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Biochemistry

Nuclear progesterone receptor is mainly heat shock protein 90-free *in vivo*

(steroid receptors/artifact/oligomeric complex/DNA binding/transient transfection)

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ABSTRACT **Heat shock protein 90 (hsp90) is associated with many steroid receptors in tissue homogenates. It is widely accepted that hsp90 regulates the binding of the receptor to the corresponding gene regulatory element. However there is no unequivocal evidence that steroid receptor–hsp90 complexes are present in the intact cells. We demonstrate here the absence of progesterone receptor (PR)–hsp90 complexes in intact target cell nuclei, using immunohistochemical and biochemical methods to determine the location and composition of the nonliganded (aporeceptor) and liganded (holoreceptor) PR complexes. In the chicken oviduct cells, both apo- and holoreceptors were nuclear, while hsp90 was exclusively cytoplasmic. When expressed transiently in HeLa cells, hsp90 was detected in the cytoplasm and PR was detected in the nucleus. Their location or staining intensity was not affected when they were coexpressed in the same cells. To confirm that the sensitivity of the immunohistochemical detection of hsp90 and PR did not differ significantly, a chimeric hsp90–PR was transiently expressed in HeLa cells. Both hsp90 and PR antigens of the chimera were detected in nuclei with the same intensity. In homogenates of the same tissue samples that were used for immunohistochemistry, the PR was complexed with hsp90. Hsp90–PR complexes were formed *in vitro* when immature bursa of Fabricius, known to contain high levels of hsp90, was homogenized in the presence of hsp90-free aporeceptor, while holoreceptor did not associate with hsp90. Our data show that nuclear PR is not complexed with hsp90 *in vivo* and suggest that the 8S-PR may be an *in vitro* artifact generated during tissue processing.**

Steroid receptors are transcription factors that regulate gene expression upon binding of their cognate ligands (1–5). The mechanism by which the ligand regulates their activity is not known. Ligand binding may induce changes in receptor structure to allow DNA binding, dimerization, and interaction with other transcription factors or cause a dissociation of an inhibiting factor. Evidence for the latter effect came from a large number of *in vitro* studies. Steroid receptors were found to be in hypotonic cell lysate in a non-DNA-binding form (sedimenting at 8 S in sucrose gradient), which is an oligomeric complex of steroid receptor and heat shock protein 90 (hsp90). Hypertonic conditions or heating triggers dissociation of the hsp90 from the complex and transformation of the receptor to a DNA-binding form (4S) (6, 7). Since the complex between steroid receptors and the hsp90 is less stable in the presence of hormone, it has been proposed that hormone binding might trigger the dissociation also *in vivo* and thus result in an active receptor (8).

There are a vast number of studies on *in vitro* transformation of steroid receptors from the non-DNA-binding form to the DNA-binding form (6). There are also many studies on the subcellular localization of steroid receptors and hsp90. However, to date there is no clear evidence indicating either that progesterone receptor (PR) and estrogen receptor (ER) are associated *in vivo* with hsp90 or that these receptors and hsp90 are located in the same subcellular compartment, a prerequisite for their association. ER and PR are nuclear proteins (9–14), whereas hsp90 is, according to most studies, located in the cytoplasm (14–16). However, it is difficult to compare the actual receptor and hsp90 distribution and concentration in different cell compartments because the liquid fixation technique used in previous studies might have caused artificial redistribution of the antigens, and sensitivities of the antibodies might be different.

To compare the distribution of the PR and the hsp90 in different cell compartments, we used an unusual fixation technique to minimize the possible relocation of soluble antigens during sample processing (13). The sensitivities of the antibodies against both the PR and hsp90 were also shown to be equal. We show here that the hsp90 is a cytoplasmic protein, undetectable in the nucleus, and that the ligand-free PR present in the nucleus cannot be complexed with hsp90. We have further shown that PR can reassociate with hsp90 when hsp90-free receptor is added during the homogenization of hsp90-containing tissue. The results suggest that the hsp90–PR complex is formed during tissue processing and that hsp90 is unlikely to interfere directly with receptor functions in the nucleus *in vivo*.

MATERIALS AND METHODS

Animals and Treatments. Immature 2-week-old chickens received daily injections of estradiol-17β (1 mg/0.1 ml of 5% ethanol/95% sesame oil) for 1 week. After a withdrawal period of 1 week, the 4-week-old chickens received a single injection of progesterone (20 mg/kg of body weight) or vehicle and were killed 1 hr after the injection. Oviduct samples were removed immediately after sacrifice.

Immunohistochemistry. The samples were frozen in liquid nitrogen, dried in vacuum, and processed for immunohistochemistry (13). Fixation with *p*-benzoquinone or formaldehyde vapor gave essentially similar results. Polyclonal antibody IgGRB (17); monoclonal antibodies PR6, PR13, and PR22 (18) against affinity-purified PR; and monoclonal antibodies BF_4, 7Dα, and 4F3 against hsp90 (19, 20) were used. Controls included presaturation with an excess of the respective affinity-purified antigen [chicken PR (cPR) or hsp90 (chsp90)], staining with nonspecific antibodies (mouse anti-human luteinizing hormone monoclonal antibody, rabbit anti-human lactoferrin), and replacing specific antibodies with buffer.

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Abbreviations: hsp90, heat shock protein 90; chsp90, chicken hsp90; HSP2, 990 N-terminal nucleotides of chsp90 cDNA; PR, progesterone receptor; cPR, chicken progesterone receptor; ER, estrogen receptor; IEMA, immunoenzymometric assay.

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Cytosols and Nuclear Extracts. These were prepared as described (10, 11). Equal aliquots of the cytosol and nuclear extracts were taken so that the final PR concentrations were comparable and quantitative. Some samples of KCl-treated cytosols were dialyzed and thereafter homogenized 1:1 (vol/vol) with bursa of Fabricius of immature chicken (not treated with estradiol), after which molybdate was added.

Recombinants. chsp90 cDNA was subcloned into the eukaryotic expression vector pSG5 (21). To make NLS-hsp90, a cDNA sequence corresponding to the nuclear localization signal (NLS) of human ER (amino acids 253–303) was amplified by PCR and ligated in front of the coding sequence of chsp90 in pSG5 (22). A fusion protein between chsp90 and cPR (cPR-HSP2) was constructed by amplifying 990 N-terminal nucleotides of chsp90 cDNA by PCR and ligating into the *Xho* I site of cPR21, which is a pSG5 expression vector containing the whole cPR coding region. The *Xho* I site is located immediately before the first ATG (23).

Transfections and Immunohistochemical Analysis of Transfected Cells. The cDNAs were transiently expressed in HeLa cells by using a standard calcium phosphate precipitation technique (22). Immunohistochemistry of formalin-fixed cells was studied with the ABC method (22). Location scoring (22) was done blind by two investigators. Immunoblotting was carried out with a standard technique using antibodies PR22 and 7Dα at a concentration of 1 μg/ml.

Sucrose Gradient Ultracentrifugation. A sucrose gradient (5–20%) was prepared in 10 mM Tris/12 mM monothioglycerol/1.5 mM EDTA/10% (vol/vol) glycerol/50 mM sodium molybdate/0.15 M KCl, pH 7.4. The tubes were centrifuged at 200,000 × g for 16 hr. Fractions (two drops) were collected by piercing the bottom of the tube. Glucose oxidase (7.9 S) sedimenting at 2.0 ml and horseradish peroxidase (3.6 S) sedimenting at 2.9 ml were used as internal standards.

PR Assays (IEMA and DCC). Samples of cytosols and nuclear extracts were assayed by sandwich immunoenzymometric assays (IEMAs) (24) specific for cPR complexes. In brief, the microtiter plates (Nunc) were coated with monoclonal antibody PR6. Samples were incubated overnight at 4°C. The second biotinylated antibody, PR22 (PR-IEMA) or 7Dα (PR–hsp90 complex IEMA), and peroxidase-labeled avidin were added. During all incubations, 20 mM sodium molybdate was present. Ligand-binding was assayed from aliquots (100 μl) of cytosol incubated with different concentrations (0.5–10 nM) of tritiated ORG-2058 (16α-ethyl-21-hydroxy-19-nor-4-pregnene-3,20-dione) with or without unlabeled 1 μM ORG-2058. Unbound ligand was separated with dextran-coated charcoal (DCC) (10, 11).

RESULTS

cPR and chsp90 Are Located in Different Cell Compartments. Since conventional liquid fixation methods in immunohistochemistry could cause artificial relocation of soluble antigens (such as steroid receptors or hsp90), we used a freeze-dry/vapor-fixation immunohistochemical method, eliminating diffusion artifacts (13). Only nuclear staining was detected in chicken oviduct tissue sections stained with different anti-PR monoclonal antibodies (Fig. 1*C*). The subcellular distribution of the cPR was not affected by progesterone administration. These findings confirm earlier studies that used a different methodological approach (10–14). In contrast, three different monoclonal antibodies against chsp90 stained the cytoplasmic compartment (Fig. 1*A*). Furthermore, chsp90 transiently expressed in HeLa cells was located in the cytoplasm (Fig. 2*A* and Table 1), whereas cPR was nuclear; coexpression of chsp90 and cPR did not affect their locations (Table 1). No change in the location of chsp90 was observed after progesterone exposure for 1 hr (Fig. 1*B* and Table 1). Note that the anti-hsp90 antibodies recognize

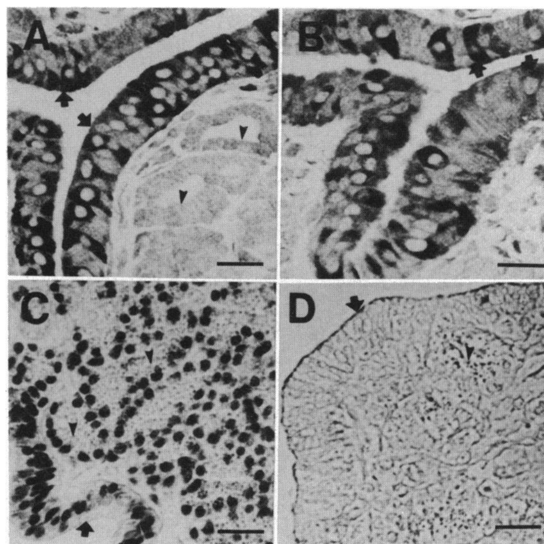

FIG. 1. Immunohistochemical location of cPR and chsp90 in the estrogen-primed immature chicken oviduct. The freeze-dry/formaldehyde vapor-fixation method was used. (*C*) Unliganded cPR was detected with the monoclonal antibody PR22 (4) at 1 μg/ml in the nuclei of epithelial, glandular, and stromal cells. (*A*) chsp90 was detected with the monoclonal antibody 7Dα at 1 μl/ml in the cytoplasm of epithelial cells and at lower concentrations in the cytoplasm of other oviductal cell types of chickens not treated with progesterone. (*B*) A similar location of chsp90 was found 1 hr after progesterone treatment. (*D*) Presaturation of 7Dα with an excess of purified chsp90 abolished all staining. Arrows point to the surface of the epithelium; arrowheads point to the glands. (Bar = 20 μm.)

both receptor-free and receptor-bound chsp90 (19, 20, 25). Staining of chsp90 was specific, since no staining was seen when the antibody was replaced by either buffer or nonspecific antibodies or when the specific antibodies were presaturated with an excess of affinity-purified chsp90 (Fig. 1*D*). Thus, we conclude that chsp90 and cPR are located in

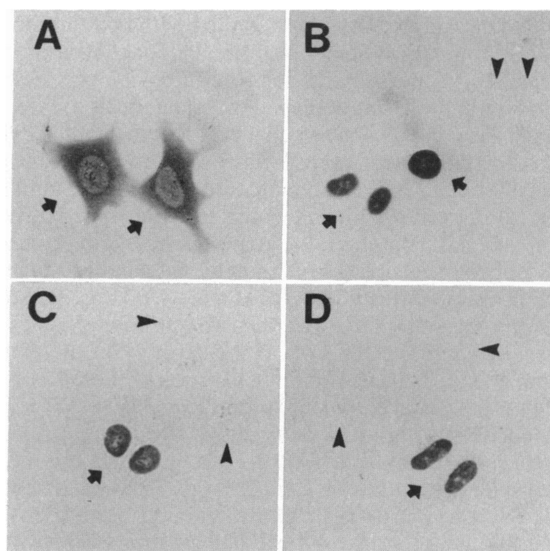

FIG. 2. Immunohistochemical location of transiently expressed chsp90, NLS-cHsp90, and cPR-HSP2. HeLa cells were transfected with either chsp90 cDNA (*A*) or with NLS-cHsp90 (*B*) and then stained with chsp90-specific 7Dα. Cells were transfected with cPR-HSP2, and the hybrid protein was detected with 7Dα (*C*) or with PR22 (*D*). It should be noted that *C* and *D* represent the neighboring areas of the same culture dish. Arrows point to the cells that express detectable amounts of the particular protein, and arrowheads point to the cells in which the protein is undetectable.

Table 1. Immunological recognition of hsp90 is independent of its intracellular location and coexpression of progesterone receptor

Expressed antigen	Antibody	R5020	% compartmentalization N	N>C	N=C	N<C
cPR	Anti-cPR	−	83	17	0	0
cPR	Anti-cPR	+	94	6	0	0
chsp90	Anti-chsp90	−	0	0	8	92
chsp90 + cPR	Anti-chsp90	−	0	0	18	82
chsp90 + cPR	Anti-chsp90	+	0	0	18	82
chsp90 + cPR	Anti-cPR	−	68	32	0	0
chsp90 + cPR	Anti-cPR	+	72	28	0	0
NLS-chsp90	Anti-chsp90	−	73	25	2	0
NLS-chsp90 + cPR	Anti-chsp90	−	90	10	0	0
NLS-chsp90 + cPR	Anti-chsp90	+	85	15	0	0
NLS-chsp90 + cPR	Anti-cPR	−	79	21	0	0
NLS-chsp90 + cPR	Anti-cPR	+	73	27	0	0

cPR and recombinant wild-type chsp90 and nuclear chsp90 (NLS-chsp90) were transiently expressed in HeLa cells. The intracellular location of proteins was analyzed when expressed alone or together in the absence (−) or presence (+) of the progestin R5020 (10 nM). The subcellular compartmentalization was classified from at least 250 cells as follows: exclusively nuclear staining (N) or nuclear staining that is more pronounced than (N>C), equal to (N=C), or less intense than (N<C) cytoplasmic staining (for details of location scoring, see ref. 22). Note that the monoclonal anti-chsp90 antibody (7Dα) used does not crossreact with the endogenous HeLa hsp90. The monoclonal anti-cPR antibody used was PR22.

different subcellular compartments and their location is not influenced by ligand binding to cPR.

To demonstrate the relative sensitivity of the immunohistochemical detection of chsp90 and cPR, we constructed a chimeric molecule between cPR and the chsp90 (cPR-HSP2). This chimera expresses an equal number of epitopes for the monoclonal antibodies PR22 (recognizing PR) and 7Dα [recognizing chsp90 but not human hsp90 (20)]. When HeLa cells were transfected with cPR-HSP2 and stained with either PR22 or 7Dα at an antibody concentration of 1 μg/ml, nuclear staining of equal intensity was detected with both antibodies (Fig. 2 *C* and *D*). When the antibody concentration was decreased to 25 ng/ml, the staining intensity was equally reduced with both antibodies. An equal decrease in the staining intensity was observed when 20 μg instead of 100 μg of peroxidase substrate per ml was used. Both the cPR and the chsp90 epitopes were undetectable when the antibody concentration was <25 ng/ml or the substrate concentration was <5 μg/ml. Immunoblot experiments with cytosolic extracts of the transfected HeLa cells revealed a protein of expected size as detected by PR22 and 7Dα (Fig. 3). Moreover, the signals obtained with the two antibodies were very similar when identical aliquots of the same cytosolic extract were used (Fig. 3). The immunodetection of hsp90 is independent of its intracellular location (Fig. 2 *A* and *B*). Full-length chsp90 (without nuclear localization signal) can be detected in the cytoplasm (Fig. 2*A*) as well as in the nuclei, when chsp90 contains the NLS of ER (Fig. 2*B*). On the other hand, coexpression of chsp90 and cPR did not affect their staining intensities or the subcellular locations (Table 1). It should be noted that the intranuclear chsp90 did not decrease the staining intensity of cPR. All of these results show that the antibodies have equal sensitivity in detecting their respective antigens. Thus, if significant amounts of nuclear chsp90 were present, it should have been detected by our assay.

Most of the PR in Hypotonic Cell Lysate Is Complexed with the hsp90. Nonliganded (aporeceptor) PR is weakly bound in the nuclei of target cells *in vivo* and leaks into the cytosol during tissue fractionation *in vitro* (6, 10). At low ionic

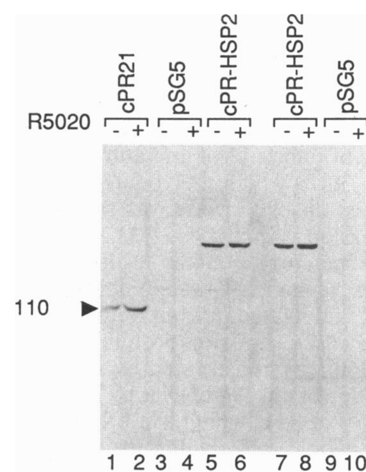

FIG. 3. Immunoblotting of equal aliquots of HeLa cell cytosol expressing cPR (cPR21) or cPR-chsp90 (cPR-HSP2) hybrid with PR22 (lanes 1–6) and 7Dα (lanes 7–10). Cells were grown either without hormone (lanes 1, 3, 5, 7, and 9) or with progestin R5020 at a final concentration of 10 nM for 8 hr (lanes 2, 4, 6, 8, and 10). Protein bands of expected size were detected for both the cPR (lanes 1 and 2) and cPR-HSP2 (lanes 5, 6, 7, and 8). The staining intensities of cPR–chsp90 with PR22 and 7Dα were similar.

strength, the cytosolic PR after tissue fractionation forms an oligomeric 8S complex with the hsp90, whereas at high ionic strength this complex dissociates. With the same tissue samples that were used for immunohistochemistry, we found association of chsp90 with cPR as detected by sucrose gradient centrifugation analysis (Fig. 4*A*) and a specific immunoenzymometric assay with PR6-7Dα, which detects heterooligomeric cPR–chsp90 complexes. In chickens not treated with progesterone, most of the cytosolic cPR (2932 ± 114 fmol/ml with ligand binding assay; 2449 ± 354 fmol/ml with PR6-PR22 IEMA) prepared in hypotonic buffer with molybdate (termed nonliganded PR) was bound to chsp90, since it sedimented at 8 S and was shifted to 10 S by the chsp90-specific monoclonal antibody 7Dα (Fig. 4*A*). However, when the cytosol was prepared without molybdate and in the presence of 0.3 M KCl, the cPR sedimented at 4 S and was not shifted by chsp90 antibodies (Fig. 4 *A* and *B*), indicating that cPR was not complexed with chsp90. When cPR was extracted from highly purified nuclei of oviductal cells of chickens that had not received progesterone, only about 1% of cPR (38 ± 1 fmol/ml with PR6-PR22 IEMA) was recovered. Progesterone treatment increased clearly the amount of the nuclear cPR (169 ± 8 fmol/ml with PR6-PR22 IEMA); however, the major part of cPR remained cytosolic (2044 fmol/ml). Progesterone treatment of the chickens decreased cPR–chsp90 complexes in cytosol from 3795 ± 308 fmol/ml (PR6-7Dα IEMA) to 506 ± 63 fmol/ml in 1 hr. The IEMA with PR6 and 7Dα showed that no chsp90–cPR complexes were present in the nuclear extracts even when receptors were extracted with molybdate, which is known to stabilize the hsp90–PR complex (6). Thus, hsp90 and PR, while present in different subcellular compartments as demonstrated by immunohistochemistry, can form complexes in soluble extracts of the same tissue.

hsp90-free PR Can Reassociate with hsp90 when Homogenized with hsp90-Containing Tissue. To study the possibility that the hsp90 and the PR could form a complex during tissue processing *in vitro*, we have prepared a chsp90-free cPR by high-salt treatment of chicken oviduct cytosol followed by dialysis. Note that both liganded and unliganded cPR remain in the 4S form after high-salt treatment and dialysis (Fig. 4*B*). When the nonliganded chsp90-free receptor was rehomogenized with the bursa of Fabricius of an immature chicken

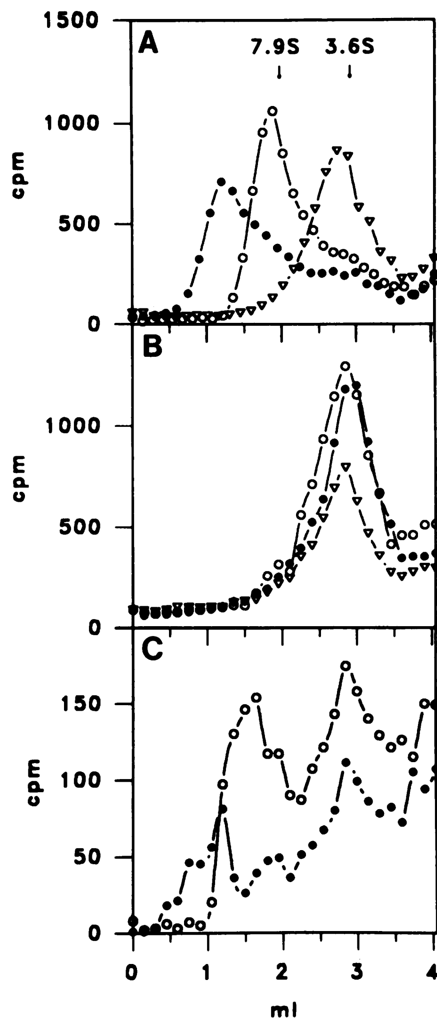

FIG. 4. Nonliganded cPR forms complexes with chsp90 *in vitro*. (*A*) Almost all of the chicken oviduct nonliganded cPR is in the 8S form in low-salt cytosol (○), and it is shifted with chsp90-specific monoclonal antibody 7Dα (●). When 0.3 M KCl is added for 2 hr, all cPR is transformed to the 4S form (▼). (*B*) KCl-treated cPR sediments at 4 S also after dialysis for desalting and contains no chsp90. ○, Nonliganded KCl-treated and dialyzed cPR, which is labeled after dialysis; ●, nonliganded KCl-treated, dialyzed cPR incubated for 4 hr at 0°C with 7Dα; ▽, liganded, KCl-treated, and dialyzed cPR. (*C*) When nonliganded aporeceptor is homogenized with immature bursa of Fabricius and thereafter labeled, about half of the binding activity sediments at 8 S (○), and this is shifted with 7Dα (●), indicating a presence of chsp90. The background labeling of the bursa cytosol is subtracted. Radioactivities (cpm) of fractions of two drops (≈150 μl) are given.

known to contain high amounts of chsp90 but no detectable cPR (11), the cPR was found to form a complex with the chsp90 (Fig. 4*D*).

Ligand Prevents PR–hsp90 Complex Formation. The formation of the chsp90–cPR complexes seems to require nonliganded apoPR (since no 8S PR was observed with liganded holoPR or with apoPR labeled *in vitro*) when it was thereafter homogenized with bursa (Fig. 5*B*). Because the nonliganded cPR can reassociate with chsp90 during homogenization (Fig. 4*C*), the ligand apparently prevents the complex formation. Indeed, when the animals were administered progesterone *in vivo* and tissue extract was prepared 1 hr later, cPR (termed holoPR) in the resulting cytosol was considerably less associated with chsp90 (Fig. 5*A*). Only the minor 8S cPR peak was shifted by chsp90 antibodies. Because the ligand prevented 8S formation *in vitro*, it can be reasoned that progesterone administration *in vivo* more likely prevents the formation of the cPR–chsp90 complex during tissue processing than causes a dissociation of a putative preexisting 8S complex.

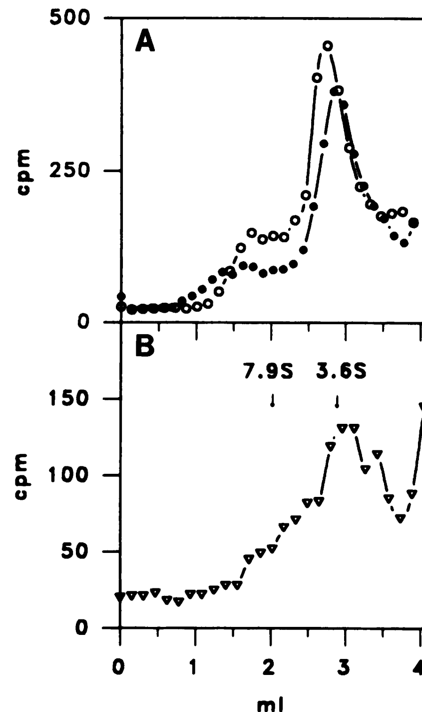

FIG. 5. Liganded cPR does not form complexes with chsp90. (*A*) One hour after progesterone injection (20 mg/kg), cPR (holoreceptor) is in the 4S form with a minute amount of 8S cPR (○). Only the 8S fraction contains the cPR–chsp90 complexes, since it is shifted with 7Dα in a sucrose gradient ultracentrifugation (●). (*B*) ApoPR (from chickens not treated with progesterone) labeled *in vitro* with radioactive ligand and treated with KCl and dialyzed (▽) sediments mainly at 4 S after homogenization with bursa of Fabricius. The background labeling of the bursa cytosol is subtracted.

DISCUSSION

Since the PR is intranuclear, hsp90 must also be nuclear if it directly interferes with the gene regulatory functions of PR *in vivo*. However, hsp90 appears to be located mainly in the cytoplasm (14–16), although both cytoplasmic and nuclear location has been reported in rabbit uterine cells (26). The reason for these conflicting results is unknown. A possible explanation may be that in the latter study liquid fixation was used, which may result in the diffusion of antigens (27, 28). Here, we have used freeze-dry and vapor-fixation techniques and thus minimized diffusion artifacts. This method clearly shows that hsp90 and PR are located in the chicken different subcellular compartments. Moreover, the sensitivity of the immunological detection of chsp90 and cPR by antibodies 7Dα and PR22, respectively, was determined to be equivalent by staining of a chsp90–cPR chimera expressing both epitopes. Therefore, the amount of intranuclear chsp90 that is supposed to be present in a ratio of 2:1 in a complex with the PR (29) should have been easily detected, if it had been present.

When unliganded PR was analyzed in chicken tissue homogenates, nearly all of the PR was associated with hsp90, whereas after progesterone treatment *in vivo*, most of the PR was free of hsp90. This has been regarded as evidence that the ligand induces dissociation of hsp90 from receptors (8, 30–32). Our results demonstrate that PR–hsp90 complex can be reconstituted when unliganded PR free of hsp90 is homogenized with hsp90-containing tissue. The reassociation does not take place, if PR is liganded. Similar reconstitutions have been reported with other steroid receptors using reticulocyte

lysate as a source for hsp90 (33–35). Taken together, the histochemical and biochemical results suggest that PR released from the nucleus during tissue fractionation associates with cytoplasmic hsp90 and that this association is affected by the ligand. Thus, ligand (agonist) binding appears to result in a change in receptor structure and may generate a receptor with a lower affinity for hsp90. It has been proposed that antagonists prevent this conformational change and stabilize the hsp90–receptor complex. The ability of the antihormone RU486 in stabilizing the hsp90–PR complex and thus inhibiting receptor–DNA interaction has been used to explain its antihormone action (36). However, it has been shown recently that the PR–RU486 complex can bind to its DNA responsive element and can activate both *in vivo* and *in vitro* transcription from some promoters (37, 38).

Our results bring 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 a 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. Indeed, it has been shown recently that steroid hormone receptors do not act as constitutive activators of transcription in nearly hsp90-free yeast cells (39). hsp90 was found to only affect, for unknown reasons, the sensitivity of the hormonal response (39). Our data do not exclude that hsp90 may have a role in posttranslational modification or chaperoning (40) of newly synthesized receptor in the cytoplasm, which would be in agreement with the findings in the yeast cell model (39). At present, it is not clear whether the glucocorticoid receptor (GR), which seems to be at least partially cytoplasmic in the absence of ligand (41), forms hsp90–GR complexes with distinct biological functions.

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