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Steroid Receptor Interactions with Heat Shock Protein and Immunophilin Chaperones*

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I. Introduction

SEVERAL nuclear receptors, including those for sex steroids, adrenocorticoids, and the dioxin class of carcinogens, are recovered from cells in large (9S) heterocomplexes that contain both heat shock proteins (hsps)[1] and immunophilins. Some components of the receptor heterocomplexes are proteins with established chaperone functions (*e.g.* hsp90 and hsp70), and one critical function of the hsp heterocomplex is to facilitate the folding of the hormone binding domain (HBD) of the receptors into a high-affinity steroid-binding conformation. hsp90 interacts directly with the HBD of the nuclear receptors, an association that appears to account for a repression of receptor function that is relieved upon subsequent binding of hormone. This ability of the hormone to control the HBD-chaperone interaction is now regarded as the earliest event in the molecular pathway of steroid hormone action.

The study of the supramolecular receptor structures has led to the observation that the chaperone proteins exist in cytosols as multiprotein heterocomplexes independent of their association with receptors. These hsp heterocomplexes

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[1] Abbreviations used are: AC88, antibody against hsp90; AR, androgen receptor; BF4, antibody against hsp90; bHLH, basic helix-loop-helix; CAT, chloramphenicol acetyl transferase; CyP, cyclophilin (cyclosporin A-binding protein); DBD, DNA-binding domain; DEAE, diethylaminoethyl; DR, dioxin receptor; EC1, antibody against FKBP52; ER, estrogen receptor; FKBP, FK506-binding protein; GR, glucocorticoid receptor; HBD, hormone-binding domain; hsp, heat shock protein; MMTV, mouse mammary tumor virus; MR, mineralocorticoid receptor; NLS, nuclear localization signal; PP5, protein phosphatase 5; PPIase, peptidylprolyl isomerase; PR, progesterone receptor; TPR, tetratricopeptide repeat.

appear to act as protein-folding machines that assemble heterocomplexes between the hsps and the receptors, as well as with some protein kinases involved in signal transduction from membrane receptors. Recently, immunopurified hsp heterocomplexes have been used to refold denatured luciferase to the catalytically active state. It now seems likely that the study of the nuclear receptor heterocomplex assembly mechanism will yield very basic information regarding more general protein-folding reactions in the cytoplasm.

The subject of nuclear receptor-hsp interactions has been reviewed briefly at various times during the past 10 yr (1–10). This review is intended as a comprehensive treatment of all work published through September of 1996. We begin with some of the earliest observations on nuclear receptor structure and the stabilization and purification of receptor complexes so that readers may appreciate the background leading to the discovery of receptor-hsp interactions. A number of assumptions about receptor behavior that were based on cytosolic steroid-binding studies performed during the 1960s and 1970s require revision in light of our knowledge of these receptor-hsp interactions. We will note these revisions as we review the background observations leading to the discovery of receptor heterocomplexes and the current state of our understanding regarding the cell-free mechanism of heterocomplex assembly. We do not review here the extensive literature on hsps or immunophilins or other protein-folding systems; appropriate reviews of these subjects are indicated in the text. However, we will discuss some interactions between hsps and nonreceptor proteins that may contribute to a more general understanding of hsp function.

II. 9S Receptors

The field of steroid receptor biochemistry started in 1958 with the synthesis of tritium-labeled estrogens by Jensen (11) and by Glascock and Hoekstra (12). Both of these laboratories demonstrated selective accumulation and retention of tritium-labeled steroid in the reproductive organs of immature female animals administered physiological amounts of hormone. Because the [^{3}H]estradiol extracted from the organs of injected animals was the unmetabolized compound (13), it was thought that the retention of steroid reflected binding to receptors located within the cells of the uterus and vagina. This organ-specific retention of estradiol was arguably the first evidence for binding of a hormone or drug to a receptor, yet even as late as 1968, some pharmacologists felt the use of the word "receptor" to describe the estradiol-binding entity was inappropriate (14).

In 1965, Noteboom and Gorski (15) demonstrated that injection of [^{3}H]estradiol to immature rats was followed by accumulation of steroid in both the nuclear-myofibrillar and cytosolic fractions of the rat uterus. The [^{3}H]estradiol was bound in the nuclear fraction in a stereospecific manner, and it was released by digestion with trypsin, suggesting that the "estrophile" [a term coined by Jensen (16) to avoid objections to the name "receptor"] was a protein. The work of Noteboom and Gorski (15) initiated the study of steroid receptors in cell-free systems, but nevertheless, attempts to demonstrate a single intracellular estradiol binding species (*e.g.* by molecular seive chromatography) had been unsuccessful. At this time, Toft and Gorski (17) prepared a 105,000 × *g* cytosolic fraction from uteri of rats injected with [^{3}H]estradiol and centrifuged the cytosol components through a gradient of 5–20% sucrose. The estradiol radioactivity sedimented in a nearly symmetrical peak at 9.5S, and, like the [^{3}H]estradiol binding studied previously in the uterine nuclear myofibrillar fractions, the cytosolic estradiol binding recovered at ~9S was stereospecific and eliminated by proteases (17). Shortly thereafter, Toft *et al.* (18) demonstrated that addition of [^{3}H]estradiol directly to cytosol prepared from uteri of untreated rats yielded a 9S complex that appeared identical to the complex obtained after *in vivo* hormone administration. This publication represents the first direct demonstration of hormone or drug binding to a receptor protein in a cell-free system.

The publication of the sucrose gradient technique triggered well over 100 papers that appeared throughout the next decade and established the existence of large (~9S) and small (~4S) forms of all the steroid receptors. These studies were summarized in several early reviews (19–24), and a recent comprehensive review by Jensen (25) may be consulted for more detail and complete references to primary sources. It is our goal in the remainder of this section to focus primarily on the observations in steroid receptor biochemistry that logically led to the discovery of receptor-hsp heterocomplexes. In various publications, the large receptor complex has been assigned sedimentation values between 8S and 10S, but to simplify the text, we will refer to the receptor heterocomplex as 9S. We will use the word *transformation* to refer to the conversion of 9S non-DNA-binding receptor to the 4S DNA-binding form. The term *activation* will refer specifically to the conversion of receptors from a form that does not bind steroid to a steroid-binding form.

A. Estrogen receptors (ERs)

The discovery of the 9S form of the cytosolic ER was rapidly confirmed by other laboratories (26–29). Importantly, it was found that treatment of cytosol with 0.3 M KCl (followed by subsequent centrifugation on a sucrose gradient containing KCl) "transformed" the cytosolic ER from a 9S to a 4S form (27–29). The discovery of this salt effect gave rise to the notion that the 9S ER was an oligomer that could be dissociated to a 4S state, a dissociation that occurred regardless of whether the receptor was free or bound with estrogen (28). Because dialysis of KCl-treated cytosol followed by sucrose gradient centrifugation in the absence of salt yielded predominantly a larger form of the ER, it was thought that the 9S to 4S transformation was reversible (27, 29). In a more direct approach, it was reported (20, 28) that the 4S peak of [^{3}H]estrogen-bound ER isolated from a KCl-containing gradient could be recovered as 9S upon recentrifugation in the absence of salt. This assumption of a "reversible equilibrium" between the 9S and 4S forms of the receptor remained an important component of models describing steroid hormone action for many years. However, these reversibility studies were compromised by poor receptor recovery and heterogeneous aggregation, and it is now known that the notion of simple reversible receptor forms was incorrect. Recent experiments demonstrate (see *Section VIII*) that the large het-

erocomplex form of the steroid receptors is reconstituted from the 4S monomeric form only via an ATP-dependent protein-folding process. The 9S form is not an oligomer of 4S units; rather, it is a receptor-hsp heterocomplex.

It was known from early autoradiography (30) and cell fractionation (15) studies that, after *in vivo* injection, the majority of [^{3}H]estrogen in the uterine cells was associated with nuclei. Jensen and co-workers (31) were able to solubilize the ER from uterine nuclei by repeated extraction with 0.3 M KCl and show that the salt-extracted nuclear receptor sedimented at 5S in sucrose gradients containing 0.4 M KCl. In 1968, the laboratories of Jensen (32) and Gorski (19, 33) demonstrated that the nuclear 5S ER was generated in a temperature-dependent manner at the expense of the cytosolic 9S ER complex. Incubation of the uterine cell nuclear fraction with [^{3}H]estradiol did not yield a steroid-receptor complex, and it was only when nuclei were exposed to cytosol containing the [^{3}H]estradiol-bound 9S complex that subsequent extraction with salt yielded the 5S receptor. These critical studies led to a very useful model of the initial events in steroid hormone action in which it was envisioned that the steroid first binds to a 9S form of the ER that is recovered in the cytosolic fraction upon cell rupture. This 9S complex was present in uterine cells before binding of steroid and was presumed to be an inactive form of the receptor. Steroid binding then, in some way, facilitated the temperature-dependent conversion of the biologically inactive 9S receptor to the biologically active form that could bind tightly to nuclei and was extractable as a 5S unit. This model is known as the "two-step" model of steroid action (32).

For a while, it was unclear whether the 5S salt-extracted nuclear ER was the same as the ~4S form produced by salt dissociation of the 9S cytosolic ER (29, 34). However, Jensen *et al.* (35) demonstrated, by sedimentation in salt-containing sucrose gradients, that the nuclear 5S receptor was clearly larger than the 4S subunit of the 9S cytosolic ER. Subsequently, it was shown in cell-free systems that estrogens promote the temperature-dependent conversion of the 4S cytosolic ER to the 5S form (36–38). At this time, it was thought by many [but not all (38)] investigators that the 4S form of the ER did not have DNA-binding activity and that the 5S ER was the DNA-binding form. Laboratories studying ERs then focused on the 4S to 5S conversion, considering this step, rather than the conversion from the 9S to the 4S form, to be the "receptor transformation" event (22).

Physical studies by the Notides' laboratory (39, 40) showed that the conversion of 4S to 5S ER was a dimerization reaction, most likely a homodimerization (41). Kumar and Chambon (42) subsequently established that the ER binds to its response element in the DNA as a ligand-induced homodimer. The exceptionally strong dimerization site located within the HBD of the ER (43) accounts for the unique ability of the ER among the steroid receptors to maintain a homodimer in the presence of salt. Although it was clear that heat transformation of cytosolic ER complexes to a DNA-binding form occurs before receptor dimerization (44, 45), the strong dimerization property of the ERs led investigators of estrogen action to focus on the equally critical receptor dimerization event rather than pursuing the structure and properties of the 9S precursor. Thus, although the 9S complex was discovered with the ER, the subsequent fundamental observations regarding receptor heterocomplex structure and function were made in laboratories studying progesterone and glucocorticoid receptors.

B. Progesterone receptors (PRs)

In 1970, PRs in cytosols prepared from chicken oviduct (46) and guinea pig uterus (47) were shown to migrate as both large (~9S) and small (4S) species in low-ionic strength sucrose gradients and, in both cases, the large species was converted to 4S at high-ionic strength. In rabbit uterine cytosol, the 4S species was observed in castrate animals, but both 9S and 4S species could be visualized after estrogen treatment (48). In some reports, only a 4S species of PR was observed in rat or rabbit uterine cytosol under low salt conditions (49, 50), giving the impression that the 9S PR complex may dissociate more readily than the 9S ER complex. Nevertheless, the chicken oviduct PR has proven to be an exceptionally useful system for study of the 9S steroid receptor heterocomplex. Because large amounts of PR protein are produced in the oviduct of the estrogen-primed chick, it became possible, once a procedure for stabilizing the 9S complex was established, to immunoadsorb large amounts of receptor and identify coadsorbed proteins by direct staining of bands resolved on denaturing gels. The coadsorbed proteins were present in sufficient quantity to permit their identification by amino acid sequencing (9). The same sequence of events that had been described for the ER — binding of steroid to a cytosolic receptor, followed by temperature-dependent transformation of the receptor to a DNA-binding form, and subsequent association of receptor with nuclei — was demonstrated for the chick oviduct PR, both in tissue slices and under cell-free conditions (51, 52).

C. Androgen receptors (ARs)

The first indications that the two-step model might apply in a general way to other nuclear receptors came from studies of androgen binding in the rat prostate. Mainwaring (53) was the first to demonstrate a 9S form of the AR in rat prostate cytosols, an observation confirmed by the Baulieu laboratory in both rat prostate (54) and muscle (55) cytosols, with salt dissociation to a 4S form being demonstrated in both cases. Selective retention of radiolabeled androgen by prostatic cell nuclei had been demonstrated in 1968 (56–58), and, in 1969, Fang *et al.* (59) showed that binding to the nuclear fraction required the presence of cytosol. The progression of AR from cytosolic 9S to salt-extractable nuclear 4S in a cell-free system was then published by Mainwaring and Peterken (60), confirming the general progression of events outlined in the two-step model.

D. Glucocorticoid receptors (GRs)

GRs were first identified in rat thymic lymphocyte cytosol by the Munck laboratory in 1966 (61), with detailed studies being published by the laboratories of Munck (62) and Schaumburg (63) in 1968. However, physical studies of the receptor awaited the introduction of tritium-labeled high affinity binders, such as dexamethasone and triamcinolone

acetonide. Baxter and Tomkins (64) were the first to report that GRs were present as 9S complexes that dissociate to 4S in rat hepatoma cell (HTC) cytosol in the presence of 0.5 M KCl. The observation was rapidly confirmed for GR in cytosols prepared from rat liver (65, 66), brain (67), and mammary gland (68). It was determined that incubation of whole cells at 37 C with glucocorticoid resulted in the majority of the specifically bound hormone being recovered in the nuclear fraction (69–71) and that the increase in specifically bound steroid in the nucleus was accounted for by the loss of specific binding from the cytosolic fraction (72). As with the other steroid receptor systems, it was shown under cell-free conditions that isolated nuclei did not bind steroid, but if cytosol containing steroid-bound receptors was warmed and then incubated with nuclei, the specifically bound steroid became associated with nuclei (70, 71, 73–76). Thus, by 1973, the two-step model had been extended to glucocorticoid hormone action.

Although it was known that cytosolic GR existed in a 9S complex (64–68) and that the temperature-transformed GR extracted from nuclei with salt was 4S (76) [owing to the fact that the homodimer must be stabilized by cross-linking to be detected as a 6S entity (77)], investigators in glucocorticoid hormone action did not focus on the dissociation of the 9S complex as the transformation event. Rather, they focused on the other half of the coin, *i.e*. the acquisition of DNA/nuclear binding activity (71–76, 78, 79), an obviously critical change for a receptor with a nuclear site of action. Indeed, in one rather influential report, it was maintained that both the untransformed and the heat-transformed, steroid-bound GR sedimented at 9S in low-salt gradients and at 4S in high salt (80). The suggestion was that the 9S form was observed only in media at unphysiologically low ionic strengths and that *in vivo* the GR exists predominantly in the 4S form (80). In mechanistic studies of GR transformation published in the late 1970s, the process was thought to involve solely a 4S receptor that could reversibly exist in a non-DNA-binding or a DNA-binding form (44, 81).

The 9S form of the GR is much less stable in cytosol preparations than the 9S forms of the ER and PR. Despite the instability of the complex and the focus of most laboratories on the acquisition of DNA-binding activity upon transformation, GR systems have been very useful in defining the composition and function of the components of the 9S complex. Actually, the instability of the 9S GR complex turned out to be an advantage. As we now know, the GR must be bound to hsp90 for the HBD to be in a high-affinity steroid-binding conformation (82). Dissociation of hsp90 from the unliganded receptor is reflected by a simultaneous loss in cytosolic steroid-binding activity (82). It was the study of this instability of cytosolic glucocorticoid-binding activity that led to the discovery of agents that inhibit the loss of steroid-binding activity, most notably molybdate and some other transition metal oxyanions (83, 84). Molybdate stabilizes the interaction of hsp90 with steroid receptors, and it permitted the purification of the untransformed PR and GR and identification of the hsp90 component of the 9S complex. A second feature of the GR that has made it particularly useful in the study of the heterocomplex is that it is essentially ubiquitous. Thus, it is present in a wide variety of cultured cell lines, some of which contain relatively high glucocorticoid-binding activity. The systems that have been especially useful are mouse L cells, which have a naturally high level of glucocorticoid-binding activity (85), and WCL2 cells, which are Chinese hamster ovary (CHO) cells that have been engineered to overexpress the mouse GR (86).

E. Mineralocorticoid receptors (MRs)

In 1963, Edelman *et al.* (87) first showed that [^{3}H]aldosterone localized to nuclei in cells of the toad bladder. In 1970, Edelman's laboratory (88) demonstrated that, after administration of [^{3}H]aldosterone to rats, stereospecifically bound steroid could be extracted from a renal chromatin fraction in a 4S form. Subsequent studies showed that a 9S complex was present in cytosols (89, 90), and from the time-course of 9S and 4S generation, it was deduced that the 9S cytosolic receptor was likely a precursor of the nuclear 4S form (89), a conclusion that agreed with *in vitro* data showing salt-mediated transformation of 9S receptor to the 4S form (89, 91). Unliganded MRs in cytosols rapidly lose their steroid- binding activity (92). It is now known that these receptors must be also associated with hsp90 to have a steroid-binding site (93), and the lability of cytosolic steroid-binding activity is due to rapid dissociation of the 9S MR to 4S. The binding of the MR to hsp90 is the least stable among the mammalian steroid receptors.

F. Dioxin receptors (DRs)

A variety of halogenated hydrocarbons bind to a receptor that mediates the induction of aryl hydrocarbon hydroxylase, a cytochrome-P-450-mediated monooxygenase involved in the metabolism of many xenobiotics. This receptor is called the *Ah* receptor, or more commonly, the dioxin receptor (for review, see Refs. 94 and 95). The DR differs from the steroid receptors in several respects, including the fact that its DNA-binding domain (DBD) contains a basic helix-loop-helix rather than a double-zinc finger structure. Nevertheless, dioxin receptors possess a number of physical properties in common with steroid receptors. After dioxin administration, for example, mouse hepatic receptors were found to accumulate in nuclei, and there was a concomitant decrease in specific dioxin-binding capacity in the cytosol (96). As with the steroid receptors, the conversion from a cytosolic to a nuclear receptor was both dioxin-dependent and temperature-dependent (97, 98). Thus, the model for dioxin receptors as it originally developed was identical to the two-step model proposed for steroid receptors (99, 100). The dioxin receptor in cytosols from several species was shown to be 9S and to dissociate to 4S in the presence of salt (98, 101). Indeed, the general properties of the glucocorticoid and dioxin receptors in rat hepatic cytosol were identical (102). However, it became apparent that the mouse dioxin receptor differs from those of rat and other species in that the 9S complex is highly resistant to salt-mediated dissociation (102, 103). This unique salt stability appears to be a property of the mouse receptor protein itself (104), and the binding of the mouse dioxin receptor to hsp90 is the most stable of all receptors examined to date. The dioxin receptor differs from

the steroid receptors in that, after ligand-mediated dissociation from hsp90, it forms a heterodimer with the ARNT protein rather than dimerizing with itself (95).

G. Antheridiol receptors

Sexual reproduction in the eukaryotic filamentous fungus *Achlya ambisexualis* is regulated by steroid pheromones. One of the steroids, antheridiol, is released from female cells and induces development and differentiation of the male sex organs or antheridia. Binding studies revealed the presence of an antheridiol receptor in cytosol prepared from *A. ambisexualis* (105). Under low salt conditions in the presence of molybdate, this receptor is 9S (105, 106). Molybdate stabilization is required to visualize the 9S form and in the presence of salt, with or without molybdate, the receptor is 4S. Of all the steroid receptors, the binding of the antheridiol receptor to hsp90 is the least stable, and the actual composition of the receptor complex remains unknown. Nevertheless, the system proved to be highly useful because it led to the purification of *Achlya* hsp90, which was used to prepare the very broad spectrum AC88 monoclonal antibody (IgG) that has been widely used in studies of hsp90 structure and function (107).

III. Receptor Transformation

It is essential to hormone action that a receptor must be able to assume at least two states — one that is inactive and one that is active — with the binding of the hormone promoting the transformation from the inactive to the active form. The key observation encompassed in the *two-step* model of steroid action was that hormone binding and transformation could be differentiated as two distinct and sequential processes. By the early 1970s, studies in both intact and cell-free systems confirmed that transformation measured either by 9S to 4S conversion or by acquisition of nuclear or DNA-binding activity was both hormone-dependent and temperature-dependent (19, 32, 33, 36–38, 51, 52, 69, 71, 73–76, 89, 99, 100). The hormone dependency of cytosolic receptor binding to DNA was consistent with the subsequent demonstration by genomic footprinting that hormone was required for receptor binding to specific response elements in intact cells (108). The requirement of hormone for both thermal conversion of the 9S receptor to 4S and concomitant acquisition of the ability to bind to a specific hormone response element was demonstrated in a cell-free system with the GR by Denis *et al.* (109) in 1988.

However, as various laboratories began to explore a variety of artifactual (*i.e.* non-hormone-dependent) methods of transforming the receptor and as different criteria were used to define transformation (*e.g.* 9S dissociation, ER dimerization, DNA binding, polyanion binding, nuclear binding, loss of negative charge), the study of cell-free transformation appeared to be illogical and, to some investigators, possibly irrelevant to biological activity. As Grody *et al.* (110) stated in a review published in 1982, "The remarkably dissimilar characteristics of transformation among different steroid receptors discussed above have so far resisted attempts at incorporation into a universal mechanistic model and have led to much confusion in the field." Nevertheless, with the exception of the differing views regarding the importance of 9S receptor dissociation *vs.* receptor dimerization cited above (*Section II.A*), certain common themes emerged, and they are summarized in reviews written before the discovery of the receptor-hsp90 complex (110–112).

A. Transformed receptors bind polyanions

Initially, investigators focused on the ability of transformed cytosolic receptors to bind to nuclei, but a variety of other assays for receptor transformation were soon published. The finding that treatment of nuclei with DNAse released transformed receptors (71, 73, 113) or prevented receptor binding to nuclei in a cell-free translocation assay (114) was consistent with DNA being at least part of the nuclear binding site. It was then determined that transformed cytosolic steroid receptors bound to chromatin preparations (115) and to purified DNA from any source, either in solution (71, 116) or immobilized to cellulose (38, 76, 117, 118). Indeed, it was shown that the receptors are transformed to a state that binds polyanions in general (78), and assays of receptor transformation were developed based on binding to phosphocellulose (119, 120), ATP-Sepharose (121, 122), and carboxymethyl-Sephadex (78, 123). Although assay of transformation using DNA-cellulose did not yield as high a percentage of receptors binding as simultaneous assays using nuclei (124), the DNA- and nuclear-binding activities paralleled each other, and conversion of the receptor to a general polyanion-binding state was accepted as a valid qualitative assay for receptor transformation. It was clear that any biologically meaningful interactions with specific DNA sequences required for gene activation would have to be detected above this background of nonspecific binding (118, 125). In the mid-1970s, many of the major laboratories in the field of steroid hormone action began gene transfer experiments to identify the hormone response elements required for gene regulation and then performed direct binding studies with partially purified receptors and hormone response elements (for reviews, see Refs. 126–130). This highly productive emphasis on the function of the transformed receptor was accompanied by somewhat diminished interest in the mechanism of receptor transformation itself.

The polyanionic binding property of transformed *vs.* untransformed receptors greatly facilitated purification of the transformed receptors, but similar purification could not be achieved with the untransformed state because the various conditions of dilution, salt, etc., employed in conventional protein purification protocols themselves promote transformation. The Gustafsson laboratory (131) used hormone- and temperature-dependent transformation to the DNA-binding state as the initial enrichment step in a protocol that yielded highly purified transformed GR (131). This purified GR was then used in a collaboration between the laboratories of Yamamoto and Gustafsson (132) to demonstrate for the first time the specific binding *in vitro* of a receptor to a DNA fragment whose transcription is regulated by hormone *in vivo*. Importantly, the Gustafsson laboratory performed limited proteolysis studies of both transformed cytosolic GR (133, 134) and the highly purified transformed GR (135, 136)

that quite accurately defined the HBDs and DBDs of the receptor several years before receptor DNAs were cloned (see Ref. 137 for review). These limited proteolysis techniques proved to be very useful in later studies localizing the hsp90-binding region on molybdate-stabilized untransformed receptors (138, 139).

B. Artifactual transforming conditions

1. Salt transformation. Cytosolic receptors can be transformed by a variety of means. As noted above, salt causes dissociation of the non-DNA-binding, 9S complex to the DNA-binding, 4S monomer (27–29, 46, 47, 54, 55, 64–68, 91, 98, 101–103, 105, 106). In the 1970s, the heteromeric nature of the 9S complex had not been established, and it was thought by many investigators that salt promoted dissociation of a 9S receptor homotetramer to the monomer (140). It is now clear, however, that salt disrupts the binding of the receptor to hsp90 (82, 141–143) and that dissociation from hsp90 is accompanied by concomitant and proportional generation of the DNA-binding state (144). The disruption of the heterocomplex by salt indicates that ionic bonds contribute significantly to the receptor-hsp90 interaction. In contrast, binding of hsp70 to peptides and proteins in general is determined by hydrophobic residues (for review, see Ref. 145), and complexes between steroid receptors and hsp70 are not disrupted by salt (141–143).

In some early studies, salt-mediated GR transformation, like temperature-mediated transformation, was thought to be hormone-dependent (78). At that time, antireceptor antibodies had not been produced, and transformation had to be assayed by centrifuging [^{3}H]steroid-bound receptors in density gradients or by binding them to DNA or nuclei. Because salt causes hsp90 to dissociate from the GR and because hsp90-free GR cannot bind steroid (82), one cannot treat the unliganded GR with salt and then bind steroid and assay for transformation. Now that antibodies are available for detection of DNA-bound receptors by Western blotting, we know that salt treatment of unliganded receptors causes transformation to the DNA-binding state.

2. Precipitation with ammonium sulfate. DeSombre *et al.* (146) first showed that precipitation of cytosolic ER with ammonium sulfate at 25% of saturation transformed the receptor, with transformation in this case being measured by dimerization to the 5S form. Transformation by ammonium sulfate was estrogen-independent and occurred at 0 C. Similar results were obtained with chick oviduct PR (52) and rat hepatic GR (78), with transformation being assayed by nuclear binding. Transformation by ammonium sulfate is now well understood. In the absence of a stabilizing agent, such as molybdate, ammonium sulfate dissociates the receptors from hsp90 and the transformed receptor is precipitated (at about 30% of saturation), yielding significant receptor purification (147, 148). Even though precipitation with 30% ammonium sulfate eliminates the hormone-binding activity of the unliganded GR (147), it has been shown by Western blotting that the hormone-free receptor is transformed to a state that binds to DNA-cellulose (148).

3. Transformation by dilution, gel filtration, and dialysis. Dilution, gel fitration, and dialysis all transform cytosolic receptors. These procedures reduce the concentration of, or eliminate, a small heat-stable cytosolic factor that, like molybdate, stabilizes the 9S receptor complex. Dilution was first shown by Higgins *et al.* (74) to transform GRs in HTC cell cytosol, and subsequent work by Milgrom's laboratory (149) demonstrated quite clearly that dilution acted by decreasing the concentration of a cytosolic inhibitor of transformation. The inhibitor was described as a small (mol wt < 500), heat-stable molecule (149). Similarly, Litwack and his colleagues (150, 151) showed that passage of cytosol through small molecular seive columns transformed the GR by removing a low molecular weight component. This factor clearly inhibits the transformation process and not subsequent binding of the transformed receptor to DNA (149, 152). The heat-stable fraction of cytosol was also shown to inhibit conversion of the GR to the more positively charged species characteristic of the DNA-binding state (153). Both dilution and gel filtration of cytosol facilitated the transformation of progesterone receptors (154) and elimination of a low molecular weight cytosolic inhibitor by dialysis was shown to transform GRs, ERs, and ARs (155).

Two laboratories maintained an interest in purifying and characterizing this small, heat-stable, transformation inhibitor (for review, see Ref. 156). Bodine and Litwack (157, 158) purified an active factor from rat liver, and physical analysis of the active fraction suggested that the inhibitor was a novel ether aminophosphoglyceride. Because the inhibitor stabilized the untransformed state of the cytosolic receptor, it was thought to possibly modulate receptor action and was called "modulator" (151, 156). The inhibitor (modulator) was subsequently separated into two isoforms that appear to interact synergistically in stabilizing untransformed receptors in cytosol (159). The proposed ether-linked aminophosphoglycerides have not yet been synthesized and shown to be biologically active; thus, their chemical structures and activities have not been proven. The purified modulator preparation has been shown to stimulate protein kinase C activity *in vitro* (160), suggesting more general effects or perhaps multiple activities in the preparation.

While studying conditions that would prevent inactivation of glucocorticoid-binding activity in cytosol, Pratt and colleagues (161, 162) found a small heat-stable, cytosolic factor that stabilized unoccupied GRs. This ubiquitous, dialyzable, anionic factor was subsequently found to inhibit receptor transformation to the DNA-binding state (163), and it was shown by a direct technique to stabilize hsp90 binding to the GR (164). The factor was stable to ashing and bound to a metal-chelating resin, leading to the conclusion that it was a metal anion (164). The factor produced the same effects on GR physical properties and function as molybdate (164), and, when the inhibitor was extensively purified from the heat-stable components of rat liver cytosol on a column matrix with high resolving properties for metal anions, it was found to coelute with the cytosolic molybdenum (165). This purified endogenous cytosolic metal anion was shown to inhibit hsp90 dissociation from the GR (165).

Although the Litwack and Pratt groups arrived at different conclusions regarding the chemical nature of the transformation inhibitor(s), both showed that their purified inhibitor preparations produced the same effects on the cytosolic GR

as molybdate (156, 158, 164). Dilution, gel filtration, and dialysis of cytosol could facilitate receptor transformation by reducing the concentration of both aminophosphoglycerides and metal anions, and it is entirely possible that there are multiple factors of different chemical composition having molybdate-like effects on receptors. It has been found, however, that rapid passage of cytosol through the Chelex-100 metal-chelating resin also makes the GR unstable, facilitating both dissociation of hsp90 and simultaneous receptor transformation to the DNA-binding state (144). This chelation method of artifactual transformation should be selective for removal of metals, perhaps arguing that the endogenous cytosolic inhibitor of prime interest is a metal anion. In any event, both laboratories have proposed that molybdate may exert its stabilizing effects on the untransformed receptor heterocomplex by occupying the binding site of the endogenous transformation inhibitor (158, 164). As we will describe below (see *Section IV.C.2*), this binding site is probably located on hsp90, rather than on the receptor.

4. Other transforming conditions. Bailly *et al.* (81) first showed that elevating cytosolic pH promoted GR transformation. It is now known that elevation of cytosol pH from 7.2 to 8.2 promotes dissociation of the GR from hsp90 (144). One can speculate that a titration of charged amino acids involved in the receptor-hsp90 interaction may be responsible for this mode of transformation, but the mechanism for the pH effect is really not known. The polyanion heparin has been reported to dissociate 9S receptor complexes (166–168), but again the mechanism of the effect is not clear. In isolated reports, a variety of small molecules, such as nucleotides (169–171) and methylxanthines (172), as well as some enzymes, such as an alkaline phosphatase (173) and an uncharacterized endogenous transforming enzyme (174), have been reported to promote receptor transformation (for review, see Ref. 112). However, these methods have not yet proven to be helpful in deriving mechanistic models of the transformation event.

C. Models of cytosolic receptor transformation

As noted in reviews of steroid receptor transformation published in the early 1980s (110, 140), diverse mechanisms had been proposed, including a conformational change in the receptor without a change in mass (44, 79), receptor dimerization (39–41), dissociation of a receptor oligomer (167, 175–179), dissociation of macromolecular or low molecular weight inhibitors (149, 152, 155, 162), receptor dephosphorylation (162, 173), and receptor proteolysis (174). The latter two possibilities, receptor dephosphorylation and partial proteolysis, were eliminated by subsequent experiments (180–182). Given the variety of conditions for bringing about transformation and the variety of ways of assaying transformation, it is not surprising that some reviewers (110) concluded "... most likely, there is no single mechanistic explanation for all these findings, and it is probably futile to insist on one."

As early as 1976, however, Atger and Milgrom (79) had published a careful study of the kinetics and thermodynamics of GR transformation that imposed important limits to be met as mechanistic models were refined. They showed that transformation was of apparent first order and that the free energy of thermodynamic activation was much higher ($\Delta G^* = 21.3$ kcal) than expected for an enzymatic process. They noted that high positive values for enthalpy ($\Delta H^* = 31.4$ kcal) and entropy ($\Delta S^* = 34$ cal/degree) were similar to those described for protein denaturation, suggesting breakage of noncovalent bonds during transformation. These kinetic and thermodynamic data for GR transformation were consistent with either a dissociation model or a model involving a change in conformation without a change in mass.

Milgrom and his colleagues (79, 81, 111) developed the strong impression that GR transformation proceeded until there was an equilibrium between transformed and nontransformed receptors, and they concluded that transformation consisted of a simple change in conformation of a 4S receptor molecule induced by the hormone. We now know that the assumption of an equilibrium was incorrect. During the transformation process, the HBD of the steroid receptor undergoes a change in its folding state, and the process is reversed only by a complex protein-folding reaction involving hsp90, hsp70, and other proteins (*Section VIII*). Thus, the steroid does not act as an allosteric modifier of receptor structure as was proposed in a number of early papers from the Tomkins laboratory (72, 183, 184). Despite the fact that Milgrom's equilibrium model of transformation turned out to be wrong, his kinetic and thermodynamic data were entirely consistent with a model for cytosolic receptor transformation in which steroid binding promotes a temperature-dependent dissociation of a 9S receptor-hsp90 complex (109, 141, 142, 144, 148, 185, 186).

Atger and Milgrom (79) made another observation of considerable potential importance. When they examined the energy changes that accompany the binding of the hormone to the receptor and the energy changes that accompany the subsequent receptor transformation at 25 C, they found that binding of steroid to the receptor requires a moderate thermodynamic activation energy ($\Delta G^* = +10.6$ kcal), but the complex corresponds to a markedly lower level of free energy ($\Delta G = -11.3$ kcal). A high energy of activation ($\Delta G^* = +21.3$ kcal) is required for receptor transformation, but the transformed receptor is at a level of free energy similar to that of the untransformed receptor ($\Delta G = -0.24$ kcal). Thus, they concluded that the overall reaction is driven mainly by binding of hormone to the receptor, which is accompanied by a large variation in free energy. We now know that only the hsp90-bound HBD of the GR is in the high-affinity steroid binding conformation, and that an important energy barrier that must be overcome in transformation of cytosolic receptor is provided by the noncovalent bonds responsible for the protein-protein interaction between the receptor and hsp90. It is now established that hsp90 is a component of the chaperone system responsible for folding of the HBD, and it is thought that the steroid receptors are anomalous with respect to other proteins in that an intermediate state in the folding process is maintained in the form of the relatively stable 9S receptor-hsp90 complex. The receptor interaction with hsp90 can be imagined as trapping the HBD in a partially unfolded state and thereby trapping some of its inherent folding energy (187). Once it occupies the steroid-binding pocket, the steroid favors the naturally folded conformation of the

HBD, and in a sense, the hormone utilizes the trapped potential energy of spontaneous folding to convert the receptor to its active state by releasing it from hsp90.

The observations of the Milgrom laboratory (44, 78, 79, 81) and most of the other observations cited above that led to various models of transformation were made with cytosolic receptors. However, the process of receptor transformation in the intact cell is undoubtedly more complex. It is highly unlikely, for example, that unliganded receptors are diffusing in Brownian fashion through the cytoplasm or within the nuclear space as 9S particles. Thus, the 9S form is most likely a minimal or "core" unit derived from a state of the receptor that is retained in one compartment or the other by association with cell structures until it binds hormone and is transformed (188). As we will describe later, several proteins in addition to hsp90 and hsp70 are present in immunoadsorbed, untransformed steroid receptor complexes (141, 142, 188), and all of these proteins are not necessarily retained in the receptor heterocomplex during gradient centrifugation. Some 9S components, such as hsp70 and a 60-kDa stress-related protein (189), are tightly bound to receptors during the folding of the HBD, but then they can cycle out of the folded complex (190, 191). Also, the receptor heterocomplex in the cell is probably dynamic in the sense that receptors are being folded and unfolded continuously, much as simultaneous folding and unfolding occurs in the cell- free systems for receptor heterocomplex assembly with hsp90 (192). Because hormone is required for steroid receptors to occupy their response elements in the genome (108), it seems that hormone-mediated transformation must, in some way, be coupled with receptor movement to these elements. This is obviously the case for receptors that are in the cytoplasm before hormone binding, but some movement through space is likely required also for receptors that have functional nuclear localization signals (NLSs) in their hormone-free state and thus await the hormone at nuclear sites of retention.

There seems little doubt but that models of receptor transformation based on purely cytosolic observations will be simplistic. However, it is only through examining hormone-mediated dissociation of more purified receptor heterocomplexes and through studying the reversal of this transformation with purified hsp chaperone systems that we will eventually develop a correct molecular model describing how the steroid drives receptor transformation. As will be described later, in more complex systems where receptors are being continuously folded and unfolded, the binding of steroid has a second effect in that it blocks reformation of the heterocomplex because the hsp chaperone system forms a stable heterocomplex only with the unliganded HBD (193, 194). Thus, in the intact cell, binding of hormone probably drives receptor transformation much as it does in cytosolic systems, but additionally, hormone binding stabilizes the HBD in a folded state such that transformation is not readily reversed by the chaperone system (192–194).

D. Physiological relevance of receptor transformation before the discovery of hsp90 binding

In reviewing potential mechanisms of transformation before the discovery of hsp90 association with receptors, Grody *et al.* (110) considered the possibility that transformation was an *in vitro* artifact — "Finally we must consider the very real possibility that the reason no satisfactory mechanism has been found to explain transformation is that none exists *in vivo*. Transformation may be purely an *in vitro* phenomenon, brought about by the artifactual association of various proteins, enzymes, or other factors with receptors as a result of cell disruption and cytosol preparation procedures." Even now, there are laboratories that consider the 9S untransformed receptor-hsp90 complex to be such an artifact of tissue processing (195). The *in vivo* validity of the receptor-hsp90 heteroligomer will be considered later, but it is useful to review here the evidence for physiological relevance of receptor transformation that existed by the early 1980s.

Initial interest in receptor transformation was driven by the role played by hormone in temperature-dependent nuclear binding of receptors in both intact cells and cell-free systems. As early as 1972, Jensen's laboratory (196) had published evidence that transformation *in vitro* converted the ER to a form that was biochemically active when mixed with nuclei. It was thought unlikely that the hormone-dependent transformation event examined in cytosol could be irrelevant to the physiological action of the hormone in the intact cell. Importantly, Munck and Foley (197) demonstrated directly that GRs underwent the same transformation change in the intact cell as in cytosol. To do this, they used the observation that the more acidic, non-DNA-binding, untransformed receptor can be separated from the more positively charged, DNA-binding, transformed species on diethylaminoethyl (DEAE) columns eluted with a phosphate buffer gradient (123, 153). Using suspensions of rat thymocytes, Munck and Foley (197) found that a rapid shift from untransformed to transformed state occurred with a half-time of <2.5 min. After injection of [^{3}H]triamcinolone acetonide to rats, Markovic and Litwack (198) demonstrated a similar shift in DEAE elution behavior of GR recovered in hepatic cytosol, with a longer half-time (10–15 min) for transformation, probably reflecting a delay due to steroid distribution after injection.

The argument for the physiological relevance of receptor transformation was buttressed considerably when Munck's laboratory (177) exposed intact cells to glucocorticoid at 37 C and ruptured them in buffer containing sodium molybdate, which blocks any subsequent receptor transformation during cytosol preparation. They clearly showed that hormone-dependent and temperature-dependent transformation of the GR from 9S to 4S occurred under physiological conditions in the cell. Miyabe and Harrison (199) showed that transformation (assayed by DEAE chromatography) occurred in a ligand-dependent manner in intact mouse pituitary tumor cells and showed that the extent of nuclear binding was proportional to the degree of receptor transformation.

In 1983, Raaka and Samuels (176) performed a very illuminating study in which they used both a dense amino acid-labeling technique and molybdate stabilization during cell rupture to show that the GR in hormone-free GH_1 rat pituitary tumor cells is 9S and is converted in the presence of hormone to 4S cytosolic and nuclear-bound forms. Because the 4S cytosolic and nuclear forms decreased upon hormone withdrawal while the 9S form increased in a man-

ner that is not dependent upon new protein synthesis, they assumed that there was an equilibrium between the 9S and 4S forms in the cell. The observations of Raaka and Samuels (176) provided strong support for the importance of hormone-dependent receptor transformation in the intact cell, but their assumptions that 1) the 9S form is a receptor tetramer, 2) the 9S and 4S forms are in an equilibrium, and 3) the steroid acts as an allosteric modifier [a model originally proposed by Samuels and Tomkins in 1970 (183)] that shifts the equilibrium to the 4S form have proven to be incorrect.

When the steroid receptors were cloned, it was possible to directly correlate biological properties of mutant receptors with their recovery as the 9S or 4S forms. Analysis of receptors produced after transfection of hormone-free cells with mutant human GR and ER cDNAs demonstrated that steroid-inducible forms of the receptor were recovered in molybdate-stabilized cytosols entirely as 9S complexes, whereas mutant receptors with constitutive activity were recovered only in the 4S form (200–202). These observations provided strong support for the argument that the 9S, non-DNA-binding form is derived from the physiologically inactive state of the receptor that is transformed by the steroid in the cell and that the 4S, DNA-binding form is derived from receptor that is active in transcriptional enhancement.

Gorski and his colleagues (203–205) raised a direct challenge to the physiological significance of the *two-step* model of steroid hormone action that should be mentioned here. Their rejection of the model is largely based on the observation that ERs were found to lie within the nuclei of hormone-free cells (206, 207), an observation that has been repeated for several steroid receptors in several cell systems (for review, see Ref. 5). The nuclear localization of an unliganded receptor obviously eliminates the requirement for a spatial translocation of the receptor from the cytoplasm to the nucleus after transformation, but as Jensen notes (208), "where the receptor is actually located has no direct bearing on the validity of the *two-step* mechanism." As noted by Schrader (209), "there are still two identifiable states of a steroid receptor an active state and an inactive one." In that hormone binding and transformation can be differentiated as distinct and sequential processes independent of receptor translocation, the *two-step* model is retained.

On the other hand, Gorski and Hansen (205) have rejected the transformation event itself, feeling that ". . . changes in the receptor induced by warming to 25–30 C ('transformation' or second step) are probably artifacts." This rejection of receptor transformation also reflects a conclusion derived by Hansen and Gorski (210, 211) from their studies of the changes in the aqueous two-phase partitioning behavior of the ER that occur with steroid binding and/or heating *in vitro*. They concluded that the major change in partitioning behavior, and thus physical properties of the ER, took place on ligand binding and not with the heating step. A major problem with this work is that transformation was not independently assayed by conversion of receptors from 9S to 4S or by acquisition of DNA-binding activity. Indeed, it was assumed that the "unoccupied ER monomer" was untransformed, yet it has been established by others that, like other steroid receptors, the 4S ER monomer is already transformed (44, 45). However, the work of Hansen and Gorski (210, 211) brings up an important point that is still not answered, which is whether the greatest conformational change in the receptor HBD occurs with steroid binding or with the subsequent temperature-dependent transformation step.

IV. Molybdate Stabilization of Receptors

One of the features that distinguished the adrenocorticoid receptors from the sex steroid receptors was the exceptional lability of their steroid- binding activity. In many cytosol preparations, the steroid-binding activity of the unliganded GR and MR decayed rapidly, whereas the steroid-bound receptor was quite stable (70, 92, 212, 213). The decay of the unliganded GR complicated the analysis of steroid-binding kinetics (214, 215), and only modest stabilization was achieved with reagents, such as EDTA or glycerol (214, 216, 217). By 1977, two factors — the redox state and, mistakenly, the phosphorylation state of the receptor — were thought to be critical for the steroid-binding activity of the GR.

A. Stabilization of steroid-binding activity

1. GR instability. Rees and Bell (218) were the first to demonstrate that one component of GR inactivation was due to disulfide bond formation that could be prevented by reducing agents such as dithiothreitol. Granberg and Ballard (219) then showed that addition of dithiothreitol-activated glucocorticoid-binding activity in cytosols prepared from rat tissues that had low endogenous reducing capacity. A series of investigations by the Pratt laboratory identified the endogenous cytosolic reducing activity as the thiol-disulfide exchange factor thioredoxin (163, 220, 221), and it is now established that the steroid-binding activity of the GR is inactivated by redox conditions that promote intramolecular disulfide bond formation (222, 223).

A recent series of studies from Simons' laboratory has demonstrated that steroid-binding activity of the GR is inactivated by the formation of disulfide bonds between cysteine SH groups that are vicinally spaced in the HBD when it is bound to hsp90 (222, 224–226). The untransformed, unliganded GR has been cleaved with trypsin to a 16-kDa fragment of the HBD that is bound to hsp90 (139) and has steroid-binding activity (227). The 16-kDa fragment contains three cysteines, of which any two can form an intramolecular disulfide (226). This cysteine cluster appears to lie in a portion of the steroid-binding pocket that is critical for binding the D ring of the steroid (228, 229), and a variety of observations indicate that a short region of the HBD containing this thiol cluster directly contacts hsp90 in the untransformed GR heterocomplex (139, 201, 230).

We now know that there are two requirements for the GR to bind steroid: 1) the redox conditions must be such that the thiols in the cluster are not oxidized to form intramolecular disulfide bonds, and 2) the receptor must be bound to hsp90. In retrospect, we appreciate that the lability of glucocorticoid-binding activity in cytosols was due both to disulfide bond formation and hsp90 dissociation. In 1979, it was only known that two distinct processes were responsible for inactivating glucocorticoid-binding activity, one that was reversed by dithiothreitol and another that was inhibited by

molybdate (162, 231). The vicinal thiol groups are unique to the GR, and the vicinal thiol-selective agent arsenite selectively inactivates the steroid- binding activity of the GR *vs.* the MR (232). The lability of steroid-binding activity of the MR in cytosol appears to reflect its less stable interaction with hsp90. Detailed information on GR thiols and steroid binding activity is available in a recent review (233).

2. The phosphorylation hypothesis leads to the discovery of molybdate stabilization. Even in cytosols with good reducing activity, the steroid-binding activity of the unliganded GR was rapidly inactivated at room temperature, and essentially all steroid-binding experiments were performed at 0–4 C. By the early 1970s, Munck and his colleagues (62, 70, 214) had shown that the ability of intact thymocytes to bind glucocorticoids was energy-dependent, seeming to be related to cellular ATP content. Other investigators then reported that exposure of mouse fibroblasts (234), thymic lymphocytes (235), or chick embryo retina (236) to the metabolic inhibitor dinitrophenol resulted in loss of glucocorticoid-binding capacity. When the metabolic blockade was removed, steroid-binding activity returned, and the return was unaffected by inhibition of protein synthesis (214, 234, 236, 237). Wheeler *et al.* (237) confirmed the very tight association between the cellular ATP level and steroid-binding activity achieved in cytosols. These observations led to the speculation that the GR might be a phosphoprotein, with ATP promoting its phosphorylation to a steroid-binding form (70, 235). Following up on this notion, Nielsen *et al.* (238) reported that incubation of L cell cytosol with highly purified alkaline phosphatase inactivated the steroid-binding activity of the unliganded GR in a manner that was clearly related to the dephosphorylating activity of the enzyme.

This report by Nielsen *et al.* (238) really led people to think that the steroid receptors might be phosphoproteins and that steroid binding might be regulated by phosphorylation. It was subsequently shown that the steroid receptors are phosphoproteins, and the study of steroid receptor phosphorylation has become a subfield of nuclear receptor research [see Orti *et al.* (239) for review]. It is now known that the GR is not phosphorylated in the HBD and the GR phosphorylation state does not affect its steroid-binding activity. Nevertheless, in the mid 1970s, two approaches were taken to address the possibility that the lability of cytosolic glucocorticoid-binding activity was due to a dephosphorylation — one approach used phosphatase inhibitors to stabilize receptors and the other approach focused on ATP-dependent generation of steroid-binding activity.

In the first approach, Nielsen *et al.* (83, 84) looked for the presence of an enzyme activity in cells that would inactivate the steroid-binding activity of unliganded GR. The 27,000–100,000 $\times$ *g* particulate fraction of mouse L cells, rat thymocytes, and rat liver was found to contain an enzyme(s) that inactivated glucocorticoid-binding activity when mixed with cytosol containing steroid-free GR. The GR-inactivating activity was extracted from the membrane fraction, partially purified (83), and several phosphatase inhibitors were tested for their ability to inhibit receptor inactivation. Ishii and Aronow (217) had previously found that certain glucose metabolites stabilized glucocorticoid-binding activity in L cell cytosol, the most effective of these inhibitors being glucose-1-phosphate. Glucose-1-phosphate had been reported to be a phosphatase inhibitor, and Nielsen *et al.* (83, 84) found that both it and another phosphatase inhibitor, fluoride, had a moderate ability to inhibit the GR-inactivating activity extracted from their particulate fraction. Molybdate, however, produced a profound inhibition of receptor inactivation. Nielson *et al.* (83, 84) also found that the three phosphatase inhibitors inhibited GR inactivation when cytosol was incubated at elevated temperature without the addition of the inactivating activity from the particulate fraction. In L cell cytosol, for example, the half-time for inactivation of steroid-binding activity at 25 C went from 30–60 min to more than 1 day, and the half-time at 37 C went from ~2 min to 2 h when 10 mM molybdate was present (83). When this work was published in 1977, it was thought that fluoride and molybdate were inhibiting a phosphatase(s) that was inactivating the GR.

In the second approach to the phosphorylation/dephosphorylation model, Sando *et al.* (240) obtained ATP-dependent reactivation of steroid-binding activity in L cell cytosol. The GR was first inactivated by incubating L cell cytosol at 25 C, molybdate was then added to prevent further inactivation, ATP was added, and the incubation was continued to permit regeneration of steroid-binding activity. Reactivation of 40–70% of the receptors was obtained by an ATP/Mg^{2+}-dependent and temperature-dependent process. When combined with the phosphatase inhibitor data, these results led Sando *et al.* (240) to conclude that regeneration of steroid-binding activity was due to a phosphorylation process, most likely phosphorylation of the receptor polypeptide itself.

Inasmuch as this conclusion was ultimately found to be wrong, why did Sando *et al.* (240) observe ATP-dependent regeneration of steroid-binding activity? It has now been shown that L cell cytosol contains the same hsp90/hsp70-based chaperone activity as the well studied system in reticulocyte lysate that has been used to form steroid receptor·hsp90 heterocomplexes (241). When immunopurified, hsp90-free GR is incubated with GR-free L cell cytosol, receptors are reassociated with hsp90, and steroid binding is regenerated (241). It is highly likely, therefore, that Sando *et al.* (240) were generating steroid-binding activity due to ATP-dependent chaperoning of the GR by the hsp90/hsp70-based protein chaperone system.

3. Molybdate stabilization is a physical effect. Molybdate stabilization of steroid-binding activity was reported for GRs in a variety of cytosol preparations (162, 168, 231, 237, 242–245) and two other transition metal oxyanions, tungstate and vanadate, were also found to be active (237, 242). The steroid-binding activity of PRs (246–251) ERs (248, 252), ARs (245, 253, 254), MRs (255), and DRs (256) was also stabilized. Because the transition metal oxyanions stabilized receptors to inactivation by nonenzymatic means, such as exposure to salt, heparin, or ammonium sulfate (147, 168, 231, 242), it rapidly became clear that the metal oxyanions were not acting as phosphatase inhibitors.

Because fluoride was a less effective stabilizer than molybdate in the original reports (83, 84), its activity was not

widely tested, although Grody *et al.* (247) showed that fluoride inhibited thermal inactivation of the PR. Much later, Housley (257) showed that receptor stabilization by fluoride requires aluminum, which in the earlier studies (83, 84, 247) was derived from the water and glassware. The requirement for aluminum is reminiscent of fluoride effects on G protein regulation of adenylyl cyclase, where the effect is also due to aluminum fluoride (258). Like molybdate, aluminum fluoride was found to stabilize the GR to inactivation by ammonium sulfate precipitation and gel filtration as well as to thermal inactivation (257). Moreover, like the transition metal oxyanions, aluminum fluoride inhibited GR transformation (assayed by DNA binding), and it was shown by a direct method to inhibit dissociation of hsp90 from the receptor (257).

By 1981, it was clear that molybdate was not acting as a phosphatase inhibitor to stabilize steroid receptors to thermal inactivation, and we know now that molybdate and fluoride both act by stabilizing the receptor·hsp90 complex. We also know that GR phosphorylation is not required for steroid-binding activity, yet addition of alkaline phosphatase to L cell cytosol inactivates steroid-binding activity (238, 257, 259, 260). How could this be? The answer lies in the fact that redox conditions change when cytosol is incubated with alkaline phosphatase.

Housley *et al.* (259) showed that the presence of both molybdate and dithiothreitol completely stabilized the steroid-binding activity of the GR when cytosol was incubated with alkaline phosphatase. In the absence of the reducing agent, steroid-binding activity was eliminated during the incubation with enzyme. If molybdate was present during the enzyme digestion, the GR could be fully reactivated by addition of dithiothreitol, but if molybdate was not present, there was no reactivation (259). At the time the work was performed, it was proposed that a conformation of the receptor, which was determined by its phosphorylation, stabilized vicinal thiol residues to maintain the receptor in its active steroid-binding form, with dephosphorylation of the receptor promoting its oxidation (259, 260). The notion was that molybdate interacted with receptor to replace the conformational effect of the phosphate moiety(ies) (260). The valid explanation, however, is quite different.

We now know that unsupplemented cytosol (*i.e.* no added reducing agent) from L cells or rat liver has relatively stable steroid-binding activity at room temperature because it maintains a high level of NADPH and consequently of reduced thioredoxin via the NADPH-dependent thioredoxin reductase reaction (220, 221). When cytosol is incubated with alkaline phosphatase, NADPH is inactivated, thioredoxin acumulates in the oxidized form, and disulfide bond formation in the vicinal thiol cluster of the GR HBD is not reversed by thiol-disulfide exchange. Thus, the receptor is inactivated due to its oxidation. If molybdate is present during the incubation with phosphatase, the GR·hsp90 complex is preserved and the steroid-binding activity can be restored, either by addition of dithiothreitol at 0 C (259) or by a short incubation at 25 C with added NADPH to regenerate the active reduced form of thioredoxin (P. R. Housley and W. B. Pratt, unpublished observation).

During the 1970s (*i.e.* before the development of anti-receptor antibodies and site-specific affinity labels), only a limited number of approaches were available to investigators studying the biochemistry of steroid receptors, and all of the approaches required the identification of the receptor through its steroid-binding property. As discussed above, a variety of salts, enzymes, and other compounds were added to cytosols to identify conditions that would inhibit or promote receptor transformation, and a similar phenomenological approach was taken to determine what would inactivate or stabilize steroid-binding activity. Incubation of cytosols with proteases, nucleases, sialidases, phosphatases, and phospholipases were reported at one time or another to affect receptor size, transformation, or steroid-binding activity (112, 140, 260–263). In retrospect, it is quite easy to see how artifacts could lead to inappropriate conclusions regarding receptor composition and structure. The phosphatase studies are one example that led to a major advance, despite the fact that the hypothesis and conclusion were wrong. Some of these observations simply led to misunderstanding. Despite the fact that artifacts were generated and erroneous conclusions were drawn, it was becoming clear that most conditions that inactivated the steroid-binding activity of the cytosolic GR also promoted its transformation and that the two processes had a common inhibitor, molybdate (242, 264).

B. Inhibition of transformation

In 1979, Nishigori and Toft (246, 265) first reported inhibition of receptor transformation by molybdate. Both thermal and salt transformation of the cytosolic PR were inhibited by molybdate, and transformation was inhibited regardless of whether it was assayed by receptor binding to ATP-Sepharose or by shift in receptor sedimention from 9S to 4S. The availability of an effective inhibitor of transformation was of broad interest and, during the next few years, many papers were published showing inhibition of GR (147, 168, 173, 242, 245, 264, 266–270), PR (167, 175, 246, 247, 249–251, 267, 268, 271), ER (245, 252, 267, 268, 272–274), AR (245, 267, 275), MR (276), and DR (256, 277) transformation in cytosol. Molybdate inhibited receptor transformation caused by salt, heparin, or ammonium sulfate precipitation, as well as thermal transformation. Tungstate and vanadate were also active (242, 265, 271, 278). The effect of molybdate was reversible, and it was effective only when added before transformation; that is, addition of molybdate after transformation neither promoted formation of 9S receptor from the 4S form nor affected DNA binding by the transformed receptor.

1. Molybdate — the physiologically relevant artifact. The profound ability of molybdate to preserve all of the steroid receptors and the DR in similar 9S complexes of M_r 300,000–330,000 suggested that this large structure might be essential to receptor function (268). Because molybdate inhibition of GR transformation in cytosol was correlated with maintenance of the 9S form of the receptor and transformation was accompanied by generation of 4S receptor, several investigators proposed that receptor transformation represents the dissociation of an oligomeric protein (176–179). At that time, it was not known whether the molybdate-stabilized species was a receptor tetramer (140) or a receptor heterocomplex.

Raaka *et al.* (279) directly tested the transformation-inhibiting effect of molybdate added to intact cells. Cells treated with molybdate had more of the 9S form and less of the 4S form after glucocorticoid treatment than control cells. Treatment of the intact cells with molybdate also reduced nuclear accumulation of GR and ER after steroid treatment. This study on molybdate effects in intact cells supported the proposal (see *Section III.D*) that molybdate inhibited a steroid-dependent change in receptor state that was related to hormone action.

2. Physical properties of molybdate-stabilized, untransformed receptors. Molybdate was shown to stabilize steroid receptors against dissociation during chromatrographic procedures of long duration (147, 269, 273, 280), and this stabilization provided a much clearer definition of the Stokes' radii and sedimentation coefficients and consequently more accurate calculation of the molecular weights of the complexes (102, 103, 140, 147, 175, 178, 179, 268, 273, 276, 277, 280, 281). Much was learned from the effect of molybdate on GR behavior during DEAE-cellulose chromatography. When cytosol containing untransformed GR is adsorbed to a DEAE-cellulose column and eluted with phosphate buffer, most of the receptor is eluted at about 0.25 M salt, which is characteristic of the untransformed state of the receptor (153, 282). In contrast, elution with KCl in a Tris buffer yields the more positively charged state characteristic of the transformed receptor, which elutes at much lower salt (123). The presence of molybdate in both the loading buffer and eluting buffer completely prevented transformation during the running of the KCl gradient, with the GR eluting from DEAE-cellulose in a well defined high salt peak that yielded a 33-fold purification of the untransformed receptor (147). It was later shown that the molybdate-stabilized, untransformed GR coelutes with hsp90 (148). hsp90 is quite acidic and when molybdate is present to stabilize the GR·hsp90 complex the charge of the hsp90 dominates and determines the overall behavior of the complex (148). When cytosol is submitted to ammonium sulfate fractionation in the presence of molybdate, the GR is precipitated out in the 45–55% range of saturation, whereas the bulk of the hsp90 is precipitated at 50–70% and transformed receptor at 20–30% of saturation (147, 148). Thus, the salting-out properties of the molybdate-stabilized, untransformed GR are more like those of hsp90 than those of the GR polypeptide, but they are not the same as the salting-out properties of hsp90.

C. Mechanism of molybdate stabilization

1. The thiol model. Little is known about how molybdate stabilizes receptor·hsp90 complexes or even the ligand(s) with which it interacts. In 1979, it was proposed that molybdate may stabilize through an interaction with thiol groups on the receptor (242, 265). Molybdate has a well known avidity for sulfur (283), and it has been shown to interact with the thiol group of cysteine (284). Molybdate has also been shown to direct the peroxide-dependent oxidation of SH groups in a variety of proteins including hsp90 and the GR (285). This thiol-specific molybdate effect shows that the metal oxyanion interacts with SH groups in the receptor heterocomplex. It has also been shown that peroxide, by promoting formation of an intramolecular disulfide bond, produces the same structural and functional effects on the GR as molybdate, vanadate, and tungstate (223, 286), suggesting that molybdate bridging with sulfur somewhere in the receptor·hsp90 complex could lead to stabilization. However, the observation that stabilization of the GR by molybdate, vanadate, or tungstate is not blocked by preincubation of receptors with enough methyl methanethiosulfonate to derivatize all of the thiols (223, 287) argues strongly against a proposal that interactions of the metal oxyanions with sulfur moieties in the receptor heterocomplex are relevant to receptor stabilization.

2. Molybdate affects hsp90. In that molybdate stabilizes complexes of hsp90 with nonreceptor proteins, it seems highly likely that the site of molybdate interaction with the complexes lies in the common hsp90 component rather than in the protein that is associated with it. For example, molybdate stabilizes complexes between hsp90 and the protein kinases $pp60^{src}$ (288, 289) and Raf (290). In the case of the $pp60^{src}$·hsp90 complex, it has been shown that vanadate, tungstate, and an endogenous cytosolic metal anion stabilize as well (289). Passage of cytosol through a metal chelating matrix markedly weakens the binding of hsp90 to the GR or to $pp60^{src}$ (144, 164, 289). This suggests that the endogenous metal anion, which copurifies with cytosolic molybdenum (165), normally stabilizes both complexes. As noted above (*Section III.B.3*), Meshinchi *et al.* (164) have suggested that molybdate and the other transition metal oxyanions bind the heterocomplex at the same site as the endogenous metal anion.

3. Molybdate may interact with an ATP site on hsp90. It has been reported that hsp90 binds azido-ATP (291, 292), and circular dichroism analysis indicates that ATP induces an increase in the β-pleated sheet content of hsp90 (293). Vanadate and molybdate induced a similar change in the conformation of hsp90 (293). Whether or not hsp90 possesses an ATPase activity or phosphorylates itself is controversial (294), and comparison of hsp90 sequences did not reveal conserved ATP-binding motifs (295). In a careful study with highly purified hsp90, Jacob *et al.* (295) were unable to demonstrate either binding to ATP-agarose or cross-linking with azido-ATP.

Given the observation of Housley (257) that aluminum fluoride produces the same stabilizing effects on the heterocomplex as molybdate, it is possible that aluminum fluoride and the transition metal oxyanions affect hsp90 at the same site and in the same manner. These agents both resemble phosphate and interact with phosphate-binding sites in proteins (296); hence their activity as phosphatase and ATPase inhibitors. *Despite the failure of careful analysis to demonstrate an ATP-binding site (295), we still feel the most reasonable proposal is that fluoride, acting as AlF_4^-, and the transition metal oxyanions, molybdate, vanadate, and tungstate, stabilize steroid receptor·sp90 complexes by binding to an ATP site on hsp90, stabilizing a conformation of hsp90 with increased affinity for the receptor.*

It is interesting to return here to a review of steroid re-

ceptor transformation and subunit structure published in 1982 (110), where at the end of a section on molybdate inhibition of transformation, it is said that "It can thus be seen from this discussion that the interaction of the transition metals with receptors has not been very helpful in elucidating the nature of transformation. Since their mechanism of inhibition of transformation is no better understood than transformation itself, they can be used to support almost any argument but only by circular reasoning." It is reasonable to predict that, with time, will come an understanding of both the mechanism of receptor transformation and the mechanism of the molybdate effect.

V. Purification of Untransformed Receptors Leads to hsp90

Because molybdate stabilizes steroid receptors in their untransformed state during protein separation procedures that normally cause transformation (*Section IV.B.2*), several laboratories were able to achieve high purification of untransformed PRs and GRs. In all cases, the most prominent Coomassie blue-stained protein in the purified preparation was a 90-kDa protein that was identified as hsp90.

A. The untransformed receptor is a heterocomplex

1. PR. Because estrogen treatment induces a very high level of progesterone-binding activity in the chicken oviduct, it was a magnificent system for receptor purification. Purification and characterization of the molybdate-stabilized, untransformed PR were achieved simultaneously in the laboratories of Toft and of Renoir and Baulieu.

In 1972, the transformed PR, comprised of two steroid-binding components designated A and B (297), had been partially purified from chicken oviduct. The A subunit was found to bind DNA, and the B subunit was thought to interact with chromatin protein (298). Both the A subunit and B subunit were purified to apparent homogeneity and shown to migrate under denaturing conditions at ~79 kDa and ~115 kDa, respectively (299–301). It is now known that both the A and the B subunits are transcribed from the same gene as a result of either alternate sites of translation initiation from a single mRNA (302) or from translation of different mRNAs arising from alternate splicing mechanisms (303). With isoforms of markedly different molecular masses, the chicken PR differs from other steroid receptors and even from the PR itself in some other species. In the early 1980s, however, the unique isoform composition of the chicken PR was important because it led to apparently conflicting structural data when the molybdate-stabilized, untransformed PR was purified.

Several resins for affinity chromatography were developed at this time, and Renoir *et al.* (304) employed affinity chromatography followed by DEAE-cellulose chromatography to achieve ~7,000-fold purification of the 9S PR from chicken oviduct cytosol. Analysis by denaturing gel electrophoresis revealed a single band migrating at M_r ~85,000 (later referred to as ~90,000). Dougherty and Toft (305) were able to distinguish two forms of the molybdate-stabilized chicken PR by elution from DEAE-cellulose: both forms sedimented at 9S but were eluted slightly differently on gel filtration, with dissociation of the larger form (component II) yielding only the B isoform and dissociation of the smaller form yielding only the A isoform of the PR. Using a deoxycorticosterone-agarose affinity resin (306), Toft's laboratory purified the molybdate-stabilized chicken PR ~6,000-fold by ammonium sulfate precipitation, affinity chromatography, and gel filtration (307). The highly purified PR sedimented at 9S and could be transformed to the 4S DNA-binding form upon removal of molybdate (308). The purified 9S receptor also resolved into two components by DEAE-cellulose chromatrography, with both components containing a single major Coomassie blue-stained polypeptide of M_r ~90,000 and an additional band of M_r ~104,000 being recovered with component II (307).

In 1982, it was thought unlikely that the major ~90-kDa polypeptide recovered by Renoir *et al.* (304) and Puri *et al.* (307) in their affinity-purified, molybdate-stabilized PR preparations was related to the ~79-kDa A and ~115-kDa B isoforms purified previously by the O'Malley laboratory (299–301). The puzzle was resolved in 1984 when the two forms of the highly purified 9S PR were shown to contain a common non-steroid-binding 90-kDa component and separate ~79-kDa and ~110-kDa steroid-binding components that were identified by site-specific cross-linking with steroid affinity ligands (309, 310). Dougherty *et al.* (311) purified the avian PR from oviduct tissue minces incubated with [^{32}P]orthophosphate and showed that the 90-kDa polypeptide was highly phosphorylated on serine residues, as was the ~110-kDa B isoform of the PR. Subsequently the A isoform was also shown to be phosphorylated (309). The 1982 paper by Dougherty *et al.* (311) stands as the first direct demonstration of phosphorylation of any hormone receptor (although in this study, hsp90 was mistakenly identified to also be a form of the PR).

The heteromeric nature of the nontransformed PR was not readily accepted. For example, Birnbaumer *et al.* (312) also showed that the 90-kDa polypeptide was not labeled by a progesterone affinity ligand, but it was their impression that the elution of the 90 kDa protein on affinity chromatography was not biospecific for progesterone. Thus, Birnbaumer *et al.* (312) concluded that the 90-kDa protein was present as an artifact of tissue homogenization and the use of particular steroid affinity resins. However, in purifying two forms of the 9S rabbit uterine PR, Renoir *et al.* (313) clearly demonstrated that the 90-kDa protein was eluted from the affinity resin in a progesterone-specific manner, and several laboratories subsequently demonstrated that the 90-kDa protein was associated with steroid receptors in intact cells (see *Section VI.H*).

2. GR. Several laboratories used the technique of affinity chromatography to purify the GR before the advent of molybdate stabilization of the untransformed state (314–317). Because the GR must be bound to hsp90 to have steroid-binding activity, it is only the untransformed GR that is retained by an affinity resin and subsequently eluted with [^{3}H]steroid. Given what we now know about the stability of the GR·hsp90 complex, further purification of the affinity column eluate by gel filtration or chromatography on DEAE-

cellulose should dissociate the complex in the absence of molybdate. Nevertheless, Failla *et al.* (314) reported a two-step, 2,000-fold purification of the unstabilized rat GR from HTC cells by sequential affinity chromatography and gel filtration, with the purified product sedimenting in a glycerol gradient as a single 9S peak. In that the purified GR was 9S and underwent temperature-dependent transformation to the DNA-binding state, the authors must have purified the GR·hsp90 complex, but no direct characterization of the purified product, such as gel electrophoresis, was provided to permit evaluation. A two-step, 7,000-fold purification of the unstabilized rat liver GR by Lustenberger *et al.* (317) had to have yielded the GR·hsp90 complex because the last step was steroid-mediated elution from the affinity resin, but again, no direct analysis of the product was provided.

The first affinity purification of the molybdate-stabilized GR was reported by Housley and Pratt in 1983 (318), with the receptor being purified from mouse L cells incubated with [^{32}P]orthophosphate. A major phosphoprotein of M_r ~90,000 and a minor phosphoprotein of M_r ~100,000 were both recovered in a stereospecific manner from the affinity resin, and neither protein was recovered upon affinity chromatography of cytosol from glucocorticoid-resistant L cells possessing only 5% of the steroid binding capacity of sensitive cells (318). Thus, as described above for the PR, it was thought that the 90 kDa protein was the major steroid-binding polypeptide. However, it was subsequently shown that only the 100-kDa phosphoprotein was labeled with the glucocorticoid-binding, site-specific affinity ligand [^{3}H]dexamethasone mesylate and that the 90 kDa non-steroid-binding component of the complex was immunologically distinct from the 100 kDa murine GR (319).

Grandics *et al.* (320) and Idziorek *et al.* (321) used affinity resins to achieve a three-step purification of the molybdate-stabilized GR from rat liver cytosol, with the extent of purification calculated by specific radioactivity of bound steroid being 4,000-fold and 11,800-fold, respectively. In both cases, the product was a 9S receptor that underwent transformation to the DNA-binding state upon removal of molybdate and incubation at 25 C. The major purified species migrated at 90 kDa on denaturing gel electrophoresis with varying recoveries of 72- to 78-kDa and 40- to 48-kDa polypeptides. These lower M_r species are produced from the full-length rat GR by endogenous proteases in liver cytosol (179, 223, 322), and they can be produced under controlled conditions by digestion with exogenous trypsin or chymotrypsin (135, 323). Stabilization with molybdate renders the rat liver cytosolic GR partially resistant to such endogenous proteolysis, but even with molybdate present at each step, significant fragmentation occurs (223, 322). Idziorek *et al.* (321) calculated that their 90-kDa band contained a substantial amount of non-steroid-binding protein, but it was some time before the Schmidt/Litwack group (320) recognized that most of the 90-kDa band purified by steroid affinity chromatography of rat liver cytosol was hsp90 (324). This is very understandable because the intact rat GR migrates in denaturing gels at ~94 kDa, essentially comigrating with a broad band of hsp90. In addition, the GR stains very poorly with Coomassie blue, whereas hsp90 stains very well and is the dominant biospecific band when affinity eluates are analyzed on denaturing gels (188, 324).

B. Antibodies against the 90-kDa protein

The development of monoclonal antibodies against the 90-kDa receptor-associated protein permitted rapid expansion of the heterocomplex model and identification of hsp90. The development of the BF4 rat monoclonal antibody by Radanyi *et al.* (325) permitted the Baulieu group to demonstrate that binding to hsp90 was a common property of steroid receptors in general (326, 327).

The first antibodies against the highly purified, molybdate-stabilized chicken PR were raised in goat and rabbit by Renoir *et al.* (328) in 1982. These antisera reacted with the 4S as well as the 9S PR, and the goat antiserum was shown to react with the purified A and B isoforms of the chicken PR (329). However, this goat antiserum also reacted with 9S PR of mouse, rabbit, and human cells (328) as well as with the 9S chicken GR (330), suggesting the presence of a common antigenic determinant among these untransformed receptors. This goat antiserum undoubtedly had one or more IgGs directed against hsp90. The highly purified 9S chicken PR was then used to prepare the rat monoclonal antibody BF4 (325), which did not interact with the 4S PR but did react with 9S PR, ER, AR, and GR in chicken oviduct cytosol (326, 327). The common component of 9S receptors recognized by BF4 was shown by immunoblotting to be a 90-kDa protein. These reports by Baulieu and his colleagues (326, 327) were very important because they were the first to present a general model in which the untransformed state of all steroid receptors consisted of a steroid-binding protein being associated with a common non-steroid-binding 90-kDa protein.

Work with monoclonal antibodies prepared by the Toft laboratory rapidly confirmed the general model of untransformed receptor heteromers and provided key information on the properties of the common 90-kDa unit (107, 331). The 90-kDa protein was purified by a heparin-agarose procedure in which it is coadsorbed when other proteins bind to heparin in the presence of molybdate. Subsequent washing of the resin with molybdate-free buffer selectively releases the 90-kDa protein. Sullivan *et al.* (331) found that two monoclonal antibodies that they prepared against the chicken oviduct 90-kDa protein interacted with 9S PR, AR, and GR in chicken oviduct cytosol. As reported by Baulieu *et al.* (326) for the BF4 antibody, the 4F3 antibody of Sullivan *et al.* (331) reacted with 90-kDa proteins in a variety of chicken tissues, such as oviduct, brain, liver, and skeletal muscle, but not in serum. Both the BF4 and the 4F3 antibodies were specific for avian 90-kDa proteins, but the AC88 mouse monoclonal antibody prepared by Riehl *et al.* (107) against heparin-agarose-purified 90 kDa protein of the aquatic fungus *Achlya ambisexualis* (see *Section II.G*) reacted with 90-kDa proteins in a wide range of tissues from both avian and mammalian sources. Unlike the BF4 and 4F3 antibodies, the AC88 antibody reacts only with the free 90-kDa protein and not with the 9S receptor heterocomplex (107), but nevertheless, its ability to react with mammalian 90-kDa proteins made it a very useful antibody for

Western blotting in subsequent studies of receptor heterocomplexes.

C. The 90-kDa receptor-associated protein is hsp90

By 1985, several facts regarding the 90-kDa receptor-associated protein had been established. 1) Its wide distribution in vertebrate tissues and its presence in an aquatic fungus, birds, and mammals (107) suggested that it was an ubiquitous protein. 2) It was located predominantly in the cytoplasm by immunohistochemical analysis (332). 3) It was abundant (107). 4) It was an acidic protein phosphorylated on serine residues (311, 318). Because hsp90 is an ubiquitous and abundant cytoplasmic phosphoprotein, it was reasonable to test the possibility that the steroid receptor-associated 90-kDa protein was hsp90.

Three papers published in late 1985 established that the receptor-associated protein was hsp90 (333–335). Catelli *et al.* (333) showed that the BF4 antibody against the chicken PR-associated 90-kDa protein reacted with a [^{35}S]methionine-labeled 90 kDa protein of heat-stressed chick embryo fibroblasts. It was also established that the 90-kDa protein in the 9S chicken PR comigrated on two-dimensional gel electrophoresis with mammalian hsp90, the physical properties of which were previously described by Welch and Feramisco (336), and the two proteins yielded the same peptide map. Schuh *et al.* (334) showed that antibodies directed against the 90 kDa protein that was coadsorbed with the oncogenic tyrosine kinase pp60$^{v\text{-}src}$ and identified as hsp90 (337) recognized the chicken PR-associated 90-kDa protein and shifted the 9S PR on gradient centrifugation. Similarly, antibodies against the PR-associated 90-kDa protein recognized the pp60$^{v\text{-}src}$-associated p90. Also, the 90-kDa proteins immunoprecipitated with the two sets of antibodies generated the same peptide maps.

Sanchez *et al.* (335) showed that the 90-kDa protein coimmunoadsorbed with the mouse GR from L cell cytosol reacted with a rabbit antiserum prepared by Kelley and Schlesinger (338) against purified chicken hsp90. This anti-hsp90 serum immunoadsorbed the 9S GR (339), and using a coimmunoadsorption assay, it was shown that hsp90 dissociated from the GR when cytosolic receptors were transformed, with dissociation being prevented by molybdate (335). Importantly, Mendel *et al.* (186) showed that hsp90 dissociated when steroid-bound GRs were transformed in intact cells.

Subsequent to these studies with the PR and GR, hsp90 was demonstrated by direct techniques to be present in untransformed ER (340), AR (341), MR (91), and DR (342, 343) complexes. In contrast, it was shown that hsp90 is not associated with newly translated thyroid hormone (344) or retinoic acid (345) receptors. Although, as of this writing, more than 30 additional members of the nuclear receptor superfamily are known to exist (346), it has not been determined whether any of them are associated with hsp90.

Despite the identification of the 90-kDa component of the 9S receptor complex as hsp90, in 1986, many (probably most) investigators quite appropriately felt that no biological significance for receptor·hsp90 interaction had been demonstrated, and it remained to be established whether or not these oligomeric proteins are artifacts of cell homogenization (347). The following section reviews details of the binding of hsp90 to receptors and the evidence for the physiological role of hsp90 in receptor function.

VI. Role of hsp90 in Receptor Function

The hsp90 family is a group of highly conserved stress proteins found ubiquitously in eukaryotes (see Refs. 348 and 349 for review). hsp90 Is the most abundant constitutive hsp in eukaryotic cells, accounting for 1–2% of cytosolic protein. Although it is essential in yeast and *Drosophila* (349), its exact function(s) in the cell has not been defined (350). Only recently has hsp90 been clearly shown to function under cell-free conditions as a molecular chaperone that facilitates the folding of various proteins (see Ref. 294 for review).

A. General properties of hsp90

In mammalian cells, two genes encode hsp90, with the human hsp90α having 86% amino acid homology with hsp90β (351). There is extensive homology with lower species; the human hsp90β, for example, is 78% homologous with the *Drosophila* 83-kDa hsp and 61% homologous with yeast hsp90 (352). The hsp90 homolog in *Escherichia coli* is a 63-kDa protein with a predicted amino acid sequence that is 42% identical to human hsp90 (353). hsp90 Purifies under nondenaturing conditions as a 165,000–180,000 dalton dimer (336), and under certain conditions, it can form oligomers of four or more units (354). In the mouse, there is a clear difference in the migration of the two gene products on denaturing gels, and the α and β forms are often called hsp86 and hsp84, with hsp84 being somewhat more abundant than hsp86 in several cultured cell types (188, 355). Using antibodies specific for hsp86 and hsp84, respectively, Perdew *et al.* (355) have shown by coimmunoadsorption that hsp86·hsp84 heterodimers exist as native complexes. Despite the fact that the Yahara laboratory has detected only $\alpha\cdot\alpha$ and $\beta\cdot\beta$ homodimers (356, 357), the data of Perdew *et al.* (355) make it clear that native heterodimers exist, although homodimers may be the dominant dimer form. Minami *et al.* (357) have shown that the C-terminal region of hsp90α is required for both homodimer formation and rescue of a lethal double hsp90 mutant of yeast, an observation consistent with the notion that hsp90 dimerization is required for function *in vivo*. In addition to being constitutively present at a high level in unstressed cells, hsp90 is heavily phosphorylated in the absence of stress and it migrates as several species on two-dimensional gels (358). It is not known how phosphorylation affects hsp90 function.

Whether or not hsp90 has an enzyme activity is somewhat controversial (294). Purified hsp90s have been reported to have ATPase activity and to undergo autophosphorylation (291, 359, 360). However, hsp90 chaperone function in cell-free assays does not require ATP (361–363) and neither recombinant human nor recombinant yeast hsp90s were found to have significant ATPase activity (364). It is known that kinases, in particular casein kinase II (365, 366), bind to hsp90 and are present in purified preparations from eukaryotes,

and it is possible that the ATPase activity of purified hsp90s is due to such contaminating enzymes (295, 364).

In unstressed cells, hsp90 is localized predominantly to the cytoplasm (332, 355, 367–372), but some hsp90 is also present in the nucleus (332, 355, 370–372). Under conditions of primary stress (372) or restress (369), hsp90 moves from the cytoplasm into the nucleus, a redistribution that is seen in some cell types but not in others (355, 370). The presence of some hsp90 in nuclei of unstressed cells is important because it has been argued that there is no nuclear hsp90 and that the PR and hsp90 cannot exist in a complex with each other in nuclei (195, 373). Some of the steroid receptors (*e.g.* PR, ER) have NLSs that are functional in their untransformed state, and they are almost entirely localized to the nuclei of hormone-free cells (206, 207, 332, 374–375). It has been shown by both cross-linking of PR in nuclear extracts (376) and by cross-linking of ER in intact cells (377) that these nuclear receptors are in heterocomplex with hsp90 in the absence of an appropriate steroid agonist. It is not known whether hsp90 performs a chaperone function in the nucleus or whether the low levels that are present reflect hsp90 that has been brought into the nucleus with other molecules, such as steroid receptors. In most studies, the hsp90 immunoreactivity in the cytoplasm has been diffusely distributed (332, 355, 367–370, 372). However, there are also reports of hsp90 localization to cytoskeleton, including actin in membrane ruffles (378), microtubules (379–382), and intermediate filaments (381, 382). The colocalization of hsp90 immunofluorescence with cytoskeleton is dependent upon both the method of cell fixation and the antibody (382). In the absence of redistribution artifacts during fixation, these reports may reflect the visualization of subpopulations of hsp90 involved in chaperoning assembly of these complex cytoskeletal structures. Alternatively, it has been proposed that cytoskeleton-associated hsp90 may reflect potential protein targeting and trafficking functions of hsp90 and its associated proteins (7, 382).

B. Stoichiometry of receptor·hsp90 complex

The first estimate of receptor·hsp90 stoichiometry was made in 1984 by Renoir *et al.* (310), who correctly deduced that the glutaraldehyde cross-linked 9S chicken PR contained one molecule of steroid-binding protein associated with two molecules of the non-steroid-binding 90-kDa protein. Using quite different approaches, Gehring and Arndt (383) and Okret *et al.* (384) deduced that the molybdate-stabilized, untransformed GR was a heteromeric complex containing one molecule of steroid-binding protein, with other components being unidentified. Denis *et al.* (385) and Radanyi *et al.* (386) subsequently determined that the 90-kDa protein released from the molybdate-stabilized GR and PR, respectively, behaved as a dimer. Lefebvre *et al.* (387) also concluded that the GR heterocomplex contained two molecules of hsp90. An asymmetric heterocomplex was suggested on the basis that native dimeric hsp90 possesses two accessible epitopes for the AC88 antibody, whereas only one epitope remains accessible in the untransformed GR (387). Using lower concentrations of antibody, others have found no reaction of AC88 with hsp90 that is in heterocomplex with receptors (107, 339). To date, the notion that the receptors are in an asymmetric complex with the hsp90 dimer has not been confirmed by other techniques, but an asymmetric complex is consistent with a model of receptor heterocomplexes derived by Gehring (388) from cross-linking studies.

Chemical cross-linking has been a particularly useful technique for defining both the composition and the stoichiometry of untransformed receptor heterocomplexes (see Ref. 388 for review). Bifunctional reagents were used to stabilize high molecular weight forms of PR (389, 390), GR (391–395), and ER (377). After cross-linking of both untransformed receptors in cytosol and steroid-free receptors in intact cells, species containing one molecule of receptor and two molecules of hsp90 were recovered. From a careful analysis of GRs submitted to progressively more extensive cross-linking, Rexin *et al.* (391) deduced the existence of another protein of ~50 kDa, proposing a heterotetrameric structure of one steroid-binding protein, two hsp90s, and one 50-kDa polypeptide. The 50-kDa protein is now known to be an immunophilin (see *Section VII.C.1*). Importantly, this heterotetrameric structure has been demonstrated in intact cells (377, 391, 394, 395), and the same structures have been demonstrated for GR, PR, and ER (376, 377, 390–395). This heterotetrameric complex is thought to be the core receptor structure that receives the hormonal signal in the cell.

There are limitations to determining stoichiometry by cross-linking. The components to be cross-linked must contain the appropriate functional moieties lying within a specific distance from each other in the heteroprotein complex. Components that do not meet this condition will be missed, and this may have happened with other receptor-associated proteins that will be discussed below. Also, it is not possible to distinguish the two hsp90 gene products in the cross-linked heterocomplex. A stoichiometry of two molecules of hsp90 to one molecule of GR was determined in a different way by Mendel and Orti (396), who immunoadsorbed receptor·hsp90 complexes from cells that were labeled to steady state with [^{35}S]methionine and calculated molecular ratios from the known methionine content of each protein. With this technique, they also determined that there was no preferential binding of hsp90α or hsp90β in the receptor heterocomplex. Their data are consistent with the immunopurified receptor being bound to homodimers of both hsp90α and hsp90β or to both homodimers and heterodimers. The data of Mendel and Orti (396) do eliminate the possibility that the GR binds only heterodimers.

Bresnick *et al.* (188) immunopurified GR heterocomplexes from [^{35}S]methionine-labeled L cells and washed the immune complexes under both mild and stringent conditions in the presence of molybdate. The ratio of hsp90 to GR under stringent washing conditions was 2:1, but under mild washing conditions the ratio was ~4:1. The higher ratio seen with mild washing may reflect the presence of higher oligomeric species of hsp90 noted by others (354, 356). In addition to two molecules of hsp90, the core [^{35}S]methionine-labeled GR heterocomplex contained a ~55-kDa protein with several isomorphs (188), which is now known to be the immunophilin FKBP52 (397).

A stoichiometry of two hsp90s to one steroid-binding protein is consistent with a model in which receptor dimeriza-

tion occurs subsequent to dissociation of the heterocomplex. From hydrodynamic analysis of complexes formed between the purified, molybdate-stabilized 9S ER and antibodies directed against either the receptor or hsp90, Redeuilh *et al.* (340) concluded that two steroid-binding subunits were associated with two molecules of hsp90. This stoichiometry differs from the stoichiometry of one molecule of ER and two molecules of hsp90 determined by Segnitz and Gehring (377) using the cross-linking method. Given that the HBD of the ER contains both its very strong dimerization site (43) as well as its hsp90-binding region, it is attractive to consider that the dimerization site becomes unmasked upon hsp90 dissociation. In the absence of compelling evidence to the contrary, an economy of logic would suggest a common heterocomplex core unit with the same stoichiometry for all of the steroid receptors (388). The presence of one steroid-binding subunit in the receptor heterocomplex requires that dimerization occurs after receptor dissociation from hsp90. Indeed, this principle had been established (see *Section II.A*) in the early 1980s when it was shown that cytosolic ER complexes were transformed to a DNA-binding form before receptor dimerization (44, 45).

C. hsp90 Binds to the receptor HBD

1. Studies with the GR. It is clear that hsp90 interacts with the HBD of the GR. In 1985, Gehring's laboratory reported that the nt^i-variant of the murine GR forms a large molybdate-stabilized complex (383). The nt^i-variant is a 40-kDa segment of the GR lacking its amino-terminal half but containing the DBD and HBD (398, 399). Pratt *et al.* (200) subsequently showed that transfected human GR deleted for the HBD does not form the 9S heterocomplex, consistent with the HBD being necessary for hsp90 binding. Denis *et al.* (138) cleaved the molybdate-stabilized rat liver GR with trypsin and isolated a 27-kDa fragment that contained the HBD and was bound to hsp90. It was subsequently shown that the GR HBD alone was sufficient to confer the property of tight hsp90 binding onto chimeric proteins (400) and that the GR HBD expressed in bacteria could be bound to rabbit hsp90 when it was incubated with rabbit reticulocyte lysate (401), which contains a receptor·hsp90 heterocomplex assembly system.

No clearly demarcated region within the GR HBD is responsible for hsp90 binding. Also, no physical properties of the HBD have been defined that even contribute to an explanation for why there is a high-affinity interaction of hsp90 with this domain and not with many other proteins. Several approaches have been used to define a minimal region of the HBD required to generate a complex with hsp90 upon transfection of mutant GR cDNAs or upon *in vitro* receptor translation in reticulocyte lysate.

Dalman *et al.* (230) translated GR-containing C-terminal deletions of various lengths and immunoadsorbed the newly translated, [^{35}S]methionine-labeled receptors with a monoclonal antibody against hsp90. A minimal region from amino acids 604–659 (mouse GR) was required for a high yield of high-affinity hsp90 binding. Using the same approach, Howard *et al.* (402) found that the region 556–604 (mouse GR) was sufficient to yield some hsp90 binding. Taken together, the two reports are consistent with the assignment of an approximately 100-amino acid region (556–659) that forms a minimal high-affinity hsp90 binding site by this technique. It is clear that a region of the HBD that lies C-terminal to this minimal site is also involved in hsp90 binding. Cadepond *et al.* (201) divided the HBD of the GR into three subregions of roughly equal length and showed that each region was sufficient to confer hsp90 binding onto a receptor fragment as determined by 9S complex formation in transfected cells. Two of these subregions sufficient for hsp90 binding contain major portions of the 100-amino acid minimal hsp90 binding site, but the third subregion lies completely outside of it, encompassing the C-terminal one-third of the HBD.

Simons and his co-workers cleaved the steroid-free, molybdate-stabilized GR with trypsin until they defined a 16-kDa core fragment of the HBD (525–661, mouse) that bound dexamethasone with a 23-fold lower affinity than the intact GR but retained the same specificity of binding as the wild-type receptor (227). This 16-kDa core steroid-binding fragment of the HBD is bound to hsp90 (139). It is important to realize that transfection of cells with cDNA encoding the core fragment does not yield a steroid-binding peptide (233), and to date, the fragment has been derived only by tryptic digestion of intact, steroid-free GR. It is well established that amino acids lying to the C-terminal side of this core steroid-binding fragment are required for forming the complete steroid-binding pocket when the HBD assumes its native tertiary conformation (see Ref. 403 for review).

Although 'minimal' hsp90-binding segments of the GR HBD have been defined, the results of Cadepond *et al.* (201) suggest that nearly the whole HBD is involved in GR association with hsp90. It will be very difficult (perhaps impossible) to define specific functional groups involved in binding, and it will also be difficult to define the exact regions of the HBD that contact hsp90. This is a general problem that relates to the ability of chaperones such as hsp70 and hsp90 to bind to a variety of proteins of widely different structure.

The steroid hormone receptors contain a highly conserved sequence of 20 amino acids (583–602, mouse GR) lying within the region of the HBD containing the minimal hsp90-binding segment of Howard *et al.* (402) and the 16-kDa 'core' steroid-binding fragment of Simons *et al.* (227). Danielsen *et al.* (404) originally speculated that this conserved region might be the site of hsp90 interaction. A mutant mouse GR (Δ574–632) deleted for this region is bound to hsp90, but the binding is less stable than that of wild-type receptor (405), suggesting that the region may define a structure of some importance in hsp90 binding. A peptide spanning 80% of the conserved sequence was found to inhibit GR·hsp90 heterocomplex assembly by the reticulocyte lysate system (230), suggesting that the peptide directly interacts with hsp90 and/or other components of the heterocomplex assembly machinery. However, this 20-amino acid region is conserved in both thyroid hormone and retinoic acid receptors, neither of which form tight complexes with hsp90 (344, 345); thus, the conserved region itself is not sufficient for detectible hsp90 binding. Because of its conservation, this sequence obviously defines a structural feature of importance for the action of steroid receptors in general, and it lies within or immediately adjacent to the steroid-binding pocket. For ex-

ample, Met610, which lies immediately to the C-terminal side of the conserved sequence, becomes covalently bound to the A ring of triamcinolone acetonide upon UV irradiation of the hormone-bound receptor (406). The fact that the 20-amino acid conserved sequence also either contacts hsp90 or lies immediately adjacent to hsp90 in the untransformed receptor heterocomplex is likely to be of considerable importance in ultimately understanding the mechanism of steroid-mediated receptor transformation.

2. Studies with the PR. Carson-Jurica *et al.* (407) transfected COS cells with mutant cDNAs for the chicken PR and found that it was necessary to delete the entire HBD of the A isoform before hsp90 binding (9S complex formation) was eliminated. Also, formation of a PR·hsp90 complex did not seem to be affected by several short deletions within the HBD. As with the GR, expression of just the HBD was sufficient for formation of a complex with hsp90 (407). In a more detailed study of chicken PR mutants expressed in quail fibroblasts, Schowalter *et al.* (408) found that three separate regions of the HBD were able to partially restore hsp90 complex formation to a PR mutant lacking the HBD. Again, deletion of individual regions within the HBD did not abolish binding, and as with the GR, it was concluded that several regions throughout the HBD are involved in PR·hsp90 complex formation (408).

Schowalter *et al.* (408) showed that chicken PR with an internal deletion of amino acids 451–521 still bound hsp90, although perhaps less stably than the intact receptor. The sequence 451–521 of the chicken PR is similar in location to the region 556–659 of the mouse GR, which, as discussed above, forms a minimal high-affinity hsp90-binding site (230, 402) and comprises most of the 16-kDa core steroid-binding fragment (227). Addition of the 451–521 segment to the C terminus of a truncated PR lacking the HBD restored hsp90 binding (408). Thus, addition of a segment that is presumably nonessential for hsp90 binding by analysis of mutant PRs with internal deletions produces a chimera that has hsp90-binding activity. This is the same phenomenon that was seen with the GR in the report by Cadepond *et al.* (201), and it underlines how difficult the analysis of the HBD·hsp90 interaction will be until crystallographic analysis of the co-complex can be performed.

3. Studies with the ER. Chambraud *et al.* (202) examined the ability of mutant forms of the human ER expressed in COS cells to form complexes with hsp90, with complex formation being assayed by recovery of 9S receptor from glycerol gradients, a technique that requires 16 h of centrifugation and therefore detects only very stable heterocomplexes. As described above for the GR and PR, deletions of individual regions within the ER HBD did not abolish binding. In contrast to the GR and PR, however, the ER HBD by itself was not sufficient for binding. A region between amino acids 251 and 271 at the C-terminal end of the DBD was also necessary for recovery of 9S ER, but, like the HBD, the 251–271 region was not by itself sufficient for 9S complex formation (202). The region 256–303 of the human ER contains a number of basic amino acids that determine the receptor's nuclear localization (409). Schlatter *et al.* (410) translated various GR and ER mutants in reticulocyte lysate and immunoadsorbed the translation products with antibodies against hsp90. Unlike the GR, in which the HBD itself was sufficient for stable binding to hsp90, the HBD of the ER was not sufficient, and an additional region N-terminal to the HBD was required for hsp90 binding.

Scherrer *et al.* (400) transfected COS cells with cDNA encoding a fusion protein of β-galactosidase and the same ER HBD segment (HE14, amino acids 282–595) that Chambraud *et al.* (202) found insufficient for recovery of a 9S complex. Under normal immunoadsorption and washing conditions, no ER HBD·hsp90 heterocomplex was seen, but when molybdate-stabilized cytosol was rapidly prepared and rapidly immunoadsorbed, heterocomplexes were readily detected. It was also shown that the β-galactosidase-HE14 fusion protein could be assembled into a complex with hsp90 when incubated with reticulocyte lysate (400). Thus, the ER HBD is sufficient for forming a complex with hsp90, but that complex is much less stable than the similar GR or PR HBD·hsp90 complexes. An additional region within the ER NLS region acts in conjunction with the HBD to form a more stable heterocomplex.

Hsp90 clearly binds directly to the ER HBD, but hsp90 has not been demonstrated to contact the additional site within the nuclear localization region of the ER, as is assumed in the model of Chambraud *et al.* (202). It is equally possible that a third component of the complex that binds both hsp90 and the receptor NLS serves to stabilize the ER heterocomplex. The principal immunophilin component of the core heterocomplex, FKBP52, binds directly to hsp90 (411), and it contains a conserved negatively charged sequence that is electrostatically complementary to the receptor NLS (412). It may very well be that the stability of the wild-type ER heterocomplex reflects a direct interaction of the immunophilin with the region 251–271 at the C-terminal end of the DBD combined with a direct interaction of hsp90 with *only* the HBD. Thus, the ER would not differ in any fundamental way from the GR and PR with respect to its site of interaction with hsp90. Regardless of whether sites outside of the HBD contact hsp90, the HBD (HE14 sequence 252–595) of the ER is sufficient for conferring both hormone-regulated binding to hsp90 (400) and hormonal control of physiological response (413) onto chimeric proteins.

4. Studies with the MR. Although detailed studies of hsp90 binding to mutant MR have not been performed, the available indirect evidence is consistent with hsp90 binding to the MR HBD. Human MR that is overexpressed in Sf9 cells is bound to insect hsp90 (414, 415). At high levels of expression, much of the MR is present in insoluble aggregates from which it can be solubilized with guanidine hydrochloride, and the solubilized MR can then be associated with hsp90 and converted to a steroid-binding state by incubation with rabbit reticulocyte lysate (414). Elimination of the HBD eliminates both MR heterooligomer formation and the self-aggregation that occurs when more MR is produced than can be bound to hsp90 (414).

5. Studies with the DR. Whitelaw *et al.* (416) constructed a series of chimeras in which various portions of the mouse DR

were linked to the N-terminal half of the human GR (including the GR DBD). The fusion proteins were translated in reticulocyte lysate, and a region between amino acids 230–421 of the DR was identified that yielded both dioxin-binding activity and stable complexes of chimera with hsp90 (416). This ligand-binding domain of the DR was subsequently translated by itself and shown to be sufficient for stable hsp90 binding (417).

There is also direct evidence that the DBD of the DR confers hsp90 binding onto a chimeric protein expressed in reticulocyte lysate (418). In contrast to the 'zinc finger' motif of the steroid receptor DBDs the DR DBD is a basic helix-loop-helix (bHLH) domain. There is evidence that hsp90 activates the DNA-binding conformation of some other transcription factors that are bHLH proteins (419, 420). This chaperoning has been demonstrated with purified hsp90 in a cell-free system without ATP and without the formation of a stable complex with hsp90. The data of Antonsson *et al.* (418) suggest that hsp90-mediated chaperoning may be required for the bHLH of the DR to assume a DNA- binding conformation. But, in contrast to the cell-free studies with other transcription factors (419, 420), the DNA-binding conformation of the DR bHLH appears to require the ATP-dependent, multicomponent (hsp90, hsp70, p60, p23) chaperone system of reticulocyte lysate. Also, in contrast to the other bHLH transcription factors, the DR bHLH complex with hsp90 is stable enough to detect by coimmunoadsorption (418).

D. Region of hsp90 that binds receptor

The problem of determining which region of hsp90 interacts with the steroid receptor HBD is much more complicated than that of determining the region of the receptor that binds to hsp90. Because the receptor·hsp90 complex is formed by a multicomponent protein chaperone system requiring at least hsp70, p60, and p23, in addition to hsp90, and because hsp90 itself has sites that interact directly with p60, p23, and possibly hsp70 (see *Section VIII.C*), in addition to a region (or shared region) interacting with the receptor HBD, a negative effect at any site of interaction will yield a decrease in or abolish receptor·hsp90 complex formation. Two laboratories have approached the problem by examining the ability of mutants of chicken hsp90 to form PR·hsp90 or GR·hsp90 complexes. To a substantial degree, the conclusions are contradictory.

Sullivan and Toft (421) translated chicken hsp90 mutants in rabbit reticulocyte lysate and incubated the lysate containing the [^{35}S]methionine-labeled hsp90 mutants with immunoadsorbed chicken PR to permit the endogenous chaperone system of lysate to form PR·hsp90 complexes. The formation of complexes is readily detected by gel electrophoresis and subsequent autoradiography of the receptor-associated hsp90. The wild-type chicken hsp90 comprises 728 amino acids, and amino acids 1–380 could be removed from hsp90 without substantial loss of PR·hsp90 complex formation. As the complex was still responsive to hormone, it must have had a steroid-binding site and been able to undergo transformation. Two regions of hsp90 (amino acids 381–441 and 601–677) were found to be particularly important for formation of a PR·hsp90 complex (421).

Cadepond *et al.* (422) coexpressed the human GR and mutant chicken hsp90s in baculovirus-infected Sf9 cells. GR·hsp90 complexes were detected by the ability of antibody specific for chicken *vs*. insect hsp90 to shift the 9S sedimentation peak of GR, the 9S peak being identified by binding with [^{3}H]triamcinolone acetonide. Complexes were also detected by GR immunoadsorption and autoradiography of [^{35}S]methionine-labeled products in the immunoabsorbate. Two deletions involving hydrophilic regions of hsp90 (amino acids 221–290 and 530–581) and a third deletion (amino acids 392–419) that removed a leucine heptad repeat were examined. It was found that the 221–290 deletion mutant did not form a GR·hsp90 complex and that the other two deletions formed "abnormal" complexes that did not bind steroid.

Although these reports from the Toft (421) and Baulieu (422) laboratories disagree on the importance of the N-terminal half of hsp90 in receptor heterocomplex formation, there is agreement to the extent that various deletions within the C-terminal half are either detrimental to or abrogate complex formation. Shaknovich *et al.* (419) have shown that a recombinant C-terminal fragment of 194 residues of murine hsp90 functions like wild-type hsp90 to promote conversion of the bHLH protein MyoD1 from an inactive to an active conformation, as assayed by sequence-specific DNA binding. This ATP-independent and cochaperone-independent function of hsp90 must be carried out by the region of hsp90 that interacts with unfolded regions of proteins, and it is reasonable to predict that this region of hsp90 is the region that contacts the receptor HBD. This C-terminal region contains the 601–677 segment that Sullivan and Toft (421) found particularly important for formation of a receptor·hsp90 complex. It should be noted here that Minami *et al.* (357) have shown that deletion of the C-terminal 49 amino acids from human hsp90α both abolishes dimer formation and biological activity as assayed by the ability to rescue a yeast double hsp90 mutant. Thus, the C-terminal ~200 amino acids of hsp90 appear to contain both the dimerization site and the region that interacts with the chaperoned protein, such as the receptor HBD. The 381–441 segment of Sullivan and Toft (421) could be a site for interaction with another component(s) of the chaperone machinery.

It is clear that the genetic approach using deletion mutants of hsp90 will be very useful for defining binding regions on hsp90 for the p23 and p60 components of the chaperone system, as it is possible to form these complexes with the individual purified proteins (see *Section VIII.C*). Additional and complementary information regarding the receptor·hsp90 complex should be obtainable by cross-linking, followed by partial proteolysis and identification by sequencing of the portions of receptor HBD and hsp90 that have been cross-linked. Given the number of players in the chaperone system and the number of closely interacting sites on hsp90 that must be involved in the formation of stable receptor·hsp90 complexes, much work will be required before the functional anatomy of hsp90 is defined.

E. Requirement of hsp90 for steroid binding

By 1980, it was known that conditions that caused receptor transformation, such as salt, dilution, and elevated pH, also

caused the unliganded GR to lose its steroid-binding activity and that both transformation and loss of binding activity were inhibited by molybdate (242, 264). After the discovery of receptor binding to hsp90, it was thought reasonable that dissociation of hsp90 from the receptor might account for loss of steroid-binding activity as well as acquisition of DNA-binding activity. Consistent with this notion, it was found that 4S unliganded GR recovered from sucrose gradients had no steroid-binding activity, whereas GR recovered in the 8S peak had normal steroid-binding activity (223). Also, transformed liganded GR was found to be unable to rebind steroid after ligand dissociation (423). Bresnick *et al.* (424, 425) demonstrated a direct relationship between the amount of GR-associated hsp90 and the glucocorticoid-binding capacity of immunopurified receptors. The GR HBD alone was sufficient to bind steroid only when it was bound to hsp90.

In the study by Bresnick *et al.* (425), there was no binding of steroid by the intact GR in the absence of hsp90. However, Nemoto *et al.* (426) found that the salt-transformed GR still possessed a weak steroid-binding activity [dissociation constant (K_D) for tramcinolone acetonide of 64 nM *vs.* a K_D of 0.5 nM for the GR·hsp90 complex]. The hsp90-free GR can be converted back to the hsp90-bound form by incubating it with reticulocyte lysate, and this is accompanied by restoration of steroid-binding activity (194). There is a linear relationship between the number of GR·hsp90 heterocomplexes that are assembled in this cell-free system and the amount of steroid-binding activity that is generated (187). When the GR HBD was expressed as a fusion protein with protein-A in *Escherichia coli*, it bound glucocorticoids with normal specificity but with markedly reduced affinity (K_D for triamcinolone acetonide ~70 nM), but translation of the HBD in reticulocyte lysate resulted in formation of HBD·hsp90 complexes that bound steroid with high affinity (401). Thus, the HBD of the GR must be bound to hsp90 to be in a high-affinity steroid-binding conformation.

The MR behaves like the GR in requiring hsp90 for the steroid-binding conformation (93, 414). The loss of mineralocorticoid-binding activity that occurs when cytosol is warmed is inhibited by molybdate and other conditions that stabilize the receptor·hsp90 complex (427). MR expressed in bacteria is not bound to hsp90 and does not bind steroid, but it can be assembled into a complex with hsp90 and activated to a steroid-binding conformation by reticulocyte lysate (93). Dissociation of hsp90 from the DR also results in loss of ligand-binding activity (428), and reassembly of the DR·hsp90 complex in reticulocyte lysate results in reactivation of ligand-binding activity (416–418, 428).

In contrast to unliganded GRs, MRs, and DR, which immediately lose their steroid-binding activity when stripped of hsp90 at 0 C, PRs may be dissociated from hsp90 without loss of steroid-binding activity as long as they are maintained at 0 C (192). However, Sullivan *et al.* (429) reported that incubation of chicken oviduct cytosol for 10 min at 37 C resulted in more than 80% loss of progesterone-binding activity without loss or cleavage of either the A or B subunit of the PR. Smith (192) then incubated the immunopurified, hsp90-free PR at 37 C and showed that steroid-binding activity was lost, but that it could be regenerated by incubation with reticulocyte lysate and reformation of the PR·hsp90 complex. Thus, it seems that the hsp90 chaperone system is required to generate a high-affinity steroid-binding conformation of the chicken PR HBD, but that this steroid-binding conformation is stable at 0 C after hsp90 removal. Warming the PR causes a conformational change (*i.e.* a change in folding) in the unliganded HBD that eliminates the steroid binding site.

Eul *et al.* (430) expressed a fusion protein of β-galactosidase and the chicken PR HBD in *E. coli*, and they demonstrated that the affinity-purified fusion protein bound [^{3}H]progesterone in the normal high-affinity manner. At this time, it is difficult to reconcile this observation with that of Smith (192) for a requirement for hsp90-dependent chaperoning in converting the PR HBD to a high-affinity steroid-binding conformation. One possibility is that the HBD of the PR can be chaperoned by the bacterial homolog of hsp90, whereas that system is not adequate to assist the folding of the GR, MR, and DR to the high-affinity ligand-binding conformation. A more likely possibility is that the steroid-binding conformation of the PR HBD is achieved without hsp90-dependent folding, but perhaps with the assistance of other chaperones, as it is translated in the bacterium. However, when the purified protein is incubated at 37 C in buffer, as in the Smith experiment (192), the HBD massively unfolds to a state that requires chaperone-assisted refolding.

As discussed for the PR, fusion proteins containing the AR HBD expressed in *E. coli* are able to bind androgens in normal high-affinity manner (431–433). Thus, there appear to be two, and possibly three, classes of hsp90-bound receptor HBDs: one class must be in the complex with hsp90 to have the high-affinity steroid-binding site (GR, MR, DR); a second class can form a steroid-binding site as the HBD is translated but requires refolding by the hsp90 chaperone machinery if it is unfolded (PR and possibly AR and ER); and a possible third class does not require hsp90-assisted folding under any conditions to assume a steroid-binding conformation (?AR, ?ER). Regardless of the effect of hsp90 on ligand-binding affinity, the binding of ligand to the binding cleft in the HBD promotes a temperature-dependent decrease in the affinity of all of these receptors for hsp90.

F. Receptor transformation and hsp90 dissociation

As discussed in *Section III*, receptor transformation measured either by 9S to 4S conversion or by acquisition of DNA-binding activity is both hormone-dependent and temperature-dependent and inhibited by molybdate. Mendel *et al.* (186) were the first to provide direct evidence that exposure of intact cells to glucocorticoid caused dissociation of the GR from hsp90. Sanchez *et al.* (148) then showed that binding of steroid to cytosolic GR promoted temperature-dependent dissociation of the receptor from hsp90 and generation of the DNA-binding state, a result confirmed by Denis *et al.* (109). Artifactual conditions that caused receptor transformation in a steroid-independent manner (*e.g.* exposure to salt, ammonium sulfate precipitation) also dissociated the GR·sp90 complex to yield the DNA-binding state. In addition, it was shown that hsp90 was responsible for the negative charge of the untransformed *vs.* the transformed GR (148). Importantly, the rate of hsp90 dissociation from cyto-

solic GR·hsp90 heterocomplexes was shown to be the same as the rate of receptor acquisition of DNA-binding activity (144).

Similar observations were made in direct studies of PR·hsp90 interaction. Kost *et al.* (141), for example, demonstrated that progesterone promoted dissociation of the avian PR from hsp90, both on *in vivo* progesterone treatment and upon addition of steroid to cytosol. The hsp90 could be dissociated from the PR with salt, and this dissociation was inhibited by molybdate (141, 142). Gustafsson, Poellinger, and their colleagues established that binding of dioxin to the DR promoted release of hsp90 with the concomitant acquisition of both the dimerization and DNA-binding activities of the receptor (428, 434–436).

Thus, the model of receptor transformation that evolved rapidly after the discovery of receptor·hsp90 heterocomplexes was that binding of steroid promoted temperature-dependent dissociation of hsp90 from the receptor HBD, resulting in unmasking of the DBD, receptor dimerization sites, and in the case of GR, MR, and DR, NLSs as well. As stated above (*Section III. C*), models of receptor transformation based purely on observations in cytosols or purified systems must be oversimplified with respect to the transformation event in the intact cell. Nevertheless, this working model of transformation is useful and heuristic.

1. How does steroid binding promote receptor transformation? As it is currently conceived, the receptor·hsp90 heterocomplex represents a normal transition state in a general protein-folding process. In the case of the great majority of chaperoned proteins, this intermediate is quite transient, but in the case of steroid receptors and the DR, the complex between the HBD and hsp90 is particularly stable, and progression from this folding intermediate has been brought under hormonal control. In this model, hsp90 is conceived as trapping the receptor HBD in a partially unfolded conformation (187, 437), which for the GR, MR, and DR is the only state in which there is an accessible steroid-binding site. Binding of steroid in the steroid-binding pocket then favors continued folding of the HBD, destabilizing its interaction with hsp90.

In the case of those receptors that require tight association with hsp90 for maintenance of the steroid-binding conformation, virtually the same progression of folding of the HBD occurs when the unliganded receptor is artifactually dissociated from hsp90; thus, the ligand-binding pocket becomes buried and is no longer accessible to steroid. There is now evidence for such a folding change occurring in the GR HBD upon hsp90 dissociation. Stancato *et al.* (438) have used a small, thiol-specific derivatizing agent to show that thiol moieties in the HBD that are accessible in the hsp90-bound state become inaccessible upon dissociation of hsp90. The fact that the change in the HBD is from accessibility to reagent in the hsp90-bound state to less accessibility when hsp90 is not bound is consistent with an internalization of thiol moieties occurring as the unliganded receptor HBD assumes a more folded conformation (438).

In this model, agonists would promote folding of the HBD and dissociation from hsp90, but if a steroid occupied the binding site and did not promote folding or even stabilized the receptor·hsp90 interaction, it could act as an antagonist. Such a mechanism has been proposed by Baulieu (439) to explain, at least in part, the action of the steroid antagonist RU38486. This proposal is based on the inactivity or only partial activity of RU38486 compared with agonists in transforming 8S GR or PR to the 4S form, both in cytosols (440–443) and in intact cells (185, 423, 444). It is difficult to evaluate the effect of RU38486 on the stability of receptor·hsp90 complexes. In some systems RU38486 is an antagonist and in others it has partial agonist activity. In mouse lymphoma cells, for example, RU38486 binds to the GR, but the receptor remains in the 8S form and does not translocate to the nucleus (423). In contrast, RU38486 does cause nuclear translocation of rat GR transfected into COS cells (445). Obviously, as noted by Baulieu (439), the effect of RU38486 is much more complicated than a simple failure to promote dissociation of receptor from hsp90, but there are systems where this may contribute to its antagonist action.

In some cases, GR nuclear transfer has been achieved without ligand. Under conditions of both chemical and thermal stress, for example, ligand-free GR becomes tightly bound to nuclei and is in a non-steroid-binding form, consistent with it being dissociated from hsp90 (446, 447). An important question asked in the 1980s was whether the only required effect of steroid hormone in producing a response was to promote receptor transformation by altering the receptor·hsp90 interaction (1). At that time, hormone-free GR and PR were shown in cell-free assays to bind selectively to hormone-regulatory elements with the same affinity as steroid-bound receptors (448, 449). Thus, it seemed possible that steroid was not required for receptor action after the initial transformation event. However, in 1990, Bagchi *et al.* (450) reported progesterone-dependent RNA synthesis from a PRE-driven promoter in nuclear extracts of human breast carcinoma cells. In this *in vitro* reconstituted system, transcriptional activation was triggered by hormone-induced binding of endogenous PR to PREs. The authors stated that the receptor existed in a 4S form in their initial salt-treated extract and was apparently dissociated from hsp90 (450). Although this observation argues that steroid is required for steps in receptor-dependent transcriptional activation beyond the transformation step, there is genetic evidence for hormone-free transcriptional activation in yeast. Kimura *et al.* (451) have shown that the GR is constitutively active in yeast with mutant Ydj1 protein. Ydj1 is a member of the DnaJ chaperone family, and it is apparently required for hsp90 suppression of GR activity in hormone-free yeast. In the presence of the Ydj1 mutation, the transcriptional activating activity of the wild-type GR is increased about 200-fold in the absence of steroid (451). This argues rather strongly that hormone is not required for wild-type GR function in yeast, except when the receptor is maintained in the inactive state by hsp90.

However, the yeast system may provide a somewhat simplified and perhaps biased result. Strong argument for a role of ligand binding beyond hsp90 dissociation comes from studies with hormone antagonists. For example, the triphenylethylene-derived antiestrogens, tamoxifen and nafoxidine, appear to promote hsp90 dissociation and DNA binding of the ER (452, 453), but they do not provide the conformational changes needed for the transcriptional acti-

vation functions of the receptor (453, 454). These ligands yield a receptor that is either inactive or partially active, depending upon the cell type and the gene promoter. Recent studies indicate that the two transcriptional activation functions of the ER, AF-1 in the amino portion of ER and AF-2 in the ligand-binding domain, synergize to provide full activity (454). This synergism plus the activity of AF-2 are blunted by the bound antiestrogen. Thus, it seems likely that ligand binding promotes degrees of conformational change and stability that influence both receptor interaction with chaperones and its association with the transcription apparatus.

2. Temperature requirement for steroid-dependent transformation. In cytosol preparations, steroids do not promote GR or PR transformation at 0 C, but at room temperature, receptor transformation is promoted by steroid (109, 141, 142, 144, 148). Inasmuch as steroid binding occurs at both 0 C and 25 C, the temperature-dependent step must occur after steroid binding. In the above model, the temperature-dependent step would be the steroid-promoted folding of the HBD that leads to dissociation of hsp90. It must be remembered that in normal cytosol preparations receptor·hsp90 disassembly is occurring in the absence of concomitant receptor·hsp90 complex assembly. In reticulocyte lysate and in intact cells, assembly is occurring simultaneously with disassembly, and the steroid-bound PR or GR HBD is not reassembled into a stable heterocomplex with hsp90 (192–194). Thus, in the more dynamic system the ligand promotes conformational changes in the HBD that negate formation of a stable complex between the receptor and the hsp90 component of the chaperone system. Whether ligand binding actually promotes hsp90 dissociation or simply waits for it to occur remains uncertain. This is an important question that can be asked in another way. Do the ligand-dependent changes in receptor conformation occur while the receptor is bound to hsp90 or after it has dissociated?

In numerous studies reported in the early 1980s, ATP at millimolar concentrations was shown to enhance 8S to 4S transformation of steroid-bound GR, PR, AR, and ER in cytosols (169–171, 173, 267, 455–460). ATP promoted transformation at 0 C, and other ribonucleotides were often active, although less so than ATP. Diehl and Schmidt (461) reported that ATP and other ribonucleotides promoted transformation of substantially purified, untransformed GR, with transformation being determined both by increased DNA binding and by shift of receptors to a form that eluted from DEAE with low salt. As in several of the studies of transformation in crude cytosol, hydrolysis-resistant ATP analogs were also effective. Although the Toft laboratory found that ATP did not promote hsp90 dissociation from immunopurified, native PR heterocomplexes under conditions in which it did promote dissociation of hsp70 (141), they found that progesterone-promoted dissociation of reconstituted PR·hsp90 complexes was most efficient in the presence of an ATP-generating system (190). At this time, there has not been sufficient direct study of hsp90 dissociation from receptor heterocomplexes to establish a direct role for ATP in the process, but it is clearly needed within the pathway of hsp90 binding, along with several additional proteins. Receptor-specific factors may also be important to the transformation process. In the case of the DR, for example, there is evidence that the Arnt protein that dimerizes with the transformed DR in the nucleus may itself play a role in releasing hsp90 from the dioxin-bound receptor (462).

G. Receptor inactivation by hsp90

Untransformed steroid receptors are unable to dimerize and they are unable to bind to DNA. Also, in most hormone-free cells, the GR (445, 463, 464), MR (465, 466), and DR (467) are localized in cytoplasm, a localization that is consistent with an inactivation of the receptors' NLS when the receptors are in the untransformed state. Because the major dimerization site is located in a region of the HBD that is part of, or adjacent to, the hsp90-binding region, it is easy to conceive how binding of hsp90 might abrogate receptor dimerization. However, it is more difficult to conceive how hsp90 abrogates receptor DNA-binding activity. Baulieu (439) originally proposed that hsp90 blocks DNA-binding activity by directly binding to the DBD. Although it was subsequently shown that hsp90 binds directly to the HBD (see *Section VI.C*), it could, possibly by virtue of its bulk, sterically block access to the DBD. At this time, it is not known whether hsp90 abrogates DNA- binding activity solely by physically masking the DBD or whether the DBD of the hsp90-bound receptor is in a different conformation from the hsp90-free receptor. It has been shown that dissociation of hsp90 from the GR increases derivatization of the DBD by a small, thiol-reactive reagent (438). In the event that hsp90 abrogates the DNA-binding activity of the GR solely by physically blocking access of DNA to the DBD, the fact that access to a small reagent is impeded suggests that there must be a rather close association of hsp90 with the DBD. It has been shown that antibodies directed against the GR NLS (468) or against the hinge region between the DBD and HBD of the PR (469) selectively recognize the hsp90-free receptors (143, 400, 468, 469). The fact that hsp90 impedes the reaction of antibody with the GR NLS suggests that interaction with the NLS-recognition protein may also be impeded, thereby accounting for the cytoplasmic localization of the untransformed receptor.

Three models have been proposed to explain how binding to hsp90 inactivates steroid receptors. The first model, which we will call the *steric interference* model, assumes that hsp90 maintains receptors in an inactive state in the cell by blocking access to the DBD. It is true that binding of hsp90 to the HBD blocks DNA-binding activity, but that does not explain the inactivation function of the HBD. Yamamoto and his colleagues (413, 470, 471) performed a very important series of experiments in which they fused the HBD of the GR or ER to proteins such as the adenovirus E1A gene product, the bacterial LexA repressor, or the Myc oncoprotein and showed the the activity of the chimeric protein was hormone regulated. This movable inactivation function of the HBD has been shown for the MR (472) as well as the GR (470, 471) and ER (413, 473–475), and hormonal regulation has been conferred to a variety of heterologous activities (for review, see Refs. 476 and 477). Yamamoto and his colleagues proposed that repression by the HBD is conferred via its association with hsp90 (470, 478), a proposal supported both by analysis of hsp90 binding by β-gal-GR chimeras (400) and by genetic

manipulation of chaperone activity (451). Because the receptor HBD carries an inactivation function that operates on activities of structurally different proteins (some without DNA-binding activity), a model of conferrable hormone regulation based solely on steric interference of receptor function by hsp90 is probably not adequate.

Yamamoto and his colleagues (470, 478) have proposed a model of functional inactivation by hsp90 that accommodates the range of regulated structures and activities observed with chimeras. They proposed that binding of hsp90 to the HBD within a protein chimera causes the polypeptide as a whole to assume an "unfolded" (*i.e.* partially unfolded) conformation that is reversed on hormone binding and hsp90 dissociation. This model assumes, then, that the function of the chimera is inactivated because hsp90 determines an unfolded conformation in the region that is regulated as well as in the HBD that is conferring the regulation. Spanjaard and Chin (479), however, have demonstrated reconstitution of GR activity by expressing as individual proteins a fragment of the GR containing the *trans*-activation and DBDs and a fragment containing the HBD, with each fragment being fused to either a c-Jun or c-Fos leucine zipper. Because each fragment of the receptor was translated and folded independently, the results of Spanjaard and Chin (479) argue strongly against a model in which hsp90 binding to the HBD causes the polypeptide as a whole to assume an "unfolded" conformation.

The third model to explain how binding of hsp90 to the HBD inactivates receptors has been called the *docking* model because it proposes that the receptors and fusion proteins containing a receptor HBD remain "docked" to the hsp90-containing heterocomplex structure until they are released by steroid-mediated reversal of their partially unfolded conformation (6, 7). In the docking model, hsp90 must determine an unfolded conformation of only the HBD. In this case the protein that is brought under hormonal control may be properly folded, but it is biologically inactive until it is released from its tight association with the hsp90 component of the chaperone protein structure, allowing it to proceed to its ultimate site of action. In the *steric interference* model, the DBD must be blocked, whereas in the *docking* model, the receptor or fusion protein must be released from the chaperone system to be active regardless of whether the DBD is blocked or not. In the case of the GR (445, 463, 464), MR (465, 466), and DR (467) the sites of docking are usually the cytoplasm, whereas for the PR (374, 375), ER (206, 207), and AR (480, 481) the docking sites are usually in the nucleus. In both cases, the receptors are associated with hsp90 and are recovered in the cytosolic fraction upon cell rupture. In the case of fusion proteins containing a receptor HBD, the docking site could be either in the cytoplasm or in the nucleus. Like the unfolding model of Yamamoto, the docking model also accommodates the ability of the HBD to confer hormone regulation onto chimeric proteins of widely different structures. In considering the docking model, it should not be thought that the hormone-responsive proteins are being "tethered" to a static cytoplasmic or nuclear structure before hormone-mediated release, as implied in some models of regulation (482). Rather, the model is a dynamic one in which the hsp90-based chaperone system is proposed to play an important role in the cytoplasmic-nuclear shuttling of the receptor (see *Section VI.I.2*), and the hormone-free receptor cannot progress from this dynamic system because it is recaptured by virtue of the unique long-lived interaction of the unliganded HBD with the hsp90 component of the system (6, 7).

H. Evidence that hsp90 is a physiologically significant regulator of receptor function

When studies of receptor·hsp90 complexes began, it was a major concern that they might be formed upon cell homogenization and not reflect a native state of receptors in cells. Both metabolic labeling studies by Howard and Distelhorst (483) and studies from Gehring's laboratory (377, 391, 392, 394), and others (395) in which steroid receptors were crosslinked to hsp90 in intact cells have demonstrated that receptors are bound to hsp90 *in vivo*. Nevertheless, this conclusion is questioned, at least for the PR, where attempts to demonstrate colocalization of hsp90 and PR in the nucleus by immunocytological methods have failed (484). These investigators have suggested that receptors are not complexed with hsp90 *in vivo* and that binding of the PR to hsp90 is an artifact generated during tissue processing (195, 484). The argument that hsp90 binding is an artifact is not consistent with genetic observations (summarized below) that establish the physiological relevance of hsp90 for receptor action [reviewed by Bohen and Yamamoto (485)].

Two of the original arguments for physiological relevance were based on correlations between hsp90 binding and the hormone responsiveness of receptors. In multiple studies of hsp90 binding to C-terminal truncated receptors or to fusion proteins, it was noted that receptors that were constitutively active were not bound to hsp90, whereas receptors that were hormone-responsive were always bound to hsp90 (200–202, 400, 407, 408, 416). Also, thyroid hormone and retinoic acid receptors, which in their unliganded state form tight complexes with nuclei that require high salt for extraction (486, 487), do not form stable complexes with hsp90 (344, 345). A major difference between these receptors and the steroid hormone receptors is that they can bind to DNA in their hormone-free state and affect transcription from their own elements or from other elements with which they interact. Because of their binding to hsp90, the steroid and dioxin receptors should have no activity unless ligand is present. An exception to this would be if receptor transformation could be brought about in a ligand-independent manner. Steroid receptor-mediated transcription has been initiated in a hormone-independent manner by treating cells with agents that affect protein phosphorylation (488) or by the activation of other signaling pathways with hormones or growth factors, such as dopamine (489, 490) or epidermal growth factor (491). We would speculate that in these instances phosphorylation of the receptor or a component of the heterocomplex either facilitated hsp90 dissociation or, perhaps more likely, reduced the efficiency of stable heterocomplex formation, thus shifting the ratio of hsp90 bound to unbound receptors.

The first evidence that hsp90 participates *in vivo* in signal transduction by steroid receptors was provided by Picard *et al.* (492) who showed that GR, MR, and ER have altered

transcriptional activating activity in yeast cells expressing ~5% of the normal amount of hsp90. At the low hsp90 level, the signaling pathways were functional but required much higher concentrations of steroid than at the normal hsp90 level. At the low hsp90 level, the receptors were predominantly in the hsp90-free state, but they were not constitutively active. Similar observations have been made in the same yeast system with a chimera of LexA and the HBD of the DR (493). Because the receptors are not constitutively active at low hsp90 levels, Picard *et al.* (492) suggested that hsp90 cannot inhibit receptor function solely by steric interference and that somehow hsp90 is required for subsequent response of the wild-type receptor. In the yeast, a truncated GR lacking the HBD was equally active at low and normal hsp90 levels, showing that transcriptional activation *per se* was not affected. Although the mechanisms underlying some of the observations made in the yeast system remain cryptic, the fact that receptor response is altered at the low hsp90 level validates the notion that hsp90 is critical for signal transduction by steroids.

In a second genetic approach, Caamaño *et al.* (494) replaced proline 643 of the rat GR with alanine. This mutation was made because Pro 643 lies in a predicted contact region for hsp90 (230) and in the steroid-binding site (227) of the GR, and it is conserved in all of the steroid receptors that bind hsp90 but not in the receptors for vitamin D, thyroid hormone, and retinoic acid, which do not bind hsp90. This GRP643A mutant binds glucocorticoids with the same stereospecificity as the wild-type receptor but with much lower affinity, and it forms a heterocomplex with hsp90 that is less stable than that of the wild-type GR, even in the presence of molybdate (494). In transcriptional activation assays, the GRP643A mutant showed only 30% of the hormonal response of the wild-type GR.

Mutations in hsp90 clearly affect the steroid-signaling pathway in yeast. Nathan and Lindquist (495) isolated eight broadly distributed temperature-sensitive point mutations in the hsp90 gene of *Saccharomyces cerevisiae*, and all eight mutations in hsp90 were found to affect glucocorticoid-induced enhancement of gene expression by the rat GR. Bohen and Yamamoto (496) used the reverse approach and screened yeast for defects in GR function. They isolated four mutants of hsp90 that interacted with the GR but showed differences in hormone responsiveness. Three of these mutant hsp90s caused defects in glucocorticoid binding *in vivo* and formed complexes with the GR that were unstable *in vitro* (497). Interestingly, different point mutations in hsp90 affected different receptors to different degrees, with GR and MR responses being generally more affected than PR and ER responses (496). It is possible this difference reflects, at least in part, the GR and MR requirement for hsp90 in maintaining a steroid-binding conformation. In a unique natural demonstration of the *in vivo* requirement of hsp90 for steroid binding, Kaufmann *et al.* (498) found that hsp90, which is present in the early germ cells of rodents, is absent from the epididymal sperm, which contain full-length GR but do not have glucocorticoid-binding activity.

Although the connections between the yeast Ydj1 protein and hsp90 binding to steroid receptors have not been worked out biochemically, mutations in the Ydj1 gene affect receptor function (451, 499). Ydj1 is the yeast homolog of the bacterial DnaJ protein (500), and it interacts with and stimulates the ATPase activity of hsp70 (501). Because hsp70 is required to form steroid receptor·hsp90 complexes (*Section VIII.B.1*), Caplan *et al.* (499) examined human AR function in yeast with a mutant Ydj1 protein. The AR was only minimally hormone-responsive in the mutant strain *vs.* wild-type yeast, but both steroid binding by the full-length AR and constitutive activity of an AR mutant lacking the HBD were the same in the mutant as in the wild-type organism. As described above (*Section VI.F.1*), Kimura *et al.* (451) isolated a mutant of the Ydj1 gene using a synthetic-lethal screen for mutations that are lethal in the presence of mutant hsp90 proteins. When the mutant Ydj1 gene was placed in yeast with an otherwise wild-type background, it was found that the basal transcriptional activity of mammalian GR and ER was increased many-fold. Thus, this Ydj1 mutant protein relaxed repression of receptor function in the absence of hormone, but hormone was still required for full activity.

Although the biochemical interpretation of the function of Ydj1 in the hormone-response pathway is cryptic (the reader is referred to Ref. 502 for comment), the experiments certainly point to a physiologically significant role for hsp70 in the pathway. Because hsp70 is required for hsp90 binding to receptors, the results indirectly support a role for hsp90 as well. Taken together, the results of all the experiments involving mammalian receptor-mediated expression in yeast (451, 492, 493, 495–497) provide elegant support for a requirement for hsp90 in the hormone response pathway *in vivo*. However, interpretation of the yeast experiments should be made with some caution, since a lowering of hsp90 function might affect basic cellular processes as well as other cell-signaling pathways (see *Section IX*) that may have an impact on steroid hormone action.

I. Potential roles of hsp90 in receptor turnover and trafficking

1. Receptor turnover. Recent studies with geldanamycin suggest that association with hsp90 may inhibit receptor degradation. Geldanamycin is a benzoquinone ansamycin that can revert transformation induced in cultured cells by oncogenic tyrosine kinases, such as $pp60^{v\text{-}src}$ (503, 504). Geldanamycin does not inhibit the kinase activity of Src, but it binds in a pharmacologically specific manner to hsp90 and inhibits the formation of the Src·hsp90 heteroprotein complex by reticulocyte lysate (505). In intact cells, geldanamycin also inhibits formation of the hsp90 heterocomplex with the serine/threonine kinase Raf-1, and Raf half-life is markedly decreased (506, 507). Geldanamycin causes a similar destabilization of the GR (508) and of the tumor suppressor p53 (509). Because geldanamycin accelerates the degradation of these proteins, it has been suggested that hsp90 binding stabilizes proteins to degradation *in vivo*.

Although this conclusion agrees with earlier *in vitro* observations finding that molybdate made the cytosolic GR more resistant to proteolysis (223, 322), the mechanism of geldanamycin destabilization of the GR is not clear.

It has been established that geldanamycin blocks the association of hsp90 with the p23 component of the receptor

heterocomplex assembly system (510). In the presence of geldanamycin, PR·hsp90 complexes can be formed by reticulocyte lysate, but they are blocked before the step where p23 binds and the resulting complexes do not bind progesterone (511). This ability of geldanamycin to block the formation of a mature receptor complex with hsp90 may indicate that proper hsp90 binding to receptors stabilizes them to degradation *in vivo*. However, it is also possible that geldanamycin accelerates receptor degradation by affecting the proteolytic mechanism itself. Whitesell and Cook (508) showed that geldanamycin-induced degradation of the GR was inhibited by cotreatment of cells with lactacystin, which is a selective inhibitor of proteasome-mediated degradation of ubiquinated proteins (512). Vedekis and his colleagues (513, 514) have provided evidence that the GR is degraded by an ATP/ubiquitin-dependent process, and hsp90 has been shown to bind to the proteasome and inhibit proteolysis in a manner that is not competitive with substrate (515, 516). Thus, it is not yet clear whether the ability of geldanamycin to increase the rate of receptor degradation reflects solely an effect on formation and dissociation of the receptor·hsp90 complex or whether it also reflects an effect on the ability of hsp90 to bind to proteasomes and inhibit their proteolytic activity.

2. Receptor trafficking. From studies of GR distribution analyzed by nuclear *vs*. cytoplasmic partitioning of bound radiolabeled steroid in thymic lymphocytes exposed to energy-limiting conditions, Munck *et al.* (70) proposed that the GR undergoes an energy-dependent cycling between the cytoplasm and the nucleus. A key component of the model is that the receptor is reutilized after it exits the nucleus into the cytoplasm, and this 1972 report contains the first evidence for the cycling and reutilization of any receptor. Munck and his colleagues (517–519) have developed an evolving kinetic model for this receptor cycling process (see Ref. 239 for review), which is supported by observations made in other systems (199, 234, 235, 237). At least part of the ATP-dependency of the GR cycle can be accounted for by the ATP-dependent reconstitution of GR·hsp90 complexes (520). Recently, studies using heterokaryons have demonstrated that hormone-free PR (521, 522), GR (523), and ER (524) shuttle between the cytoplasm and the nucleus (see Refs. 525–527, for review of nuclear-cytoplasmic shuttling).

It is now clear that steroid receptors are constantly passing into and out of the nuclei of hormone-free cells, and that under steady-state conditions most of the GR, MR, and DR molecules are detected in the cytoplasm (445, 463–467), whereas most of the PR, ER, and AR (206, 207, 374, 375, 480, 481) are in the nucleus. It is not clear what determines the steady-state localization. The mouse GR, for example, is localized in the cytoplasm of mouse L cells (464), whereas the same mouse receptor expressed in CHO cells is nuclear (143, 528). Similiarly, the human AR is predominantly nuclear when expressed in HeLa cells, whereas it is mainly cytoplasmic when expressed in COS-1 cells (529). Although the cellular factors that determine receptor localization are unknown, it is clear that localization of the hormone-free receptor in the cytoplasm *vs*. the nucleus does not reflect a fundamental difference in the mechanism of receptor action.

Because hormone-free receptors are associated with hsp90 regardless of whether they are in the cytoplasm or in the nucleus (377, 391, 392, 394, 395), it seemed reasonable to propose that they traffic through the cytoplasm and the nucleus in association with hsp90 (6). The question that arises is whether hsp90 and other proteins in the receptor heterocomplex actually play a role in the trafficking process. In the model of Pratt (7), the hsp90-containing heterocomplex may act as a transport particle, or *transportosome*, with the immunophilin component of the heterocomplex being responsible for targeting receptor movement (412, 530, 531).

The idea that receptors shuttle in association with hsp90 is supported by experiments in which chicken hsp90 was targeted to the nucleus by fusion with the nucleoplasmin NLS (532). When expressed by themselves in COS-7 cells, the hsp90-NLS localized to the nucleus and PR or GR mutants lacking the NLS localized to the cytoplasm. However, when the receptor mutants lacking an NLS were coexpressed with hsp90-NLS, there was complete nuclear localization of the receptors. The interpretation, then, is that the receptors undergo "piggyback" transport with hsp90-NLS (532). However, it must be remembered that steroid receptor·hsp90 heterocomplexes are dynamic in the sense that they are constantly dissociating and being reformed in the cell at 37 C (192); thus, the piggyback movement might involve interaction of a receptor with several hsp90-NLS molecules, making it likely that movement through the cytoplasm is saltatory.

Building on the observation of Raaka *et al.* (279) that treatment of intact cells with molybdate retards steroid-induced conversion of receptors from 9S to 4S, Yang and DeFranco (533) introduced sodium molybdate into cells with a liposome-mediated delivery system. They found that molybdate stabilized GR·hsp90 complexes *in vivo* and inhibited hormone-dependent nuclear import of the GR. The molybdate treatment trapped both the GR and the PR in the cytoplasm of cells chronically exposed to hormone, suggesting that the receptors can export from nuclei but cannot be reimported into nuclei in the presence of molybdate. This is entirely consistent with Munck's model of the receptor cycle where reassociation of receptors with hsp90 in the cytoplasm is required for recycling to the nucleus (239, 520). Because molybdate stabilization caused receptors to accumulate in the cytoplasm, Yang and DeFranco (533) proposed that association of receptors with hsp90 must be dynamic to permit the access of macromolecular components that participate in the delivery of receptors to their nuclear sites of action. Another observation that suggests hsp90 may be involved in receptor trafficking itself or in receptor attachment to a trafficking machinery is that brief treatment of cells containing steroid-bound GR with the hsp90-specific agent geldanamycin inhibits dexamethasone-induced movement of the GR from the cytoplasm to the nucleus (534).

In considering the possibility that steroid receptor translocation requires an, as yet, undefined movement machinery, it is a reasonable notion that some sort of cytoskeletal network must serve as a scaffold for such directed movement (4, 7). In this respect, it is interesting that several immunocytochemical studies have found cytoplasmic GR (535–537) and

vitamin D receptors (538) to localize along fibrillar structures, at least some of which have been identified as microtubules (536, 537, 539). In biochemical studies, the 9S GR heterocomplex has been reported to bind to actin filaments (540), and GR transformed *in vitro* has been associated with a protein complex containing elements (tubulin, actin, and vimentin) of all three cytoskeletal networks (4, 541). The fact that microtubule-disrupting agents do not inhibit cytoplasmic-nuclear translocation of PR (542) or GR (530) or hormone-mediated transcriptional activation by the GR (543) argues against trafficking along microtubules, or possibly for a redundancy of trafficking systems along multiple cytoskeletal networks. In contrast to the situation with the GR, microtubule-disrupting agents do inhibit both nuclear (539) and mitochondrial (544) accumulation of the vitamin D receptor, and they inhibit 1,25-dihydroxyvitamin D_3-dependent modulation of gene transcription (544). At this time, studies of the potential role for cytoskeleton in steroid receptor movement have not defined a movement pathway, and the status of the notion is unresolved (see Ref. 545 for review). If hsp90 is involved in cytoplasmic trafficking of receptors, then a redundancy of pathways is possible, in that hsp90 has been localized in various reports to all three cytoskeletal networks (378–382).

It is easy to envision that untransformed receptors might undergo cytoplasmic-nuclear shuttling in association with hsp90, but it is difficult to envision how steroid-transformed receptors might traffic by a similar mechanism. It is difficult, at least, to envision given the model provided from biochemical analysis in which the receptor dissociates from hsp90 and the immunophilins upon transformation. However, it may be that in the intact cell, the conformational change that occurs in the HBD upon steroid binding converts a receptor's very tight interaction with hsp90 to a weak interaction typical of many proteins that constitutively utilize the same machinery for targeted trafficking (10).

VII. Other Proteins Recovered in Native Receptor Heterocomplexes

By the late 1980s, it was clear that other proteins were present in immunoadsorbed receptor heterocomplexes in addition to hsp90. Two of these proteins, hsp70 and p23, are required for assembly of stable receptor·hsp90 heterocomplexes, and their roles in the assembly process will be considered in *Section VIII*. Another essential member of the chaperone system, p60, is only present in receptor heterocomplexes as a transient participant during their assembly and is not recovered in native receptor heterocomplexes immunoadsorbed from cytosols. p60 and two other potential assembly components, p48 and hsp40, will be discussed only in *Section VIII*. Several immunophilins and a protein phosphatase that are recovered in receptor heterocomplexes bind via their tetratricopeptide repeat (TPR) domains to a TPR-binding domain on hsp90. These include the mammalian FK506-binding immunophilins FKBP51 and FKBP52, the 50-kDa and 54-kDa avian immunophilins, the mammalian cyclosporin A-binding immunophilin CyP-40, and the protein-serine phosphatase PP5. Because these proteins all bind to the same site on hsp90, they exist in independent heterocomplexes with cytosolic receptors. By 1991, it was clear that the proteins identified in native receptor heterocomplexes existed in cell cytosols in multiprotein complexes with hsp90 independent of the presence of receptors (189, 546–548). In this section, we review first the individual proteins recovered in native receptor heterocomplexes and then the organization of these hsp90 heterocomplexes.

A. hsp70

In eukaryotes, members of the conserved hsp70 family of proteins are present in the cytoplasm and within organelles where they act as chaperones of protein folding and translocation (for review, see Refs. 549–552). Two forms exist in the cytoplasm, a 73-kDa form that is constitutively expressed (hsc70) and a 72-kDa stress-inducible form (hsp70). Although the dominant form associated with steroid receptors is hsc70, we will use the generic term hsp70 hereafter. Members of the hsp70 family bind in an ATP-dependent manner to unfolded regions in proteins or to hydrophobic peptides, and ATP hydrolysis facilitates release of the protein (553). Eukaryotic homologs of the bacterial DnaJ protein, such as Ydj1 (discussed above in *Section VI.H*), stimulate this ATPase activity (501). It has been shown that hsp70 has a promiscuous ability to bind to a wide variety of newly synthesized proteins (554). Thus, it binds to proteins regardless of structure by recognizing regions of hydrophobic character. In the unstressed cell, hsp70 is diffusely located in both the cytoplasm and the nucleus, but in the heat-shocked cell there is a concentration of hsp70 in nucleoli, as well as an increase of the protein in both cytoplasm and nucleus (555).

In a purification of the transformed GR from rat liver cytosol published in 1984, Wrange *et al.* (135) noted the copurification of a 72-kDa protein that did not bind hormone, react with anti-receptor antibody, or bind to DNA. At the time, it was unclear whether the unidentified 72-kDa protein was a functional subunit of the receptor or a contaminant (556), but it is now clear that this was the first demonstration of hsp70 association with a steroid receptor (324). In 1987, Estes *et al.* (557) reported that a 76-kDa non-steroid-binding protein that reacted with a monoclonal antibody against hsp70 copurified with both untransformed and transformed human PR. Kost *et al.* (141) then showed that both hsp70 and hsp90 were copurified with untransformed PR from chick oviduct cytosol, whereas transformed PR isolated from nuclear extracts was bound only to hsp70. Hsp70 was subsequently identified as a component of the untransformed GR heterocomplex in CHO cells (143) and the human AR heterocomplex in prostatic carcinoma cells (341, 558), and it was recovered with a fusion protein of β-galactosidase and the ER HBD expressed in COS-1 cells (400).

Unlike hsp90, hsp70 is not dissociated from receptors by either hormone treatment or salt (141–143, 559). Although it remains associated with the transformed receptor when it binds to DNA (559), its presence does not affect DNA binding activity (560). Kost *et al.* (141) found that the PR·hsp70 complex could be disrupted by incubating the immunopurified PR heterocomplex with ATP at 23 C, consistent with the observation that ATP hydrolysis facilitates hsp70 release

from the chaperoned protein (553). The stoichiometry of hsp70 with respect to the receptor is not well established, but the estimates available suggest that it is present in substoichiometric amounts (142, 324, 556). The site of hsp70 interaction with the receptor is the HBD (400, 408). As with the binding of hsp90, Schowalter *et al.* (408) found it necessary to delete essentially the entire HBD before PR binding to hsp70 was eliminated. Also, three separate regions of the HBD were able to partially restore hsp70 binding to a PR mutant lacking the HBD. Because hsp90 can be dissociated with salt, leaving receptor bound only to hsp70 (142, 143), it is clear that hsp70 binds directly to the HBD and not indirectly via another protein. In the study of Schowalter *et al.* (408), the binding sites for hsp70 and hsp90 could not be separated and it is thought that they both interact with the HBD through interactions at multiple locations or through some structural quality (hydrophobicity) that is distributed throughout the HBD.

Although hsp70 is required for assembly of receptors into a complex with hsp90 (190, 191), it is not known whether hsp70 plays a role in receptor action or cycling after the heterocomplex is formed. In contrast to the more weakly bound components, hsp90 and the immunophilin FKBP52, hsp70 has not been recovered in cross-linked receptor heterocomplexes (377, 390, 393, 395). Based upon this failure and the fact that some hsp70 is bound to immunomatrix independent of receptor (393), Gehring has argued that hsp70 nonspecifically sticks to the receptors as a persistent contaminant during purification procedures and is not a genuine structural component of the GR·hsp90 complex beyond a transient role in assembly (388). At this time, no other role for hsp70 in receptor action has been defined. Hsp70 has been carefully looked for and not found (at least not beyond trace levels) in GR heterocomplexes immunopurified from mouse L cells (143, 188) where the hormone-free receptor is cytoplasmic (464), yet it is present in mouse GR heterocomplexes immunopurified from CHO cells where the receptor is nuclear (143). This prompted the speculation that hsp70 may be somehow involved in receptor events occurring in the nucleus (143). Hsp70 is required for nuclear import of a number of proteins (561), but it is apparently not required for import of the GR. Yang and DeFranco (562) used a digitonin-permeabilized cell system to demonstrate that an antiserum against hsp70 blocked nuclear import of SV40 large T antigen but not import of GR. This assay is restricted to nuclear import and not to other nuclear events in receptor action and cycling. It is still puzzling that, to our knowledge, hsp70 has not yet been found in receptor heterocomplexes immunopurified from cells in which the untransformed receptors are clearly cytoplasmic in their localization.

There is no evidence that the hsp70 recovered in native receptor heterocomplexes is the hsp70 that was involved in the heterocomplex assembly. Hsp70 binds to many proteins and it can bind to receptors in the absence of other proteins. It has been shown, for example, that hsp70 homologs from bacteria (DnaK) and the endoplasmic reticulum (BiP) bind to GR in a manner that is nonproductive with respect to heterocomplex assembly (563). Gehring's (388, 393) reservations with respect to hsp70 not being a necessary structural component of the untransformed receptor are correct to the extent that several GR heterocomplexes [*e.g.* mouse L fibroblasts, WEHI mouse lyphoma cells, rat thymocytes, HeLa cells, rat NRK fibroblasts (143, 188, 393, 395, 562)] are not associated with hsp70 and are, of course, fully functional with respect to hormone response. His reservations are also correct in that hsp70 binding to receptors does not necessarily reflect a physiologically meaningful interaction (563). It is entirely possible that the hsp70-bound receptors represent a fraction of the receptors that are in the process of heterocomplex assembly or are for some reason (perhaps pertaining to their nuclear location) held up in the assembly process. At this time, we feel it would be inappropriate to dismiss the presence of hsp70 in receptor heterocomplexes simply because it has not been cross-linked to the receptors in native receptor heterocomplexes.

B. p23

When rapid, gentle procedures were first used to immunoadsorb receptor heterocomplexes, a 23-kDa protein was isolated with the avian PR (142) and the murine GR (188). Smith *et al.* (142) showed that p23 behaved on two-dimensional gels as a very acidic protein with some charge heterogeneity, and like hsp90, it was released from the PR upon progesterone treatment *in vivo* or salt treatment *in vitro*. Johnson *et al.* (564) prepared antibodies against p23 and showed that it was present in all tissues tested and in several species from yeast to humans. A full-length human cDNA was isolated and found to be unique. Antibodies against p23 immunoprecipitated the protein in complex with hsp90 and could be used to isolate PR complexes from chick oviduct cytosol (564). Johnson and Toft (565) found that immunoadsorption of p23 from reticulocyte lysate in the absence of PR resulted in coisolation of a complex containing hsp90 and immunophilins. It has now been shown that p23 binds directly to hsp90 through a process that requires ATP (510, 566). It seems that the p23·hsp90 association must be highly conserved, because purified human p23 forms a complex with plant hsp90 when it is added to wheat germ extract (567). Again, complex formation requires ATP (567). It is curious that neither p23 (565) nor hsp90 (295) contain consensus ATP-binding sites and the site of ATP binding, and perhaps hydrolysis, required for forming the p23·hsp90 complex is not yet determined.

C. Immunophilins

The immunophilins are ubiquitous and conserved proteins that bind immunosuppressant drugs, such as FK506, rapamycin, and cyclosporin A (for review, see Refs. 568–570). All members of the immunophilin family have peptidylprolyl isomerase (PPIase) activity, suggesting that they may play a role in protein folding in the cell (571). The immunophilins may be divided into two classes; the FKBPs are binding proteins for compounds like FK506 and rapamycin, and the cyclophilins (CyPs) bind cyclosporin A. The immunosuppressant drugs occupy the prolyl isomerase sites on the immunophilins and inhibit the *cis-trans* isomerization of peptidyl-prolyl bonds *in vitro*. As yet, there is only limited evidence that immunophilins play a role in protein folding

in vivo (572). Almost all of the information available on immunophilins relates to the low molecular weight members, such as FKBP12 and CyP-18, which are thought to be the cellular components responsible for inhibition of T cell activation.

Three higher molecular weight immunophilins have been discovered as components of steroid receptor complexes. Unlike the small immunophilins, these larger immunophilins possess several TPR domains and a calmodulin-binding domain in their C-terminal half. TPR domains were first identified by Sikorski *et al.* (573) as degenerate consensus sequences of 34 amino acids. The TPR domains are often arranged in tandem repeats, and they are thought to be sites where intra- and intermolecular interactions occur (for review, see Ref. 574). It has been shown that hsp90 binds a variety of proteins that contain from three to seven TPR domains, including the three immunophilins in steroid receptor complexes, the phosphatase PP5, the stress-related protein p60, and some proteins not found in steroid receptor heterocomplexes (531). Thus, it is predicted that hsp90 has a universal TPR domain-binding region that permits it to bind multiple proteins, with the TPR proteins recovered in steroid receptor heterocomplexes being a subset of these proteins (531). The binding of immunophilins to hsp90 via TPR domains is conserved in plants as well as in the animal kingdom (567), suggesting that this may be a basic protein interaction critical to the function of the high molecular weight immunophilins.

1. The FKBPs. FKBP52 was discovered in 1984 by Faber and his co-workers when they used the partially purified molybdate-stabilized, 9S, rabbit PR as an antigen to prepare the EC1 monoclonal antibody (575). This antibody recognized a 59- kDa rabbit protein and it bound to the 9S but not to the 4S PR (576). In addition to immunoadsorbing the 59-kDa protein, EC1 coimmunoadsorbed a 92-kDa protein (577) that was subsequently shown to be hsp90 (546). The EC1 antigen, which at that time was called p59, was shown to be associated with PR, AR, ER, and GR, establishing it as a common component of untransformed receptor heterocomplexes (577, 578). Sanchez *et al.* (546) identified the EC1-reacting protein in human IM-9 lymphocyte cytosol as a 56-kDa protein with several isomorphs that was present in a cytosolic complex with hsp90 and some hsp70. Because both heat shock and chemical stress increased its rate of synthesis, Sanchez called the protein hsp56 (579).

In 1992, it was established both by binding to an FK506 affinity matrix and by amino acid sequence that this receptor-associated protein was an FKBP. The initial observation relating to affinity isolation of FKBP52 was made when Fretz *et al.* (580) synthesized FK506 and rapamycin affinity matrices and isolated a ~60-kDa protein from human Jurkat cell extracts. Yem *et al.* (581) then showed that this 60-kDa protein possessed the N-terminal sequence previously reported by Sanchez *et al.* (546) for p59/hsp56. A peptide derived from this 60-kDa protein was homologous to a region near the C terminus of both FKBP12 and FKBP13 (581). Tai *et al.* (582) used the FK506 affinity matrix to isolate a 59-kDa protein from IM-9 lymphocyte cytosol that reacted with the EC1 antibody. Importantly, hsp90, hsp70, and the GR were all coisolated with the FKBP (582). In a different approach, Wiederrecht *et al.* (583) purified a 51 kDa [^{3}H]dihydroFK506 binding protein from Jurkat cells that by sequence analysis was similar or identical to p59/hsp56.

The first cloning of FKBP52 was by Lebeau *et al.* (584) who used the EC1 antibody to screen a rabbit liver cDNA library and clone the rabbit protein. The cloned cDNA coded for an open reading frame of 458 amino acids containing a segment between amino acids 41 and 134 of the protein that had 55% amino acid homology to the PPIase FKBP12. As noted by Tai *et al.* (582), all 10 amino acids in the FK506-binding site of FKBP12 were conserved in the rabbit p59/hsp56. Subsequently, human (585) and mouse (586) cDNAs were cloned and expressed. The human protein had a calculated M_r of 51,810 and Peattie *et al.* (585) called it hFKBP52. The purified, bacterially expressed human protein was shown to have PPIase activity that was inhibited by FK506, and Northern analysis demonstrated expression in all human tissues tested (585). FKBP52 is expressed in all mammalian species tested, and in cultured cells, FKBP52 gene expression appears to respond to mitogenic growth factors in a manner similar to the delayed early response genes (587).

Unfortunately, various laboratories have called the protein by various names, including p59 (577), p56 (546), hsp56 (579), FKBP51 (583), hFKBP52 (585), FKBP59 (582), HBI (588), and p59-HBI (589), with HBI referring to hsp-binding immunophilin. In an attempt to standardize the nomenclature, we are using the human designation FKBP52 to refer to the protein in all species. It is important to note that two proteins of 50 and 54 kDa reported by Smith *et al.* (142) to copurify with the chicken PR are also FKBPs. Peptides derived from both the avian p50 and p54 possessed high homology with rabbit FKBP52 (590), and both proteins bound to an FK506 affinity resin (591). The avian p50 is recognized by rabbit antisera against mammalian FKBP52 and seems to be its homolog in the chicken (591, 592). The avian p54 is a distinct protein, and cloning and sequencing of the cDNA for the human homolog of FKBP54 (593) shows its identity with mouse FKBP51 (594, 595). We will use the term FKBP51, matching its calculated molecular weight. This novel immunophilin has been reported in both avian (591) and human (596) PR heterocomplexes.

A structural modeling of rabbit FKBP52 by Callebaut *et al.* (588) reveals three globular domains followed by a C-terminal portion containing a predicted calmodulin-binding domain. The N-terminal domain possesses the highest homology (49%) with FKBP12 (588). When expressed alone, domain I has PPIase activity *in vitro* (589), and a proteolytic fragment comprising domain I binds FK506 (597). The full-length FKBP52 appears to behave as a dimer (583), and domain I alone behaves as a dimer (597), suggesting that it may possess the dimerization site. Domain II has less homology (28%) with FKBP12 (588) and virtually no PPIase activity (589). A consensus nucleotide-binding sequence was detected in domain II (588), and bacterially expressed domain II was shown to bind in a specific manner the azido analogs of ATP and GTP (598). Domain III comprises three TPR domains, and deletion of this domain abrogates binding to hsp90 (599). The predicted calmodulin-binding sequence of the C terminus of FKBP52 is apparently functional, as the

protein is retained by calmodulin-Sepharose in the presence of calcium and is eluted with EGTA (600).

When the stoichiometry of FKBP in receptor heterocomplexes was determined on the basis of the size of cross-linked complexes, a ratio of one molecule of receptor and two molecules of hsp90 to one molecule of FKBP52 was obtained for GR (393), PR (390), and ER (377). However, when stochiometry of immune-purified avian PR heterocomplexes was determined from stained gels, the ratio was one molecule of PR to 0.56 molecule of FKBP52 (*i.e.* avian p50) (142). A ratio of 0.55 molecule of FK506-binding sites to steroid-binding sites was determined for immunoadsorbed GR heterocomplexes (397). The cross-linking method gives an accurate FKBP52 stoichiometry for a cross-linked product of a particular size detected with a steroid affinity label, but this technique does not differentiate between receptor complexes that contain FKBP52 and receptor complexes that contain other molecules of a similar size instead of FKBP52. This has become an important issue because it is now clear that there are multiple receptor heterocomplexes, depending upon the protein (FKBP52, FKBP51, CyP-40, PP5) that is occupying the common TPR domain-binding site on hsp90 (531, 601–603).

From their cross-linking study, Renoir *et al.* (578) concluded that in the untransformed receptor complex FKBP52 contacted hsp90 but not the receptor itself. However, Gehring and co-workers (391, 393) have isolated small amounts of a 150-kDa product from the cross-linked GR heterocomplex that likely contains the GR plus an immunophilin, suggesting proximity of the receptor and the immunophilin in the heterocomplex (388). Salt-mediated dissociation of immunophilins from receptors is inhibited by molybdate and tungstate (142, 411, 578, 601). Despite the fact that molybdate interacts with hsp90 (see *Section IV.C.2*), it does not stabilize an FKBP52·hsp90 complex unless the receptor is also present (411). Purified hsp90 and purified FKBP52 bind to each other in a reversible equilibrium, and purified FKBP52 forms a complex with the hsp90-bound GR but not with the hsp90-free GR (411). Thus, any potential direct interaction between the FKBP and the receptor protein must be much weaker than the FKBP·hsp90 interaction.

2. CyP-40. CyP-40 was discovered in 1990 by Ratajczak *et al.* (604) who used an estrogen-derivatized affinity column to purify ER from calf uterine cytosol and copurified hsp90 and two proteins of M_r 38,000 and 22,000. When the Ratajczak laboratory cloned and sequenced the cDNA encoding the 38-kDa protein (605), they found it to be the same as a 40-kDa protein isolated by Kieffer *et al.* (606) by adsorption of calf brain cytosol onto a cyclosporin A affinity column. The calf brain protein was called CyP-40, and in a second report Kieffer *et al.* (607) cloned the human cDNA. Cyp-40 has now been identified in mammalian GR (531, 601, 608) and chicken PR (596) heterocomplexes. CyP-40 is more weakly bound to the GR heterocomplex than FKBP52 but, like FKBP52, it is stabilized in the heterocomplex by molybdate (601).

The N-terminal half of CyP-40 has a high homology with CyP-18 and contains the PPIase site, and the C-terminal half shares about 30% identity with domain III of FKBP52 (605, 607). Like FKBP52, CyP-40 possess a potential calmodulin-binding site at the extreme C terminus. Ratajczak *et al.* (605) were the first to recognize that the homologous regions in the C-terminal half of FKBP52 and CyP-40 contain three TPR domains. Radanyi *et al.* (599) were the first to show that the FKBP52 TPR domain mediates its binding to hsp90, and Hoffmann and Handschumacher (609) then showed that the TPR domain of CyP-40 mediates binding to hsp90.

3. A common immunophilin-binding site on hsp90. It has been known for several years that isolation of FKBP52 from cytosol by immunoadsorption (546) or by FK506 affinity chromatography (582) yielded coretention of hsp90 and that immunoadsorption of hsp90 (547) or passage of cytosol through a matrix of immobilized hsp90 (360) yielded coretention of FKBP52. However, these complexes contained hsp70 and sometimes other proteins; thus, direct binding studies were performed. Using purified proteins, it was shown that FKBP52 (411) and CyP-40 (601) bind directly to hsp90, CyP-40 competes for FKBP52 binding to hsp90 (601), and FKBP52 competes for CyP-40 binding to hsp90 (602). Immunoadsorption of cytosol with anti-FKBP52 antibody or passage of cytosol through an FK506 affinity matrix yielded coretention of hsp90 but not CyP-40, and immunoadsorption with anti-CyP-40 or passage through a cyclosporin A affinity column yielded coretention of hsp90 but not FKBP52 (531, 601, 608). These observations all support the conclusion that there is a common immunophilin-binding region on hsp90 and that the immunophilins exist in separate complexes with hsp90.

There is good evidence that the major determinant for immunophilin binding to hsp90 is its TPR domain. Radanyi *et al.* (599) showed that bacterially expressed mutants of FKBP52 lacking the three TPR units did not bind hsp90 in a gel retardation assay. Hoffmann and Handschumacher (609) prepared fusions of glutathione *S*-transferase with deletion mutants of CyP-40 and measured hsp90 retention by the fusion proteins immobilized on glutathione-agarose. They found that a CyP-40 fragment containing the three TPR domains and the C-terminal calmodulin-binding domain bound hsp90. Ratajczak and Carrello (602) used a similar approach and concluded that most efficient retention of hsp90 required the TPR domains plus short flanking regions of acidic and basic residues clustered at the N- and C-terminal ends of the TPR domain. Finally, it has been shown that binding of both FKBP52 and CyP-40 to hsp90 is competed by the purified fragment of human CyP-40 comprising its three TPR domains (531) and by a fragment of rat PP5 comprising its four TPR domains (603). As noted by Ratajczak and Carrello (602), it is likely that regions bordering the TPR domains will participate in the binding to hsp90, but the TPR domains are critical.

There appears to be a relationship between the number of TPR domains in a protein and its hsp90-binding affinity. Owens-Grillo *et al.* (531) have shown, for example, that proteins with six or seven TPR domains bind more tightly than FKBP52 or CyP-40, each of which has only three TPR domains. It is also clear that TPR domain proteins that are not immunophilins, such as PP5, p60, and the mitochondrial

import protein Mas70p, bind to the same region of hsp90. For example, p60 has six to eight TPR domains, and it competes for the binding of all of these proteins to hsp90 (531). Thus, it has been proposed that hsp90 has a site that acts as a universal TPR acceptor, permitting it to bind to multiple proteins (531). It has also been shown that a plant FKBP binds to wheat hsp90 in a manner that is competed by the human CyP-40 fragment containing its TPR domains (567). This suggests that the ability of hsp90 to bind TPR domain proteins to this site may be a conserved interaction important for its biological function.

4. Effects of immunosuppressants on steroid receptor functions. When they are bound by cyclosporin A or FK506, the small immunophilins, CyP-18 and FKBP12, competitively bind to and inhibit the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin (610). Calcineurin inhibition prevents activation of specific transcription factors, such as NF-AT and NF-IL2A, involved in lymphokine gene expression required for T cell activation (611). Thus, the effect on calcineurin is felt to underlie the molecular basis of cyclosporin A and FK506 immunosuppression. CyP-40 competes for binding of CyP-18 to calcineurin in the presence of cyclosporin A, and cyclosporin A-bound CyP-40 inhibits calcineurin protein phosphatase activity, albeit more weakly than cyclosporin A-bound CyP-18 (607). On the other hand, FK506-bound FKBP52 does not complex with or inhibit the phosphatase activity of calcineurin (583, 612). Thus, it seems unlikely that calcineurin phosphatase inhibition is related to the function of the large immunophilins that complex with hsp90.

FK506 and cyclosporin A can bind to FKBP52 and CyP-40 when they are in the GR and PR heterocomplexes, and the immunosuppressants do not affect heterocomplex composition (397, 582, 608, 613). Neither FK506 (397) nor cyclosporin A (601) affect GR heterocomplex assembly by cells or by the reticulocyte lysate system. Thus, it is clear that peptidylprolyl isomerase activity of the immunophilins is not required for GR·hsp90 heterocomplex assembly and proper folding of HBD by the hsp90-associated protein-folding system. FK506 was not found to affect steroid binding by the GR (397), and only the most minimal reduction in K_d (~50% for both the agonist Org258 and the antagonist RU38486) has been reported for the PR (613).

FK506 treatment of L-929 cells stably transfected with the mouse mammary tumor virus (MMTV)-chloramphenicol acetyl transferase (CAT) reporter plasmid (LMCAT cells) does not modify enhancement of gene expression caused by a high concentration (10^{-6} M) of dexamethasone (397), but at lower concentrations of dexamethasone (10^{-8} or 10^{-7} M), FK506 was found to potentiate expression (614). FK506 alone had no effect on the basal level of CAT expression, hormone-regulated enhancer sequences were sufficient for the FK506-mediated potentiation of dexamethasone action, and the effect was blocked by the antagonist RU38486 (615). A similar enhancement of dexamethasone-induced CAT gene expression was shown with cyclosporin A in LMCAT cells (608). FK506 was also shown to potentiate reporter gene induction in yeast containing the human PR (615). Milad *et al.* (596) found that cyclosporin A potentiated the response of T47D human breast carcinoma containing MMTV-CAT to the synthetic progestin R5020. In contrast to its effect in T47D cells, cyclosporin A inhibited a PR/reporter gene system in L cells. FK506 had no effect on progestin action but it potentiated glucocorticoid action in T47D cells (596). Thus, the effects of the immunosuppressants vary with both the cell type and the hormonal system that is tested.

Although it seems clear that both classes of immunosuppressant drugs can modify biological responses to progestins and glucocorticoids, there is no evidence, as yet, that their effects are due to any action on the immunophilins in receptor complexes. Indeed, it is very likely that FK506 potentiation of steroid-mediated expression does not involve immunophilins at all but, instead, reflects the ability of FK506 to increase the intracellular concentration of steroid by competing for an outward transport system that clears certain steroids from the cell. In L cells, for example, where both FK506 (614) and cyclosporin A (608) potentiate dexamethasone response at subsaturating levels of hormone, there exists an energy-dependent outward transport system for dexamethasone and some other steroids (616, 617). In yeast, it has been shown that an ATP-binding-cassette transporter selectively decreases the intracellular levels and response to some steroids (618), much as the multidrug transporter (Mdr) can be responsible for decreased sensitivity to some anticancer drugs (for review, see Ref. 619). In fact, Burgeois *et al.* (620) have shown that Mdr1 transports dexamethasone and can determine cell sensitivity to the steroid. Mdr1 also binds and transports FK506 and cyclosporin A (621, 622), both of which can reverse multidrug resistance (623, 624). Kralli and Yamamoto (625) have shown that FK506 potentiates dexamethasone responsiveness in both yeast and L cells, and in L cells, they showed that FK506 increases dexamethasone accumulation without altering the hormone-binding properties of the GR. This rather nonspecific ability of the immunosuppressants to increase the intracellular concentration of steroid likely explains the potentiation of steroid response observed in various cell systems.

5. Potential role for immunophilins in protein trafficking. The notion that receptor association with hsp90 may be required for its trafficking or for receptor attachment to a trafficking machinery has been discussed above (see *Section VI.I.2*). Pratt *et al.* (412) proposed that the FKBP52 component of the receptor heterocomplex may play a role in targeting receptor movement to the nucleus. It was noted that FKBP52 contains a sequence of eight amino acids [EDLTDDED, rabbit (584)] with six negatively charged residues that is located in a short-hinge segment between domains I and II predicted by Callebaut *et al.* (588). This sequence is retained with conservative replacements in human (585) and mouse (586) FKBP52, and it is electrostatically complementary to the receptor NLSs [*e.g.* the NL1 sequence RKTKKKIK of rat GR (445)]. Renoir *et al.* (592) raised an antibody against this conserved negatively charged sequence, and, consistent with a possible role for FKBP52 in targeting receptor movement, Czar *et al.* (530) showed that injection of the antibody into L cells impeded subsequent dexamethasone-mediated shift of the GR into the nucleus.

The fact that FKBP52 is localized predominantly in the

nucleus (371, 626–628) is also consistent with a possible role for the immunophilin in nuclear targeting of receptor movement. It was shown by confocal imaging in a variety of cell types that FKBP52 is distributed in a mottled pattern throughout all planes of the nucleus but is excluded from nucleoli (626–628). In CHO cells that are overexpressing the mouse GR, the untransformed receptor is also distributed in a mottled pattern throughout the nucleus (528). It has been shown by double labeling and confocal imaging that the GR and FKBP52 colocalize in the same nonrandom pattern throughout the nonnucleolar space of the CHO cell nuclei (627). The minority of FKBP52 that is cytoplasmic colocalizes with microtubules, and the distribution of FKBP52 is not affected by FK506 (627, 628). Perrot-Applanat *et al.* (628) have performed immunofluorescence localization with two different anti-FKBP52 peptide antibodies, one of which yielded a stronger signal in the cytoplasm than in the nucleus, with the reverse being the case for the other antibody. This suggests that FKBP52 may have sites that are masked in one locus but open in another. The cytoskeletal staining of FKBP52 suggests associations with organized networks and would be consistent with the notion that the immunophilin plays a role in receptor trafficking through the cytoplasm to the nucleus.

Although the immunophilins have not yet been shown to bind directly to the receptor proteins themselves, it seems that the receptors determine, to some extent, the proteins that are bound to hsp90 and recovered in the receptor heterocomplex. For example, the native GR·hsp90 heterocomplex contains FKBP52, but p50, another protein that binds to a site on hsp90 that is distinct from the TPR-binding site (603), has not been detected in the receptor heterocomplex (290, 548). However, the native Raf·hsp90 heterocomplex contains p50 but not FKBP52 (290, 629). Thus, the GR and the Raf have made different choices with respect to the hsp90-bound proteins that are recovered in their respective heterocomplexes.

As outlined above, the cellular localization of FKBP52 is consistent with a proposed role in nuclear targeting of receptor movement. Like $pp60^{src}$, which is also complexed with hsp90 and p50 (for review, see Ref. 630), Raf must traffic from its cytoplasmic sites of translation to the inner surface of the plasma membrane where it functions as part of a multicomponent system for normal mitogenic signal transduction through activation of mitogen-activated protein kinase (for review, see Ref. 631). p50 was recently identified as the mammalian homolog of the yeast cell cycle control protein cdc37 (632, 633), and another term for it is $p50^{cdc37}$ (632). p50 does not contain TPR domains, but it appears to bind to a site near the TPR-binding site on the surface of hsp90 (603) such that an immunophilin and p50 cannot be simultaneously bound to hsp90 (531). The cellular localization of p50 is consistent with the notion (290, 531) that it targets movement of Raf and Src to their sites of action. The p50 extends on cytoskeletal fibrils from a concentration in the perinuclear region out to the cell periphery where some of it is localized at the inner surface of the plasma membrane (531). In contrast to FKBP52, which is predominantly nuclear but excluded from nucleoli, CyP-40 localizes predominantly within nucleoli, perhaps consistent with a role for targeting protein traffic to that organelle (531).

To date, no function for the immunophilins in receptor heterocomplexes has been defined. In some cases there may be no function. CyP-40, for example, is found in only a small number of GR·hsp90 heterocomplexes compared with FKBP52, and its cellular localization is not consistent with an action on the receptor. It has been proposed that the immunophilins may play a role in receptor heterocomplex assembly. However, GR heterocomplexes have been assembled with purified proteins in the absence of immunophilins (634). If the major role of the receptor-associated immunophilins were to chaperone protein folding in the cell, then it follows that the nucleus is the major site of FKBP52 protein-folding action and the nucleolus is the major site of CyP-40 action because that is where the majority of each protein is located. A role for the immunophilins in receptor folding/heterocomplex assembly does not seem consistent with the known cell biology of the receptors. The notion that TPR domains are somehow involved in protein targeting is derived from observations in several systems (for review, see Ref. 635), and in the absence of evidence to the contrary, a targeting role for the immunophilin components of receptor heterocomplexes should be considered.

6. PP5 and the potential for redundancy of immunophilin action. Both an FKBP52 homolog (636) and a CyP-40 homolog (637) have been recovered in complexes with hsp90 in yeast. Because yeast are suitable for the genetic studies that will be required for determining the function of immunophilins *in vivo*, it is important to note that none of the immunophilins examined to date appear to be essential for the viability of haploid yeast cells (570). The lack of essentiality may reflect a level of redundancy that covers the loss of one or more of these proteins, and this could seriously complicate defining the role of immunophilins in receptor action. With respect to the receptor heterocomplexes, the function of FKBP52 could be redundant with that of PP5, a newly identified component of receptor heterocomplexes.

Several laboratories have isolated cDNA clones for a protein-serine phosphatase designated PP5 (638–640). PP5 has four TPRs in its N-terminal domain and a C-terminal catalytic domain with protein phosphatase activity. It is recovered from cell lysates in native complexes with hsp90 via its TPR domain, and it is recovered from L cell cytosol with the native GR·hsp90 heterocomplex (641). Both intact PP5 and its expressed TPR domain compete for immunophilin binding to hsp90, and native hsp90 immunophilin complexes do not contain PP5 (603). These observations are consistent with mutual competition between immunophilins and PP5 for a common binding region on hsp90. Like FKBP52, PP5 is localized predominantly in nucleus where it is distributed in a similar mottled pattern and is excluded from nucleoli (639). Interestingly, PP5 is retained by an FK506 affinity matrix (603), suggesting that it has a weak FK506 binding affinity. PP5 is present in untransformed GR heterocomplexes from cells where the receptors are cytoplasmic as well as from cells where they are nuclear (603). In contrast to the low levels of CyP-40 that are recovered with native GR heterocomplexes, there are 0.35 molecule of PP5 per molecule of L cell GR (603).

VIII. The Receptor Heterocomplex Assembly Mechanism

Early attempts to form receptor·hsp90 complexes, *e.g.* by eliminating the salt after dissociation of cytosolic heterocomplexes or by mild urea treatment and slow renaturation of purified receptor and hsp90, were unsuccessful because the binding is not a simple equilibrium interaction between the two components (141, 193, 642). There are, however, three reports in which ~9S receptor complexes were formed under cell-free conditions in an ATP-independent manner. In one report by Inano *et al.* (643), purified ER was mixed at 40 C with a ~1000-fold excess of hsp90 in a buffer containing Chaps detergent, dimethylformamide, and thiocyanate, and the mixture was then dialyzed against molybdate buffer. Although the product was ~9S by density gradient analysis, it was later reported by the same laboratory (644) that association by this method with hsp90 strongly stimulated the binding of the ER to its response element, suggesting that the heterocomplex they formed did not behave like the native 9S receptor. In seemingly direct contrast, Sabbah *et al.* (645) reported that purified hsp90 inhibits the binding of the ER to its response element, much as purified hsp90 had been reported earlier to inhibit GR binding to DNA-cellulose (646). Sabbah *et al.* (645) also found that purified hsp90 caused dissociation of ER that was prebound to response elements, generating a form of the ER that sedimented at 9S. The ER binds to its response element as a tightly associated homodimer, and in the work of Sabbah *et al.* (645) it was not determined whether the 9S complex released from DNA possessed the same stoichiometry (1 receptor:2 hsp90) as determined by cross-linking for native complexes (Ref. 377; see *Section VI.B*) or whether it represented binding of an ER dimer to a dimer of hsp90 (340). Third, Tuohimaa *et al.* (484) reported that homogenization of unliganded hsp90-free PR with bursa of Fabricius at 4 C produced a complex that sedimented at ~9S, but no attempt was made to determine whether the complex had the properties of the untransformed receptor heterocomplex.

A. The receptor·hsp90 heterocomplex assembly activity in reticulocyte lysate

Because early attempts to form a 9S GR·hsp90 complex under cell-free conditions were unsuccessful, Denis and Gustafsson (647) translated the rat GR in rabbit reticulocyte lysate and showed that the translation product behaved as a native 9S complex in that it had steroid-binding activity and did not bind to DNA. They also showed that dexamethasone promoted the temperature-dependent conversion of the cell-free translated GR to the DNA-binding form and that molybdate prevented conversion of the 9S complex to 4S. Dalman *et al.* (642) then used a direct assay to show that rat GR became bound to hsp90 just at the termination of receptor translation in reticulocyte lysate. The newly translated GR bound steroid with the same affinity as the native receptor, and again, it was in a non-DNA-binding form that could be transformed by steroid to the DNA-binding state. Schlatter *et al.* (410) translated the GR HBD in lysate, showing that it alone was sufficient for formation of the heterocomplex with hsp90. Although the wild-type rat GR translated in rabbit reticulocyte lysate was functionally indistinguishable from the native 9S receptor, the same GR translated in wheat germ extract behaved like a 4S receptor, in that it did not bind steroid and it bound to DNA without requiring transformation (344, 642). Thus, it was assumed that the wheat germ lysate could not assemble the GR·hsp90 heterocomplex. We now know that heterocomplexes with plant hsp90 are assembled, but it appears that the rate of their disassembly in wheat germ lysate is very rapid with respect to the rate of their disassembly in rabbit reticulocyte lysate (241).

At the time these studies were performed, the Pratt laboratory assumed that binding of the GR to hsp90 was somehow linked to the process of receptor translation in reticulocyte lysate. The Toft laboratory made a major advance by showing that immunoadsorbed chicken PR that had been stripped of chaperone proteins by incubation with salt and ATP could be assembled into a heterocomplex with rabbit hsp90 and hsp70 when the immune pellet was incubated with reticulocyte lysate (193). Thus, the reticulocyte lysate contained a system that would assemble a receptor·hsp90 heterocomplex independent of protein translation. Smith *et al.* (193) showed that the PR HBD was required for heterocomplex assembly in this lysate system and that no heterocomplex formation could be detected if the PR was bound by progesterone. It is still not clear whether progesterone binding to the receptor negates recognition of the HBD by the chaperone proteins or whether heterocomplexes are formed between hsp90 and the steroid-bound receptor, but they are more rapidly disassembled than complexes between hsp90 and the hormone-free receptor.

Because the GR must be bound to hsp90 for its HBD to be in a steroid-binding conformation (see Section *VI.E*), generation of glucocorticoid-binding activity could be used as a functional assay to show that the receptor HBD in the heterocomplex assembled in reticulocyte lysate was in the same "folding" state as the HBD of the native GR·hsp90 heterocomplex. Scherrer *et al.* (194) showed that the hormone-free mouse GR became associated with both rabbit hsp90 and hsp70 upon incubation with reticulocyte lysate, and heterocomplex assembly was accompanied by restoration of steroid-binding activity and conversion of the receptor to the non-DNA-binding state. In addition to hsp90 and hsp70, receptor heterocomplexes assembled in reticulocyte lysate contained the p23 and immunophilin (FKBP52) components of native receptor heterocomplexes (190, 397). Thus, by both functional and structural criteria, the rabbit reticulocyte lysate contained a system that assembled receptors into heterocomplexes that were indistinguishable from native 9S, untransformed receptors.

B. Components of the heterocomplex assembly system

The initial studies of heterocomplex assembly in reticulocyte lysate had shown only that the process was temperature-dependent (193, 194), but it was soon demonstrated that ATP and Mg^{2+} were required (187, 190, 648). It was also shown that dialysis of reticulocyte lysate inactivated its heterocomplex-reconstituting activity and that both ATP/Mg^{2+} and a monovalent cation were required to restore the activity (187).

Indeed, heterocomplex assembly was shown to have a strict monovalent cation selectivity, with K^+, NH_4^+, and Rb^+ permitting assembly and Na^+ and Li^+ being inactive (187).

The generation of steroid-binding activity in reticulocyte lysate was directly related to the extent of GR·hsp90 complex formation (187). Later, Smith (192) showed that hsp90-free PR lost its steroid-binding activity when incubated at elevated temperatures and that the binding activity could be restored by assembling the PR·hsp90 complex with reticulocyte lysate. Thus, generation of steroid-binding activity is now being used with both receptors as a rapid functional assay to determine conditions required for heterocomplex assembly that can be later confirmed by the much slower procedure of assaying chaperone binding to the receptors.

Because very large amounts of PR can be immunoadsorbed from oviduct cytosol of estrogen-treated chickens, both heterocomplex intermediates and the final components of the heterocomplex can be detected by Coomassie blue staining of the electrophoretically resolved proteins in receptor immune pellets. This cannot be readily done with the GR, and the assembly of PR heterocomplexes by reticulocyte lysate has been the most useful system for determining the components of the assembly process. Using this approach, Smith *et al.* (190) found that a 60-kDa protein (p60) was bound when the PR immune pellet was incubated with reticulocyte lysate that was not supplemented with ATP. When an ATP-regenerating system was added to the lysate, the amount of PR-associated p60 and hsp70 declined, and the amount of hsp90 and p23 increased (190). In a careful study of the time course of heterocomplex assembly, Smith (192) found that p60 and hsp70 were recovered with the PR immune pellet immediately, with peak binding observed at ~4 min and recovery of both proteins decreasing as the incubation was continued at 30 C. In contrast, recovery of p23 was not evident until 2 min, and it rapidly reached a plateau from which there was no decline.

These observations formed the basis for the model shown in Fig. 1 where the receptor forms an initial tight complex with hsp90·p60·hsp70 (step 2) that is followed by dissociation of all of the p60 and variable amounts of the hsp70 and by the tight binding of p23. It is still not clear whether p23 becomes tightly bound to the complex before p60 dissociates or after, as indicated in Fig. 1 (step 3). In his kinetic study, Smith (192) also identified a 48-kDa protein that was recovered with the PR immune pellet with the same time course as hsp70 and p60. Below, we review what is now known about the role of each of these proteins in heterocomplex assembly.

1. hsp70. Because receptor heterocomplexes assembled in reticulocyte lysate contained hsp70 as well as hsp90 (187, 190, 193, 194) and because at least a portion of hsp70 and hsp90 in cytosols and reticulocyte lysate was known to exist in hsp heterocomplexes independent of receptors (189, 190, 546–548), it was reasonable to ask whether hsp70 was required to bind hsp90 to the receptor. Smith *et al.* (190) showed that addition of a monoclonal anti-hsp70 antibody to reticulocyte lysate inhibited PR·hsp90 heterocomplex assembly. Hutchison *et al.* (191) depleted lysate of hsp70 by adsorption with ATP-agarose, which binds hsp70 with high affinity. The resulting hsp70-depleted lysate did not assemble GR·hsp90 complexes but it could be reactivated by adding purified hsp70. Purified hsp70 alone can bind to the GR, but there is no activation of steroid-binding activity unless hsp90 and the other components of the heterocomplex assembly system are present (191). It is not known whether the binding of purified hsp70 alone to the receptor is related to the binding of hsp70

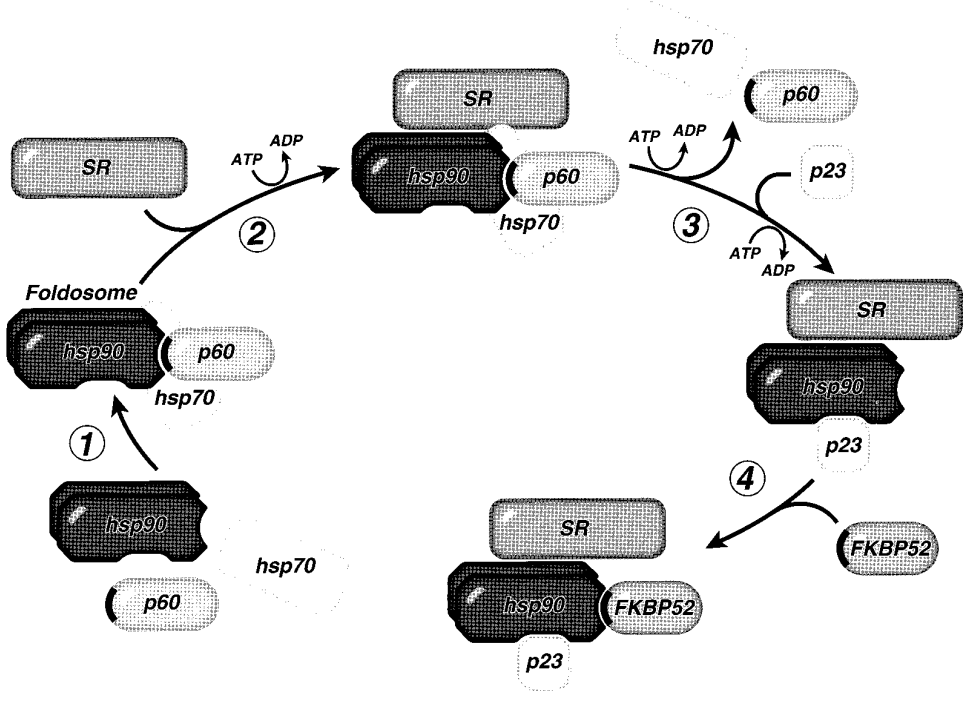

FIG. 1. Model of receptor·hsp90 heterocomplex assembly. The model presents the minimal system for steroid receptor heterocomplex assembly discussed in detail in *Section VIII*. ATP-dependent association of the receptor (SR) with the hsp90·p60·hsp70 folding complex (*foldosome*) yields an intermediate (SR·hsp90·p60·hsp70) complex that is unstable in the absence of p23 or molybdate. A region on hsp90 binds TPR domain proteins, such as p60 and the immunophilins (*e.g.* FKBP52, CyP-40, etc.), with the TPR domain being indicated by the *solid black crescent* on each protein. When p60 leaves the intermediate complex, this site is free to associate with the immunophilin.

as a component of the complete heterocomplex assembly system (563).

It is thought that the monovalent cation selectivity of receptor·hsp90 heterocomplex assembly is related to the role of hsp70 in the process. Schlossman *et al.* (649) first showed that the clathrin-uncoating ATPase activity of hsp70 requires a monovalent cation, and Palleros *et al.* (553) have shown that the ATP-induced dissociation of hsp70 from the protein being chaperoned requires K^+.

2. p60. Smith *et al.* (189) immunopurified hsp90 from oviduct cytosol, coisolating p60, which was then purified by electrophoresis and used to generate peptides for N-terminal sequencing. The sequences showed high homology with a 63-kDa human protein (referred to by the code name IEF SSP 3521) originally identified by Honoré *et al.* (650) as a protein that is up-regulated by viral transformation. The yeast homolog of p60 is the nonessential stress protein Sti1 (651). In a reconstituted reticulocyte lysate system, immune depletion of p60 inactivates heterocomplex assembly, and assembly activity is reactivated by the addition of bacterially expressed human p60 (634). Thus, like hsp70, p60 is required for receptor·hsp90 heterocomplex assembly.

At least part of the role of p60 in the assembly process is to form a tripartite complex containing hsp90, hsp70, and p60 as illustrated in step 1 of Fig. 1. It was noted by Smith *et al.* (189) that immunoadsorption of either hsp90 or hsp70 from reticulocyte lysate resulted in coimmunoadsorption of both the other hsp and p60. Then, it was shown that the F5 monoclonal antibody prepared against p60 coimmunoadsorbed hsp90 and hsp70 (189). It is important to note that purified hsp90 and hsp70 do not bind to each other unless a third factor from reticulocyte lysate is present (411). That factor is now known to be p60 (652), which, like the yeast Sti1 protein (653), contains six to eight TPR domains (650). Direct protein binding studies have shown that p60 competes for the binding of immunophilins to the TPR domain-binding region of hsp90 (531), and mutational analysis of p60 indicates that p60 binds independently to hsp70 via an N-terminal TPR region and to hsp90 via a central TPR-containing region (652).

When the three purified proteins (hsp90, hsp70, and p60) are mixed together, they form a tripartite complex in an ATP-independent manner as shown in step 1 of Fig. 1 (654). The mixture of purified hsp90, p60, and hsp70 is sufficient to convert the GR to the steroid-binding state in an ATP-dependent reaction as illustrated in step 2 of Fig. 1 (654). The complex that is formed is unstable in the absence of p23, and formation of a steroid-binding GR heterocomplex by purified hsp90, p60, and hsp70 is best observed when molybdate is present during assembly to stabilize the complex and when steroid is present during assembly to trap the steroid-binding state of the receptor as each unstable heterocomplex is formed.

3. p23. As discussed in *Section VII.B*. p23 is a unique protein that was originally identified as a component of immunopurified native receptor heterocomplexes, and it forms an ATP-dependent complex with hsp90 independent of the presence of steroid receptors that is stabilized by molybdate. Immunoprecipitation of p23 from cytosols and reticulocyte lysate yields coimmunoprecipitation of hsp90 and variable amounts of hsp70 as well as immunophilins (564, 565). When the PR is incubated with reticulocyte lysate, the resulting heterocomplex contains p23 (190, 193). Immune depletion of p23 from lysate inactivates receptor·hsp90 heterocomplex assembly, and assembly is reactivated by the addition of purified p23 (555).

In a reconstituted system consisting of purified p23, hsp90, hsp70, and lysate from bacteria expressing p60, p23 was required for GR·hsp90 heterocomplex assembly (634). However, as discussed above, functional (*i.e.* steroid binding) GR·hsp90 heterocomplexes can be formed in the absence of p23 when molybdate is present. In the absence of molybdate, assembly of a stable GR·hsp90 heterocomplex requires p23, and all four components (hsp90, hsp70, p60, and p23) must be present simultaneously (634, 654). In wheat germ extract where GR·hsp90 complexes are assembled at a rate similar to reticulocyte lysate but where disassembly is very rapid (241), the addition of purified human p23 markedly increases recovery of a stable GR·plant hsp90 heterocomplex containing p23 (655). At present, it appears that the best notion regarding p23 action is that its binding to hsp90 stabilizes the association of hsp90 with the receptor.

4. p48. The 48-kDa protein recovered at early times of PR heterocomplex assembly in reticulocyte lysate (192) must play some role in the assembly process, but a requirement for p48 has not yet been demonstrated and we have not included it in the scheme of Fig. 1. Prapapanich *et al.* (656) cloned the human cDNA encoding p48 and found the amino acid sequence to be 90% identical to a rat hsc70-interacting protein (Hip) discovered by Höhfeld *et al.* (657). Hip binds to the ATPase domain of hsc70, stabilizing the ADP state of hsc70 that has a high affinity for substrate protein (657). The p48/Hip appears to be ubiquitously expressed, and when it is immunoadsorbed from reticulocyte lysate, substantial amounts of hsp70 and smaller amounts of hsp90 and p60 are coadsorbed (656).

5. hsp40. Mammalian cells contain homologs of the bacterial DnaJ protein, which functions together with DnaK (hsp70) in chaperoning protein folding. Four human DnaJ homologs that range from 38 to 45 kDa have been identified, and it has been suggested that the mammalian DnaJ homologs be called the hsp40 family (658). As discussed in *Section VI.H*, mutations in the yeast DnaJ homolog Ydj1 affect steroid receptor function (451, 499). Thus, it is to be expected that hsp40 will be involved at some point in the receptor-chaperoning process, but to date, hsp40 has not been found either in heterocomplex intermediates or in native heterocomplexes.

C. Effect of geldanamycin on heterocomplex assembly

As discussed in *Section VI.I*, the antibiotic geldanamycin binds directly to hsp90 (505), and it has become a useful reagent for studying hsp90 actions, both *in vivo* and *in vitro*. Johnson and Toft (510) examined the effects of molybdate and geldanamycin on the formation and stability of p23·hsp90 and PR·hsp90 heterocomplexes. When p23·hsp90 complexes were formed in rabbit reticulocyte lysate in the presence of ATP and molybdate, these complexes were com-

pletely disrupted by geldanamycin treatment. Thus, it was concluded that geldanamycin blocked the binding of p23 to hsp90 (510). Geldanamycin affected the formation of PR·hsp90 heterocomplexes in reticulocyte lysate by increasing recovery of hsp70 and p60, decreasing recovery of hsp90, and eliminating p23 and immunophilins (510). At this stage, the notion was that geldanamycin blocked the formation of a p23·hsp90 complex that would normally exchange with the hsp90·p60·hsp70 complex bound to the PR in step 2 of Fig. 1; thus, receptor heterocomplex assembly would be unable to progress from the PR·hsp90·p60·hsp70 intermediate.

Smith *et al.* (511) examined the effect of geldanamycin on PR heterocomplex assembly by reticulocyte lysate and the effect of geldanamycin treatment of intact COS cells on the composition of PR heterocomplexes subsequently recovered from cytosol. In both cases they found that PR immune complexes contained increased amounts of hsp70 and p60, with decreased hsp90, and no p23. In both the lysate and the intact cell, geldanamycin treatment rapidly inactivated the steroid-binding capacity of the PR. Subsequently, it was shown that geldanamycin treatment of intact L cells also yielded a GR·hsp90 heterocomplex containing p60 and hsp70, but without steroid-binding activity (534), and that geldanamycin treatment of cells expressing Raf-1 yielded a Raf-1·hsp90 heterocomplex containing p60 and hsp70 (507).

Like Johnson and Toft (510), Smith *et al.* (511) thought it likely that the geldanamycin arrest of PR assembly was due to the antibiotic's ability to block p23 binding to hsp90. It should be noted, however, that in a reconstituted system where GR heterocomplex assembly is carried out in the absence of p23, geldanamycin blocks assembly such that the receptor accumulates at a GR·hsp90·p60·hsp70 complex that does not have steroid-binding activity (654). Thus, geldanamycin binding to hsp90 both blocks receptor heterocomplex assembly with the accumulation of a p60-containing intermediate state and blocks p23 binding to hsp90, but receptor heterocomplex assembly is not necessarily blocked at the p60-containing intermediate only because p23 cannot bind to hsp90.

D. The concept of a dynamic heterocomplex assembly machine

Because the hsp90, hsp70, and immunophilin components of receptor heterocomplexes were found to be coimmunoadsorbed from cytosols and reticulocyte lysate in multiprotein hsp complexes independent of the presence of receptors (189, 546–548), Scherrer *et al.* (659) investigated whether the proteins required for receptor heterocomplex assembly were preassociated in reticulocyte lysate. A large protein complex was isolated from reticulocyte lysate by fractional precipitation with ammonium sulfate (30–50%) and chromatography through Sepharose CL-6B. This complex, which contained both hsp90 and hsp70, had a very low but demonstrable GR heterocomplex-reconstituting activity (659). However, it was also found that a component of lysate present in the 50% ammonium sulfate supernatant was required for extensive GR·hsp90 heterocomplex reconstitution activity (659), and this component was later identified as p23 (655).

Hutchison *et al.* (660) then sought to determine whether all of the factors required for GR·hsp90 heterocomplex assembly were prebound to hsp90. When hsp90 was immunoadsorbed from reticulocyte lysate with a monoclonal antibody, the washed immunopellet, which was shown to contain hsp90, hsp70, and p60, contained all the factors required for GR·hsp90 heterocomplex assembly and activation of steroid-binding activity (660). When the hsp90 immune pellet was washed extensively, it lost a weakly bound protein required for receptor activation (660), and this protein was later identified as p23 (655). This led the Pratt laboratory to conclude that the components required for assembly of the GR into a functional heterocomplex with hsp90 are preassociated in a self-sufficient protein-folding structure that could be called a *foldosome*.

This notion that the receptor became bound to a multiprotein unit acting as a protein-folding machine was, to some degree, contrary to the conclusion of Smith (192), who proposed an ordered assembly pathway based on the observation that p60 and hsp70 were recovered transiently with the PR at early times of assembly, whereas p23 was recovered at a later time. The model presented in Fig. 1 contains elements of both the foldosome model and an ordered process. The foldosome is depicted as the hsp90·p60·hsp70 complex. It has been shown that immunoadsorbed p60 with its coimmunoadsorbed proteins activates the GR to the steroid-binding state and that addition of purified human p23 to the p60 immune complex potentiates this activation (654). The hsp90·p60·hsp70 complex may or may not contain p23, depending upon the conditions of its isolation. For example, Johnson and Toft (510) have shown that incubation of reticulocyte lysate with both ATP and molybdate yields a complex containing substantial amounts of all four proteins. Thus, p23 could probably be shown entering the reaction scheme of Fig. 1 at step 1, or step 2, or step 3, and given the dynamic nature of the various complexes, interaction with p23 may occur at all steps. However, the level of p23 in PR immunoprecipitation is not appreciable until later times of reconstitution, corresponding to step 3.

Because much of this cellular p23 appears to be bound to hsp90 independent of the presence of steroid receptors, some models of receptor heterocomplex assembly have suggested that the hsp90 that binds to the receptor upon its association with the hsp90 · p60 · hsp70 complex is replaced by another hsp90 that enters subsequently in association with p23 (510, 511). However, as purified p23 potentiates GR activation by p60 immune complexes, this substitution of one receptor-bound hsp90 for another is not necessary, and p23 is shown entering the complex by itself in the scheme of Fig. 1.

In Fig. 1, it is assumed that dissociation of p60 from an intermediate state of the complex opens up a TPR domain-binding region on hsp90 that can be occupied by FKBP52 or one of the other TPR domain proteins recovered with receptor heterocomplexes (FKBP51, CyP-40, PP5). This assumption is based upon the observation that immunoadsorption of one TPR domain protein yields coimmunoadsorption of hsp90 but not another TPR domain protein and the observation that p60 competes for TPR protein binding to hsp90 (531). Thus, when p60 is dissociated, immunophilins can bind in equilibria that are determined by their rel-

ative abundance and binding affinities. This may actually occur as soon as p60 dissociates in step 3 of the model. We have shown FKBP52 binding at step 4 to indicate that its binding is not necessarily linked to the binding of p23. The final product produced in step 4 is the untransformed receptor heterocomplex, which may also contain substoichiometric amounts of hsp70. As discussed above (*Sections VI.I.2* and *VII.C.5*), these complexes are in a constant cycle of assembly and disassembly in the intact cell, where their formation may be important for receptor trafficking.

E. Heterocomplex assembly with hsp90 is a very basic and conserved process

Although most studies of cell-free assembly of receptor·hsp90 complexes have used rabbit reticulocyte lysate, assembly can be carried out in concentrated cytosols prepared from human, monkey, mouse, and insect cells, as well as in wheat germ extract (241). Thus, the hsp90-based heterocomplex assembly system appears to be ubiquitous.

Schena *et al.* (661) showed that GR expressed in plant cells could activate a reporter gene linked to hormone response elements, provided that the plant cells were treated with glucocorticoid. Thus, it could be predicted that plants can form a GR·hsp90 heterocomplex with the appropriate steroid binding conformation and that the receptor can traffic to its site of action in the plant cell. What is perhaps surprising is that components of the plant and animal receptor·hsp90 assembly systems function so well in association with the heterologous proteins. For example, purified wheat hsp70 has the same ability as purified mouse hsp70 to reconstitute the GR·hsp90 assembly activity of hsp70-depleted rabbit reticulocyte lysate (241), and recombinant plant hsp90 is functional at GR·hsp90 heterocomplex assembly in a system containing purified rabbit hsp70 and human p23 and p60 (K. D. Dittmar, P. Krishna, and W. B. Pratt, work in progress). Also, purified human p23 stabilizes GR·plant hsp90 complexes formed in wheat germ extract (655). The human p23 also binds to plant hsp90 in an ATP-dependent manner that is blocked by geldanamycin (567). Thus, despite approximately 600 million years for evolutionary divergence between the plant and animal systems, at least three components have been functionally conserved. The fact that the heterocomplex assembly system shown in Fig. 1 is both ubiquitous and conserved suggests that assembly of protein complexes with hsp90 is fundamental to the biology of the eukaryotic cell.

It is important to realize that all components of this heterocomplex assembly system are not required for chaperoning of protein folding *in vitro*. Purified hsp90 prevents the aggregation of denatured proteins, thus assisting their refolding to their native state independent of any requirement for the presence of other proteins or ATP (361, 662). Schumacher *et al.* (663) have shown that p60, hsp90, and hsp70 immune complexes isolated from reticulocyte lysate are able to renature thermally denatured firefly luciferase in an ATP-dependent manner. Importantly, a mixture of purified hsp90 and hsp70 was able to carry out ATP-dependent renaturation of luciferase with about one-third the activity of whole reticulocyte lysate. Recently, it has been shown that this refolding depends on the presence of a DnaJ protein as well (664). Luciferase renaturation by reticulocyte lysate is inhibited by geldanamycin, and the inhibition is overcome by addition of hsp90 (665). Because several of the proteins in the heterocomplex assembly system can act as chaperones by themselves and because the combination of two or three components of the system can promote ATP-dependent protein renaturation, it is not clear whether the complete receptor·hsp90 heterocomplex assembly system exists purely as a system for chaperoning protein folding. Rather, it must be asked why is this protein heterocomplex being made?

IX. Other Proteins That Are Bound to hsp90

Because hsp90 is a ubiquitous, abundant, and essential cellular chaperone, it is likely to participate in the folding or processing of many proteins. To date, hsp90 has been reported in association with 14 transcription factors and 14 protein kinases, as well as some cytoskeletal and regulatory proteins. As reviewed above (*Sections VII* and *VIII*), hsp90 complexes (with or without receptors or protein kinases) have been reported to contain various amounts of hsp70, p60, immunophilins, PP5, p50, and p23 in various combinations, depending upon the conditions of the assay.

Although many proteins bind to hsp90, and it is likely that many more will be reported, there are potentially seven known or likely protein interaction sites on hsp90. One site is a substrate site, *i.e.* a region that interacts with the proteins that are being chaperoned (*e.g.* steroid receptors, protein kinases). This site is likely located in the C-terminal half of hsp90 (see *Section VI.D*), and it binds proteins of different structure without any specific binding motif. A second site interacts with the p23 component of the assembly system, and this site is dependent upon regions in the N-terminal half of hsp90 (D. O. Toft, work in progress). In the center of hsp90 there is a Ca^{2+}-calmodulin binding site (666), and at the C terminus there is a dimerization site (357). There is at least the potential for a site on hsp90 that interacts with hsp70. Although purified hsp70 and hsp90 do not bind to each other, they form a tripartite complex when p60 is present (see *Section VIII.B.2*), and it is possible that p60 brings hsp90 and hsp70 into direct contact with each other. There is a region of hsp90 that interacts with a variety of TPR domain proteins, including p60, the immunophilins, and PP5 (see *Section VII.C.3*). Finally, there is a region lying near the TPR-binding site on the surface of hsp90 that binds $p50^{cdc37}$ (see *Section VII.C.5*).

It is not known how the protein interaction sites are distributed on hsp90, and the stoichiometries have not been worked out. Thus, in the absence of receptor, we do not know whether an hsp90 dimer binds two molecules of p60, immunophilin or p50, or whether each molecule of hsp90 contributes a half-site for binding and one molecule of each protein is bound per dimer of hsp90. Dimerization of hsp90 may be required for chaperoning *in vivo* (357), and it is possible that the 'substrate' site is only complete when hsp90 is a dimer.

Table 1 lists a number of 'substrate' proteins that have been demonstrated to have a functional and/or physical interaction with hsp90. Most of these interactions have not been

TABLE 1. 'Substrate' proteins for hsp90

Substrate protein	Reference	Substrate protein	Reference
Transcription factors		Protein kinases	
Glucocorticoid receptor	335	Tyrosine kinases	
Progesterone receptor	333, 334	v-Src, c-Src	288, 673, 674
Estrogen receptor	327, 340	v-fps	675
Androgen receptor	327, 341	v-yes	675
Mineralocorticoid receptor	91	v-fes	675
v-erbA	667	v-frg, c-frg	676, 677
Dioxin receptor	342, 343	lck	677, 678
Sim	668	Wee1 kinase	679
MyoD1[a]	420	Sevenless PTK[b]	680
E12[a]	420	Heme-regulated eIF-2α	681, 682
Heat shock factor	360	eEF-2 kinase	683
Tumor promotor-specific binding protein	669	Casein kinase II	363, 364, 366
Hepatitis B virus reverse transcriptase	670	v-Raf, c-Raf, Gag-Mil	290, 684, 685
p53 tumor suppressor mutant	671, 672	MEK	507
		PI-4 kinase[c]	686
Other proteins			
Actin	378	Proteasome	515, 516
Tubulin[d]	379	$G_{\beta\gamma}$ complex	688
Centrin	687		

[a] Only functional interaction has been demonstrated.
[b] Interaction suggested on the basis that hsp90 mutation impairs function.
[c] hsp90 copurifies with the protein.
[d] Interaction may be indirect via hsp90-bound immunophilin, rather than a direct interaction with the 'substrate' site on hsp90.

studied in detail, and some may not require the entire receptor heterocomplex assembly system for interaction with hsp90. While the steroid receptors and Src-related kinases still represent the largest groups of hsp90-associated proteins, several unrelated transcription factors and protein kinases can now be included. Most of these proteins are regulatory in nature and most are phosphoproteins. It is tempting to speculate that such proteins have a particular need for hsp90. However, at this stage it is also possible that investigators have neglected to study substrates that are not regulatory or phosphorylated. It is interesting that the other hsp90-associated proteins (*i.e.* those that are not transcription factors or protein kinases) all exist, at least part of the time, in multiprotein complexes. It is tempting to speculate that hsp90 may play a role in the formation of these complexes.

Some of the proteins in Table 1 have been noted earlier and have been identified using approaches similar to those used to identify steroid receptor and $pp60^{v\text{-}src}$ heterocomplexes. Others, such as Wee1 and sevenless protein kinases, were recognized based on genetic evidence. Introduction of receptors into yeast that have been modified to express hsp90 at either a high or low level may reveal requirements for hsp90 chaperoning in a variety of signaling pathways. For example, Holley and Yamamoto (689) introduced retinoic acid receptors into yeast and found high-affinity ligand-binding activity when hsp90 levels were normal but no ligand-binding activity at low hsp90 levels. Retinoic acid receptors do not form stable complexes with hsp90 that are detectable by coimmunoadsorption (345), but a chaperoning action involving transient interaction must be required to achieve their proper folding into a retinoid-binding conformation that is then stable in the absence of continued association with the chaperone system. Recently, the hsp90-binding drug, geldanamycin, has been used effectively to demonstrate hsp90 associations with mutants of the p53 tumor suppressor (509), the cellular serine/threonine kinase c-Raf-1 (506), and the reverse transcriptase of hepatitis B virus (670). This drug should be very useful for the identification of additional proteins because it provides an easy approach for testing hsp90 interactions in intact cells where it appears to cause inactivation and/or down-regulation of hsp90-associated proteins (see *Section VI.I.1*).

Other than the steroid receptors, the most studied hsp90 interactions are those with the oncogenic tyrosine kinase $pp60^{v\text{-}src}$ (Src) and the heme-regulated eIF-2α kinase. The Src·hsp90 heterocomplex was the first hsp90 interaction demonstrated, and there are many similarities with steroid receptor·hsp90 heterocomplexes.

1. Src and Raf heterocomplexes with hsp90 and p50. The $pp60^{v\text{-}src}$ protein of the Rous sarcoma virus is a membrane-associated tyrosine kinase that induces oncogenic transformation of cells (for review, see Ref. 690). In 1981, it was reported that immunoprecipitation of v-Src yielded coimmunoprecipitation of hsp90 and a 50-kDa protein, p50 (673, 674). The heterocomplex is formed when Src is translated, and newly synthesized Src remains transiently associated with hsp90 and p50 in a cytosolic complex until the kinase localizes to cell membranes, where Src dissociates from the complex (691, 692). These findings led to the suggestion that hsp90 and p50 are involved in the transport of Src to cell membranes (see Ref. 630 for review). Possibly because only a small portion of the Src is in transit to the membrane at any time, only a small portion of the total cellular Src is recovered in the cytosolic fraction complexed with hsp90 and p50 (673, 691).

Several observations lead to the conclusion that formation of the Src·hsp90 heterocomplex is critical for Src function. For

example, large proportions of certain temperature-sensitive Src variants that are partially defective at cellular transformation exist in multiprotein complex with hsp90 and p50 (693, 694). In yeast, expression of v-Src produces growth arrest, and Xu and Lindquist (695) showed that lowering the level of hsp90 expression relieved cell cycle arrest and rescued growth in yeast cells expressing v-Src. Genetic experiments by Lindquist's laboratory have shown a tight linkage between hsp90 requirements for steroid receptor activity and for v-Src activity. For example, Nathan and Lindquist (495) showed that point mutations affecting hsp90 function with the GR in yeast also affect v-Src activity. Also, in yeast expressing a mutant Ydj1 protein in which the basal transcriptional activity of mammalian GR and ER was increased many-fold (see *Section VI.H*), the Ydj1 mutation rescued v-Src lethality (451).

Biochemically, the Src·hsp90 and receptor·hsp90 heterocomplexes are very similar. Both heterocomplexes are stabilized by molybdate, vanadate, tungstate, and an endogenous cytosolic metal factor (289), and both heterocomplexes are formed by the reticulocyte lysate heterocomplex assembly system (288). The serine/threonine kinase Raf-1, with which Src interacts directly (507), also exists in native heterocomplexes with hsp90 and p50 that can be assembled in reticulocyte lysate (290). Like the immunophilin component of receptor·hsp90 heterocomplexes, the p50 component of Src·hsp90 and Raf·hsp90 heterocomplexes binds directly to hsp90 (531) at a site that is different from the immunophilin-binding site (603). Interestingly, the Raf·hsp90 heterocomplex binds [^{3}H]FK506 (629), suggesting that there may be multiple complexes, one containing p50 and others containing immunophilins. Geldanamycin has the same effect on Src and Raf heterocomplexes (505–507) that it produces on PR and GR heterocomplexes (508, 511, 534).

2. Association of heme-regulated eIF-2α kinase with hsp90. Shortly after the identification of hsp90 associations with $pp60^{v\text{-}src}$ and steroid receptors, a third very distinct example of hsp90 interaction, involving a key initiation factor for protein synthesis, was recognized. Protein synthesis is inhibited in rabbit reticulocyte lysate that is deficient in heme. This is accomplished by activation of a serine/threonine protein kinase, heme-regulated inhibitor (HRI), that phosphorylates the initiation factor eIF2 on the α-subunit. When phosphorylated, eIF2 forms an inactive complex with another factor, eIF2B, that is less abundant and is necessary for the recycling of eIF2 to the active GTP-bound form. The resulting lack of eIF2B inhibits protein synthesis. That this regulatory system is related to hsp90 was indicated by the studies of Hardesty and co-workers (681, 696), who showed that a 90-kDa protein that copurified with HRI was hsp90. They also showed that hsp90 had a stimulatory effect on HRI activity. A physical interaction between HRI and hsp90 was documented in 1989 by Matts and Hurst (682) who showed that an HRI complex could be adsorbed using an antibody to hsp90. They also presented evidence that this complex was an inactive form of HRI and that the complex dissociated when HRI was activated by heme deficiency. From these results, it was suggested that the activity of HRI might be regulated in a manner that was similar to that of steroid receptors. Support for this notion was presented by Matts *et al.* in 1992 (697) when the HRI complex was shown to contain two additional proteins, hsp70 and FKBP52, that are common to steroid receptor complexes. Thus, HRI would appear to reside in this inactive complex when heme levels are adequate and a lack of heme would in some way initiate an activation process to yield active, dissociated HRI. However, this system may have additional complexities inasmuch as multiple active and inactive states of HRI have been described, and the actual site for heme action in this system is unclear.

X. Summary

We have provided a historical perspective on a body of steroid receptor research dealing with the structure and physiological significance of the untransformed 9S receptor that has often confused both novice and expert investigators. The frequent controversies and equivocations of earlier studies were due to the fact that the native, hormone-free state of these receptors is a large multiprotein complex that resisted description for many years because of its unstable and dynamic nature. The untransformed 9S state of the steroid and dioxin receptors has provided a unique system for studying the function of the ubiquitous, abundant, and conserved heat shock protein, hsp90.

The hormonal control of receptor association with hsp90 provided a method of manipulating the receptor heterocomplex in a manner that was physiologically meaningful. For several steroid receptors, binding to hsp90 was required for the receptor to be in a native hormone-binding state, and for all of the receptors, hormone binding promoted dissociation of the receptor from hsp90 and conversion of the receptor to the DNA-binding state. Although the complexes between tyrosine kinases and hsp90 were discovered earlier, the hormonal regulation of steroid receptor association with hsp90 permitted much more rapid and facile study of hsp90 function. The observations that hsp90 binds to the receptors through their HBDs and that these domains can be fused to structurally different proteins bringing their function under hormonal control provided a powerful linkage between the hormonal regulation of receptor binding to hsp90 and the initial step in steroid hormone action.

Because the 9S receptor·hsp90 heterocomplexes could be physically stabilized by molybdate, their protein composition could be readily studied, and it became clear that these complexes are multiprotein structures containing a number of unique proteins, such as FKBP51, FKBP52, CyP-40, and p23, that were discovered because of their presence in these structures. Further analysis showed that hsp90 itself exists in a variety of native multiprotein heterocomplexes independent of steroid receptors and other 'substrate' proteins. Cell-free systems can now be used to study the formation of receptor heterocomplexes. As we outlined in the scheme of Fig. 1, the multicomponent receptor·hsp90 heterocomplex assembly system is being reconstituted, and the importance of individual proteins, such as hsp70, p60, and p23, in the assembly process is becoming recognized.

It should be noted that our understanding of the mecha-

nism and purpose of steroid receptor heterocomplex assembly is still at an early stage. We can now speculate on the roles of receptor-associated proteins in receptor action, both as individuals and as a group, but their actual functions are still vague or unknown. We can make realistic models about the chaperoning and trafficking of steroid receptors, but we don't yet know how these processes occur, we don't know where chaperoning occurs in the cell (*e.g.* Is it limited to the cytoplasm? Is it a diffuse process or does chaperoning occur in association with structural elements?), and, with the exception of the requirement for hormone binding, we don't know the extent to which the hsp90-based chaperone system impacts on steroid hormone action.

It is not yet clear how far the discovery of this hsp90 heterocomplex assembly system will be extended to the development of a general understanding of protein processing in the cell. Because this assembly system is apparently present in all eukaryotic cells, it probably performs an essential function for many proteins. The bacterial homolog of hsp90 is not an essential protein, but hsp90 is essential in eukaryotes, and recent studies indicate that the development of the cell nucleus from prokaryotic progenitors was accompanied by the duplication of genes for hsp90 and hsp70 (698). We envision that a chaperone function of hsp90 that is nonessential in bacteria has been used to perform additional functions, such as trafficking of proteins to and from the nucleus, that are fundamental to the eukaryotic cell. It is likely that, like hsp70, hsp90 binds to unfolded regions of proteins, giving it the power to interact, albeit transiently, with hundreds or thousands of proteins regardless of structural features or binding motifs. Because the steroid receptors have evolved a persistant interaction with the 'substrate' (*i.e.* chaperoning) site of hsp90, they have provided us with a glimpse into a fundamental heterocomplex assembly process that is normally so dynamic that it might not otherwise have been visualized. Thus, just as the description of steroid receptor genes led to the discovery of a superfamily of transcription factors, the ultimate legacy of the study of untransformed steroid receptors may be much more fundamental and far reaching than an understanding of how the hormones transform their receptors.

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