Steroid Receptor Folding by Heat-Shock Proteins and Composition of the Receptor Heterocomplex

William B. Pratt, Kevin A. Hutchison, and Lawrence C. Scherrer

Over the past 2 years, reports from several laboratories have supported the proposal that the steroid receptors are bound through the hormone-binding domain to a protein complex that contains three heat-shock proteins—hsp90, hsp70, and hsp56. This receptor–heat-shock-protein heterocomplex accounts for the behavior of the classic 9 S, non-DNA-binding form of the adrenocorticoid, sex hormone, and dioxin receptors. The receptor heterocomplex has now been reconstituted by an enzymatic system in reticulocyte lysate. This represents the first in vitro system for reversing receptor transformation, and this ability to reconstitute the receptor heterocomplex promises rapid advances in our understanding of how these receptors are folded, transported, and regulated by hormone in the cell. (Trends Endocrinol Metab 1992;3:326–333)

Twenty-six years have elapsed since Toft and Gorski (1966) first demonstrated that the estrogen-binding activity in rat uterine cytosol sedimented at 9 S when analyzed by sucrose gradient centrifugation. The composition of this very large (320–350 kD) form of all of the steroid receptors remained undefined until it was found that molybdate ion could stabilize the receptor in its 9 S, untransformed state (Leach et al. 1979). When molybdate-stabilized receptors were purified, it became clear that the 9 S receptors were heterocomplexes containing a steroid-binding protein and a common 90-kD non-steroid-binding protein (Joab et al. 1984) that was identified soon thereafter as the 90-kD heat-shock protein, hsp90 (Catelli et al. 1985; Sanchez et al. 1985; Schuh et al. 1985).

William B. Pratt, Kevin A. Hutchison, and Lawrence C. Scherrer are at the Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

This 9 S heterocomplex form of the steroid receptors is recovered from hormone-free cells, whereas receptors recovered from hormone-treated cells are usually dissociated from hsp90 and in a 4 S form (see Pratt [1987] for review). In the case of the glucocorticoid receptor (GR) (Pratt et al. 1988; Cadepond et al. 1991), the progesterone receptor (PR) (Carson-Jurica et al. 1989), and the estrogen receptor (ER) (Chambraud et al. 1990), it has been shown that constitutively active, mutant receptors are not bound to hsp90, whereas mutant receptors that are steroid inducible are. Thus, it is argued (see Pratt [1990] for review) that the 9 S receptor heterocomplex is derived from the physiologically inactive state of the receptors that is activated by hormone in the cell, and the 4 S, dissociated form is derived from receptor that is competent for transcriptional activation.

In the past 3 years, it has become clear that the heterocomplex form of the steroid receptors contains two other heat-shock proteins, hsp70 and hsp56, in addition to hsp90. A heterocomplex containing these three heat-shock proteins exists in cytosol independent of the presence of steroid receptors (Sanchez et al. 1990a; Perdew and Whitelaw 1991). Hsp70 and hsp90 are thought to be involved in protein folding and trafficking in the cell (Rothman 1989), and it is a reasonable speculation that the 9 S heterocomplex is derived from receptors that are attached to such a protein transport system (Pratt 1992). As suggested in the diagram presented in Figure 1, it has been proposed (Pratt 1992) that the heat-shock-protein heterocomplex may act as a protein transport particle, or *transportosome*, which we define as a very small heteroprotein structure to which the steroid receptors remain attached with high affinity while they shuttle through the cytoplasm and into the nucleus. In the absence of hormone, the receptors remain *docked* to this complex and, when they are exposed to hormone, they dissociate from hsp90 and progress from the protein-trafficking system to the high-affinity binding sites where transcriptional activation occurs. In contrast to the steroid and dioxin receptors, more primitive members of the steroid–thyroid hormone receptor family, such as retinoic acid or thyroid hormone receptors, do not bind stably to hsp90 and, in the hormone-free cell, these receptors proceed directly to high-affinity nuclear binding sites without docking (Dalman et al. 1990 and 1991b).

In the 2 years since the last review of this subject (Pratt 1990), we have learned a lot about the proteins that make up the core heterocomplex, and a system has been developed for enzymatically reconstituting the untransformed steroid receptor heterocomplex with the use of rabbit reticulocyte lysate. This lysate system is a promising system for studying the mechanisms by which protein folding/unfolding processes are involved in the assembly of heteroprotein units. In the first half of this review, we present a summary of the components of the heterocomplex, emphasizing the most recent work. The reader is referred to a detailed review (see Pratt [1993]) of the

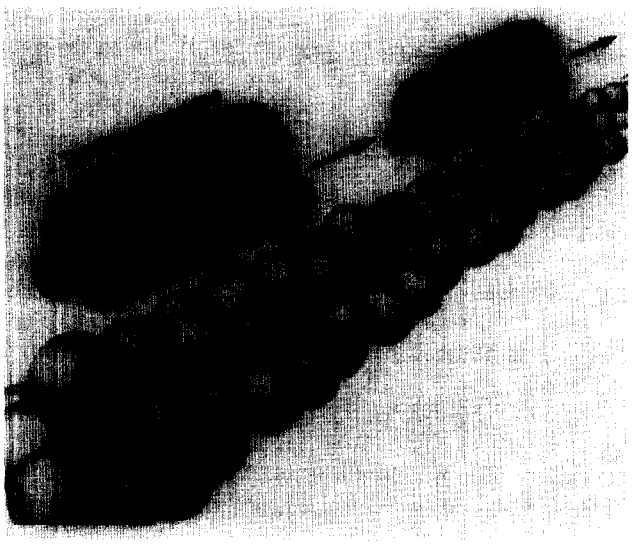


Figure 1. The heat-shock-protein complex as it may function in the transport of steroid receptors (SR) through the cytoplasm to the nucleus. As discussed in the text, several receptors that are nuclear in their hormone-free state (for example, progesterone and estrogen receptors) are associated with significant amounts of hsp70 as well.

literature over the past decade for a more complete discussion of all the work that has contributed to our current understanding of heterocomplex composition for different receptors, stoichiometry of the proteins in the complex, and the physiologic relevance of receptor attachment to the heterocomplex. The second half of this review is devoted to heterocomplex assembly and manipulation of the functional state of the GR in vitro through association with heat-shock proteins.

• The Heat-Shock-Protein Complex

When unliganded steroid receptors are immunoadsorbed under very gentle conditions designed to preserve as much of the structure of the native heterocomplex as possible, several proteins coimmunoadsorb with the receptors in addition to hsp90. For example, Toft's laboratory (Kost et al. 1989; Smith et al. 1990a) has identified proteins of 70, 54, 50, and 23 kD in the chicken PR complex, and our laboratory has identified proteins of 70, 56, and 23 kD in murine GR complexes (Bresnick et al. 1990; Sanchez et al. 1990b). That some of these proteins exist together in a complex independent of the receptor was indicated by similar coimmunoadsorption studies with anti-hsp antibodies. Immunoadsorption of the 56-kD receptor-associated protein (hsp56) from human IM-9 cell cytosol with a monoclonal antibody yielded coimmunoadsorption of hsp90, hsp70, and an acidic 23-kD protein (Sanchez et al. 1990a). Similar immunoadsorption of rat hepatocyte cytosol with a monoclonal antibody against hsp90 yielded coimmunoadsorption of hsp70, hsp56, and a 50-kD protein that was subsequently shown to be the same as the pp50 component of the $pp60^{v\text{-}src}$–hsp90 heterocomplex (Perdew and Whitelaw 1991; Whitelaw et al. 1991).

Those proteins that have been identified as components of the untransformed GR heterocomplex isolated from cells where the hormone-free GR is cytoplasmic are included in the transport form of the receptor shown in Figure 1. The hormone-free PR is located in the cell nucleus, as is the overexpressed murine GR in CHO cells and, in both cases, the heterocomplex also contains hsp70. We suggest that this heat-shock-protein complex may act as a general transport particle for a variety of proteins in addition to $pp60^{src}$ and the steroid receptors. During evolution, the steroid receptors may have exploited the general role of hsp90 as a chaperone component of the protein-trafficking system such that they evolved higher-affinity binding to hsp90 that is under hormonal control.

Hsp90

Hsp90 is the most abundant of the heat-shock proteins, being present at ~1% of cytosolic protein. It is ubiquitous, essential, and highly conserved, and it is a well-established component of glucocorticoid, mineralocorticoid, estrogen, progestin, androgen, and dioxin receptor heterocomplexes (Pratt 1987 and 1990). In all cases when these receptors are bound to hsp90, they cannot bind DNA in vitro and, when hsp90 is dissociated, they bind to DNA with high affinity. The binding of hsp90 to the steroid receptors is stabilized remarkably well by molybdate, vanadate, and tungstate. This effect is not specific to steroid receptors; the $pp60^{src}$–hsp90–pp50 complex is stabilized as well, and it is thought that molybdate acts by binding to hsp90 and affecting its conformation (Hutchison et al. 1992c). Hsp90 is a dimeric protein and it binds to the receptors as a dimer. Rexin et al. (1992) have shown that the hormone-free GR can be cross-linked to both hsp90 and hsp56 in intact cells, with the stoichiometry of the complex being one GR, two hsp90, and one hsp56.

Hsp90 binds to the hormone-binding domain (HBD) of the GR (Pratt et al. 1988; Cadepond et al. 1991) and the PR (Carson-Jurica et al. 1989; Schowalter et al. 1991). In addition to the HBD, a short sequence at the COOH-terminal end of the DNA-binding domain is required for stable ER binding to hsp90 (Chambraud et al. 1990). This sequence contains the ER nuclear localization signal, and it is a likely point of contact for either hsp90 or another protein in the complex. Howard et al. (1990) and Dalman et al. (1991a) examined hsp90 binding by mutant GRs truncated at the COOH terminus and defined a minimal hsp90-binding region of ~100 amino acids (556–659, mouse GR) within the hormone-binding domain that is sufficient for stable hsp90 binding. Chakraborti and Simons (1991) have cleaved the molybdate-stabilized GR with trypsin to produce a 16-kD "core" fragment of the HBD (575–671, mouse GR) that retains the ability to bind steroid and is bound to hsp90. This core steroid-binding fragment demonstrates that there is an intimate association between the hsp90-binding site and the steroid-binding pocket.

Although one can define a minimal hsp90-binding site by this approach, Cadepond et al. (1991) and Schowalter et al. (1991) have shown that portions of the HBD to the COOH-terminal side of this minimal site are also sufficient for

hsp90 binding to the GR and PR. This observation is important because it indicates that there are either multiple contact sites for hsp90 within the HBD or, perhaps more likely, as Schowalter et al. (1991) have proposed, that hsp90 binding depends upon a general property of the unfolded protein that involves a diffuse region of the HBD. Thus, it seems highly unlikely that a discrete motif will be identified that defines an hsp90-binding site. In considering a potential general chaperone role for hsp90 in binding to many proteins, there may be some property or properties of the unfolded state that are common to proteins of very different structure.

The existence of a region in the HBD common to both hsp90 binding and the steroid-binding pocket is consistent with the observation made in many laboratories that binding of steroid agonist to its receptor promotes temperature-dependent dissociation of hsp90 from the receptor, both in cytosol and in intact cells (see Pratt 1990 and 1993). In the case of two receptors, the GR (Bresnick et al. 1989; Scherrer et al. 1990; Hutchison et al. 1992b) and the dioxin receptor (Pongratz et al. 1992), the HBD must be bound to hsp90 for it to have the appropriate conformation for high-affinity steroid binding. This feature differentiates these receptors from the sex steroid receptors, which apparently have a normal steroid binding site in the absence of hsp90.

Hsp70

The hsp70 family of proteins are thought to catalyze protein assembly and are known to bind to newly synthesized but incompletely assembled oligomeric enzymes, but not to the fully assembled product (see Rothman [1989] for review). Hsp70 has protein unfoldase activity that is important for protein translocation across the membranes of organelles.

Kost et al. (1989) demonstrated that a 70-kD protein associated with the chicken PR is the constitutive form of hsp70. Hsp70 has subsequently been found in untransformed progesterone and estrogen receptor nuclear heterocomplexes from other species (see, for example, Onate et al. [1991]). With the exception of the overexpressed GR in CHO cells, which is also localized to the nucleus in the unliganded state, hsp70 has not been recovered as a significant component of native, untransformed GR heterocomplexes (Sanchez et al. 1990b; Rexin et al. 1992). Sanchez et al. (1990b) have suggested that hsp70 may be associated with nuclear receptor heterocomplexes because it is part of the mechanism involved in transporting the receptor heterocomplex across the nuclear membrane, much as hsp70 unfoldase activity is implicated in protein passages across membranes of the endoplasmic reticulum and mitochondria.

Like hsp90, hsp70 is bound to the HBD of the PR (Schowalter et al. 1991) and ER (Scherrer et al., submitted manuscript). The PR or GR can be transformed to the DNA-binding state in vitro with dissociation of hsp90 but not hsp70 (Smith et al. 1990a; Sanchez et al. 1990b); however, hsp70 is not involved in receptor binding to DNA (Onate et al. 1991). As described below, unfoldase activity of hsp70 is thought to be crucial for association of hsp90 with steroid receptors.

Hsp56

Hsp56 is a novel heat-shock protein and immunophilin that was discovered during studies of receptor-associated proteins. Tai et al. (1986) prepared the EC1 monoclonal antibody by using the partially purified, molybdate-stabilized rabbit PR as antigen. They found that EC1 reacted with a 59-kD, non-steroid-binding rabbit protein but also caused the coimmunoadsorption of untransformed progestin, estrogen, androgen, and glucocorticoid receptors. Subsequently, we showed that EC1 immunoadsorbed a 56-kD protein from human IM-9 cell cytosol and coimmunoadsorbed hsp90 and hsp70 (Sanchez et al. 1990a). Because two of the proteins in this complex were heat-shock proteins, Sanchez (1990) heat shocked IM-9 cells and demonstrated that the rate of synthesis of the 56-kD protein was increased. Thus, the EC1 antigen is a novel heat-shock protein and it can be called hsp56. This protein is still called p59 in some literature, but hsp56 is more descriptive and more representative of the M_r of the major protein band in most species.

Hsp56 is now known to be an immunophilin of the FK506-binding class. The immunophilins are a family of proteins that bind immunosuppressants like cyclosporin A, FK506, and rapamycin in a high-affinity and specific manner. All of the members of the family have rotamase (peptidyl-prolyl *cis-trans* isomerase) activity in vitro, and it is thought that the immunophilins, like hsp70 and hsp90, play major roles in protein folding and protein trafficking in the cell (see Walsh et al. [1992] for review).

Hsp56 was found to be an immunophilin when Yem et al. (1992) found that a ~60-kD protein from human Jurkat cells that was retained by a matrix of immobilized FK506 had an NH_2-terminal sequence identical to the sequence we had previously published for human hsp56 (Sanchez et al. 1990a). There was also homology to the low molecular weight FK506-binding proteins FKBP-12 and FKBP-13. In simultaneous work, Lebeau et al. (1992) used the EC1 antibody to screen a rabbit liver cDNA library and clone the cDNA for hsp56, which possessed a deduced amino acid sequence homology to rotamase. Subsequently, Tai et al. (1992) isolated an ~59-kD protein from rat and human cells by using immobilized FK506 and rapamycin. The NH_2-terminal sequence of the human protein was again identical to the sequence that we reported for hsp56. Very importantly, Tai et al. (1992) showed that the FK506 affinity matrix selectively retained hsp90, hsp70, and the GR in addition to hsp56.

It is of intrinsic interest that the three heat-shock proteins—hsp90, hsp70, and hsp56—have now been shown by several methods to exist together in a cytosolic complex, suggesting perhaps that they act together in a spatially organized and temporally coordinated manner. FK506 is known to inhibit the rotamase activity of immunophilins (Walsh et al. 1992), stimulating the speculation on the part of several authors that FK506 may affect steroid receptor function. We have found, however, that FK506 does not affect GR function, steroid-mediated transcriptional activating activity, or reconstitution of the GR into the heat-shock protein heterocomplex (Hutchison et al., submitted manuscript). Thus, the role of hsp56 in the complex is currently unknown.

p23

Johnson and Toft (1992) have prepared a monoclonal antibody against the 23-kD protein reported by Smith et al. (1990a) to be associated with the chicken PR.

The chicken p23 is an acidic phosphoprotein that is apparently unique and is not a heat-shock protein. The 23-kD protein reported by Bresnick et al. (1990) to be a component of the murine GR heterocomplex is recognized by this monoclonal antibody (P.R. Housley, personal communication). A very significant advance should accompany the cloning and sequencing of the cDNA for p23.

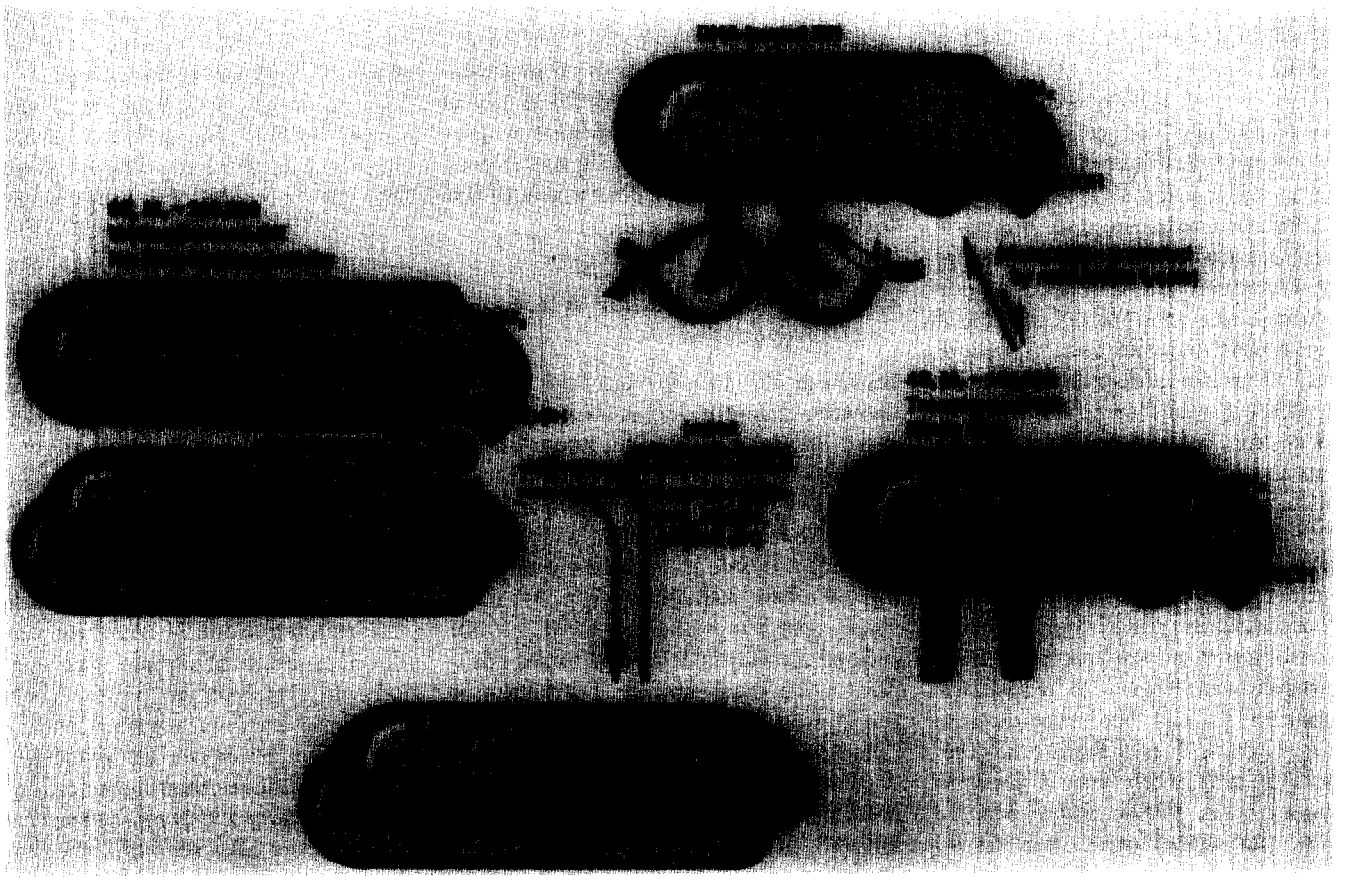
- **GR Translated in Reticulocyte Lysate Is Bound to hsp90**

Steroid receptors are not in a free-binding equilibrium with hsp90. In cytosol, receptors can be dissociated from hsp90 with salt or by binding of steroid and heating and, in the intact cell, dissociation occurs as a consequence of hormone binding to the receptor. Regardless of how dissociation has occurred, the receptors appear to have undergone a change in conformation (indicated by the inward folding of the HBD in Figure 2) such that the hsp90-binding site is eliminated. Because efforts to rebind hsp90 to receptors in vitro were unsuccessful, it was asked whether receptor translated in vitro was bound to hsp90.

Denis and Gustafsson (1989) were the first to show that rat GR translated in rabbit reticulocyte lysate behaved as a 9 S receptor. The newly translated receptor did not bind DNA, but it was converted to the 4 S, DNA-binding form by binding it with dexamethasone and heating. Thus, it seemed likely that the rat receptor had combined with hsp90 in the lysate to produce a hybrid rat GR–rabbit hsp90 complex that functioned like a native 9 S receptor isolated from the cell.

Dalman et al. (1989) developed a direct method of assaying the binding of in vitro–translated GR to hsp90. The rat GR was translated both in rabbit reticulocyte lysate, which contains ~2 μM hsp90, and in wheat-germ extract, which does not contain protein reacting with anti-hsp90 antibodies of broad-spectrum reactivity within the animal kingdom. It was found that GR translated in reticulocyte lysate was bound to rabbit hsp90, bound steroid with high affinity, and was in a non-DNA-binding form that could be transformed to the DNA-binding state. In contrast, GR translated in the plant extract was not bound to a plant protein, had no steroid-binding activity (despite the fact that the full-length GR was translated), and was translated in the DNA-binding form (Dalman et al. 1989 and 1990). Thus, the GR translated in the reticulocyte lysate behaved like the normal heterocomplex on the left in Figure 2, and the GR translated in the plant system behaved like the

Figure 2. Model of dissociation of the glucocorticoid receptor (GR)–hsp90 complex and its reconstitution by a protein-folding system in reticulocyte lysate. Hsp90 binds to the hormone-binding domain of the receptor to form a 9 S complex without DNA-binding activity. The complex can be stabilized by molybdate, as indicated by the *small globe* with the $M^{=}$. Addition of salt, dilution of cytosol, or increasing the pH promotes hsp90 dissociation and simultaneous transformation to the DNA-binding state (indicated by the 4 S receptor with the exposed "zinc fingers" on the right). Physiologically, this dissociation is promoted by steroid. Either immunoadsorbed or DNA-bound unliganded receptor is reassociated with hsp90 in an ATP-dependent reaction directed by rabbit reticulocyte lysate, to reconstitute the non-DNA-binding, untransformed receptor. From Scherrer et al. (1990), Fig. 4. Reproduced by permission of the American Society for Biochemistry and Molecular Biology.



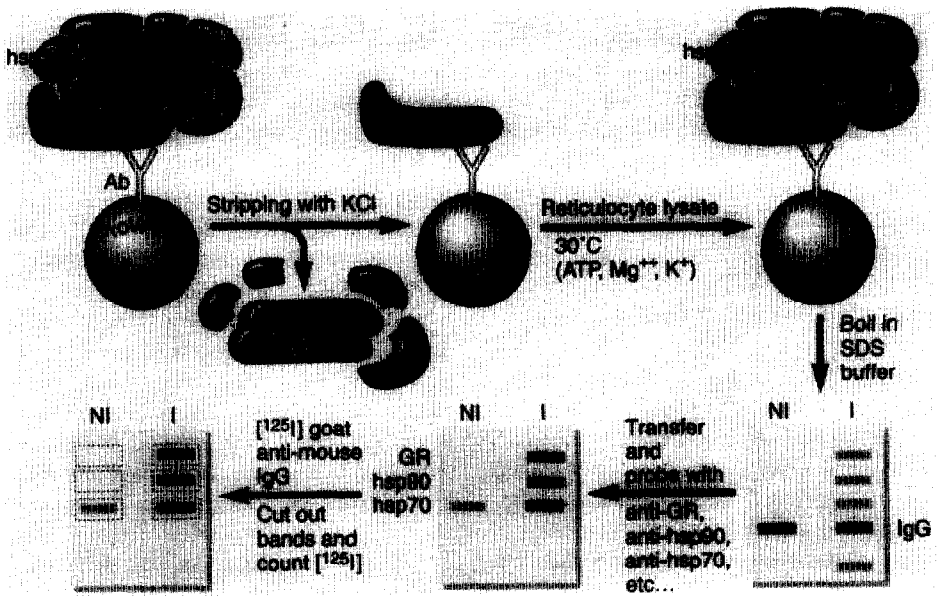


Figure 3. Cell-free reconstitution of the glucocorticoid receptor (GR) heterocomplex with heat-shock proteins. Receptor heterocomplexes are immunoadsorbed to a pellet of protein A–Sepharose with the BuGR monoclonal antibody, and receptor-associated proteins are stripped off with 0.5 M KCl. After several washes, the pellet containing the immunopurified receptor is incubated with rabbit reticulocyte lysate for 10–30 min at 30°C in the presence of an ATP-generating system and 100 mM KCl. The immunopellet is then washed several times with a buffer containing 20 mM molybdate to stabilize the heterocomplex. The proteins in the reconstituted heterocomplex are resolved by denaturing gel electrophoresis and identified by immunoblotting with specific antibodies. Control samples with nonimmune IgG are always run in parallel to demonstrate that the proteins in the heterocomplex are present in a manner that is specific for the receptor. NI, nonimmune sample; I, immune sample; and Ab, BuGR antibody.

dissociated 4 S receptor at the right of the figure.

Schlatter et al. (1992) recently demonstrated that the hormone-binding domain is itself sufficient for hsp90 binding to the newly translated GR, an observation consistent with the data on hsp90 binding in intact cells. Dalman et al. (1989) examined hsp90 binding by partially translated wild-type GR and found that receptors that had been translated through the minimal hsp90-binding site in the HBD, but had been released from the ribosome before complete translation to the COOH terminus, were not bound to hsp90. It was also found that the full-length GR bound to hsp90 at the completion of receptor translation or very shortly thereafter. Taken together, these observations raised the possibility that association of the receptor HBD with hsp90 was linked to translation termination. It has been shown that members of the hsp70 family interact cotranslationally with many proteins (Beckman et al. 1990), and it seemed possible that association with hsp90 might be obligatorily coupled to receptor translation. If binding to hsp90 occurs at the termination of receptor translation in the intact cell, this would imply a coupling of protein translation, protein folding, and protein binding to the proposed transportosome component of the trafficking system (Figure 1).

• In vitro Reconstitution of Receptor Heterocomplex

Smith et al. (1990b) were the first to show that hsp90 association with steroid receptors in reticulocyte lysate is not obligatorily coupled to receptor translation. They incubated immunoadsorbed, hormone-free chicken PR with rabbit reticulocyte lysate and demonstrated binding of rabbit hsp90 and hsp70 to the avian receptor. Scherrer et al. (1990) formed a mouse GR–rabbit hsp90 complex in the same manner. Formation of the steroid receptor heterocomplex by reticulocyte lysate is clearly a temperature-dependent and ATP-dependent enzymatic process that requires a monovalent cation (K^+ or NH_4^+) (Smith et al. 1992; Hutchison et al. 1992b). An important requirement for heterocomplex reconstitution is that the receptor be hormone free.

The method of heterocomplex reconstitution as we perform it with the GR is diagrammed in Figure 3. Receptor heterocomplexes are immunoadsorbed to a pellet of protein A–Sepharose. Depending upon the source of the receptor, hsp70 may or may not be present in the native immunoadsorbed complex. After dissociating the receptor-associated proteins with salt and washing the immunopellet several times with buffer, the immunopurified receptor is incubated with reticulocyte lysate. After a few minutes of incubation at 30°C, the immunopellets are washed again with a buffer containing molybdate to stabilize the heterocomplex while eliminating unbound reticulocyte proteins. The reconstituted GR heterocomplex has been shown to contain hsp56 (Hutchison et al., submitted manuscript) as well as hsp70 and hsp90 (Hutchison et al. 1992b). Proteins identified in the reconstituted chicken PR heterocomplex are hsp90, hsp70, and p23 (Smith et al. 1992).

The Reconstituted Heterocomplex Behaves Like the Native Complex

Although one can demonstrate the presence of appropriate proteins in a reconstituted receptor heterocomplex, that does not demonstrate that they are arranged in the appropriate manner to function like the native heterocomplex. The GR is useful for examining the function of the reconstituted complex because hsp90 must be bound appropriately to the HBD for it to bind steroid. Thus, reconstitution of the complex should convert the GR from a non-steroid-binding state to a steroid-binding conformation. That this is the case is shown in Figure 4. Lane 1 shows the immunoadsorbed receptor after stripping of associated proteins with salt. As shown in the autoradiogram in the bottom panel, this form of the receptor does not bind the site-specific affinity label [^{3}H]dexamethasone 21-mesylate. Lane 3 shows an identical sample after incubation with reticulocyte lysate. There is now hsp90 associated with the GR, and the receptor binds steroid. There is considerable evidence that the generation of steroid-binding activity is due to reassociation of the GR with hsp90. For example, generation of steroid-binding activ-

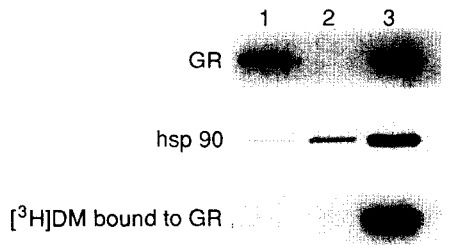


Figure 4. Reconstitution of the glucocorticoid receptor (GR) heterocomplex restores the receptor to the steroid-binding conformation. *Lane 1* shows GR that was immunoadsorbed from mouse L cell cytosol and salt stripped of associated proteins as shown in the diagram of Figure 3. The stripped immunopellet was divided into two portions, with one portion being assayed for GR and hsp90 by Western blotting and the other portion used for assay of steroid binding by incubating with the site-specific affinity ligand [^{3}H]dexamethasone 21-mesylate. Receptors incubated with affinity label were resolved by gel electrophoresis and autoradiography. *Lane 3* shows an identical sample that was stripped and then incubated with reticulocyte lysate to reassociate it with hsp90 and reactivate it to the steroid-binding state. *Lane 2* shows a nonimmune sample that was stripped and incubated with lysate in the same manner. Adapted from Dalman et al. (1991a), Fig. 2. Reproduced by permission of the American Society for Biochemistry and Molecular Biology.

ity is blocked by peptides that block association of hsp90 (Dalman et al. 1991a), and there is a direct relationship between the extent of hsp90 associated with the receptor and the number of specific binding sites that are generated (Hutchison et al. 1992b).

Steroid receptors in the native 9 S heterocomplex do not bind to DNA, whereas hsp90-free receptors bind to DNA with high affinity. We have shown that hormone-free glucocorticoid receptors that have been transformed with salt and bound to DNA are released from DNA by incubating them with reticulocyte lysate (Scherrer et al. 1990). Receptor release is ATP dependent and the released receptor has been reassociated with hsp90. This reaction is indicated as a two-step event in Figure 2. But it is not yet known whether the receptor is first released from DNA and then bound to hsp90 or if binding to hsp90 occurs as part of the release. In either case, the enzymatic system in reticulocyte lysate has converted the receptor from the DNA-binding form back to the non-DNA-binding form, and this represents the first demonstration of the reversal of receptor transformation. The liganded receptor is not released from DNA.

The general observation that the liganded receptors are not reassociated with hsp90 is consistent with the overall model of steroid hormone action in which the steroid favors a receptor conformation that results in hsp90 dissociation. In the reconstitution system, it is clear that the HBD is sufficient for association with hsp90. For example, no reconstitution with heat-shock protein was obtained with a PR mutant lacking the HBD (Smith et al. 1990b). Also, the HBD of the GR is sufficient to render fusion proteins with β-galactosidase capable of being formed into a heterocomplex (Scherrer et al., submitted manuscript).

Reconstitution of the pp60$^{v\text{-}src}$-hsp90–p50 Complex

Like steroid receptors, the oncogenic tyrosine kinase pp60$^{v\text{-}src}$ is recovered from cells in a heterocomplex with hsp90. The pp60$^{v\text{-}src}$ heterocomplex also contains a 50-kD protein that is associated with hsp90 in uninfected cells (Whitelaw et al. 1991). In intact cells, pp60$^{v\text{-}src}$ becomes associated with these proteins as soon as it is translated and remains in the heterocomplex while it is transported to the cell membrane. We have shown that the reconstitution method diagrammed in Figure 3 can be employed with immunopurified pp60$^{v\text{-}src}$ to form a heterocomplex of the viral tyrosine kinase with hsp90, hsp70, and p50 (Hutchison et al. 1992a). As with the steroid receptors, the complex is formed in a temperature-dependent and ATP-dependent manner and is stabilized by molybdate. This implies that the reticulocyte lysate has a general system (or systems) for carrying out protein-heterocomplex assembly.

• Mechanism of Receptor Heterocomplex Assembly

At this time, we know little about how the receptor heterocomplex is formed. For example, it is not yet known whether the heterocomplexes are formed in lysate by a process of ordered addition of individual proteins or whether the receptors become attached to a preformed complex. The fact that hsp90, hsp70, and hsp56 exist in a complex independently of the receptor is consistent with the possibility that the receptor HBD becomes attached to a preformed complex. Recently, we have found that a protein complex (200–250 kD) that was partially purified from rabbit reticulocyte lysate by fractionation with ammonium sulfate and Sepharose CL-6B chromatography reconstitutes a GR–hsp90 complex (Scherrer et al. 1992). The efficiency of reconstitution is very low compared with unfractionated reticulocyte lysate, but the GR that is rebound to hsp90 is converted to a steroid-binding state in an ATP-dependent manner. The partially purified protein complex from reticulocyte lysate was shown to contain hsp90, hsp70, and hsp56. Again, these observations are consistent with a model in which receptors are attached to a preformed complex.

A Role for hsp70 in Complex Assembly

The current models of heterocomplex assembly (Smith et al. 1992; Pratt et al. 1992) are certainly incomplete, but they assume that hsp90 and hsp70 are preassociated and act together as a unit, with the protein unfoldase activity of hsp70 playing a critical role in initiating the process. Hsp70 has an established protein-unfolding activity in other systems (Rothman 1989), and there are several reasons for assuming a role for this activity in receptor heterocomplex formation. First, both Toft's lab and our group have found that reassociation of the PR, GR, or pp60$^{v\text{-}src}$ with hsp90 is always accompanied by association with hsp70 (Smith et al. 1992; Hutchison et al. 1992a and b; Scherrer et al. 1992). Second, reconstitution of the complex has the same monovalent cation requirement as reported for the clathrin-unfolding activity of hsp70 (Hutchison et al. 1992b). Third, Smith et al. (1992) have shown that pretreatment of reticulocyte lysate with a monoclonal antibody against hsp70 inhibits the assembly of the PR–hsp90 complex.

The heterocomplex assembly model assumes at least two steps: in the first step, hsp70 recognizes some general feature(s) of the folded state of the HBD (see transformed GR in Figure 2) and facilitates ATP-dependent unfolding and, in the second step, the hsp90 component of the complex stabilizes the unfolded state of the HBD. The receptor is now docked to the heat-shock protein com-

plex until binding of steroid promotes dissociation from the hsp90 component. Other proteins and other steps are undoubtedly required. Indeed, our experience with GR reconstitution by the partially purified heat-shock protein complex from reticulocyte lysate demonstrates that other components are required (Scherrer et al. 1992). Smith et al. (1992) have identified a 60-kD protein that is present during assembly of the chicken PR heterocomplex by reticulocyte lysate, but appears to be released in an ATP-dependent manner and is not present in the final PR heterocomplex. Further definition of this protein may contribute greatly to our understanding of heterocomplex assembly.

• Envoi

Over the past 2 1/2 decades, principles and techniques from other areas of biology have been used to develop a tremendous amount of information regarding the structure and function of steroid receptors. Because of rapid advances made since the cloning of these proteins and the discovery of the receptor–heat-shock-protein heterocomplex, the steroid receptor proteins themselves have become powerful tools for probing the mechanisms of basic biologic processes, such as protein folding, protein transport, and regulation of protein function. This has generated the feeling among molecular endocrinologists that the field of steroid receptor investigation has changed from being to a large extent conceptually derivative of other fields to being a field of investigation that is developing general principles that will be applicable to a variety of biologic systems.

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