Journal of Cellular Biochemistry 74:458–467 (1999)

Only a Small Portion of the Cytoplasmic Progesterone Receptor Is Associated With Hsp90 In Vivo

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Abstract In cell extracts all of the nonliganded steroid receptor molecules are found as an oligomeric complex with Hsp90 and other proteins. In previous studies we have shown that Wild-type Hsp90 and progesterone receptor (PR) are located in different cell compartments (Tuohimaa et al. [1993] Proc. Natl. Acad. Sci. USA 90:5848–5852). In the present work we studied whether PR and Hsp90 can efficiently associate provided they are present in the same cell compartment. The association of Hsp90 with PR in vivo was studied by nuclear cotranslocation and immunohistochemistry with an antibody (αD) which can distinguish between the oligomeric and dissociated form. Upon expression of a cytoplasmic mutant of PR with Wild-type (cytoplasmic) Hsp90 and Wild-type (nuclear) PR with NLS-Hsp90 (a Hsp90 with a nuclear localization signal), we noted that the epitope of αD in PR was exposed in both cases. Also, in vivo crosslinking and treatment of cells with substances which stabilize the oligomeric complex in vitro were inefficient in demonstrating or inducing a similar oligomeric receptor form detectable in vitro in cell homogenates. However, when the cytoplasmic PR mutant (ΔPR) was coexpressed with a nuclear form of Hsp90 (NLS-Hsp90), a portion of PR was cotranslocated into the nucleus. This would indicate that steroid receptors are indeed associated with Hsp90 in intact cells, but the Hsp90-associated receptor pool represents only a small portion of the receptors. This suggests that the majority of oligomeric complexes seen in cell extracts are formed during cell fractionation. J. Cell. Biochem. 74:458–467, 1999. © 1999 Wiley-Liss, Inc.

Key words: Hsp90; progesterone receptor

Steroid receptors are transcription factors whose activity is regulated by ligand binding [Gronemeyer and Laudet, 1995]. According to a current view, nonliganded steroid receptors exist as oligomeric complexes with different proteins (RAP, receptor-associated proteins) of which the majority are heat shock proteins [Smith and Toft, 1993]. The oligomeric form is regarded as a "poised" conformation, inactive but highly responsive to the triggering ligand [Bohen et al., 1995]. Ligand binding renders the oligomeric complex less stable in vitro and it has thus been postulated that also in vivo ligand-induced dissociation of the complex is required for activation of the steroid receptors [Baulieu, 1987; Groyer et al., 1987]. In contrast to steroid receptors, retinoic acid receptors (RAR), thyroid receptors (TR), and the vitamin-D receptor (VDR) have not been found as stable oligomeric complexes and it has been proposed that their interaction with RAPs is transient, taking place immediately after synthesis [Dalman, 1990, 1991; Whitfield et al., 1995]. The concept of a stable oligomeric complex in the case of steroid receptors is based mainly on in vitro results: initially from experiments made with hypotonic cell extracts and subsequently by reconstituting the complex in reticulocyte lysate [Toft and Gorski, 1966; Grody et al., 1982; Smith et al., 1990]. Since the oligomeric complex can be reconstituted by cell lysate the question arises whether this is formed in vivo in intact cells or whether it is formed when cells are lysed during cell fractionation [Ylikomi et al. 1998].

In order to study the possible complex formation in situ in intact cells, we have raised an antibody (αD) which can distinguish between

Grant sponsor: Sigrid Juselius Foundation; Grant sponsor: Novo Nordisk Funden; Grant sponsor: Medical Research Fund of Tampere University Hospital.

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Received 12 May 1998; Accepted 17 February 1999

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the oligomeric and dissociated forms [Pekki et al., 1995]. The epitope of the antibody is located in a domain which is hidden in the oligomeric form (domain D of the PR) and the antibody thus recognizes only the dissociated form in vitro. We have used this antibody for immunohistochemical studies and shown that in tissue sections it also recognizes the nonliganded receptor in the cell nucleus although all of the receptors in the cytosol prepared from these tissues are found in oligomeric complex form [Pekki et al., 1995]. The results thus indicated that the majority of nuclear receptors are in dissociated conformation. There is, however, some evidence that cytoplasmic receptor forms can interact with Hsp90, since the glucocorticoid receptor (GR), which is at least partially a cytoplasmic protein, can be crosslinked to Hsp90 in intact cells [Rexin et al., 1991]. Also a mutant PR in which a nuclear localization signal has been deleted can be made to translocate partially into the nucleus by cotransfecting it with a NLS-Hsp90 (a Hsp90 molecule to which a nuclear localization signal has been added), although a Wild-type PR cannot cotranslocate a Wild-type Hsp90 [Kang et al., 1994; Tuohimaa et al., 1993].

The present work comprised a further investigation into whether progesterone receptors and Hsp90 are associated if they are located in the same subcellular compartment. We demonstrated that the Hsp90 and PR are indeed associated in vivo in intact cells but that the associated receptors represent only a small pool of all PR molecules. All these findings are consistent with the notion that Hsp90 and most other oligomeric from-associated proteins are chaperones which interact transiently with their substrate proteins to aid their folding and structural changes. It is thus conceivable that all nuclear receptors (not just RAR and TR) are only transiently associated with these chaperoning molecules in vivo.

MATERIALS AND METHODS

Expression Vectors

Wild-type chicken progesterone receptor (cPR21) and Wild-type chicken Hsp90 expression vectors have been described elsewhere [Turcotte et al., 1990; Tuohimaa et al., 1993]. NLS-Hsp90 contains a nuclear localization signal of human estrogen receptor (amino acids 253–303) in front of the coding sequence of chicken Hsp90 and has been further characterized by Tuohimaa et al. [1993]. The nuclear localization signal-deficient chicken progesterone receptor (PR35; amino acids 454–486 deleted) has been characterized by Ylikomi et al. [1992].

Cell Culture and Transfections

COS cells were grown on glass coverslips in Dulbecco‘s modified Eagle medium supplemented with 5% fetal bovine serum, which was dextran-charcoal treated. Cells were transfected by lipofection using Lipofectamine reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer‘s instructions. The cells were incubated with the serum-free medium Optimem (Life Technologies), containing the DNA-liposome-complexes for 24 h at 37°C. The cells were incubated in medium with 10% of fetal bovine serum treated with dextran-charcoal for 2 h, the medium was changed and the cells further incubated for 24–48 h. Progesterone or promegestone (R5020) was added to some of the cell culture dishes immediately after adding the liposomes at final concentrations of 20 nM and 10 nM, respectively.

Cell Treatments

Crosslinking in intact cells was effected either with EGS (ethylene glycolbis [succinimidyl-succinate]; Pierce Chemical Co., Rockford, IL) or with paraformaldehyde. The EGS crosslinking procedure was performed as follows. The cell culture dishes were incubated on ice for 4 h and washed with ice-cold PBS. EGS (a final concentration of 10 mM in PBS and 5% DMSO) was then added to the cells. After an incubation of 1 h the reaction was stopped by adding lysine (a final concentration of 100 mM dissolved in PBS) and continuing incubation for an additional hour. The cells were then washed with PBS and fixed with paraformaldehyde. EGS stock solution was prepared by diluting 10 mg of EGS in 100 ml of DMSO; it was freshly made immediately prior to incubation. Paraformaldehyde crosslinking was carried out by adding paraformaldehyde directly to the culture dishes (at a final concentration of 1.5%) and continuing incubation for 5 min at 37°C before final fixation in 4% paraformaldehyde for 15 min.

H_2O_2 treatment was proceeded according to Tienrungoj et al. [1987]. The cells were incubated on ice for 4 h and washed with cold PBS. They then incubated with 10 mM NaN_3 in PBS (to inactivate catalase) on ice for 30 min followed by incubation with H_2O_2 in PBS (final

concentration 10 mM) for 30 min on ice. After peroxide incubation the cells were directly fixed in cold 4% paraformaldehyde for 15 min without prewashing in PBS.

Molybdate treatment was according to Raaka et al. [1985]. The tissue culture dishes were taken to room temperature, sodium molybdate added at a final concentration of 30 mM, and the cell incubated at room temperature for 1 h. They were then incubated on ice for 4 h and washed with PBS containing 30 mM sodium molybdate. After washing, they were fixed with cold 4% paraformaldehyde also containing 30 mM sodium molybdate.

Combined molybdate, H_2O_2 and EGS treatment was carried out as follows. The cells were incubated with 30 mM sodium molybdate at room temperature, incubated in cold, and washed with PBS containing molybdate as outlined above. They were then incubated with NaN_3 and by H_2O_2 as above followed by a wash with cold PBS containing molybdate. The cells were then crosslinked with EGS as above, washed with molybdate containing PBS and fixed with cold 4% paraformaldehyde also containing 30 mM sodium molybdate for 15 min.

Histochemical Techniques

In order to compare immunostaining of the two antibodies in the same cells and to verify the coexpression of transfected PR and Hsp90 in the same cells we used a double immunofluorescence labelling technique with αD/PR22 and αD/7Dα antibodies. After fixation the cells were washed in PBS for 10 min, incubated in 0.5% Triton-x-100 in PBS for 40 min at room temprature, and washed in PBS for 10 min. There after they were then incubated in 10% normal rabbit and horse serum in PBS for 30 min, and after removal of excess serum the primary antibodies were added and sections incubated overnight at 4°C. The monoclonal antibodies PR22 and 7Dα were used at a final concentration of 1 ug/ml and the polyclonal antibody αD at a dilution of 1:200. PR22 and αD antibodies as well as αD and 7Dα were mixed and applied to the neighboring areas of the same culture dish. The next day the cells were washed in PBS for 10 min, incubated with secondary antibodies, namely biotinylated anti-mouse IgG (from goat, Amersham, Arlington Heights, IL) 1:400 and Fluorescein labeled anti-rabbit IgG 1:200 in PBS for 40 min. These secondary antibodies were added to the sections simultaneously. The cells were then washed in PBS for 10 min and incubated with Rhodamin-labeled avidin D 1:100 in PBS for 30 min followed by washing in PBS for 10 min and mounting in fenylendiamin-glycerin (50 mg fenylendiamin was diluted in 5 ml PBS, filtrated and added to 45 ml glycerin. pH 8.0 was adjusted at 9.0. with 0,5M carbonate buffer). Double-labeled specimens were analyzed with a Carl Zeiss LSM 410 invert Laser Scan Microscope equipped with helium-neon (543 nm) and argon (488 nm) lasers. Antigen colocalization was confirmed by single and overlay image display of the selected optical sections.

RESULTS

The Major Portion of Cytoplasmic PR Is Not Associated With HSP90

PR is a nuclear protein which can be rendered cytoplasmic by deleting the nuclear localization signal (NLS). In the present work we used a progesterone receptor mutant, PR35, in which two of the four nuclear localization protosignals had been deleted [Ylikomi et al., 1992]. The deletion does not affect the interaction of Hsp90 and PR in vitro [Schowalter et al., 1991; Kang et al., 1994]. The mutant PR was expressed in COS cells and the possible in vivo association of cytoplasmic PR with Hsp90 studied by immunohistochemistry using a double staining method with two different PR antibodies: PR22, which detects both oligomeric and dissociated receptor, and αD, which detects only the dissociated form. Results were analyzed by confocal microscopy.

When the PR35 expression was studied with PR22 in progesterone nontreated COS cells cytoplasmic staining was seen. When the cells were stained with the αD antibody identical cytoplasmic staining was detected, indicating that the epitopes for both antibodies are readily exposed (Fig 1A,B). In extracts prepared from cells expressing a similar mutant, PR has been detected as an oligomeric complex [Kang et al., 1994]. In order to establish whether overxpression of Hsp90 affects the immunostaining, we cotransfected the Wild-type Hsp90 with the cPR35. Since the transfected Hsp90 is of chicken origin it can be detected separate from the endogenous Hsp90 by the chicken Hsp90-specific antibody 7Dα. When the cells were stained

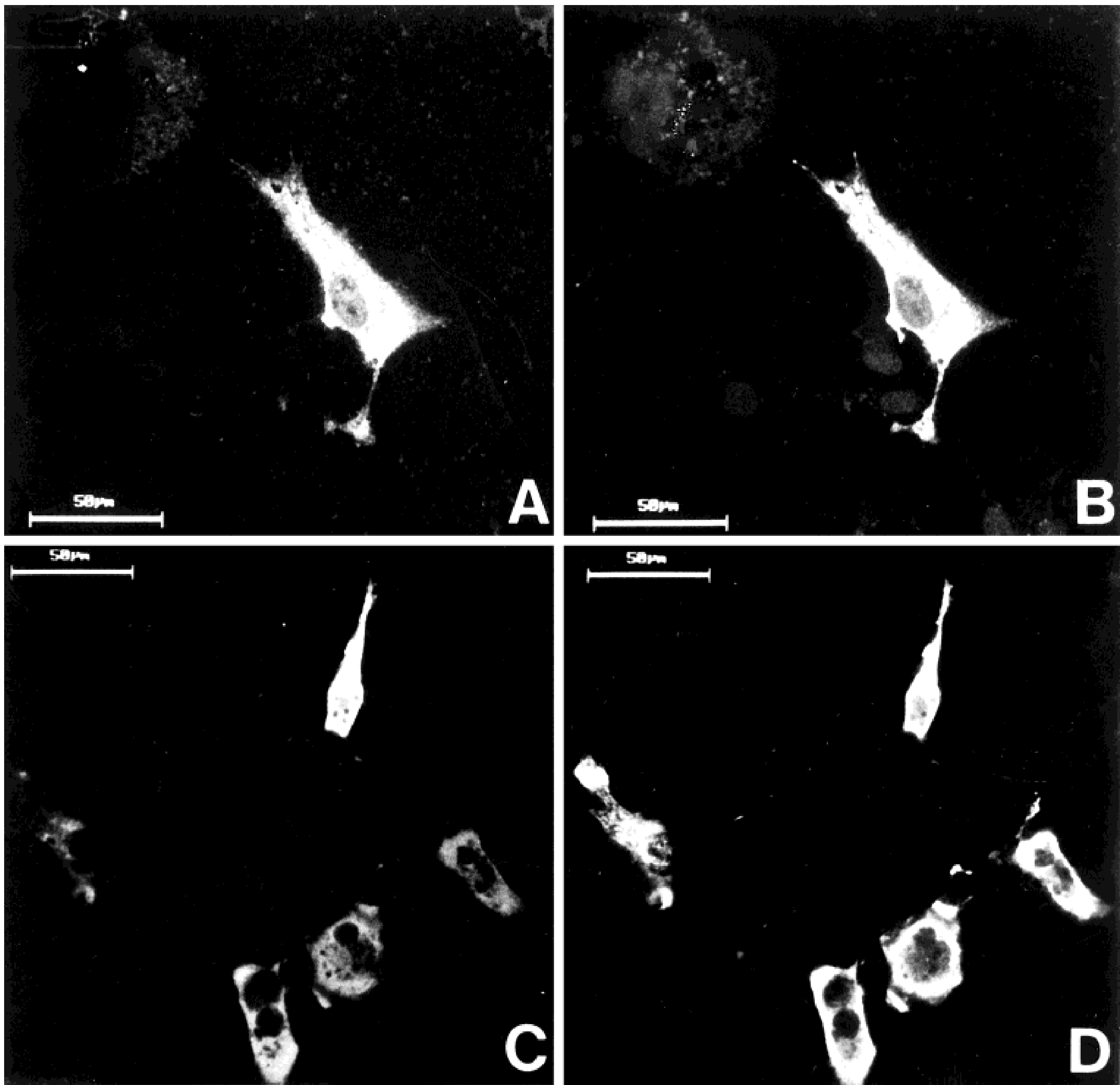

Fig. 1. Detection of cytoplasmic progesterone receptor with the αD and PR22 antibodies. Nuclear localization-deficient PR was transfected in COS cells and the expression detected with αD **(A)** and with PR22 **(B)**. The effect of the overexpression of Hsp90 on the immunostaining was studied by cotransfecting the PR mutant with Wild-type Hsp90. Cotransfected cells were stained with αD **(C)** and with PR22 **(D)**. All of the stainings were carried out by double immunofluorescence technique in order to compare the staining in the same cells and the results were analyzed by confocal microscopy. The figures were selected to represent the majority of stained cells.

with PR22 and αD, considerably lower staining intensity was seen with αD, which would suggest that a portion of the transfected Hsp90 interacts with the PR (Fig. 1C,D).

Nuclear HSP90 Is Not Associated With the PR in Detectable Amounts

Hsp90 is a cytoplasmic protein which can be made to translocate into the nucleus by attachment of a nuclear localization signal (NLS-Hsp90) [Tuohimaa et al., 1993]. It was sought now to ascertain, by coexpressing PR with the NLS-Hsp90, whether a Wild-type PR can form a complex with Hsp90 if both proteins are located in the nucleus. When cells transfected with wt-PR were stained with PR22 and αD, marked nuclear staining was observed with both antibodies (Fig. 2A,B). Cotransfecting

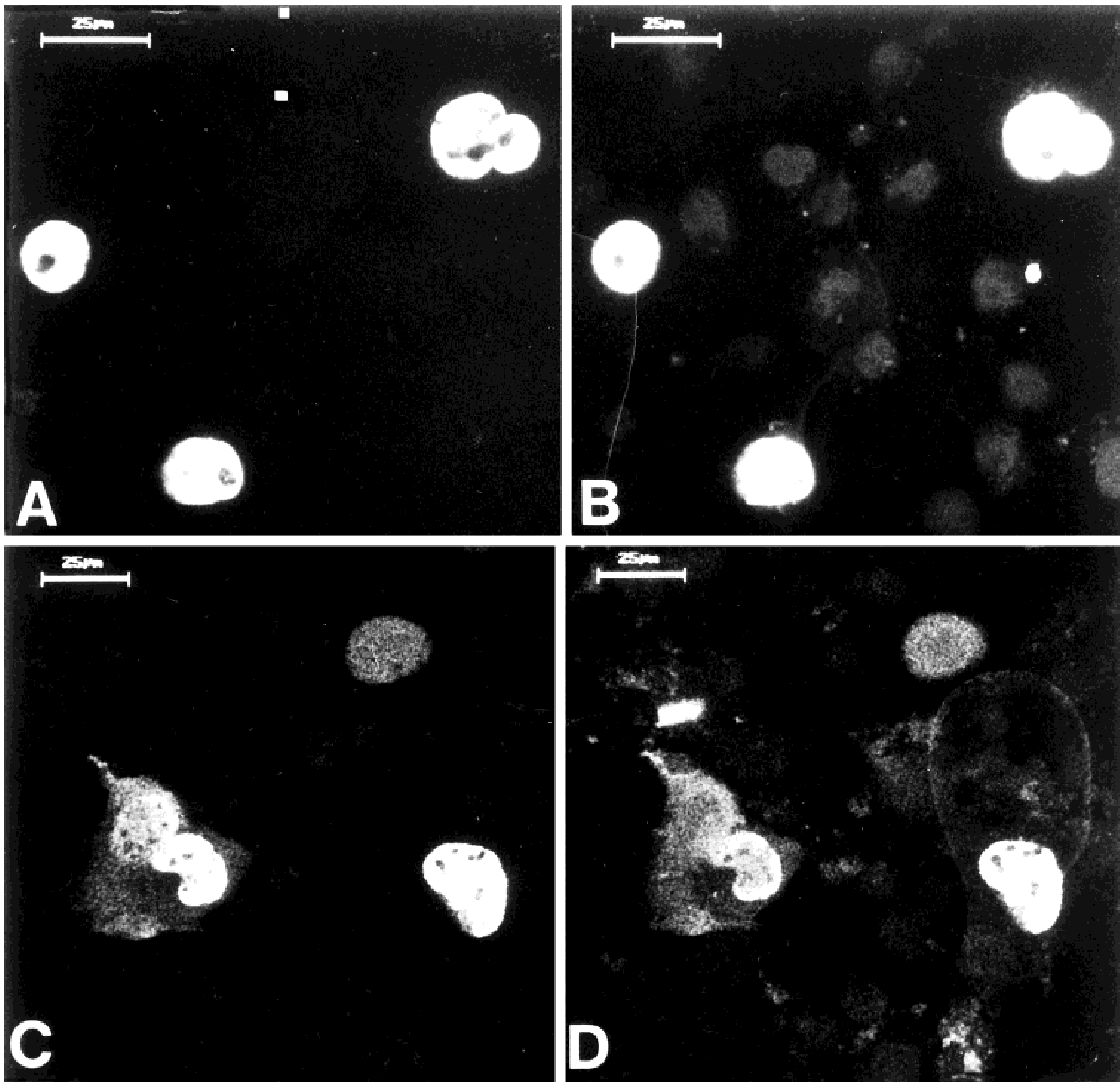

Fig. 2. Detection of the nuclear progesterone receptor with the two PR antibodies. wtPR was transfected in COS cells and the expression detected with αD **(A)** and with PR22 **(B)**. The effect of nuclear colocalization of the Hsp90 was studied by cotransfecting wtPR with a nuclear Hsp90 (NLS-Hsp90). Cotransfected cells were stained with αD **(C)** and PR22 **(D)**. All the stainings were carried out by double immunofluorescence technique in order to compare the staining in the same cells, and the results were analyzed by confocal microscopy. The figures were selected so as to represent the majority of stained cells.

NLS-Hsp90 with wt PR did not change the intensity of αD staining, indicating that the epitope for αD is as readily accessible in the nucleus as it is in the cytoplasm (Fig. 2C,D). In order to verify that both proteins are coexpressed in the same cells we also stained the cotransfected cells with 7Dα (which recognizes the chicken Hsp90) and PR22, and noted that most of the transfected cells expressed both antigens.

Effect of Hormone on the Association of HSP90 and PR

According to a current view hormone binding causes a dissociation of the oligomeric complex. We therefore studied the effect of ligand on the staining of the cytoplasmic and nuclear forms of the PR. No difference in immunostaining with αD could be detected when cells grown in the absence of ligand were compared with those grown in its presence.

Interaction of Hsp90 and PR can also be studied by monitoring the ability of NLS-Hsp90 to translocate cytoplasmic PR into the nucleus. We exploited this system to study the effect of ligand binding on the interaction of the two proteins. Cells coexpressing a cytoplasmic PR mutant (CPR35) and Wild-type Hsp90 were immunostained with PR22 and, in order to quantify the nuclear colocalization, the immunostaining was classified into four categories: N, cells which contained only nuclear staining; N > C, cells which also contained cytoplasmic staining; N = C, cells in which the intensity of nuclear and cytoplasmic staining was equal; N < C, cells in which the intensity of cytoplasmic staining exceeded nuclear staining [see details in Ylikomi et al., 1992]. The analysis was carried out with light-microscopy, not with confocal microscopy (Table I). With cPR35 most of the staining fell in the categories N = C and N < C, indicating that the mutant did not show nuclear accumulation. When NLS-Hsp90 was cotransfected with cPR35, nuclear accumulation (categories N plus N > C) increased from 1% to 33%, but the majority (64%) of the cells still fell into the categories N = C and N < C with only a few cells in N (Table I). In the presence of ligand, the nuclear accumulation of cPR35 increased due to activation of the proto-NLS in the ligand binding domain [Ylikomi et al., 1992]. When the nuclear colocalization was studied in the presence of ligand a marginal increase in nuclear accumulation was observed: cells in the N or N > C categories increased from 20% to 32% (Table I).

Effect of In Vivo Crosslinking on the Oligomeric Complex

In vivo crosslinking by bifunctional crosslinkers or by paraformaldehyde has been used to study the in vivo association of proteins. We "in situ fixed" cells by adding paraformaldehyde to the growth medium and analyzed PR staining with αD. The epitopes were readily detectable with both PR antibodies and there was no detectable change in the staining when compared with sections fixed after termination of the cell culture (Fig. 3A,B).

TABLE I. Effect of NLS-Hsp90 on the Nuclear Transport of a Nuclear Localization-Deficient PR Mutant[a]

PR mutant	N	N > C	N = C	N < C
apo cPR 35	0	1	19	80
apo cPR35 + NLS-hsp90	3	33	33	31
holo cPR35	2	18	20	60
holo cPR35 + NLS-hsp90	1	31	30	38

[a]Apo cPR35 and holo cPR35, nuclear localization signal-deficient chicken progesterone receptors in nonliganded or liganded form, respectively. NLS-Hsp90, a chimeric molecule between a Hsp90 and a nuclear localization signal of the estrogen receptor. Immunostaining was performed with PR22.

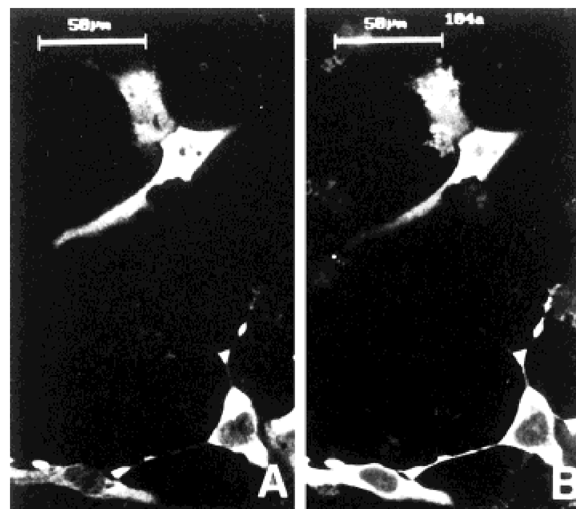

Fig. 3. The effect of in vivo crosslinking on the PR immunostaining was studied by treating cells with paraformaldehyde **(A,B)**. Cells were stained with αD (A) and with PR22 (B). Double immunofluorescence technique was employed. The figures were selected to represent the majority of stained cells.

We also used a bifunctional crosslinker EGS (ethylen glycol bis[succinimidylsuccinate]) to crosslink PR in intact cells. Like paraformaldehyde crosslinking, also crosslinking with EGS did not affect the staining of the two PR antibodies.

Effect of Oxidative Stress and Molybdate on the Oligomeric Complex In Vivo

The oligomeric complex can be rendered more stable in vitro by the addition of molybdate to the cytosol or by increasing the oxidative status of the cytosol [Nishigori and Toft, 1980; Tienrungroj et al., 1987]. The stabilized complex can resist heat and hypertonicity-induced dissociation in vitro. There is evidence that treatment of cells in vivo with molybdate results in a more stable complex in the cytosol prepared from these cells [Raaka et al., 1985]. We thus undertook to study whether molybdate or oxidative stress can affect αD staining.

We added sodium molybdate to culture medium and cultured cells for 1h. The cells were

washed and fixed in the presence of molybdate. The H_2O_2 treatment was carried out by first washing the cells with cold PBS followed by treating them with NaN_3 to inactivate catalase, and subsequently incubating them with 0.1–10 mM H_2O_2 on ice for 30 min. The cells were then either directly fixed in paraformaldehyde for immunohistochemistry or treated with EGS before paraformaldehyde fixation. We also combined the molybdate, H_2O_2, and EGS treatments. None of these treatments affected the immunostaining with αD.

DISCUSSION

In previous works we have shown that Hsp90 and PR are located in different cell compartments, Hsp90 in the cytoplasm and PR in the nucleus [Tuohimaa et al., 1993; Pekki, 1991]. Here we show that transiently expressed PR and Hsp90 are also located in different cell compartments. There is evidence, however, that steroid receptors are associated with Hsp90, since when NLS deficient PR was transfected with a NLS-Hsp90 (an Hsp90 with a nuclear localization signal), the mutated PR was seen to accumulate in the nucleus with the NLS-Hsp90. The association is not particularly efficient, however, since the nuclear colocalization was not very significant. Similar results have previously been obtained, but the magnitude of colocalization of PR was not quantified [Kang et al., 1994]. Since the oligomeric complex cannot be transported to the nucleus, the nuclear accumulation is probably due to retention by the NLS-Hsp90 of a shuttling PR mutant in the nucleus [Yang and Defranco, 1996]. In the present work we also studied the effect of ligand on the colocalization and demonstrated that agonist binding slightly reduces the Hsp90-PR interaction. In the cotranslocation experiment the nuclear transport kinetics were not measured and the experiment gave no indication of the magnitude of the receptor and Hsp90 association. During a 2- to 3-day transfection experiment even a minimal association would contribute to a marked cotranslocation of a molecule which has a tendency to accumulate in the nucleus by merit of its DNA-binding ability and reduced nuclear export ability due to the deletion of the nuclear localization signal (the NLS of the PR has been shown to control both nuclear import and export). It should also be noted that Wild-type PR cannot significantly change the location of Wild-type Hsp90, indicating that without a heterologous NLS the Wild-type Hsp90 is a cytoplasmic protein whose location is not affected by nuclear receptors [Tuohimaa et al., 1993].

To study further the possible association of Hsp90 with PR in intact cells we have raised an antibody which can distinguish the oligomeric form and the dissociated form [Pekki et al., 1995]. In previous work we have used this antibody to study endogenous PR by immunohistochemistry on tissue sections. It was established that the epitope of the nonliganded PR is readily detectable in the cell nucleus although the PR extracted from these tissues is in oligomeric form and not detectable by the antibody. We also showed that the fixation used for the preparation of histochemical sections does not affect the oligomeric complex [Pekki et al., 1995]. These results are consistent with the finding that the PR is a nuclear protein and not associated with a cytoplasmic Hsp90. There are, however, receptors which are located, at least partially, in the cytoplasm and might thus be associated with Hsp90 [Wikström et al., 1987]. For this reason we sought to establish whether PR and Hsp90 would be associated if they were located in the same cell compartments. We coexpressed cytoplasmic forms of Hsp90 and PR in COS cells and studied their possible association by means of the αD antibody. Both PR22 and αD recognized the cytoplasmic PR and there was no increase in staining intensity when liganded receptors were studied with the αD antibody, which would be expected if the dissociation or association were regulated by ligand binding. Cotransfecting the Hsp90 with the mutated PR, however, reduced the staining compared that with PR22. The results indicate that the interaction was not detectable with the cytoplasmic form of the PR and the endogenous Hsp90, but could be triggered in the cytoplasm by overexpressing Hsp90. This cannot be explained by the possibility that the amount of transfected receptor exceeds the amount of endogenous Hsp90, since all of the transfected receptors have been found as an oligomeric complex in the cytosol of transfected cells [Chambraud et al., 1990; Carson-Jurica et al., 1989]. In contrast, no association of the Wild-type PR and the NLS-Hsp90 could not be detected in the nucleus.

The stability of the oligomeric complex can be increased by transition metal ions (molybdate and tungstate), by increasing oxidative conditions (H_2O_2) and by crosslinking [Nishigori and Toft, 1980; Tienrungroj et al., 1987; Raaka et al., 1985; Alexis et al., 1992; Rexin et al., 1991]. There are several works where GR has been crosslinked to RAPs when intact cells were treated with various crosslinking substances [Alexis et al., 1992; Rexin et al., 1991]. In two studies a true nuclear receptor ER (nonliganded GR has been shown to be at least partially cytoplasmic protein) has been crosslinked in intact MCF-7 cells [Rossini and Camelli, 1994; Segnitz and Gehring, 1995]. The results of these two studies were controversial. In one of them ER was crosslinked only with an undefined 50k protein [Rossini and Camelli, 1994], while in the other, Hsp90 together with a p59 were found crosslinked with the receptor [Segnitz and Gehring, 1995]. One of the important differences between the works was that in the latter the cells were incubated for 2 h on ice before the crosslinkers were administered. Cold prevents nuclear transport and shuttling nuclear proteins become cytoplasmic, and it cannot be excluded that these authors may not have studied nuclear receptor but the one accumulated in the cytoplasm [Guiochon-Mantel et al., 1991]. These results suggest that at least cytoplasmic receptors can be crosslinked to Hsp90 in vivo. We thus decided to stain the in vivo crosslinked cells with the αD antibody. We used two different crosslinkers (paraformaldehyde and EGS), and we expressed both the Wild-type proteins and the cytoplasmic or nuclear forms of the proteins, but were unable to demonstrate the oligomeric complex. We were also unable to demonstrate the complex after treatment of transfected cells with molybdate and oxidative stress alone or in combination with in vivo crosslinking. This failure to detect the oligomeric complex after stabilizing the complex in vivo suggests that the fraction of PR which is associated with the RAPs is small. The association can be detected by cotranslocation experiments, since it represents nuclear accumulation over an extended time span (2–3 days), but not in αD immunohistochemistry, in which a large pool of Hsp90-free receptors dominates the staining.

Taking together the histochemical experiments on the location of steroid receptors and Hsp90 as well as the cotranslocation experiments, it can be concluded that the magnitude of the Hsp90 and PR association is small, and that since the wt Hsp90 is a cytoplasmic protein, the association takes place in the cytoplasm. It should be noted that the present experiments were conducted with cells which do not express endogenous PR. However, a biochemical experiment on the association between RAPs and steroid receptors, using reticulocyte lysate and transiently expressed receptors in various cell types, has shown to reflect association in target cell lysates. The finding raises a question of the origin of the oligomeric complex found in cell extracts and reticulocyte lysate, where all of the nonliganded receptor molecules are associated with Hsp90. The majority of the receptor-associated proteins belong to chaperoning proteins which bind to hydrophobic regions of unfolded proteins to prevent their aggregation and aid proper folding of the substrate proteins. The hormone binding domain of nonliganded steroid receptors is highly hydrophobic and they are thus good targets for the Hsp interaction [Hansen and Gorski, 1985; Alnmeri and Litwack, 1993; Ylikomi et al., 1998]. It is conceivable that nonliganded steroid receptors are associated with chromatin constituents and, are released during cell fractionation, and their hydrophopic surfaces are exposed to an aqueous milieu. This induces Hsp90 binding, which prevents receptor aggregation, and generates the oligomeric complex. Ligand binding causes a conformation change which renders the receptor more hydrophilic, and thus liganded receptors do not associate in cell extracts with Hsps, or the association is so weak that it cannot be detected in vitro [Wurtz et al., 1996; Hansen and Gorski, 1985]. In support of such a conception there is ample biochemical evidence demonstrating that nonliganded receptor monomers can be reconstitututed to the oligomeric complex in various cell lysates (reticulocyte, mouse L cell, insect cell and plant cell) and homogenates [Smith et al., 1990; Tuohimaa et al., 1993; Stancato et al., 1996]. It has moreover been demonstrated that most of the RAPs form stable complexes with various denatured proteins in vitro which would further support the notion that the formation of a stable oligomeric complex with Hsps is an in vitro phenomenon [Freeman et al., 1996].

ACKNOWLEDGMENTS

We are indebted to Hinrich Gronemeyer for the cPR cDNA. We are grateful to Maria Catelli for the Hsp90 cDNA, to David Toft for monoclonal antibodies (PR22 and 7Dalpha).

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