Evidence for the existence of an oligomeric, non-DNA-binding complex of the progesterone receptor in the cytoplasm

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Steroid receptors are found as a hetero-oligomeric complex in cell extracts. Due to the dynamic interaction between receptor-associated proteins and receptors, it is difficult to study the oligomeric complex in living cells. Here this was attempted in cells in which the interaction was stabilized by introducing molybdate into the cells or by incubating the cells at low temperature. The complex was studied with an antibody (αD) recognizing only the dissociated form of the chicken progesterone receptor (PR) and with antibodies (PR22, PR6). Recognizing also oligomeric forms of the receptor. When wild-type chicken PR was transfected, all antibodies showed nuclear staining. Molybdate or cold treatment of cells resulted in cytoplasmic accumulation of the PR as detected with PR22/PR6. αD, however, stained predominantly the nuclear PR in treated cells. These findings suggest that when the oligomeric complex of the PR is stabilized in intact cells in vivo and then crosslinked with paraformaldehyde, a portion of the cytoplasmic receptor is seen as an oligomeric complex, whereas, in the nucleus, most, if not all receptor molecules are in dissociated form.

Key words: heat shock protein 90, progesterone receptor, oligomeric complex, immunohistochemistry, molybdate.

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Steroid receptors belong to a large family of nuclear receptors functioning as ligand-regulated transcription factors. Receptor function is mediated by receptor-interacting proteins; co-activators interact with agonist-occupied receptors and mediate transcription activation while co-repressors interact with non-liganded or antagonist-occupied and mediate transcription repression. Ligand binding induces a considerable change in the structure of the ligand-binding domain (LBD), affecting the surface exposition of the receptor which dictates the interaction of the cofactors [Moras and Gronemeyer 1998, Renaud *et al.* 1995, Wurtz *et al.* 1996]. The earliest known receptor-interacting protein is Hsp90 [Dougherty *et al.* 1984]. It is known to interact *in vitro* with the LBD of the steroid receptor in a ligand-dependent manner: it binds to non-liganded steroid receptor but not to agonist-occupied receptor. Non-liganded steroid receptor LBDs act as intramolecular repressors of the activation functions of steroid receptors as well as of heterologous proteins when covalently linked to them [Picard *et al.* 1988]. Deletion of the LBD generates a constitutively active steroid receptor which does not form a stable oligomeric complex with Hsp90 *in vitro*. In view of these data, it has been proposed that the Hsp90 interaction is responsible for the repressor function of non-liganded LBDs [Scherrer *et al.* 1993]. Binding of Hsp90 interferes with the DNA binding of the receptor, which has been regarded as one possible mechanism by which Hsp90 functions as a corepressor. Hsp90 is also required for proper ligand binding of the glucocorticoid receptor [Bresnick *et al.* 1989]. It has been proposed that the oligomeric form of steroid receptors represents a poised conformation, inactive but ready to interact with the ligand. Ligand binding is thought to dissociate the complex and allow receptor binding to DNA [Baulieu 1987, Bohen *et al.* 1995]. It should be noted that such a mechanism is confined solely to steroid receptors, as other nuclear receptors, such as retinoid-, thyroid- and vitamin-D receptors apparent-

ly do not associate with Hsp90 [Dalman *et al.* 1990, Dalman *et al.* 1991, Whitfield *et al.* 1995].

Heat shock proteins are one of the most highly conserved group of proteins and are expressed in organisms ranging from bacteria to practically all eukaryote cell types [Bardwell and Craig 1984, Hunt and Morimoto 1985]. Although termed heat shock proteins, most of them are constitutively expressed at significant levels at all physiological temperatures [Kelley and Schlesinger 1982] [Welch and Feramisco 1982]. Their expression is enhanced in consequence of a wide variety of environmental assaults such as increased temperature, metabolic poisons, alcohol and toxins [Burdon 1986]. The response to such noxious conditions is now more often referred to as a stress response and the proteins in question as stress proteins. Intracellular accumulation of abnormally folded polypeptides constitutes a trigger for the initiation of the stress response [Welch 1992]. Stress proteins belong to a large family of unrelated protein classes called chaperones [Hemmingsen *et al.* 1988]. These molecular chaperones promote correct folding of a newly synthetized polypeptide into a functional protein by recognizing and binding non-native proteins, thus preventing aggregation and retaining the proteins in the productive folding pathway. The folding takes place sequentially through several intermediates. Chaperones are involved in all stages of protein metabolism: protein folding, oligomeric assembly, transport to a particular subcellular compartment and targeting proteins for degradation [Ellis and van der Vies 1991].

There is evidence that Hsp90 is a cytoplasmic protein and that there is not a sufficient amount of Hsp90 molecules in the nucleus to form complexes with nuclear steroid receptors [Tuohimaa *et al.* 1993]. There is, however, evidence that the glucocorticoid receptor (GR), which is predominantly cytoplasmic in its unoccupied form, can be crosslinked in vivo with Hsp90, which would imply that steroid receptors may be associated with Hsp90 in the cytoplasmic compartment. [Rexin *et al.* 1991]. At elevated temperature, the interaction of the receptor-associated proteins and steroid receptors is a dynamic process; the complex is dissociated with a half-life of 5 minutes and the assembly is reinitiated. [Smith 1993]. At low temperatures, the energy-dependent nuclear import is prevented, whereas the nuclear export is still functional [Guiochon-Mantel *et al.* 1991]. Cold treatment also stabilizes the oligomeric complex [Grody *et al.* 1982]. It is difficult to study the association in vivo in living cells. We have raised an antibody, termed αD, against the hinge domain (domain D) of the chicken PR. This antibody recognizes only the dissociated form and not the oligomeric complex of the PR in tissue homogenates [Pekki *et al.* 1995]. In order to study the oligomeric complex in intact cells in vivo, we used this antibody and showed that the epitope is fully detectable by immunohistochemistry, even though all the receptors extracted from these tissues are in oligomeric form and are thus not recognized by the antibody. However, inability to detect the complex could be attributable to the fore-mentioned dynamic interaction. In the present work we focused on the oligomeric complex in cells in which the dynamic interaction was stabilized by either molybdate or cold treatment, both of which have been shown to stabilize the complex. The results suggest that most receptors are in dissociated form in the nucleus.

Materials and Methods

Cell culture, transfection and expression vectors

Cos-7 cells were grown on glass coverslips and maintained in DMEM (Life Technologies, Gaitersburg) supplemented with 5% charcoal-stripped fetal bovine serum. The cells were transfected by lipofection using Lipofectamine reagent (Life Technologies) according to manufacturer`s instructions. They were incubated with serum-free Optimem (Life Technologies) containing the DNA-liposome-complexes for 24h at 37°C and thereafter in medium with 10% charcoal-stripped fetal bovine serum for 2h; the medium was changed and the cells further incubated for 24h. Wild-type chicken progesterone receptor (cPR21) and wild-type chicken Hsp90 expression vectors were used and have been described elsewhere [Tuohimaa *et al.* 1993, Turcotte *et al.* 1990].

Cold treatment

Immediately after 24h incubation following transfection, the cells were rinsed with DMEM supplemented with 5% charcoal-stripped FBS at 4°C and thereafter incubated on ice for 4h. Following cold treatment, they were rinsed three times with PBS and fixed in 4% paraformaldehyde.

Molybdate treatment

After 24h incubation, the transfected cells were washed three times with PBS, then switched to phenol red-free DMEM plus 10% charcoal-stripped FBS and incubated for 8-10h. Immediately preceding molybdate addition, cells were rinsed with PBS. Molybdate was added to attain a final concentration of 40 mM and lipofectamine reagent (2 mg/mL; Life Technologies) was diluted 1:333 in phenol red-free and serum-free DMEM and the mixture incubated at room temperature for 40 min. The lipofectamine/molybdate solution was then applied to the cells and they were incubated overnight at 37 C. Thereafter, the cells were rinsed with PBS three times and fixed in 4% paraformaldehyde.

Antibodies

We used two monoclonal antibodies, PR22 and 7Dα, and polyclonal antibody αD. PR22 is a mouse monoclonal antibody which recognizes the chicken PR [Sullivan *et al.* 1986]. We raised polyclonal antibody (αD), which can distinguish between the oligomeric and dissociated form of the chicken PR [Pekki *et al.* 1995]. Monoclonal antibody 7Dα is chicken Hsp90-specific, recognizing both free Hsp90 and the Hsp90 in the oligomeric PR complex [Schuh *et al.* 1985]. The mouse monoclonal antibody PR6 raised against PR recognizes only the B component of PR [Sullivan *et al.* 1986].

Histochemical techniques

In order to compare the immunostaining of the two antibodies in the same cells, we used a double immunofluorescence labelling technique. The polyclonal antibody αD was combined with one of the monoclonal antibodies (αD/PR22, αD/PR6 or αD/7Dα). 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 temperature and washed in PBS for 10 min. They were then incubated in 10% normal rabbit and horse serum in PBS for 30 min. PR 22 and αD, PR6 and αD antibodies as well as αD and 7Dα were mixed and applied to sections and incubated O/N. The monoclonal antibodies PR22 and 7Dα were used at a final concentration of 1 μg/mL, PR6 at a final concentration of 1:500 and the polyclonal antibody αD in a dilution of 1:200. The next day, cells were washed in PBS for 10 min, incubated with secondary antibodies, namely a biotinylated anti-mouse antibody (from goat, Amersham) dilution of 1:400 and an anti-rabbit-antibody Fluorescein dilution of 1:200 in PBS for 40 min. The 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 1:100-150 in PBS for 30 min followed by washing in PBS for 10 min and mounting in phenylenediamine-glycerine (50 mg phenylenediamine was diluted in 5 mL PBS, filtrated and added to 45 mL glycerine. pH was adjusted to 9.0 with 0.5M carbonate buffer). The results were analyzed by confocal microscopy.

Confocal microscopy

A confocal microscope, Bio-Rad MRC1024 (Bio-Rad, Cambridge, Mass) connected to a Zeiss Axiovert 135M (Carl Zeiss Jena GMbH, Göttingen, Germany) inverted microscope was used. The laser was argon/krypton (American Laser Corporation, Salt Lake City, Utah). FITC-stained samples were imaged by excitation at 488 nm with a 506-541 nm band pass emission filter. TRITC-stained exitation at 568 nm long pass emission filter. The different fluorophores were imaged separately to ensure pictures with no excitation/emission wavelength overlap; the separate images were merged into a single image later.

Results

Molybdate stabilization

The hetero-oligomeric complex of the PR was studied in Cos-7 cells which were transfected with the whole length B-form of the chicken PR cDNA. The receptor was detected using double immunohistochemistry with two antibodies. These antibodies have different epitopes: PR22 and PR6 recognize an epitope in the aminoterminal region of the PR and can detect both oligomeric and dissociated forms of the PR, whereas αD recognizes an epitope (D-domain) which is masked in the hetero-oligomeric complex and which thus recognizes only the dissociated form. For cell fixation, we used a crosslinking fixative, paraformaldehyde, and immunostaining was studied by confocal microscopy. When transiently expressed, PR was studied with PR22 (Figure 1D) and PR6 (Figure 1C) (in cells not treated with molybdate); in most of the cells only nuclear staining was detected. As also previously observed, about 15% of cells showed faint

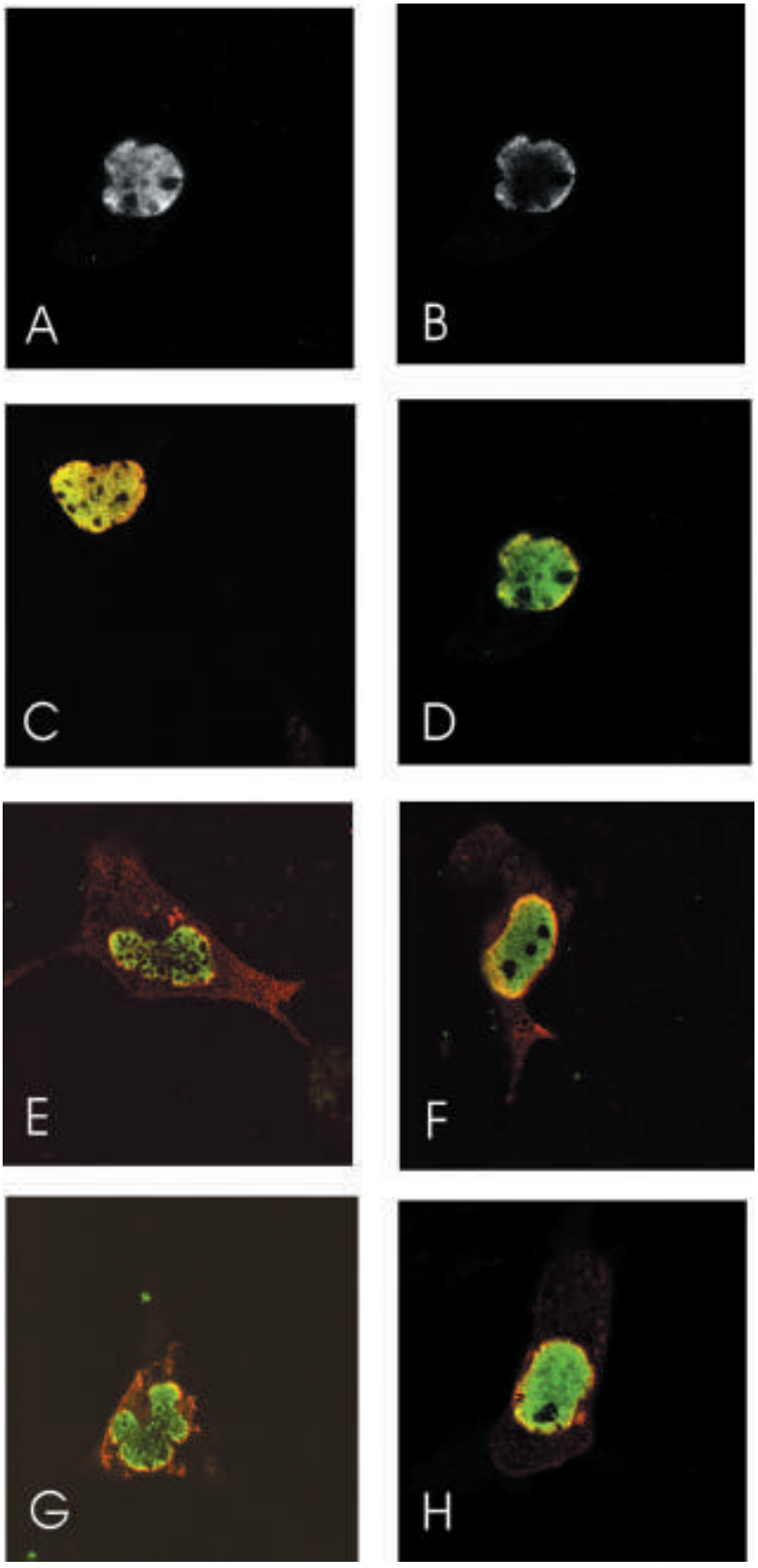

Figure 1. The detection of progesterone receptor with antibodies PR22, PR6 and αD. The whole length B-form of the chicken PR cDNA was transfected into Cos Cos-7 cells. Double immunofluorescence staining of PR were performed with antibodies PR22 (rhodamin) and αD (fluorescein) shown as single stains in a) αD and b) PR22 or in d) as double staining. c) Double immunostaining with antibody pair PR6 (rhodamin)/αD (fluorescein). We used molybdate e) PR6 (rhodamin)/αD (fluorescein), f) PR22 (rhodamin) /αD (fluorescein) and cold treatment g) PR6/αD h) PR22/αD to stabilize the oligomeric complex. The stainings were carried out by double immunofluorescence technique and the results analyzed by confocal microscopy.

cytoplasmic staining when stained with the PR22. When the same cells were stained with the αD antibody, only nuclear staining was detected. Interestingly, PR22 stained predominantly the nuclear periphery whereas αD stained both the nuclear periphery, and the center (Figure 1A,B). Since the nuclear envelope cannot be visualized, it cannot be excluded that part of the staining was perinuclear (cytoplasmic).

Molybdate is known to stabilize oligomeric steroid receptor complexes *in vitro* in cell lysates [Raaka *et al.* 1985]. The relative impermeability of the plasma membrane to molybdate has hampered its use for stabilizing receptor oligomers *in vivo.* However, Yand and DeFranco have successfully used liposomes to deliver divalent molybdate anion to living cells for stabilization of oligomeric receptor complexes [Yang and DeFranco 1996]. When PR-transfected Cos-7 cells were grown in the presence of molybdate and stained with PR22 or PR6, PR was seen to accumulate in the cytoplasm (Figures 1E,F). The nuclear periphery was also stained as in the nontreated cells. When the same cells were stained with αD, staining was seen predominantly in the nucleus, with faint cytoplasmic staining (Figures 1E,F). Co-transfection of Hsp90 into the cells together with the PR did not change the staining pattern (*data not shown*). The transiently transfected Hsp90 was seen to be located exclusively in the cytoplasm.

Cold treatment

When PR-transfected Cos-7 cells were incubated at 4°C for 4 hours in the absence of the ligand and the PR visualized with PR22 and PR6, intensive cytoplasmic staining was seen (Figures 1G,H). As in the molybdate-treated cells, the nuclear periphery was also stained. When the same cells were stained with αD, intense nuclear staining was detected, with faint cytoplasmic staining (Figures 1G,H). Co-transfection of the Hsp90 with the wild type PR did not alter the staining pattern.

Discussion

In the present work we used an antibody, αD, raised against a region of PR which is required for the stability of the oligomeric form. The antibody does not recognize the native oligomeric form in the cytosol. The major components of the oligomeric complex in the cytosol include the receptor, Hsp90, p23 and one of the immunophilins [Johnson and Toft 1994]. Since p23 and the immunphilins are though to be associated with the complex through the Hsp90, it is most probably the latter which is responsible for masking the αD epitope. In a previous immunohistochemical study, we showed that αD can recognize endogenous PR in the nucleus of chicken oviduct cells, indicating that the epitope is readily accessible, and furthermore, that most of the PR molecules in the cell nucleus are in dissociated form [Tuohimaa *et al.* 1993]. In addition, we have previously shown that fixation does not cause artificial exposure of the epitope [Pekki and Tuohimaa 1989]. When PR-transfected Cos-7 cells were treated with molybdate or incubated in cold to stabilize the oligomeric complex, a portion of the nuclear receptor was seen to be translocated into the cytoplasm. The cytoplasmic receptor was detected with PR22 or PR6, whereas αD showed nuclear staining. In the nucleus, the PR22 stained preferentially the nuclear periphery, and to a lesser extent, the central parts of the nucleus. The reason for this weak staining in the nuclear center is not clear but the same phenomenon has been reported by Chandran and DeFranco [Chandran and DeFranco 1992]. The αD antibody, in contrast, shows marked staining throughout the nucleus except for the nucleoli. The strong staining of the nuclear PR with αD indicates that the epitope is readily exposed in the nuclear PR. Taken together, these results suggest that only a fraction of receptors can form complexes with Hsp90 in the cytoplasm, whereas most, if not all of the nuclear receptors are Hsp90-free. Coexpression of Hsp90 did not alter the staining pattern, indicating that the results were not due to an insufficient amount of endogenous Hsp90. The transiently expressed chicken Hsp90 was located in the cytoplasm, which is consistent with the location of the endogenous Hsp90 in the chicken oviduct.

There are four lines of evidence suggesting an association of Hsp90 with steroid receptors *in vivo* in living cells: overexpression of unliganded LBD of PR and GR, but not liganded LBD, activates endogenous heat shock factor, proposing that the nonliganded LBD of steroid receptors can interact with heat-shock proteins *in vivo* [Xiao and DeFranco 1997], *in vivo* crosslinking studies and cotranslocation studies with mutated receptors and experiments performed with NLS-tagged Hsp90 [Alexis *et al.* 1992, Kang *et al.* 1994, Rexin *et al.* 1992]. In crosslinking studies, where living cells were treated

with a bifunctional crosslinker agent, Hsp90 and p53 could be identified as GR binding partners [Alexis *et al.* 1992, Rexin *et al.* 1992]. Conflicting results have been reported for the crosslinking of ER in intact MCF-7 cells. Rossini and Camellini (1994) reported that an unidentified 50 kD protein but not Hsp90 was associated with the ER, whereas Segnitz and Gehring (1995), showed that both Hsp90 and p59 can be crosslinked to ER in intact cells. The reason for this discrepancy remains unclear, but it should be noted that in the experiment by Segnitz and Gehring, the cells were incubated for 2 hours at 0°C prior to crosslinking. Such treatment is known to inhibit nuclear translocation and result in cytoplasmic accumulation of steroid receptors [Guiochon-Mantel *et al.* 1991]. The possibility cannot thus be entirely excluded that the crosslinked species does not represent a cytoplasmic complex. In cotranslocation studies, a recombinant Hsp90, carrying a heterologous nuclear localization signal, cotransported a fraction of cytoplasmic steroid receptor mutant into the nucleus when coexpressed in the same cells [Kang *et al.* 1994, Passinen *et al.* 1999]. These authors reasoned that if NLS-Hsp90 could transport the cytoplasmic receptor into the nucleus, intact steroid receptors should reciprocally co-transport cytoplasmic Hsp90 into the nucleus. However, it has since been demonstrated that this is not the case, since overexpressed wild-type PR was incapable of significantly altering the cytoplasmic location of wild-type Hsp90. These results, together with those of the *in vivo* crosslinking studies and data presented in the present work, indicate a cytoplasmic association *in vivo* between steroid receptors and Hsp90, but do not demonstrate receptor-Hsp90 interaction in the cell nucleus.

In crosslinking studies, non-liganded ER has been detected as a complex with chromatin proteins [Rossini and Camellini 1994]. In living cells in vivo, apo-receptors are probably associated with chromatin constituents [Wrenn and Katzenellenbogen 1990] and do not require Hsp90 for their stability. Interestingly, high-mobility group chromatin proteins 1 and 2 can interact with steroid receptors, but not with retinoic acid receptors or vitamin D receptors, and enhance DNA binding [Boonyaratanakornkit *et al.* 1998]. Analogously, purified Hsp90, but not Hsp70, can stimulate DNA binding of partially denatured ER [Sabbah *et al.* 1996]. The interaction with these chromatin proteins might serve as a support for the receptor to keep the hormone-binding domain as a functionally active conformation and thus complement the requirement of the Hsp90 interaction in vivo in the nucleus. Upon homogenization, the steroid receptors are released and their hydrophobic surfaces become exposed to an aqueous milieu. This induces Hsp90 binding, prevents receptor aggregation, and generates 8S heterooligomers. Holoforms of steroid receptors, retinoic acid receptors and thyroid receptors, whose LBDs are more hydrophilic, are less amenable targets for Hsp interaction and thus cannot be seen as oligomeric complexes in tissue extracts [Hansen and Gorski 1985, Wurtz *et al.* 1996]. It may thus be concluded that the majority of progesterone receptors are in the nucleus associated with chromatin proteins. Upon homogenization, these highly hydrophobic molecules are released into an aqueous milieu which triggers the formation of the stable oligomeric complex, which in vivo in intact cells, can be seen as a transient complex after receptor synthesis and possibly also in the cytoplasm during receptor shuttling.

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