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Reappraisal of the Role of Heat Shock Proteins as Regulators of Steroid Receptor Activity

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ABSTRACT: Almost 30 years have passed since the original demonstration that steroid receptors, comprising a subfamily of the nuclear receptor (NR) superfamily, exist as large (6-8S) non-DNA-binding complexes in hypotonic extracts (cytosol) of target cells; later such complexes were shown to correspond to a heterooligomer composed of receptor, heat shock (Hsp), and other proteins. Subsequently, an impressive number of studies have dealt with the composition of the "nonactive" complex, its dissociation and/or reassembly *in vitro*, possible functions of the non-receptor components, and their subcellular compartmentalization. While there is little dispute about the chaperoning role of some Hsps in such a complex, there is still no final proof of an association *in vivo* of NRs and Hsps in the nuclei of target cells, which is requisite for a direct regulatory involvement of Hsps in NR function. Here we critically review the various models that have been put forward to attribute a biological function to the NR-Hsp90 interaction, evaluate the corresponding experimental data, and integrate recent concepts originating from the structural and functional analyses of NRs.

KEY WORDS: heat shock protein, steroid receptor, chaperoning, nuclear localization.

Abbreviations: anti-D, antibody against the PR that distinguish the oligomeric and the dissociated form of the receptor; **AR,** androgen receptor; **DBD,** DNA-binding domain; **ER,** estrogen receptor; **GR,** glucocorticoid receptor; **Hsp,** heat shock protein; **LBD,** ligand binding domain MR, mineralocorticoid receptor; **NLS,** nuclear localization signal; **NR,** nuclear receptor; **PR,** progesterone receptor; **RAP,** receptor-associated protein; **RAR,** retinoic acid receptor; **RXR,** retinoic X receptor; **SR,** steroid receptor; **TIF,** transcription intermediary factor; **TR,** thyroid receptor; **VDR,** vitamin-D receptor

I. INTRODUCTION

Steroid receptors are transcription factors whose activities are mainly regulated by ligand binding. Recent studies have unraveled the mechanism whereby ligands turn receptors active. Apparently, ligand binding induces a transconformation of the receptor ligand-binding domain (LBD) that generates the surface(s) for interaction with the basal transcription machinery, either directly and/or indirectly via transcription intermediary factors (TIFs)/coactivators. Simultaneously, this structural change destabilizes the binding interfaces established between some apo-NRs and transcriptional corepressors.[1,2,2a,b] Recently, some of these coactivators have been shown to act as, or recruit, histone acetylases, and corepressors have been shown to form complexes with histone deacetylases (for reviews see Refs. 3 to 5). Several putative coactivators have been cloned that are proposed to mediate ligand-induced transactivation by nuclear receptors (for reviews see Refs. 2b, 6, and 7). While the concept of positively acting transcriptional mediators originated from transcriptional interference or "squelching" studies,[8–10] the existence of receptor-associated inhibitory factors that maintain apo-receptors in an inactive state had been proposed soon after the existence of steroid receptors was demonstrated. Toft and Gorski[11] and Jensen et al.[12] showed that a specific and high-affinity binding species for estrogen existed as a large non-DNA-binding complex in hypotonic cell extracts sedimenting in sucrose gradients at 8S. Dissociation of this "untransformed" form to a 4S species was shown to be requisite for receptor DNA interaction (for review see Ref. 13). This 4S form could be obtained directly from nuclear extracts of ligand-exposal target tissue, suggesting the dissociation of a receptor inhibitory factor after ligand binding.

The composition of the "untransformed" receptor complex was resolved only after steroid receptors could be purified. These studies revealed a heterooligomer that contains in addition to one receptor molecule several other proteins of which the heat shock protein Hsp90 appeared to be responsible for the inhibition of *in vitro* DNA binding.[14–17] The oligomeric receptor form could be dissociated by heat and by hypertonic conditions[14,18] (for review, see Ref. 13). *In vitro*, the complex was unstable in the presence of ligand, and it was proposed that hormone binding triggers the dissociation also *in vivo*, thereby generating an active receptor.[19] According to this model a major function of the ligand is to change receptor conformation to dissociate the oligomeric complex and allow DNA-binding of the receptor. Note, however, that such a mechanism would be confined only to steroid receptors, as other nuclear receptors, such as the retinoid (RAR), thyroid hormone (TR), and vitamin D3 (VDR) receptors, apparently do not associate efficiently with Hsp90.[20–22]

Heat shock proteins constitute an evolutionarily highly conserved group of stress-induced proteins present in species ranging from bacteria to eukaryotic.[23,24] Despite their strong transcriptional induction after heat shock, most Hsps are expressed constitutively at physiological temperatures at significant levels.[25,26] Hsp expression is increased under a wide variety of stress conditions, such as metabolic poisons, alcohols, and toxins in addition to increased temperature (for review see Ref. 27). This stress response is triggered by the intracellular accumulation of abnormally folded polypeptides (for review see Ref. 28). Stress proteins belong to a large family of unrelated classes of proteins, commonly referred to as "chaperones"[29] (for review see Refs. 30 to 32). These proteins stabilize the native structure of other polypeptides by controlled

binding to them and release of the properly folded protein. Chaperones are involved in all phases of a protein's lifetime: folding, assembly of oligomers, transport to a particular subcellular compartment, and targeting for proteolytic degradation (for review see Ref. 33).

Although the original *in vitro* experiments suggested that steroid receptor function is repressed by Hsp90 interaction, genetic analysis in yeast revealed rather a positive effect of Hsp90 expression on the transcriptional activity of steroid receptors.[34] Indeed, the role of Hsp90 in nuclear receptor signaling has remained largely elusive, and apparently contradicting reports have been published. Here we critically review these various results with the aim of balancing previous discussions about Hsp90-receptor interaction and functionality (for review see Refs. 35 to 38). In addition, in considering the recently solved 3-dimensional structure of nuclear receptor LBDs, we discuss the characteristics of the putative Hsp90-binding surfaces and the possibility that electrostatic forces could account for an "artifactual" interaction between steroid receptor apo-LBDs and Hsp90. In this review we put particular emphasis on the critical evaluation of data supporting or contradicting the evidence of the hetero-oligomeric Hsp-receptor complex *in vivo* relative to data obtained *in vitro*.

II. RECEPTOR-ASSOCIATED PROTEINS (RAPs)

Apart from the NR TIFs/co-activators and corepressors, whose function in receptor-mediated transcription activation and repression, respectively, is becoming increasingly better understood[39–46] (for review see Refs. 2b, 6, and 7), other proteins were recognized earlier to associate with NRs. In hypotonic cell extracts (cytosol) steroid receptors were found as large oligomeric complexes composed of one receptor molecule and several receptor-associated proteins. The first of these RAPs, a 90-kDa protein, copurified with steroid receptors and was later identified as a heat shock protein (Hsp90).[14,47–50] Until 1989 Hsp90 was considered to be the only RAP. In recent years, however, several other proteins (Hsp70, p60, p59, p56, Hsp56, p54, p50, p48, p23) have been detected in these complexes[15,17,51–53] (and for review see Ref. 38).

A. Hsp90

Hsp90 is an abundant, ubiquitously expressed protein found in organisms ranging from *E. coli* and fungi to man. It is upregulated by effectors as diverse as heat shock, mitogenic stimuli, and estrogens.[54,55] The term Hsp90 is often used to refer to its homologues in man, mouse, and chicken (MW 90 kDa), as well as yeast (Hsp82, MW 82 kDa). In man, mouse, and chicken, two closely related genes (Hsp90α and Hsp90β) have been identified, both of which are equally able to interact with steroid receptors.[56–59] Hsp90 is found as a dimer in extracts from different tissues.[60] In the presence of steroid receptors an additional oligomeric complex is found that contains two molecules of Hsp90 per receptor molecule.[14,47] There is, however, evidence that this 2:1 ratio, commonly observed in density gradient centrifugation and gel filtration analyses, represents only a core unit that may derive from a larger structure. When the oligomeric complex was analyzed after a minimal washing procedure, a ratio of 4:1 was observed for the Hsp90-glucocorticoid receptor complex.[61]

Hsp90 has been shown to interact *in vitro* also with several signaling molecules other than steroid receptors. A direct biochemical association with Hsp90 in cell extracts or in reticulocyte lysate has been

demonstrated for the c-Raf serine/threonine kinase,[62] tyrosine kinase pp60 v-src,[63] casein kinase II,[64] the heme-regulated protein kinase,[65] calmodulin,[66] the dioxin receptor,[67] Fes protein-tyrosine kinase,[68] and the heat shock transcription factor Hsf1.[68] Similarly, as in the case of steroid receptors, also the oligomeric complexes of these signal transducers contain other Hsp chaperones, including Hsp70, p60, and Hsp56/FKB59. The role of these components in the oligomeric forms of a particular signaling molecule is not known. However, as for NRs,[34] Hsp90 appears to be important for the proper functioning of some kinases, because its mutation reduces their activity in yeast.[69,70] Notably the pleiotropic chaperoning action of Hsp90 has been interpreted recently to facilitate morphological evolution essentially by "buffering" the consequences of polygenic mutations.[70a]

B. Hsp70 and p60

The Hsp70 family is also an abundant, ubiquitous set of proteins found in all organisms, ranging from *E. coli* to man. Most cells carry both heat-inducible and constitutive forms of Hsp70.[71] Hsp70 proteins have been described to play a role at various stages of protein metabolism[72] (for review see Ref. 73). Unfolded and folding intermediates of unstable proteins or proteins prone to aggregation occur unavoidably as intermediates in the synthesis, transport, assembly, and degradation. Hsp70 functions via binding and release of solvent-exposed hydrophobic regions of unfolded proteins to prevent their aggregation and reestablish proper folding.[74] In tissue extracts Hsp70 is found as a complex with Hsp90 and p60.[75] Hsp70 has been recovered as a significant component of the oligomeric form of some, but not all steroid receptors extracted from tissues. In cytosol, Hsp70 copurifies with the progesterone receptor (PR) in a stoichiometric ratio but not with the majority of untransformed glucocorticoid receptor (GR).[76] However, Hsp70 is always present in receptor heterooligomeric complexes reconstituted in reticulocyte lysates.[52] Only a trace amount of p60 copurifies with PR obtained from target cell cytosol, but for Hsp70 nearly stoichiometric levels of p60 are found in PR complexes generated in reticulocyte lysate.[77] The function of p60 is unknown. It is an evolutionarily highly conserved ubiquitous protein exhibiting sequence identity with a 63-kDa human protein whose expression is upregulated after viral transformation and exhibits sequence homologies to the yeast stress-induced protein STI1.[78]

C. Immunophilins

Mammalian steroid receptor complexes contain a protein that has been variously termed p59[79,80] and p56.[81] The expression of this protein is heat shock responsive and, hence, it has been termed Hsp56.[82] This protein is a member of the immunophilin class of proteins that bind immunosuppressive agents like rapamycin and FK506 with high affinity and specificity. Accordingly, immunophilins are also referred to as FK binding proteins (FKBP) and p59/Hsp56 additionally has been termed FKB59,[83] FKB52,[84] FKB51,[85] or HBI (Hsp binding immunophilin).[86] Within preparations of the oligomeric form of estrogen receptor (ER), GR, and PR a further 40-kDa protein, named Cyp-40, which shares identity with p59 has been identified.[87–89] Cyp-40 can compete with Hsp56 for binding to Hsp90, thus suggesting that Hsp90 might have a common immunophilin binding site.[89] Oligomeric avian PR preparations contain two proteins, p50 and p54, which are apparently distinct members of the FKBP family.[90] Although it

is now clear that p59 binds directly to Hsp90, it is still unresolved whether p59 interacts directly with steroid receptors. Renoir et al. proposed that unlike Hsp90 and Hsp70, p59 interacts with Hsp90 rather than the receptor itself.[80] Rexin et al.[91] have, however, reported that p59 can be cross-linked to GR even in the absence of Hsp90. As p59 is a more loosely associated component of the oligomeric complex than Hsp90, the exact stoichiometry is difficult to assess, but it has been estimated that immunopurified complex contains one molecule of p59 per Hsp90.[92] All of the members of this family have rotamase activity and thus might act as chaperones (for review see Ref. 28). Some of the FKBP family members are thought to mediate immunosuppressive effect (to inhibit T-cell proliferation) of FK506, which appears to be unrelated to the chaperoning functions.[93]

D. p48/Hip

An ubiquitously expressed protein, p48, has been detected in the oligomeric form of human PR in cell-free assembly in rabbit reticulocyte lysate. It can also be coimmunopurifid with Hsp70 and to a lesser extent with Hsp90 and p60.[51] Hip is a rat homologue of p48 and it interacts with ATPase domain of hsc70.[94] Hip interacts with hsc70 in an ADP-sensitive manner and the interaction requires DanJ/Hsp40 to promote Hsp70-Hip complex formation. It has been proposed that Hip might prolong the time window during which hsc70 interacts with unfolded polypeptides and may support the efficient cooperation of the Hsp70 system with downstream chaperones like Hsp90 and TriC.[94] Note in this respect that Yid1, a yeast DanJ homologue, is required for mammalian steroid receptors to function in yeast, but it has not been shown to be associated with the oligomeric complex.[95,96]

E. p23

The smallest protein that copurifies with chicken PR and murine GR, a 23-kDa protein,[53,97] was initially thought to be a member of the low-molecular-weight heat shock proteins (24 to 27 kDa). It is, however, not induced by heat shock and cloning revealed that this highly acidic protein shares no homology with any other known protein.[53] The only common functional motifs present in p23 are multiple putative phosphorylation sites. Cross-reacting antibodies indicate that it is an ubiquitous protein conserved from yeast to man.[53] As Hsp70 and p60,[81,82,98] p23 is also associated with Hsp90 in tissue extracts whether the receptors are present or not.[53] p23 is apparently essential for the reconstitution of the oligomeric complex, as it has been reported that depletion of p23 from reticulocyte lysate prevents the formation of the oligomeric complex.[97]

III. PROPOSED ROLES OF RECEPTOR-RAP INTERACTION

A. DNA Binding

The concept of an activation step being required to confer onto NRs the ability to interact with DNA originated from studies in which steroid receptors were extracted as cytosolic preparations from target tissues as 8S complexes that were incapable of binding to DNA (for review see Ref. 13). Hsp90 is the component of the oligomeric form that was believed to maintain the receptor in the "nonactivated" state.[99] None of the other above-described RAPs interfere with DNA binding of the receptor since; for example, Hsp70 has been found in receptor forms that are capable of DNA binding.[16,100] Supportive evidence for a role of Hsp90 in

modulating the DNA binding activity of steroid receptors (SRs) has been provided by experiments showing that reticulocyte lysate and purified Hsp90 can inhibit GR and ER binding to cognate elements.[101,102] It is generally accepted that a stable oligomeric complex (sucrose gradient centrifugation and immunoprecipitation) can be isolated only from cells containing apo-SRs, that is, not exposed to hormone. Initially, it has been proposed that the main, and perhaps the only, role of the hormone is to promote the dissociation of receptor-Hsp90 complexes[103] and thereby generate a transcriptionally active receptor. However, experiments in yeast have demonstrated convincingly that the mere removal of Hsp90 from the complex is not sufficient for receptor activation.[34] *In vitro* transcription experiments also support this view.[104] It is currently believed that the key event leading to the formation of a transcriptionally competent NR involves a ligand-induced allosteric change of the receptor, which generates the surfaces for the binding of coactivators and leads to dissociation of corepressors.[1,2,2a,105] This transconformation is independent of Hsp90, because it corresponds to a general mechanism of NR activation, including non-Hsp90-binding receptors.[2,105]

Notably, Hsp90 has also been reported to enhance the DNA binding of some proteins. For example, Myo-D1 binding to its response element could be stimulated by purified Hsp90 even though no stable complex could be observed between the two proteins.[106] Indeed, even the DNA-binding activity of partially denatured ER can be enhanced by purified Hsp90.[107] In these cases it has been assumed that Hsp90 functions as a chaperone to generate and maintain the native conformation of these transcription factors. Thus, studies addressing the impact of Hsp90 on the DNA binding function of SRs should carefully dissect any Hsp90 chaperoning of (partially) denatured receptors (that are likely not to bind DNA) from the interaction of Hsp90 with properly folded receptors, which may not be trivial.

B. Hormone Binding

Hsp90 binding to GR is required for high-affinity hormone binding *in vitro*.[101,108] GR produced in *E. coli* is not associated with Hsp90 and binds hormone with reduced affinity.[109] *In vitro*-translated GR is associated with Hsp90 and binds hormone with normal affinity.[109] However, GR is apparently unique in this respect, because no such requirement was observed for progesterone or androgen receptors that bind their cognate ligands with the same affinity in the absence or presence of Hsp90.[110,111] Note also that a great number of NRs, which do not associate with Hsp90 (e.g., retinoid, thyroid, or vitamin D3 receptors,[20–22] bind their ligands with affinities comparable to those receptors that interact with the heat shock protein.

C. Antihormone Action

The antiglucocorticoid RU486 was reported to stabilize the oligomeric forms of GR and PR *in vitro*, as judged from an increased resistance to heat- or salt-induced dissociation.[112,113] Thus, the antiglucocorticoid appears to retain the receptor in the oligomeric form. It has been proposed that this mechanism contributes to antihormone action, because it results in a receptor that is believed to be incapable of activating target genes (for review see Ref. 114). However, RU486-bound PR can interact *in vivo* with its response elements, as it activated transcription significantly, albeit weaker than in the presence of an agonist.[115,116] Indeed, it was not possible to

monitor DNA binding of the GR-RU486 or PR-RU486 complexes by genomic footprinting.[116a,116b] It is also interesting to note that not all antagonists stabilize the oligomeric form. When the aldosterone antagonists spironolactone and progesterone were bound to mineralocorticoid receptor (MR), the oligomeric form was less stable than with the agonist (aldosterone).[117] Thus, the stability of the oligomeric form does not necessarily correlate with the antagonistic potential of the ligand. Rather it appears that in some cases antagonists induce association of recently recognized corepressor proteins (NCor-R and SMRT), originally identified as factors interacting with Hsp90-noninteracting apo-NRs such as RAR and TR (for review see Ref. 6). This opens the interesting possibility that, even in the case of ER and PR, antagonists may actively repress target genes by recruiting co-repressors.

D. Chaperoning

Correct folding of a newly synthesized polypeptide into a functional protein requires a complex machinery of chaperones that, by interacting with their substrates, aid folding. These molecular chaperones recognize and bind nonnative proteins, thus preventing aggregation and keeping proteins in the productive folding pathway. The folding takes place sequentially through several intermediates. Chaperones like Hsp70 and Hsp40 bind cotranslationally to growing polypeptide chains and prevent premature (mis) folding. The proteins are then transferred to so-called "downstream" chaperones (like Hsp60 and Hsp90) that are thought to mediate proper folding.[94,118] The role of Hsp90 in *de novo* protein folding is not well understood. Under heat shock conditions Hsp90 prevents irreversible intermolecular interactions of nonnative proteins and apparently stabilizes proteins against inactivation.[119]

Most of the RAPs belong to chaperoning proteins. The potency of different RAPs as molecular chaperones has been studied by incubating denatured proteins *in vitro* with individual RAPs or with mixtures of RAPs. With the exception of p60, all RAPs have been shown to form stable complexes *in vitro* with denatured proteins and, thus, have the potential to solubilize aggregation-prone and denatured proteins.[119–123] Moreover, the combination of several RAPs can further increase the efficiency of renaturation. For example, together Hsp40/DanJ, Hsp90 and p23 can yield a 30 to 50% renaturation of the native activity of guanidine hydrochloride-denatured β-galactosidase *in vitro*. Concerning Hsp90 alone, different results have been reported. On the one hand, it has been shown that purified Hsp90 can restore 40 to 60% of the activity of denatured citrate synthetase and the Fab fragment of a monoclonal antibody *in vitro*,[123] but on the other hand it was not able to refold denatured β-galactosidase to the functional state.[122] It is interesting to note that the reconstitution of the activity of the denatured Fab fragment and citric acid synthetase *in vitro* by Hsp90 does not require ATP, which, however, is required for the formation of a stable oligomeric complex with steroid receptors.[123–125] Together, all these observations indicate that most RAPs can either alone or in combination with each other form stable complexes, aid protein folding, and eventually restore the biological activity of denatured proteins.

There is evidence that RAPs can chaperone NRs. Most direct evidence comes from experiments showing that purified Hsp90, but not Hsp70, could restore DNA binding of partially denatured ER *in vitro*.[107] Furthermore, ligand binding of GR is compromised when Hsp90 is dissociated from the receptor complex *in vitro*.[108] Importantly,

the same is true in the case of sperm cells that are deficient of Hsp90.[126] In both cases chaperoning of Hsp90 may account for these observations. Steroid receptor activity is also compromised in yeast cells that are Hsp90 or DanJ/Hsp40 deficient or carry mutations in these genes.[34,95,96] Notably, Hsp90 deficiency did not reduce the maximal receptor activity, rather it reduced the responsiveness at a given ligand concentration; full induction could still be achieved with increasing the ligand concentration. This suggests that inefficient chaperoning leading to improperly folded receptors could account for the observed phenomenon.[34,127] Note also that the transactivation efficiency of GR, which other than ER requires Hsp90 for high-affinity ligand binding *in vitro*, was more affected in Hsp90-deficient yeast cells than that of ER.[34] Importantly, Hsp90 deficiency not only reduces the transactivation efficiency of steroid receptors but also that of RAR that does not form heterooligomeric complexes *in vitro*.[128] Notably, Hsp90 mutants that compromised receptor activity were still competent to interact with GR *in vitro*, further supporting the idea that receptor chaperoning and the formation of an oligomeric complex *in vitro* are separable events.[127]

Various immunophilins (intracellular binding sites for immunosuppressive agents) have been observed as components of heterooligomeric forms of steroid receptors. Immunophilins have peptidyl-propyl isomerase (also termed rotamase) activity and can catalyze the cis-trans isomerization of proline-peptide bonds (for review see Ref. 28). Because immunosuppressive agents like cyclosporine A and FK-506 can inhibit the rotamase activity and alter receptor-mediated transactivation, it was tempting to suggest that their effect is mediated through the oligomeric complex.[129–131] Unfortunately, there are conflicting results concerning the effect of these immunosuppressants on the properties of the heterooligomeric steroid receptor. Addition of FK506 or rapamycin to PR-containing cytosol was found to stabilize the oligomeric PR complex and increase the affinity of progesterone binding.[132] In contrast, these compounds had no effect on the properties of the GR complex.[92] In considering the potential effects of FK506 on receptor activity, it has to be taken in to account that this compound is also an inhibitor of calcineurin (a calcium-calmodulin-dependent phosphatase) and, furthermore, can regulate the steroid transport into cells.[130,133] Thus, at present there is insufficient evidence that immunosuppressants act directly through the steroid receptor heterooligomers, and it cannot be excluded that the observed effects are related to altered phorphorylation and/or steroid transport.

E. Nuclear Transport and Shuttling

Although nuclear receptors contain nuclear targeting signals of their own, it has been proposed that certain RAPs are required for nuclear localization signal (NLS)-mediated nuclear transport. Hsp56 may play a role in the nuclear translocation of steroid receptors, as intracellular injection of antibodies against a negatively charged sequence of rabbit Hsp56 (that is electrostatically complementary to a prototypic NLS) impeded Dex-mediated cytoplasmic-nuclear trafficking of GR.[134] Antibodies against other epitopes of Hsp56 did not affect GR localization.

Hsp70 has been shown to be involved in translocation of proteins across the mitochondrial and endoplasmic reticulum membranes.[135,136] Protein import in these organelles requires its unfolding and refolding, and it has been postulated that Hsp70 "chap-

erones" these folding events (for review see Ref. 74). Nuclear proteins are not believed to become unfolded during import; thus, it is unlikely that Hsp70 binding to steroid receptors is the target for chaperoning during the transport process (for review see Ref. 137). Hsp70 has been found in both the cytoplasmic and nuclear compartments and shuttles between them.[138] Injecting antibodies against Hsp70 or depleting Hsp70 from cytosol inhibits nuclear transport of SV-40 large T antigen and nucleoplasmin but not GR, indicating a distinct involvement of Hsp70 in the transport of these various nuclear proteins.[139,140] The mechanism by which Hsp70 assists nuclear transport is not known, but the observation that human Hsp70 expression complements a SV40 T antigen NLS mutation leading to cytoplasmic location has been taken as evidence suggesting that a Hsp70-induced structural alteration might be required for the nuclear translocation of some proteins.[141] There is no evidence that Hsp90 could be involved in nuclear transport. Interestingly, molybdate-stabilized oligomeric GR did not translocate into the nucleus, suggesting that dissociation of the oligomeric complex is requisite for nuclear transport.[140,142]

Steroid receptors, as with most nuclear proteins, shuttle between cytoplasm and nucleus.[143–146] Proteins preferentially accumulate within a specific subcellular compartment as a result of the rates of nuclear import and export. There are conflicting results about the subcellular distribution of the apo-GR locating exclusively in the cytoplasm,[147] both in the cytoplasm and nucleus[148–150] or exclusively in the nucleus.[151,152] These different results may, at least in part, reflect a peculiarity of GR, whose NLSs differ from those of PR and ER in that GR has a stronger inducible NLS in the LBD and the apo-LBD of the GR effectively masks the constitutive NLS in the hinge region.[153,154]

The biological significance of the nuclear-cytoplasmic shuttling of NRs is not clear. It is possible that (1) the cytoplasmic receptor may have a distinct biological function, as proposed by Verdi and Campagnoni[155] and Migliaccio et al.,[156] or (2) repeated chaperoning in the cytosol might stabilize some structurally unotable nuclear proteins. There is evidence that both nuclear export and import are NLS mediated, because non-nuclear proteins to which a heterologous NLS is attached can acquire a shuttling ability.[146] Rapidly shuttling proteins carry an additional signal required for nuclear export, distinct from the nuclear localization signal.[157] There is no evidence of the involvement of RAPs in nuclear export/shuttling.

IV. FEATURES OF THE Hsp90 INTERACTION SURFACE(S) OF STEROID RECEPTORS

A. Two Surfaces at Opposite Sides of the LBD Are Required for Hsp90 Interaction

Receptor sequences required for Hsp90 association have been determined by mutagenesis and peptide competition experiments. The amino terminal region A/B and the DNA-binding domain (DBD) are dispensable for Hsp90 interaction. For PR, GR, and androgen receptor (AR) the hormone binding domain (LBD) is sufficient for interaction, whereas for ER, in addition to the LBD, a sequence of the hinge region D stabilizes the Hsp90-ER complex.[158–163] As the canonical crystal structure of the LBD of five members of the nuclear family has been determined[1,2,2a,105,164,164a,164b,164c] (for a recent review see Ref. 2a) it is now possible to locate the Hsp90-interacting surfaces. Two distinct LBD sequences have been

found to be important for Hsp90 interaction, one encompasses the end of the helix H3 and helix H4, and the other comprises the β-turn, helix H6, and the beginning of the helix H7 (Plate 1*). These regions are located at opposite sides of the LBD and contribute to the formation, or are in the vicinity of the hydrophobic ligand binding cavity. That the interacting surfaces are located at opposite sides of the receptors could account for the observation that two molecules of Hsp90 interact with one receptor molecule. Very little is known about NR sequences involved in the interaction with other RAPs. One study concerning the regions of PR required for the binding of Hsp70 indicates that, in principle, the same sequences are required for Hsp90 and Hsp70 binding.[158]

B. Hydrophobicity May Dictate Receptor-Hsp90 Interaction

The surface hydrophobicities of steroid receptors and Hsp90 have been studied by using hydrophobic interaction chromatography and phase partitioning,[165–167] demonstrating that both Hsp90 and apo-SRs are highly hydrophobic. Analysis of GR and ER mutants showed that the LBD is the major contributor to receptor hydrophobicity.[167] Thus, steroid receptor LBDs should be good targets for Hsps, whose principal function is to cover hydrophobic regions of other proteins to aid in proper folding, conserve the native structure, and prevent aggregation.

The LBD of the estrogen receptor appears to be the major determinant for the poor solubility of overexpressed recombinant ER. When the full-length receptor was overexpressed in the baculovirus system, the majority of the protein formed insoluble aggregates, while the deletion of the LBD yielded soluble truncated receptors.[168] Incubation of these ER aggregates with reticulocyte lysate reconstituted the functional oligomeric form of the receptor,[168] suggesting that the binding of Hsp90 (and other Hsps) results in masking of the hydrophobic regions that prevents receptor aggregation. In keeping with this hypothesis, the hydrophobicity of the Hsp90-ER complex has been shown to be lower than that of its components.[165] It is interesting to compare in this context the modeled LBDs of GR with that of RAR, a non-Hsp90 binding receptor. Plate 2 shows the electrostatic surface potential of one of the two Hsp90-interacting surfaces of the two receptor LBDs. In the apoform helices H3 and H4 are clearly less charged with GR than with RAR, indicating that this Hsp90-interacting surface is more hydrophobic with GR than with RAR. Most pronounced are the differences in the C-terminal region of helix H4 where RARs possess a positively charged residue (Arg or Lys also observed in the RXR subgroup), whereas GR has a neutral residue in the corresponding position (Gln, as all steroid receptors, except ER). Thus, it is possible that retinoic acid receptors do not form oligomeric complexes in reticulocyte lysates or cytosols because their LBDs contains less hydrophobic patches. The same feature may account for the observation that RAR LBDs are less prone to aggregation than those of ER and GR. It would be interesting in this respect to investigate whether mutant retinoid receptor LBDs can be generated that acquire Hsp90 binding affinity *in vitro*.

C. Ligand Binding Alters the Structure of the Hsp90 Interaction Surfaces of the Nuclear Receptors

Agonist binding destabilizes heterooligomeric steroid receptor complexes, whereas antagonists either stabilize or

* Plates 1 and 2 appear following page 452.

destabilize the complex depending on the ligand used.[19,112,117] Crystallographic analysis has revealed that ligand binding causes a profound change in the structure of the LBD,[1,2,2a] including a transconformation of helix H12, which in its holoconformation closes like a "lid" on the entrance of the ligand binding pocket. This event markedly decreases the surface hydrophobicity of this Hsp90-interacting region (Plate 2). Ligand binding also alters the structure of the second Hsp90 interacting surface (Plate 1). The β-sheet and the helix H6 are shifted and the omega loop (1–3) is flipped over these structures. Together, these changes result in a more compact and less hydrophobic structure. As mentioned above, the decreased surface hydrophobicity after ligand binding has been experimentally confirmed by two phase partitioning experiments and hydrophobic interaction chromatography.[167,169] It is tempting to speculate that an altered structure and surface hydrophobicity of SRs after ligand binding makes them less favorable targets for Hsp90 interaction. Such a model would be compatible with the observation that ligand exposure *in vivo* results in the disruption of the heterooligomeric 8S complex. *In vitro*, ligand binding is insufficient for disruption of the 8S complex and the "transformation" to the 4-5S form requires exposure to high ionic strength at 4°C or thermal treatment (30 min at 28°C) (for review see Ref. 13). These observations suggest that *in vitro* at low-temperature Hsp90 binding is stabilized and ligand binding may not induce a complete transformation of the LBD, unless the Hsp90 interaction is weakened by approaching physiological temperature. That the structure of the fully transformed holo-receptors do not provide Hsp90 binding surfaces is supported by the observation that holo-steroid receptors do not reassociate with Hsp90.[101,124,170]

V. HOW IS THE OLIGOMERIC STEROID RECEPTOR COMPLEX FORMED?

It has been difficult to study the conditions required for the formation of the 8S complex, because it does not form by simply mixing the individual proteins. Recently, reconstitution has been achieved in reticulocyte lysates using both purified Hsp90-free or *in vitro*-synthesized apo-receptor.[52,171] The structure of the reconstituted complex is very similar to the 8S complex found in cytosolic extracts, except for higher binding levels of Hsp70 and p60.[77] Only a trace amount of p60 is co-immunoprecipitated with PR, whereas nearly stoichiometric levels of p60 are found to be complexed with PR in the reticulocyte lysate.[75]

In vitro assembly of the oligomeric complex requires hydrolyzable ATP, Mg^{++}, K^{+}, and a near-physiological temperature.[124,125] The sequence of events leading to its formation *in vitro* has been studied in detail with the chicken PR.[17] When purified chicken PR is incubated in reticulocyte lysate, it rapidly associates with Hsp70 followed by the association of Hsp90, Hip, and p60 generating an "intermediate complex". By 5 to 10 min PR is found associated with Hsp90, p23, and one of the immunophilins ("the mature complex"). At elevated temperature the mature complex dissociates with a half-life of 5 min and the assembly pathway is reinitiated. Under steady state conditions most of the PR exists as the mature complex.[17] The observation that the oligomeric complex can be readily reconstituted in cell lysates prompts the question whether it is indeed formed *in vivo* within intact cells or rather generated *in vitro* after cell fractionation.

A. Are Hsps and Nuclear Receptors Associated *In Vivo*?

1. PR and Hsp90 Are Located in Different Subcellular Compartments

Immunohistochemical studies employing various fixation techniques and light as well as electron microscopy have shown that both endogenous and transiently expressed exogenous Hsp90 is cytoplasmic (Figure 1;[170,172]). There is, however, a study reporting a simultaneous cytoplasmic and nuclear location.[173] It is not known whether the divergent results are due to cell-specific differences or related to the methods used. Notably, in one study Tuohimaa et al.[170] used anti-Hsp90 and anti-PR antibodies of similar detection sensitivity, as was deduced from the comparison of Hsp90-PR fusion proteins expressing both epitopes. Because there are two to four molecules of Hsp90 per molecule of steroid receptor,[47,61] the concentration of Hsp90 in the nucleus should exceed by severalfold the concentration of the receptor and should have been detected easily. Thus, there is an apparently insufficient amount of Hsp90 in the nucleus to form an oligomeric complex with all steroid recep-

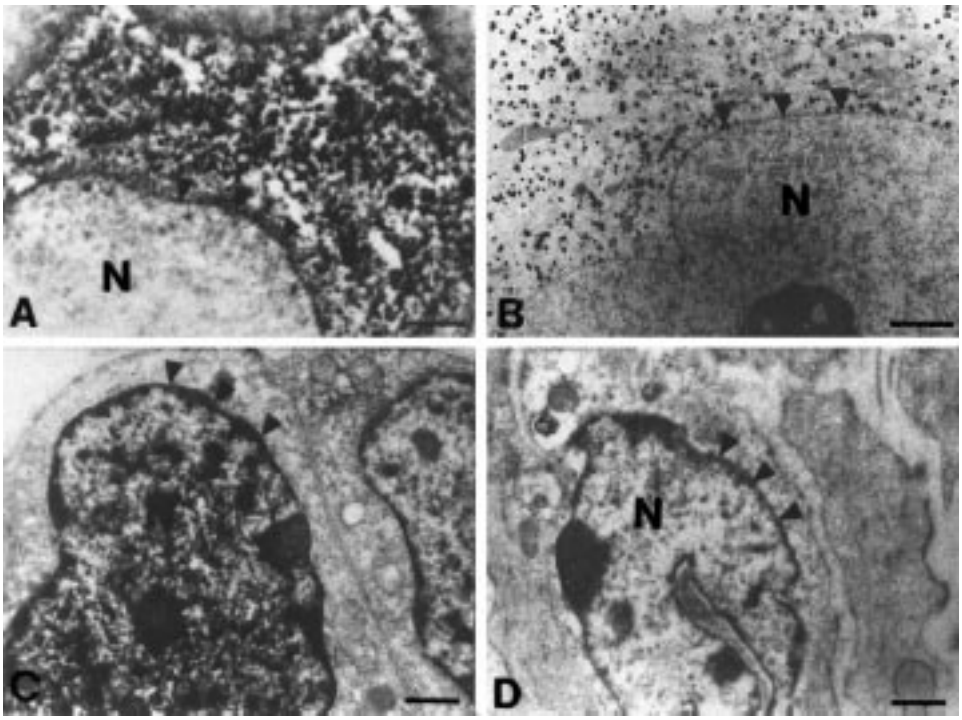

FIGURE 1. Progesterone receptor and Hsp90 are located in different subcellular compartments. The intracellular location of Hsp90 and PR were studied in chick oviduct cells by preembedding immunoelectron microscopy using monoclonal antibodies. Antibodies were detected by the avidin-biotin technique using either peroxidase or 5 nm gold particle labeling. Technical details can be found in References 172 and 196. Immunoperoxidase (A) and immunogold (B) staining of Hsp90 in epithelial cells. Note that Hsp90 staining is exclusively cytoplasmic. (C) Immunoperoxidase staining of PR shows only nuclear staining. (D) No staining is seen in control sections in which the antibodies were presaturated with purified Hsp90 protein and purified B-subunit of PR. (N = nucleus; arrowhead = nuclear envelope.)

tor molecules, even not taking into consideration other Hsp90-interacting proteins.

2. Cytoplasmic Receptors and Hsp90 Can Be Cross-linked In Vivo

In vivo the association of steroid receptors and Hsps has been studied by using three different approaches: (1) *in vivo* cross-linking,[174,175] (2) cotranslocation studies with mutated receptors and NLS-tagged Hsp90,[176] and (3) immunohistochemistry with antibodies against epitopes that are masked in the oligomeric complex (Figure 2;[177,178]). When intact cells were treated with a bifunctional cross-linking agent and the receptor-associated proteins were analyzed, Hsp90 and p59 but not Hsp70 could be identified as GR binding partners.[174,175] Conflicting results have been reported for the cross-linking of ER in intact MCF-7 cells. Rossini and Camellini (1994)[179] reported that only an unidentified 50-kDa protein but not Hsp90 was associated with ER.[179,180] Segnitz and Gehring (1995), however, showed that both Hsp90 and p59 can be cross-linked to ER in intact MCF-7 cells.[181] The reason for the difference between the two studies is not clear. It should be noted, however, that the experimental protocol of Segnitz and Gehring[181] involved an incubation of the cells for 2 h at 0°C prior to cross-linking. Such a treatment is known to inhibit nuclear translocation and results in cytoplasmic accumulation of SRs.[145] Thus, in the absence of parallel immunohistochemical data demonstrating a nuclear location, it cannot be entirely excluded that the cross-linked species does actually correspond to the cytoplasmic complex.

3. NLS-Hsp90 Can Transport Cytoplasmic PR Mutant Into the Nucleus

It has been demonstrated that recombinant Hsp90, artificially targeted to the nucleus by a heterologous NLS, cotransported a fraction of cytoplasmic steroid receptor mutants into the nucleus.[176,178] Kang et al.[176] reasoned that if NLS-Hsp90 could transport the cytoplasmic receptors into the nucleus, intact steroid receptors should reciprocally co-transport cytoplasmic Hsp90 to the nucleus. However, it had already been demonstrated that this is not the case, because overexpressed wild-type PR was incapable of significantly altering the cytoplasmic location of wild-type Hsp90.[170] Together with the *in vivo* cross-linking data these experiments indicate a cytoplasmic association *in vivo* between steroid receptors and Hsp90, but they do by no means demonstrate receptor-Hsp90 interaction in cell nuclei.

4. In Vitro Nuclear Transport Assays PR, but not Hsp90, Is Translocated Into the Nucleus

Nuclear transport can be studied *in vitro* by incubating the proteins with purified nuclei or by introducing them into digitonin-permeabilized cells.[182,183] Both systems have been shown to reflect important aspects of nuclear transport in intact cells in that they are ATP- and NLS-dependent.[182,183] With the digitonin system nuclear localization can be visualized by immunohistochemistry; using purified nuclei, the nuclear transport can be quantified by immunoblotting. When the oligomeric complex of PR was studied in these *in vitro* systems, PR was

efficiently translocated into the nuclei, while Hsp90 remained in the cytoplasm exclusively.[184] This indicates that the association of Hsp90 with PR is transient and takes place only in the cytoplasm. The "mature oligomeric complex" seen in reticulocyte lysate thus may represent a short-lived intermediate that dissociates prior to (or during) nuclear translocation. Given that these systems accurately reflect nuclear transport *in vivo*, these data would argue against a nuclear translocation of the entire receptor-Hsp90 complex in intact cells and indicate its dissociation prior to nuclear transport of the receptor.

5. Results Obtained with Antibodies That Distinguish between the Oligomeric and Dissociated Forms Argue Against Association of Hsp90 and PR in Intact Cells

An antibody (termed anti-D) that has been raised against a PR epitope inaccessible in the oligomeric complex recognized the "activated" progesterone receptor *in vitro* in cell extracts.[177] Tissue sections from animals that were not treated with progestins showed a strong nuclear staining, indicating that the epitope is exposed and not hidden by Hsp90 (Figure 2;[177]). However, in cytosol prepared from the same tissue, all of the receptors were in the oligomeric form and did not react with the same antibody.[177] To study whether receptors and Hsp90 can efficiently associate provided they are present in the same cell compartment, cytoplasmic PR mutants carrying a deletion of the NLS were transiently expressed in cultured cells and evaluated with the anti-D antibody. The PR mutant was readily detected by the antibody, indicating that a major fraction of the receptor molecules was Hsp90 free.[178] Similarly, the antibody also recognized the wild-type nuclear PR when it was coexpressed with nuclear Hsp90 (a Hsp90 linked to a nuclear localization signal).[178] Thus, the efficiency of Hsp90-receptor association in cytosolic extracts *in vitro* is clearly distinct

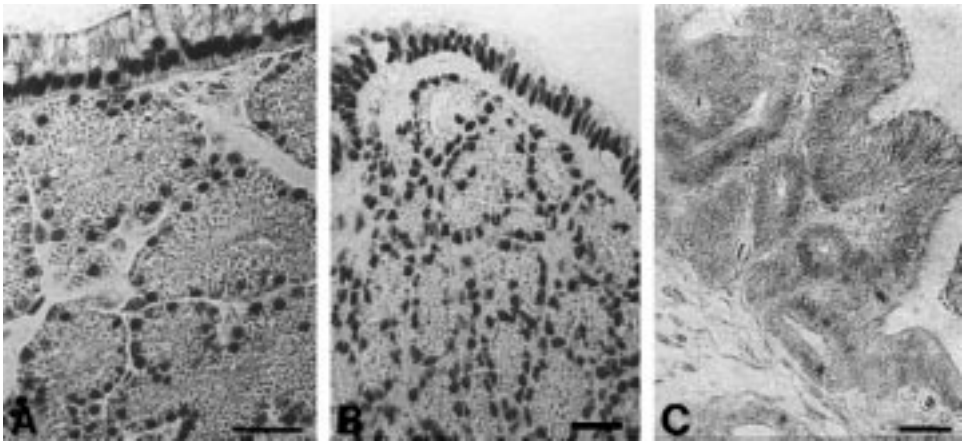

FIGURE 2. A PR antibody (anti-D) directed against an epitope that is hidden in the oligomeric PR complex detects the apo-PR in tissue sections. An antibody (anti-D) was raised against an epitope in Hsp90-free PR that is hidden in the oligomeric form and becomes accessible after Hsp90 dissociation. For details see Pekki et al.[177] (A) Immunohistochemical staining of chick oviduct with this antibody shows that the epitope is fully detectable in the cell nucleus indicating that the apo-PR is free of Hsp90. (B) An identical staining pattern can be seen with an antibody that detects both the oligomeric and dissociated form. (C) The specificity of the immunostaining was confirmed by histochemical staining with antibodies presaturated with overexpressed PR protein.

from that *in vivo* in either the cytoplasmic or nuclear compartments. These antibody studies indicate that a major fraction of cytoplasmic (mutant) and nuclear (wild type) steroid receptors is Hsp90 free; however, they do not exclude that a minor fraction of the receptors forms Hsp90 complexes, as the pool of Hsp90-free receptors will dominate immunostaining.

B. Hsps and Nuclear Receptors May Associate during Cell Fractionation

As discussed earlier, the oligomeric complex can be reconstituted from a purified steroid receptor *in vitro* in reticulocyte lysate. Interestingly, reticulocyte lysate is also able to solubilize and functionally reconstitute insoluble receptor aggregates formed during baculovirus overexpression.[168] The mechanism how the lysate reconstitutes the oligomeric complex from purified receptor or from aggregated receptor is not known. When the oligomeric form can be reconstituted in reticulocyte lysate, a fresh cytosol (during homogenization) may have the same capacity to support complex formation. In fact, reconstitution of PR hetero-oligomers has been observed with various cell extracts as well as when apo and dissociated receptor was homogenized with tissue that does not contain PR.[170,185] Reconstitution was not seen with holo-receptor. Homogenization thus may liberate factors similar to those present in the reticulocyte lysate, leading to the assembly of the oligomeric structure.

VI. CONCLUSIONS

A. Hsp90 May Act as Chaperone

The evidence that Hsp90 can act as a chaperone is based on its ability to restore *in vitro* the DNA-binding activity of denatured ER and MyoD1, and the activities of denatured citric acid synthetase, immunoglobulin Fab fragment, and β-galactosidase.[106,107,122,123] Defects in nuclear receptor and tyrosine kinase-mediated cell signaling due to mutations or reduced amounts of Hsp90[34,69,70,127] suggest that Hsp90 is involved in chaperoning in intact cells. Also, the observation that only a fraction of cytoplasmic steroid receptors is associated with Hsp90 *in vivo* (see above) is in agreement with the idea that it functions as a chaperone for these proteins. Importantly, it should be pointed out that chaperoning of receptor does not correlate with its ability to form a stable oligomeric complex *in vitro*, because RAR, which does not generate oligomeric complexes in cytosolic extracts, nevertheless require Hsp90 for efficient trans-activation.[21,128] Analogously, Hsp90 can also chaperone MyoD1 without forming a detectable complex *in vitro*.[106]

B. Hsp90 Does Not Act As a Bona Fide Corepressor

The unoccupied LBD acts as intramolecular repressors of the activation functions of nuclear receptors. Steroid receptor LBDs can in addition "silence" the functions of heterologous proteins when covalently linked to them[186] (for review see Ref. 187). Deletion of the LBD generates constitutively active steroid receptors that do not form stable oligomeric complexes with Hsp90 *in vitro*. From these data, it has been proposed that Hsp90 interaction is responsible for the repressor function of apo-LBD.[188–190] Recent observations, however, do not support this conclusion. For example, Lee et al. (1996)[191] found that the ER apo-LBD represses the activity of VP16-GAL when linked to it, but this chimera does not interact with Hsp90. Conversely, White et

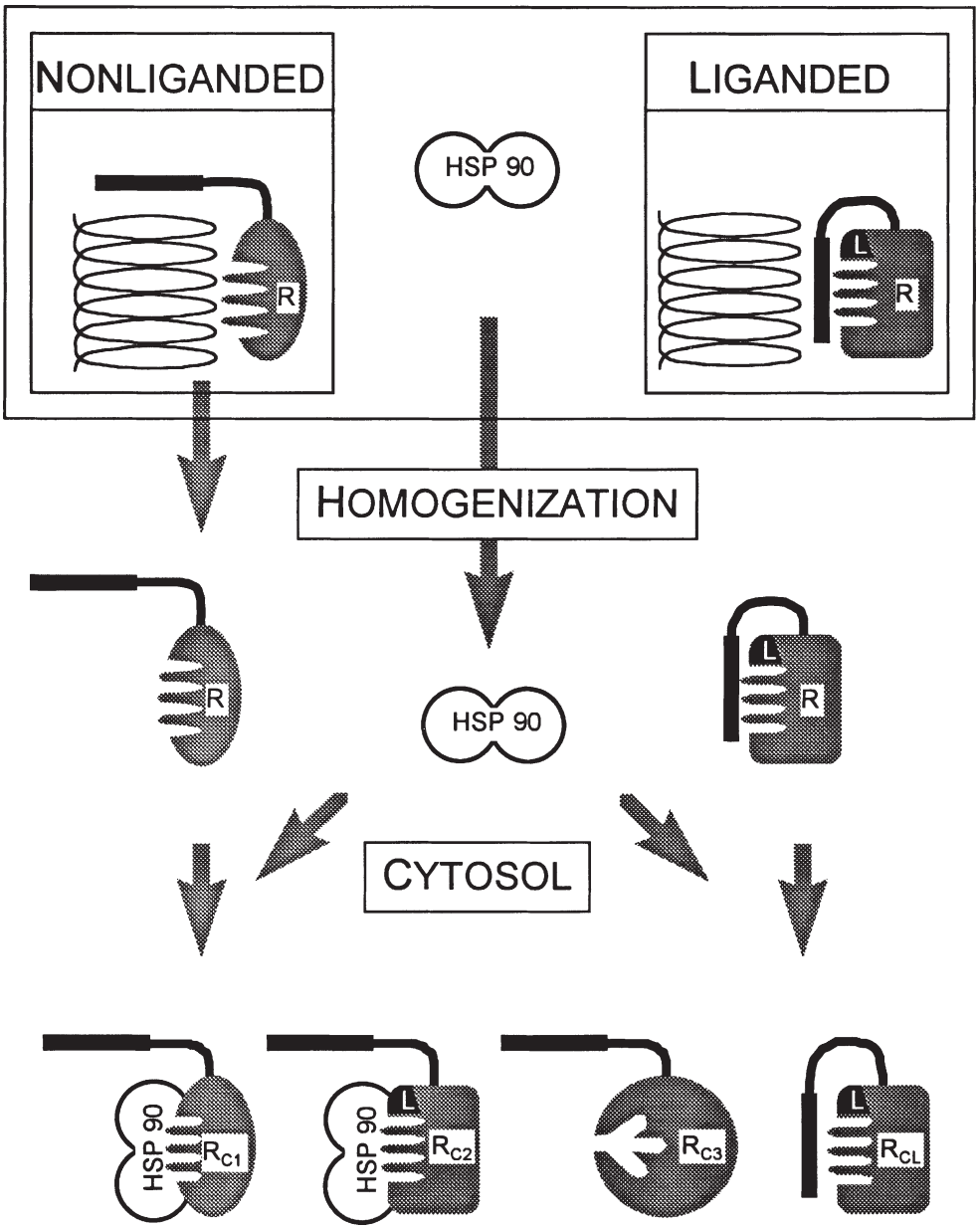

FIGURE 3. A model for the formation of the oligomeric complex of steroid receptor during tissue fractionation. Hsp90 and steroid receptors are located in different cell compartments. Both holo- and apo-receptor molecules are nuclear proteins, whereas Hsp90 is cytoplasmic. The apo-receptor is a hydrophobic molecule and may be associated with chromatin constituents in the cell nucleus. This association probably covers the hydrophobic regions of the LBD. During homogenization the receptor is released into an aquous milieu and these hydrophobic regions become exposed, inducing the binding of Hsp molecules, thus creating the 8S heterooligomeric form of the receptor (R_{C1}). Ligand binding *in vivo* causes a transconformation in the Hsp90 interacting surfaces and renders LBD less hydrophobic. Thus, holo receptors remain Hsp90 free after extraction (R_{CL}). If Hsp90 is dissociated from the cytosolic receptor, for example, by salt treatment, some hydrophobic LBDs may become unstable in aqueous milieu and adopt a conformation that does not allow ligand binding with wild-type characteristics (R_{C3}). This might explain why some steroid receptors, such as GR, are unable to bind ligand if they are expressed in bacterial systems. Heterooligomeric forms of steroid receptors can bind ligand *in vitro* without promoting dissociation of Hsp90 if the incubation is carried at in low temperature or the oligomeric form is stabilized by molybdate (R_{C2}). Under these conditions transconformation may be incomplete and the ligand-bound heterooligomer (R_{C2}) probably adopts a structure that is different from that of the holo receptor in the Hsp90-free form (R_{CL}).

al. (1997)[192] have shown that a point mutation in helix H12 of the LBD can generate a constitutively active ER, but this molecule interacts efficiently with Hsp90 *in vitro*. Thus, Hsp90 cannot be considered a *bona fide* corepressor, such as N-CoR or SMRT.[42,43]

C. The heterooligomer: An *In Vitro* Artefact?

Even though Hsp90 can act as a chaperone, there is no strict correlation between the formation of the oligomeric complex *in vitro* and the chaperoning or intramolecular repressor functions of Hsp90. Therefore, it is justified to ask whether alternative mechanisms could account for the observation that some apo-receptors (steroid receptors) are found as stable oligomeric complexes in cell extracts. Mainly based on immunohistochemical results, it has been proposed that the association Hsp90 with steroid receptors is an artefactual phenomenon occurring *in vitro* during tissue fractionation.[170,177,193] In support of this notion, there is ample biochemical evidence for the *in vitro* formation of such oligomeric complexes. It has been demonstrated that purified apo-receptor monomers can be reconstituted to the oligomeric complex in various cell lysates (reticulocyte, mouse L cell, insect cell, and plant cell) and homogenates.[52,170,171,185] Thus, the oligomeric complex seen in the cytosol of target cells may not necessarily represent a complex formed *in vivo* in intact cells, but may be formed during tissue fractionation. What could be a possible origin for this association? Hsps and other stress proteins prevent the aggregation of other proteins by covering their hydrophobic surfaces. Homogenization of cells and tissues in hypotonic buffer releases proteins from their normal microenvironment, their interactions with other cell constituents are altered or disrupted and hydrophobic regions could be exposed. The apoforms of steroid receptors contain highly hydrophobic LBDs and are likely to be good targets for Hsp interaction. In contrast, the retinoic acid receptors, thyroid receptors, and the holo-forms of steroid receptors, whose LBDs are more hydrophilic, appear to be not as good targets for Hsp interaction (see above and Plate 2). Indeed, it has been demonstrated that most of the RAPs form stable complexes with various denatured proteins *in vitro*, further supporting the notion of the formation of stable oligomeric complexes with Hsps is an *in vitro* phenomenon.[122]

D. A Model of the Formation of the Oligomeric Complex *In Vitro*

A hypothetical model that could explain the formation of the oligomeric complex during cell fractionation is shown in Figure 3. Apo-receptors, possibly associated with chromatin constituents,[194] are released during cell fractionation and their hydrophobic surfaces are exposed to an aqueous milieu. This induces Hsp90 binding, prevents receptor aggregation, and generates the 8S heterooligomer. LBDs that are less prone to aggregation (e.g., RAR, RXR) are not targets for Hsp interaction under such conditions. After ligand exposure *in vivo*, the receptor undergoes a transconformational process that disrupts the Hsp90 interface due to conformational electrostatic alterations of the cognate receptor surface(s), which does not allow the reassociation of Hsp90 and holo-receptor. *In vitro*, at low temperature, the 8S complex can bind ligand

without causing Hsp90 dissociation, but the structure of the receptor LBD is distinct from that of the holo-receptor.

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