoblot experiments with cytosolic extracts of the transfected HeLa cells revealed a protein of expected size as detected by PR22 and D7a, and the band was detected with equal intensity with PR22 and D7a [19]. These

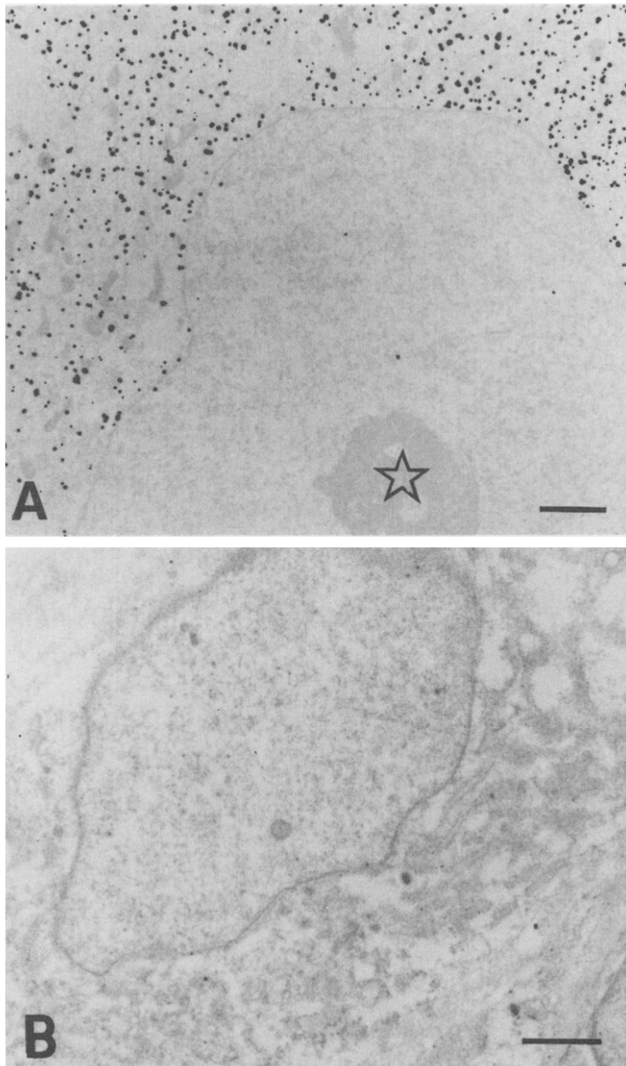

Fig. 2. Immunogold staining of hsp90 with antibody D7a in estrogen-withdrawn chick oviduct epithelial cells. Gold particles (5nm) are silver-intensified. (A) Hsp90 is in the cytoplasm. Bar = 500 nm. (B) The specificity of the immunostaining was studied by histochemical staining with antibody D7a presaturated with purified 90K. No staining is seen. Bar = 1 μm.

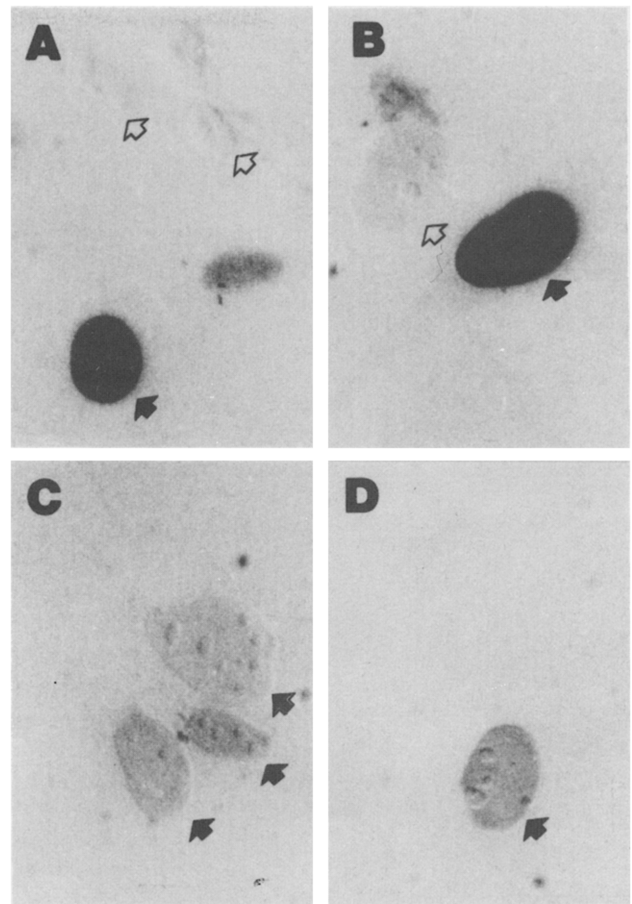

Fig. 3. Relative detection sensitivities of the monoclonal anti-PR (PR22) and anti-hsp90 (D7a) antibodies. A fusion protein between hsp90 and PR (cPR–hsp2) was transiently expressed in HeLa cells. When the cells were stained with PR22 (A) and D7a (B) at an antibody concentration of 1 μg/ml, nuclear staining of equal intensity was detected. When the antibody concentration was decreased to 25 ng/ml, the staining intensity became equally weak with PR22 (C) and D7a (D). It should be noted that figure pairs A/B and C/D represent adjacent areas of the same culture dish. Filled arrows point to PR–hsp90-expressing cell nuclei and open arrows to cell nuclei with nondetectable amounts of antigen.

results demonstrate that the antibodies are of equal sensitivity in detecting their respective antigens.

DISCUSSION

It is widely accepted that hsp90 binds *in vitro* to most of the nuclear hormone receptors and regulates the binding of steroid receptors to the corresponding gene regulatory element [26]. It has been proposed that hormone binding triggers the dissociation of hsp90 from the receptors and this results in an active receptor [5]. The oligomeric form of PR comprises one molecule of PR and two molecules of hsp90. To ensure that all PR molecules are complexed with hsp90 there should be a severalfold excess of hsp90 in the nucleus compared to PR. In the present work we have shown that PR is exclusively intranuclear, whereas hsp90 is a cytoplasmic protein. The results are in agreement with those of earlier immunohistochemical studies showing that unliganded and liganded PR are intranuclear [10–14, 17, 19], whereas hsp90 is located in the cytoplasm [10, 17, 19]. To study the possible influence of the different sensitivities of the antibodies to detect their respective antigens we constructed a chimeric hsp90-PR, which was transiently expressed in HeLa cells. Both hsp90 and PR antigens of the chimera were detected in the nuclei with the same intensity, which demonstrates that the sensitivity of the immunohistochemical detection of hsp90 and PR did not differ significantly. This indicates that if significant amounts of nuclear hsp90 were present in intact cells, the protein should have been detected by our method. However, our immunohistochemical findings suggest that PR and hsp90 are not associated in the intact cell nucleus.

Because all PR molecules are complexed with hsp90 in tissue homogenates (8S-PR), it is possible that they form a complex during tissue processing. To study this possibility, we prepared an hsp90-free PR by high salt treatment of the chick oviduct cytosol followed by dialysis [19]. After dialysis, all hsp90 molecules remained dissociated from PR (4S-PR). When the non-liganded hsp90-free PR was rehomogenized with the bursa of Fabricius of an immature chick, known to contain high amounts of hsp90 but no detectable PR, the PR was found to form a complex with the hsp90. The results are summarized in Fig. 4. The formation of the hsp90–PR complexes seems to require non-liganded PR, since no 8S-PR was observed when the liganded PR was homogenized with bursa. This would suggest that the ligand binding can prevent the complex formation. It can therefore be reasoned that progesterone administration *in vivo* is more likely to prevent the formation of a hsp90–PR complex during tissue processing than cause the dissociation of a putative pre-existing 8S complex. Our results call into question the proposed role of hsp90 in the activity of PR and, possibly, other steroid receptors. Due to its cytoplasmic compartmentalization, hsp90 cannot be in complex with PR in the nucleus and thus act as a direct inhibitor of PR functions by, for example, interfering with DNA binding or transcription activation. Our results do not exclude the possibility that hsp90 may have a role in posttranslational modification or chaperoning [27] or cytoplasmic transport [28] of newly synthesized receptor in the cytoplasm. Furthermore, it is not excluded that PR during the shuttling [29] could be associated with hsp90 in the cytoplasm.

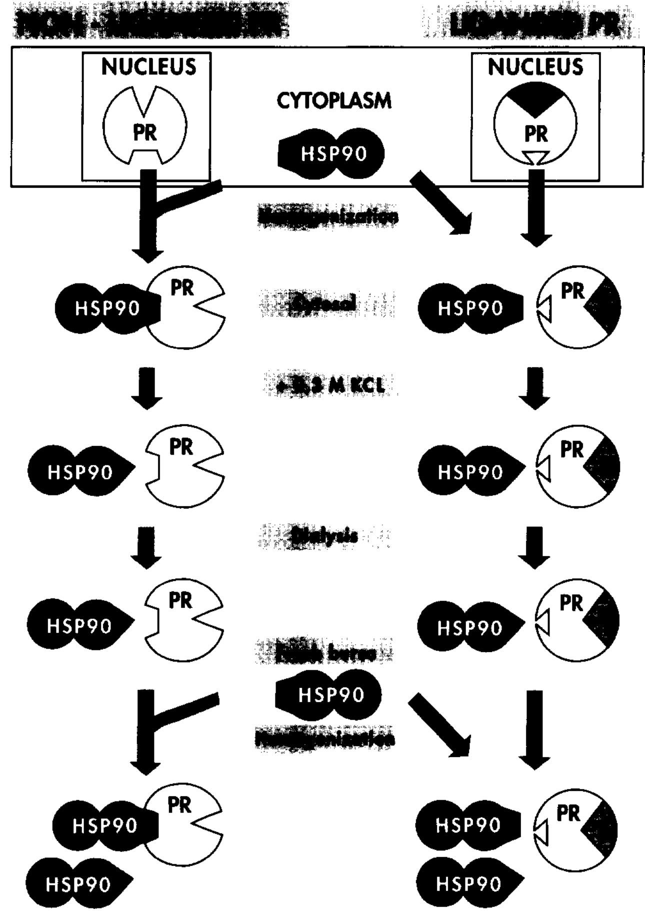

Fig. 4. A schematic summary of the present results and the homogenization study [19]. In the intact cells PR and hsp90 are not complexed. In the cytosol the nonliganded PR complexes with hsp90 due to the homogenization. Treatment of the cytosol with 0.3 M KCl for 2 h causes a dissociation of the hsp90 from the nonliganded PR. When the cytosol is rehomogenized with a fresh immature chicken bursa of Fabricius, only the non-liganded PR reassociates with hsp90. The reassociation does not take place, when PR is liganded. The results suggest that PR released from the nucleus during tissue fractionation associates with cytoplasmic hsp90 and this association is probably prevented by structural changes caused by the ligand.

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