

## Chaperone functions of the heat shock proteins associated with steroid receptors

William B. Pratt and Michael J. Welsh

*Mammalian steroid receptors exist in hormone-free cells in a heterocomplex that contains the three heat shock proteins hsp90, hsp70 and hsp56. Some protein kinases, including pp60<sup>v-src</sup> and v-Raf, exist in similar cytosolic heterocomplexes containing hsp90 and a 50 kDa protein of unknown function, pp50. The four proteins—hsp90, hsp70, hsp56 and pp50—exist together in a heterocomplex independent of the presence of steroid receptors and protein kinases. Both the receptor and the protein kinase heterocomplexes can be formed by a protein folding-heterocomplex assembly system in reticulocyte lysate that carries out an hsp70-dependent attachment of the proteins to the preformed heat shock protein complex. Association of receptors with this structure occurs at the termination of receptor translation and is critical for maintenance of the receptors in a transcriptionally inactive state in the absence of hormone. We discuss how this preformed protein folding structure may be involved in the subsequent targeted trafficking of steroid receptors through the cytoplasmic space to the nucleus.*

**Key words:** heat shock proteins / protein folding / protein trafficking / steroid receptors

ALMOST 10 YEARS AGO, it was established that the large form (~9S) of steroid receptors that is recovered in cytosols prepared from hormone-free cells is actually a heterocomplex containing the ubiquitous heat shock protein hsp90 in addition to the steroid binding proteins.<sup>1,2</sup> In a number of studies performed over the next 5 years, it was established that this association with hsp90 is a critical factor for maintenance of the receptors in a transcriptionally inactive state, with hsp90 binding tightly to the hormone binding domain (HBD) of the receptors and binding of steroid to the HBD somehow promoting dissociation of the receptor from the heat shock protein.<sup>3-6</sup> Indeed, a technique that has proven useful for answering a variety of questions in cell biology consists of fusing the HBD of a steroid

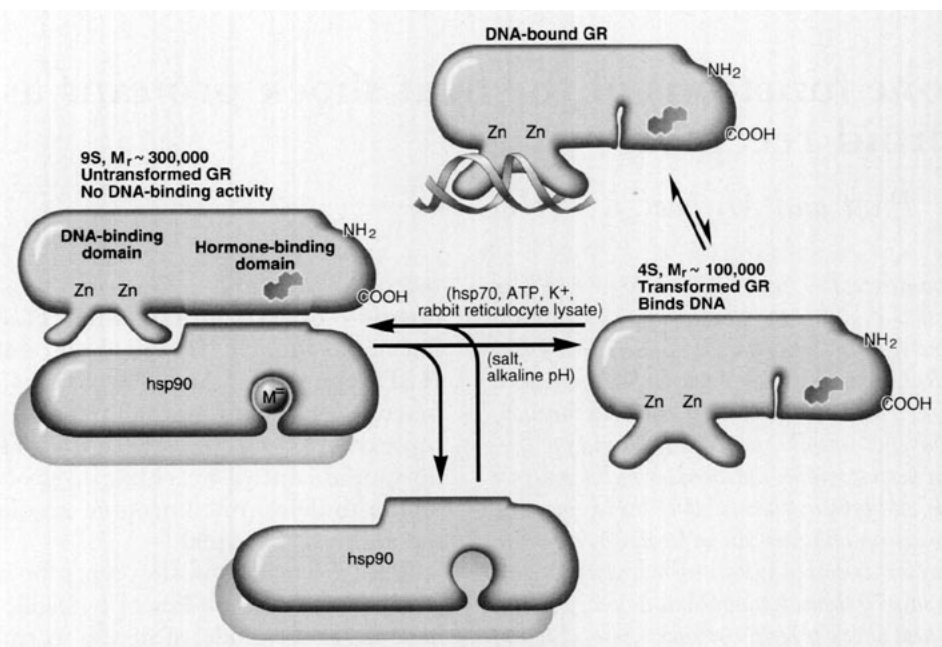
receptor to another protein, thus bringing the function of structurally different proteins under hormonal control.<sup>7</sup> In the absence of hormone, the HBD maintains the heterologous protein in an inactive state and steroid treatment reverses this repression. This ability of the HBD to confer hormonal control on chimeric proteins reflects its ability to determine hormone-regulated binding of the chimera to hsp90.<sup>8</sup>

The discovery of the receptor-hsp90 complex permitted direct studies of cytosolic receptors that have led to the model of steroid receptor transformation presented in Figure 1. Both under physiological conditions and in cytosols, binding of hormone to the receptor heterocomplex promotes the temperature-dependent dissociation of receptors from hsp90.<sup>10-14</sup> When cytosolic receptors are bound to hsp90 they are unable to bind to DNA, but dissociation of hsp90 is accompanied by the simultaneous conversion of the receptor to the DNA-binding state.<sup>11-13</sup> In cytosols, dissociation of receptors from hsp90 can be brought about in a nonsteroid-dependent manner by simply adding salt or increasing cytosolic pH, and the transition metal anions molybdate, vanadate and tungstate inhibit dissociation of the receptor-hsp90 complex.<sup>1</sup> The ability of molybdate to stabilize the receptor in its heterocomplex form has facilitated cell-free analysis of heterocomplex composition because, in the absence of the metal oxyanion, the complex spontaneously dissociates as it is purified.

It has been established that steroid receptors are bound to hsp90 in intact hormone-free cells,<sup>15,16</sup> and the heterocomplex dissociation model of steroid receptor transformation is now presented in biochemistry and cell biology texts essentially as shown in Figure 1. However, this popular model is overly simplified because mammalian steroid receptor heterocomplexes have recently been found to be multiprotein units that contain at least two other heat shock proteins in addition to hsp90. The study of these steroid receptor heterocomplexes and their cell-free assembly has provided very basic insights into the chaperone functions of hsp70 and hsp90, and work published in the last 3 years has led to heuristic

*From the Departments of Pharmacology and Anatomy and Cell Biology, The University of Michigan Medical School, Ann Arbor, MI 48109, USA.*

©1994 Academic Press Ltd  
1043-4682/94/020083 + 11\$8.00/0



**Figure 1.** Dissociation model of glucocorticoid receptor (GR) transformation to the DNA-binding state. Hsp90 is bound to the hormone binding domains of the steroid receptors and the presence of hsp90 abrogates DNA-binding activity. The receptor-hsp90 complex is stabilized by molybdate (indicated by the *small globe* with the  $M^-$ ), which interacts with an ATP site on hsp90 and induces a conformational change in the hsp.<sup>9</sup> Dissociation of the untransformed receptor-hsp90 complex is induced physiologically by steroid binding. In cytosols, dissociation can be induced by salt or alkaline pH yielding simultaneous transformation to the DNA-binding state (indicated by receptor with exposed 'zinc finger' on the right). Hormone free receptors can be reconstituted into a heterocomplex with hsp90 by incubating them with rabbit reticulocyte lysate.

models of receptor folding and trafficking that may have general application to a broad spectrum of proteins that, like the steroid receptors, undergo targeted movement through the cytoplasm. In this review we will summarize the work leading to these receptor folding and trafficking models.

### The heat shock protein (hsp90-hsp70-hsp56) complex

The constitutive form of hsp70 was first demonstrated to be a component of the chicken progesterone receptor heterocomplex<sup>17</sup> and it has subsequently been identified in native mammalian progesterone (PR),<sup>18</sup> glucocorticoid (GR)<sup>19</sup> and estrogen (ER)<sup>8</sup> receptor heterocomplexes. Native GR heterocomplexes isolated from some cytosols (e.g. L cell, HeLa)<sup>19,20</sup> do not contain hsp70 but steroid receptor heterocomplexes reconstituted under cell-free conditions with reticulocyte lysate always contain

hsp70.<sup>21,22</sup> The hsp70 proteins (constitutive and stress-induced) are involved in protein folding-unfolding reactions, they are important for protein translocation across membranes of organelles, and they are thought to catalyze protein assembly (for review, see ref 23). Like hsp90, hsp70 binds to the hormone binding domain of steroid receptors,<sup>5,8</sup> but, in contrast to hsp90, hsp70 is not removed on receptor transformation<sup>14,19</sup> and its presence does not affect receptor DNA binding activity.<sup>18</sup>

The third receptor-associated heat shock protein hsp56 was discovered when a monoclonal antibody (EC1) prepared against crudely purified, molybdate-stabilized, untransformed rabbit PR was found to react with a ~59 kDa rabbit protein that was a component of progesterone, estrogen, androgen and glucocorticoid receptor heterocomplexes.<sup>24</sup> This EC1 antibody immunoadsorbed a ~56 kDa protein of unique sequence from human lymphocyte cytosol<sup>25</sup> and its translation rate was found to increase with stress.<sup>26</sup> The literature on hsp56 has

recently been reviewed in detail.<sup>27</sup> Hsp56 was discovered to be an immunophilin when a protein that bound to FK506- and rapamycin-Affi-Gel-10 matrices was found to have the same NH<sub>2</sub>-terminal sequence as hsp56.<sup>28,29</sup> Immunophilins are proteins that bind immunosuppressive drugs like cyclosporin A, FK506, and rapamycin (for review, see ref 30), and they all have peptidylprolyl isomerase (PPIase) activity that is inhibited by the immunosuppressive agents which bind directly to the PPIase site. The rabbit<sup>31</sup> and human<sup>32</sup> cDNAs for hsp56 have been cloned and the human protein produced in *Escherichia coli* was shown to have PPIase activity inhibited by FK506.<sup>32</sup>

Other proteins that are recovered in native steroid receptor complexes include a unique, highly acidic 23 kDa protein of unknown function,<sup>2</sup> and two proteins of 50 kDa and 54 kDa, which have been recovered with the immunoadsorbed untransformed chicken PR.<sup>14</sup> Partial amino acid sequencing showed 80% identity of p50 and 60% identity of p54 with regions of rabbit hsp56<sup>33</sup> and both proteins were shown to bind to immobilized FK506.<sup>34</sup> The results of immunoadsorption and Western blotting experiments suggest that p50 is the avian homolog of mammalian hsp56 and that p54 is the avian homolog of a new mammalian immunophilin distinct from hsp56.<sup>34</sup>

In what may turn out to be an observation of broad significance in developing an understanding of how many proteins are folded in the cytoplasm, it was found that the three heat shock proteins, hsp90, hsp70 and hsp56, exist together in a cytosolic complex independent of the presence of steroid receptors. This was first shown by immunoadsorbing hsp56 from human lymphocyte cytosol and demonstrating co-immunoadsorption of hsp90 and hsp70.<sup>25</sup> Similarly, immunoadsorption of mouse hepatocyte cytosol with a monoclonal antibody against hsp90 yielded co-immunoadsorption of hsp70, hsp56 and a 50 kDa protein that was shown to be the same as the pp50 component of the pp60<sup>v-src</sup>-hsp90 heterocomplex.<sup>35,36</sup> More recently, it has been found that purification of hsp56 from human cytosol by affinity chromatography on an FK506-coupled matrix yields co-isolation of hsp90 and hsp70,<sup>29</sup> thus confirming the existence of the hsp complex by another method. Co-immunoadsorption experiments have also revealed the presence of a ~60 kDa 'stress-related' protein in the hsp heterocomplex.<sup>37</sup> This protein is seen only in transient association with steroid receptor complexes

as an apparent intermediate formed during cell-free heterocomplex assembly by the protein folding system in reticulocyte lysate<sup>21</sup> (to be described below).

It is important to note that each of the three heat shock proteins in the hsp heterocomplex is a chaperone protein. In that hsp56 has peptidylprolyl isomerase activity, it is by definition a protein folding enzyme. Hsp70 has well characterized protein unfoldase activity<sup>23</sup> and hsp90 has been shown to promote protein folding by binding to and suppressing the aggregation of proteins *in vitro*.<sup>38</sup> The existence of these three heat shock proteins in the same multiprotein complex suggests that they may function together in a temporally and spatially organized manner.

### GR folding and heterocomplex assembly

It is important to note that steroid receptors are not in a free binding equilibrium with hsp90, and simply mixing purified hsp90 with purified steroid receptor does not produce a complex. It seems that after hsp90 has dissociated from the glucocorticoid receptor, the hormone binding domain undergoes a rapid change in its folding state such that the determinants for its tight binding to hsp90 are no longer exposed. This folding change is indicated in Figure 1 by the invagination in the HBD of the hsp90-free form of the receptor. In the case of the unliganded glucocorticoid receptor, the consequence of hsp90 dissociation is to eliminate the high affinity steroid binding site. If the untransformed receptor is already bound with steroid, then the steroid remains bound when hsp90 dissociates and it is transformed to the DNA-binding state, but if the GR is hormone-free when hsp90 dissociation occurs, then the steroid binding conformation is eliminated. This requirement for the receptor to be bound to hsp90 for it to have a steroid binding site was shown first for the GR<sup>39,40</sup> and it pertains for dioxin<sup>41</sup> and mineralocorticoid<sup>42</sup> receptors as well. The progesterone receptor also requires hsp90 to form a steroid binding site, however loss of steroid binding activity after hsp90 dissociation from the hormone-free receptor is very temperature-dependent. At 0°C, the hsp90-free progesterone receptor remains in a steroid binding configuration, whereas incubation of the hsp90-free receptor at 30°C is accompanied by rapid loss of steroid binding activity that is reconstituted when hsp90 is reassociated with the

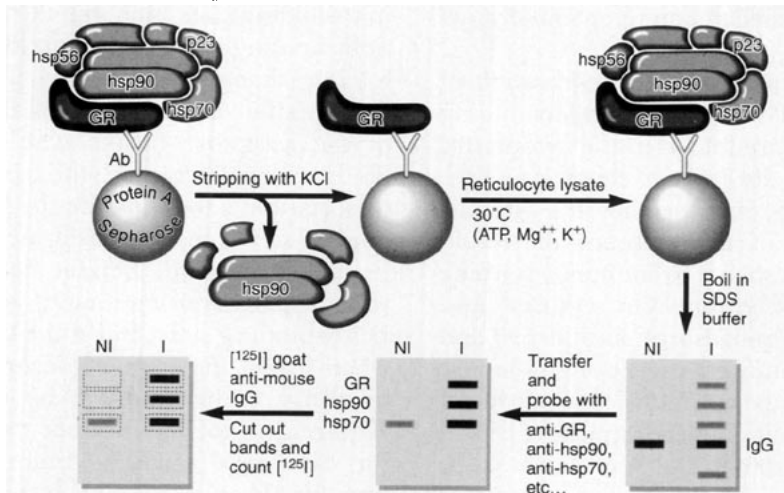
receptor by incubating it with the protein folding system of reticulocyte lysate.<sup>43</sup>

The first evidence for formation of a steroid receptor-hsp90 complex under cell-free conditions was obtained in an *in vitro* translation system. It was shown that GR translated in rabbit reticulocyte lysate was 9S, was co-immunoadsorbed with hsp90, and it behaved like untransformed receptor in that it was in a steroid-binding conformation and without DNA binding activity.<sup>44,45</sup> The GR-hsp90 complex formed in reticulocyte lysate appeared to be exceptionally stable at 30°C, a temperature at which the complex in other cytosols spontaneously and rapidly falls apart. It is now clear that the receptor-hsp90 complexes formed in reticulocyte lysate are disassembled at 30°C as in cytosols but they are also rapidly reassembled into heterocomplex form.

The dynamic nature of heterocomplex assembly in rabbit reticulocyte lysate was first appreciated when Smith *et al*<sup>46</sup> showed that incubation of immunoadsorbed, hsp90-free chicken progesterone receptor with reticulocyte lysate resulted in binding of rabbit hsp90 and hsp70 to the avian receptor. The HBD of the receptor was required for heterocomplex formation and the process was both temperature- and ATP-dependent.<sup>21,46</sup> Heterocomplexes were

formed if the receptors were unliganded but not if the steroid binding site was occupied by progesterone. Similarly, Scherrer *et al*<sup>40</sup> incubated immunopurified, hsp90-free L cell GR with reticulocyte lysate and reconstituted the GR-hsp90 complex.

GR heterocomplex assembly by reticulocyte lysate is illustrated in Figure 2.<sup>47</sup> Hormone-free GR heterocomplexes are immunoadsorbed to a pellet of protein A-Sepharose and the associated proteins are stripped off by incubating with salt and washing with buffer. Uniquely, hsp70 is not stripped off the GR with salt;<sup>19</sup> however, we routinely immunoadsorb native L cell heterocomplexes which do not contain hsp70. The native heterocomplex in the immunopellet has steroid binding activity but the salt-stripped immunopellet has no steroid binding activity. Incubation of the stripped immunopellet with reticulocyte lysate reconstitutes a heterocomplex containing hsp56<sup>48</sup> and p23 (P. Housley, personal communication) as well as hsp90 and hsp70.<sup>22,40</sup> The newly-assembled mouse GR-rabbit hsp heterocomplex has now regained high affinity steroid binding activity and has been converted from a DNA-binding form back to a non-DNA-binding form.<sup>40</sup> Thus, the reticulocyte lysate protein folding-heterocomplex assembly system has completely



**Figure 2.** Cell-free assembly of the GR heterocomplex with heat shock proteins. Receptor heterocomplexes are immunoadsorbed to a pellet of protein A-Sepharose with a monoclonal antibody (Ab) and receptor-associated proteins are stripped off with 0.5 M KCl. Depending on the cell type, hsp70 may or may not be present in the native heterocomplex. After several washes, the pellet containing the immunopurified receptor is incubated with rabbit reticulocyte lysate at 30°C in the presence of an ATP-generating system and 100 mM KCl. The immunopellet is then washed several times with a buffer containing 20 mM molybdate to stabilize the heterocomplex. The proteins in the reconstituted heterocomplex are resolved by denaturing gel electrophoresis and identified by immunoblotting with specific antibodies. Control samples with nonimmune IgG (NI) are always run in parallel with immune samples (I) to demonstrate that the proteins in the heterocomplex are present in a manner that is specific for the receptor. (From ref 47.)

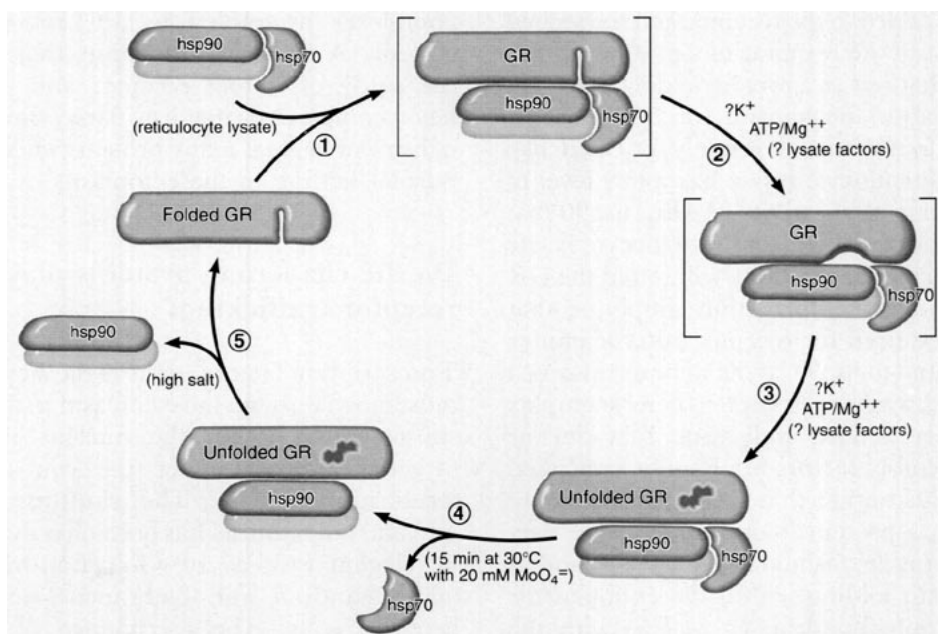
reversed receptor transformation. A direct relationship exists between the amount of hsp90 that is bound to receptors in the immunopellet and the number of specific glucocorticoid binding sites formed by the reticulocyte lysate,<sup>22</sup> and generation of steroid binding activity is blocked by peptides that block hsp90 reassociation with the receptor.<sup>49</sup>

### Unraveling the mechanism of heterocomplex assembly

Some of the requirements for lysate-directed heterocomplex assembly have been defined. Formation of a steroid receptor complex with hsp90 requires ATP/Mg<sup>2+</sup> and a monovalent cation.<sup>21,22</sup> The step(s) requiring monovalent cation is unclear but it is highly selective in that K<sup>+</sup>, Rb<sup>+</sup> and NH<sub>4</sub><sup>+</sup> permit heterocomplex assembly while Na<sup>+</sup> and Li<sup>+</sup> do not.<sup>22</sup> The same monovalent cation selectivity pertains with chaperonin-mediated protein folding reactions<sup>50</sup> and for the protein unfoldase activity of hsp70.<sup>51</sup> Hsp70 is always present in steroid receptor-hsp90 complexes assembled in reticulocyte lysate,<sup>21,22,40,46</sup> and Smith *et al*<sup>21</sup> have shown that pretreatment of lysate with a monoclonal antibody against hsp70 inhibits formation of a progesterone receptor-hsp90 complex, suggesting that hsp70-mediated protein unfoldase activity might

be required to bind hsp90 to receptors. This notion has been proven in a system where reticulocyte lysate was passed through an ATP-agarose matrix that removed hsp70 and inactivated heterocomplex assembly. Addition of purified hsp70 to this hsp70-depleted lysate restored its ability to form a GR-hsp90 complex and reactivate the receptor to the steroid binding form (K.A. Hutchison, K.D. Dittmar, M.J. Czar, W.B. Pratt, submitted manuscript).

The model in Figure 3 summarizes our current understanding of the protein folding-heterocomplex assembly system. In step 1, a folded form of the GR without steroid binding activity binds to the preformed heat shock protein heterocomplex. For convenience, only two proteins are presented in the complex shown in the figure. Hsp56 is part of the final receptor heterocomplex and, like hsp90, it is present in an hsp70-dependent manner but this probably reflects the fact that hsp56 is bound to hsp90 and rides it in piggyback fashion into the complex. It is unlikely that the PPIase activity of hsp56 is required for assembly of the receptor heterocomplex because assembly is not affected by the PPIase inhibitor FK506.<sup>48</sup> Another protein that is not presented is the 60 kDa stress-related protein which has been identified as a transient component of the progesterone heterocomplex formed in reticulocyte lysate and is probably one of the *lysate factors* required for complex assembly.<sup>2</sup>



**Figure 3.** Model of GR heterocomplex assembly. Details of the model are described in the text. (From ref 47.)

After formation of the initial complex (indicated in brackets), we propose that hsp70 mediates an unfolding of the receptor HBD (step 2) and that hsp90 stabilizes this unfolded state (step 3). The receptor is now in a conformation that binds steroid. At this point, hsp70 can leave the complex (step 4) and, so long as molybdate is present to stabilize the binding of hsp90 to the HBD, the receptor remains in the high affinity steroid binding conformation. The mechanism of hsp70 exit from the complex is not well understood but it appears to be promoted by ATP. In that the native untransformed GR recovered from L cells is bound to hsp90 but not hsp70, it is likely that a similar cycling of hsp70 out of the complex occurs in intact cells. If the immunoadsorbed GR-hsp90 complex is suspended in buffer containing salt but no molybdate, hsp90 dissociates and the HBD immediately converts back to the non-steroid-binding conformation. The receptor can then be recycled again through the entire heterocomplex assembly process.

### General speculations on the role of the hsp complex in protein folding

There are two general mechanisms by which receptor heterocomplex assembly could occur. One is via an ordered sequence of reactions in which soluble components are added to the receptor and the second is by attachment of the receptor to a preformed hsp heterocomplex that acts as a protein folding machine. We favor the latter mechanism for two reasons. First we have found that a crudely purified hsp complex from reticulocyte lysate has a low level of heterocomplex assembly activity.<sup>52</sup> Also, hsp90 that has been immunoadsorbed from reticulocyte lysate with a monoclonal IgM antibody will refold the GR to a steroid binding conformation, implying that all the factors required for receptor heterocomplex assembly are bound to hsp90. If the immunoadsorbed hsp complex is washed, receptor heterocomplex assembly activity is lost, indicating that one or more of the assembly factors are loosely associated components. This notion that several proteins are preassociated in a protein folding unit where they act in a cooperative fashion appears in models describing protein folding within the endoplasmic reticulum and mitochondria, as well as with the cytoplasmic protein folding process outlined in Figure 3.

Obviously, a protein folding structure involving highly abundant heat shock proteins has not evolved solely to fold steroid receptors. Thus, it is reasonable to predict that *reticulocyte lysate* may have a general ability to form heterocomplexes with a variety of proteins. Another protein that forms native complexes with hsp90 is the oncogenic tyrosine kinase pp60<sup>v-src</sup>. The cytosolic form of pp60<sup>v-src</sup> is recovered from cells in a tight complex with hsp90 that also contains a 50 kDa phosphoprotein of unknown function, pp50 (for review, see ref 53). As mentioned above, pp50 is a component of the heat shock protein heterocomplex.<sup>36</sup> It has been shown that reticulocyte lysate forms a pp60<sup>v-src</sup>-hsp90-pp50 heterocomplex in the same manner as it forms steroid receptor complexes.<sup>54</sup> Like the steroid receptor heterocomplexes, the pp60<sup>v-src</sup> heterocomplex is stabilized by molybdate.<sup>55</sup> Investigators have focused on hsp90 binding to steroid receptors and to pp60<sup>v-src</sup> because in both cases the binding is of high affinity. However, weaker complexes can be detected between hsp90 and some other protein kinases. For example, the serine/threonine kinase v-Raf, which is a protein kinase implicated in normal mitogenic signal transduction, exists in a native heterocomplex with hsp90 and pp50 and this complex can be reconstituted by reticulocyte lysate.<sup>56</sup> Hsp70 has the promiscuous ability to bind to a wide variety of proteins of very different structure,<sup>57</sup> and hsp70 is a component of both pp60<sup>v-src</sup> and Raf heterocomplexes assembled by the reticulocyte lysate system. A general prediction that evolves from the study of steroid receptor and protein kinase heterocomplexes is that hsp70 may direct the binding of hsp90 to a great many proteins while they undergo normal folding in the cytoplasm.

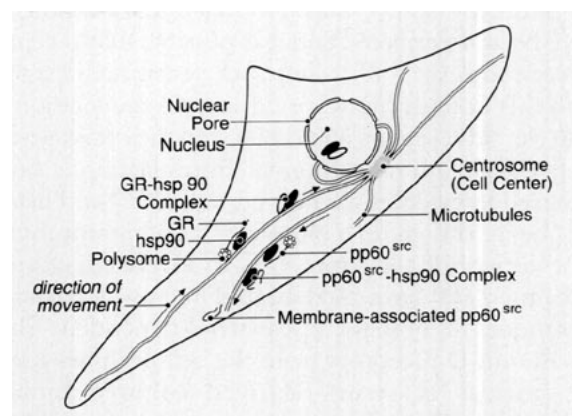
### Do the chaperone proteins play a role in receptor trafficking?

Transcription factors, such as the steroid receptors, must move through the cytoplasm to the nucleus and subsequently within the nucleus in a precisely targeted manner under the control of nuclear localization signals. The shuttling of receptors into and out of nuclei has been directly demonstrated in elegant studies of Guiochon-Mantel *et al*<sup>58</sup> and Chandran and DeFranco<sup>59</sup> using transient heterokaryons. The mechanism of this receptor trafficking is unknown. Indeed, the mechanisms by which proteins in general move throughout the

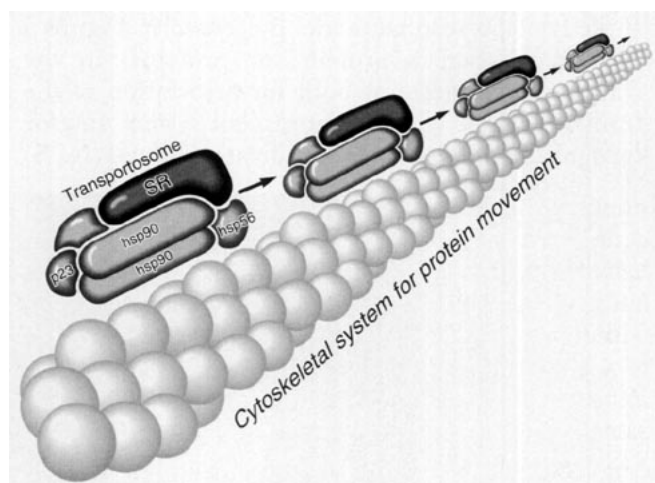
cytoplasm from their sites of translation to sites of action in the nucleus or at the inside of the plasma membrane are unknown. It has been a general notion that both protein chaperones and immunophilins are involved in protein trafficking, but details of their possible involvement have not been worked out.

The idea that hsp90 could in some way be involved in protein trafficking has evolved from the observation that pp60<sup>v-src</sup> binds to hsp90 and pp50 at the termination of its translation and it remains associated with the proteins while it undergoes trafficking through the cytoplasm to plasma membrane.<sup>53</sup> Whether the role of hsp90 is simply to stabilize pp60<sup>v-src</sup> until it becomes membrane-bound or whether hsp90 actually functions as a component of the trafficking process is unknown. Steroid receptors also bind to hsp90 at the termination of their translation,<sup>45</sup> but their trafficking is in the retrograde direction toward the nucleus (Figure 4). Several observations have led to the speculation that the hsp complex may act as a transport particle (a *transportosome*) to which the receptors remain bound while they move through the cytoplasm.<sup>60</sup> In this respect it is important to note that both hsp90 and hsp56 have been identified in steroid receptor heterocomplexes isolated from hormone-free cells where the receptors are cytoplasmic as well as from cells where the receptors are nuclear.<sup>60</sup> The mouse GR in hormone-free L cells, for example, is predominantly localized in the cytoplasm by indirect immunofluorescence<sup>61</sup> and is associated with both hsp 90 and hsp56,<sup>48</sup> whereas the mouse receptor overexpressed in CHO cells is localized in the nucleus<sup>19</sup> and it is also associated with hsp90 and hsp56.<sup>56</sup> As the same proteins are associated with receptors in both compartments, it is logical to conclude that the receptors must have traveled from their cytoplasmic sites of translation to the nucleus while attached to both of these members of the heat shock protein complex, with the complex itself perhaps acting as a *transportosome* as illustrated in Figure 5. Indeed, the GR expressed in CHO cells has been shown by confocal microscopy to be localized in all planes of the nucleus and at the center of the nucleus as well as at its periphery.<sup>62</sup> Thus, it seems possible that receptors may also move within the interior of the nucleus while associated with hsp90 and hsp56.

It is important to ask what kind of movement system could be responsible for the transport of hsp90-bound proteins, such as the steroid receptors,



**Figure 4.** Bidirectional movement of hsp90-bound proteins. Both pp60<sup>v-src</sup> and the GR bind to hsp90 at or near the termination of their translation and remain bound to hsp90 while they move to their sites of action at the plasma membrane or in the nucleus, respectively. Systems exist for transport of vesicles along microtubules in both directions (i.e. in an anterograde direction away from the nucleus and in a retrograde direction toward the nucleus). It is proposed here that proteins also travel along such pathways while they are attached to a common transport particle as illustrated by the *transportosome* in Figure 5. The direction of movement would be determined by targeting signals inherent to the transported protein, such as the nuclear localization signals of steroid receptors. (From ref 60.)



**Figure 5.** The steroid receptor (SR) in association with the heat shock protein heterocomplex as it may function as a *transportosome* for protein movement in the intact cell. (Modified from ref 60.)

pp60<sup>v-src</sup> and v-Raf, through the cytoplasm. We have indicated in the diagram of Figure 4 that such movement might occur along cytoskeletal pathways. Both microtubule- and microfilament-based systems

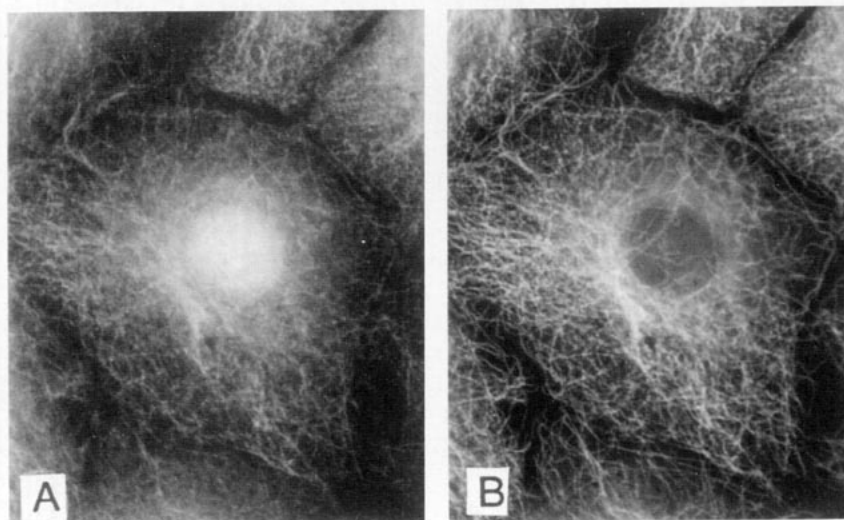
for bidirectional cytoplasmic transport of organelles have been described<sup>63</sup> and it is possible that receptor heterocomplexes traffic along such systems. Consistent with this notion, there is evidence for association of both steroid receptors and hsp90 with the cytoskeleton. The GR in human gingival fibroblasts has been localized to cytoplasmic microtubules,<sup>64,65</sup> and hsp90 has been shown to colocalize with microtubules in a variety of cell types.<sup>66,67</sup> In a related system, Barsony *et al*<sup>68</sup> have used a rapid microwave fixation technique to observe a calcitriol-dependent shift of vitamin D receptors from the cell periphery into the nucleus of serum-deprived cultured human fibroblasts. They described a fascinating response in which there is first a clumping of receptors at the cell periphery with 15-45 s, followed by alignment of clumps along fibrils within 30-45 s, perinuclear accumulation of clumps within 45-90 s, and intranuclear accumulation of receptors within 1-3 min. The vitamin D receptors have now been shown to move through the cytoplasm along tubulin-containing filaments,<sup>69</sup> consistent with the general notion of a microtubule-based system for protein movement.

### Does hsp56 mediate the direction of receptor trafficking?

If the transportosome model presented in Figure 5 is correct, then a protein (or proteins) in the complex must account both for association of the transportosome with the movement system and for recognition of the nuclear localization signal (NLS)

determining the direction of receptor movement. The immunophilin component (hsp56) of the receptor heterocomplex has some properties that suggest it could be involved in determining the directionality of trafficking. Hsp56 has been localized by immunofluorescence to filamentous cytoplasmic structures in several cell types.<sup>70</sup> Its localization is compared with the localization of tubulin in rat endothelial cells in the immunofluorescence photographs shown in Figure 6. As shown in the figure, a considerable portion of the hsp56 in the cell is found in the nucleus but the cytoplasmic hsp56 colocalizes with microtubules. This pattern of hsp56 localization in the cell is consistent with a role in nuclear protein trafficking.

We noted<sup>56</sup> recently that pp60<sup>v-src</sup> and v-Raf, both of which traffic predominantly toward the cell membrane (Figure 4), are recovered from cells in heterocomplexes containing hsp90 and pp50, *but not hsp56*, whereas steroid receptor heterocomplexes, which traffic in the opposite direction to the nucleus, contain hsp90 and hsp56, *but not pp50*. This led to the notion that hsp56 and pp50 may be involved in attachment of the protein trafficking complexes to retrograde and anterograde movement systems respectively.<sup>56</sup> Particles have been shown to move in a retrograde direction through the cytoplasm along microtubules in a process that is driven by tubulin-associated dynein motors (see ref 71 for dynein review). It is of potential interest, therefore, that immunoadsorption of hsp56 from several cell cytosols has been found to yield co-immunoadsorption of both intermediate and heavy chains of dynein, suggesting



**Figure 6.** Comparison of immunofluorescence produced by an anti-hsp56 antibody (A) and an anti-tubulin antibody (B) in the same rat endothelial cell.



that hsp56 may interact either directly or through another bridging protein with the dynein motor (J.K. Owens-Grillo, A.W. Yem, K.L. Leach, M.R. Deibel, W.B. Pratt, submitted manuscript).

It has been difficult to conceive how nuclear localization signals determine the direction of protein movement. These signals are characterized only as clusters of positively charged amino acids,<sup>72</sup> and an NLS-recognition protein would require a negative charge cluster to bind the signal. Some component of the transportosome must come into direct contact with the nuclear localization signal of the steroid receptor for the model of Figure 5 to be correct. As we have noted in a recent review of hsp56, it may not be entirely fortuitous that hsp56 has a conserved anionic sequence (with 6 out of 8 consecutive amino acids being negatively charged) linking its first two predicted domains.<sup>27</sup> Thus, hsp56 not only binds to hsp90 in an equilibrium manner, but it has the additional potential for binding directly to the receptor NLS. By binding to this second site, the affinity of hsp56 for the steroid receptor-hsp90 complex would be increased and one can then envisage how the NLS could determine dynein-mediated movement of the receptor through the cytoplasm to the nucleus.

In that all of the steroid receptors contain nuclear localization signals, one can readily ask why all of the receptors are not immediately carried to the nucleus. We have looked at the ability of an antibody directed against the NL1 nuclear localization signal of the GR to interact with  $\beta$ -galactosidase-GR fusion proteins, and we find that when hsp90 is tightly bound to the GR HBD, the NL1 site is blocked.<sup>8</sup> Thus, in many cells the GR is cytoplasmic until it is bound by steroid. Binding of steroid reduces the affinity of the GR for hsp90, and, either through a conformational change or unmasking, access of the NLS-recognition protein (hsp56?) to NL1 is permitted. Thus, in many cells there is hormone-dependent translocation of the GR from the cytoplasm to the nucleus.<sup>73,74</sup>

## Conclusion

The study of receptor-associated proteins has provided a role for two of these proteins, hsp70 and hsp90, in chaperoning the folding of the hormone binding domain of the steroid receptors. Hsp70 and hsp90 appear to act together as part of a preformed heat shock protein complex that may act as a protein folding unit to which both steroid receptors and some

protein kinases become attached in an hsp70-dependent manner. Hsp70 can then leave the heterocomplex, with the notion being that the heterocomplex can then act as a transport particle to which the proteins remain attached while they undergo trafficking through the cytoplasm. Thus, in the cytoplasm, the processes of protein folding and protein trafficking may be linked functions of the same hsp-containing structure.

## Acknowledgement

The authors' work reported herein was funded by NIH grants CA28010 and DK31573 to W.B.P. and ES06265 to M.J.W.

## References

1. Pratt WB, Hutchison KA, Scherrer LC (1992) Steroid receptor folding by heat shock proteins and composition of the receptor heterocomplex. *Trends Endocrinol Metab* 3:326-333
2. Smith DF, Toft DO (1993) Steroid receptors and their associated proteins. *Mol Endocrinol* 7:4-11
3. Pratt WB, Jolly DJ, Pratt DV, Hollenberg SM, Giguere V, Cadepond FM, Schweizer-Groyer G, Catelli MG, Evans RM, Baulieu EE (1988) A region in the steroid binding domain determines formation of the non-DNA-binding 9S glucocorticoid receptor complex. *J Biol Chem* 263:267-273
4. Denis M, Gustafsson JA, Wikstrom AC (1988) Colocalization of the Mr 90,000 heat shock protein with the steroid binding domain of the glucocorticoid receptor. *J Biol Chem* 263:18520-18523
5. Schowalter DB, Sullivan WP, Maihle NJ, Dobson ADW, Conneely OM, O'Malley BW, Toft DO (1991) Characterization of progesterone receptor binding to the 90- and 70-kDa heat shock proteins. *J Biol Chem* 266:21165-21173
6. Cadepond F, Schweizer-Groyer G, Segard-Maurel I, Jibard N, Hollenberg SM, Giguere V, Evans RM, Baulieu EE (1991) Heat shock protein 90 as a critical factor in maintaining glucocorticosteroid receptor in a nonfunctional state. *J Biol Chem* 266:5834-5841
7. Picard D (1993) Steroid-binding domains for regulating the functions of heterologous proteins *in cis*. *Trends Cell Biol* 3:278-280
8. Scherrer LC, Picard D, Massa E, Harmon JM, Simons SS, Yamamoto KR, Pratt WB (1993) Evidence that the hormone binding domain of steroid receptors confers hormonal control on chimeric proteins by determining their hormone-regulated binding to heat-shock protein 90. *Biochemistry* 32:5381-5386
9. Csermely P, Kajtar J, Hollosi M, Jaisovszky G, Holly S, Kahn CR, Gergely P, Soti C, Mihaly K, Somogyi J (1993) ATP induces a conformational change of the 90-kDa heat shock protein (hsp90). *J Biol Chem* 268:1901-1907
10. Mendel DB, Bodwell JE, Gametchu B, Harrison RW, Munck A (1986) Molybdate-stabilized nonactivated glucocorticoid-receptor complexes contain a 90-kDa nonsteroid-binding phosphoprotein that is lost on activation. *J Biol Chem* 261:3758-3763

11. Sanchez ER, Meschinchi S, Tienrungraj W, Schlesinger MJ, Toft DO, Pratt WB (1987) Relationship of the 90-kDa murine heat shock protein to the untransformed and transformed states of the L cell glucocorticoid receptor. *J Biol Chem* 262:6986-6991
12. Groyer A, Schweizer-Groyer G, Cadepond F, Mariller M, Baulieu EE (1987) Antigluco-corticoid effects suggest why steroid hormone is required for receptors to bind to DNA *in vivo* but not *in vitro*. *Nature* 328:624-626
13. Denis M, Poellinger L, Wikstrom AC, Gustafsson JA (1988) Requirement of hormone for thermal conversion of the glucocorticoid receptor to a DNA-binding state. *Nature* 333:686-688
14. Smith DF, Faber LE, Toft DO (1990) Purification of unactivated progesterone receptor and identification of novel receptor-associated proteins. *J Biol Chem* 265:3996-4003
15. Howard KJ, Distelhorst CW (1988) Evidence for intracellular association of the glucocorticoid receptor with the 90-kDa heat shock protein. *J Biol Chem* 263:3474-3481
16. Rexin M, Busch W, Segnitz B, Gehring U (1992) Structure of the glucocorticoid receptor in intact cells in the absence of hormone. *J Biol Chem* 267:9619-9621
17. Kost SL, Smith DF, Sullivan WP, Welch WJ, Toft DO (1989) Binding of heat shock proteins to the avian progesterone receptor. *Mol Cell Biol* 9:3829-3838
18. Onate SA, Estes PA, Welch WJ, Nordeen SK, Edwards DP (1991) Evidence that heat shock protein-70 associated with progesterone receptors is not involved in receptor-DNA binding. *Mol Endocrinol* 5:1993-2004
19. Sanchez ER, Hirst M, Scherrer LC, Tang HY, Welsh MJ, Harmon JM, Simons SS, Ringold GM, Pratt WB (1990) Hormone-free mouse glucocorticoid receptors overexpressed in Chinese hamster ovary cells are localized to the nucleus and are associated with both hsp70 and hsp90. *J Biol Chem* 265:20123-20130
20. Rexin M, Busch W, Gehring U (1991) Protein components of the nonactivated glucocorticoid receptor. *J Biol Chem* 266:24601-24605
21. Smith DF, Stensgard BA, Welch WJ, Toft DO (1992) Assembly of progesterone receptor with heat shock proteins and receptor activation are ATP mediated events. *J Biol Chem* 267:1350-1356
22. Hutchison KA, Czar MJ, Scherrer LC, Pratt WB (1992) Monovalent cation selectivity for ATP-dependent association of the glucocorticoid receptor with hsp70 and hsp90. *J Biol Chem* 267:14047-14053
23. Rothman JE (1989) Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell* 59:591-601
24. Tai PK, Maeda Y, Nakao K, Wakim NG, Duhring JL, Faber LE (1986) A 59-kilodalton protein associated with progestin, estrogen, androgen and glucocorticoid receptors. *Biochemistry* 25:5269-5275
25. Sanchez ER, Faber LE, Henzel WJ, Pratt WB (1990) The 56-59-kilodalton protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70- and 90-kilodalton heat shock proteins. *Biochemistry* 29:5145-5152
26. Sanchez ER (1990) Hsp-56: a novel heat shock protein associated with untransformed steroid receptor complexes. *J Biol Chem* 265:22067-22070
27. Pratt WB, Czar MJ, Stancato LF, Owens JK (1993) The hsp56 immunophilin component of steroid receptor heterocomplexes: could this be the elusive nuclear localization signal-binding protein? *J Steroid Biochem Mol Biol* 46:269-279
28. Yem AW, Tomasselli AG, Heinrikson RL, Zurcher-Neely H, Ruff VA, Johnson RA, Deibel MR (1992) The hsp56 component of steroid receptor complexes binds to immobilized FK506 and shows homology to FKBP-12 and FKBP-13. *J Biol Chem* 267:2868-2871
29. Tai PK, Albers MW, Chang H, Faber LE, Schreiber SL (1992) Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. *Science* 256:1315-1318
30. Walsh CT, Zydowsky LD, McKeon FD (1992) Cyclosporin A, the cyclophilin class of peptidylprolyl isomerases, and blockade of T cell signal transduction. *J Biol Chem* 267:13115-13118
31. Lebeau MC, Massol N, Herrick J, Faber LE, Renoir JM, Radanyi C, Baulieu EE (1992) P59, and hsp90-binding protein: cloning and sequencing of its cDNA and preparation of a peptide-directed polyclonal antibody. *J Biol Chem* 267:4281-4284
32. Peattie DA, Harding MW, Fleming MA, DeCenzo MT, Lipke JA, Livingston DJ, Benasutti M (1992) Expression and characterization of human FKBP52, an immunophilin that associates with the 90-kDa heat shock protein and is a component of steroid receptor complexes. *Proc Natl Acad Sci USA* 89:10974-10978
33. Smith DF, Bagenstoss BA, Marion TN, Rimerman RA (1993) Two FKBP-related proteins are associated with progesterone receptor complexes. *J Biol Chem* 268:18365-18371
34. Smith DF, Albers MW, Schreiber SL, Leach KL, Deibel MR (1993) FKBP54, a novel FK506 binding protein in avian progesterone receptor complexes and HeLa extracts. *J Biol Chem* 268:24270-24273
35. Perdew GH, Whitelaw ML (1991) Evidence that the 90-kDa heat shock protein (hsp90) exists in cytosol in heteromeric complexes containing hsp70 and three other proteins with  $M_r$  63,000, 56,000 and 50,000. *J Biol Chem* 266:6708-6713
36. Whitelaw ML, Hutchison KA, Perdew GH (1991) A 50-kDa cytosolic protein complexed with the 90-kDa heat shock protein (hsp90) is the same protein complexed with pp60<sup>v-src</sup> hsp90 in cells transformed by the Rous sarcoma virus. *J Biol Chem* 266:16436-16440
37. Smith DF, Sullivan WP, Marion TM, Zaitso K, Madden B, McCormick DJ, Toft DO (1993) Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. *Mol Cell Biol* 13:869-876
38. Wiech H, Buchner J, Zimmerman R, Jakob U (1992) Hsp90 chaperones protein folding *in vitro*. *Nature* 358:169-170
39. Bresnick EH, Dalman FC, Sanchez ER, Pratt WB (1989) Evidence that the 90-kDa heat shock protein is necessary for the steroid binding conformation of the L cell glucocorticoid receptor. *J Biol Chem* 264:4992-4997
40. Scherrer LC, Dalman FC, Massa E, Meshinchi S, Pratt WB (1990) Structural and functional reconstitution of the glucocorticoid receptor-hsp90 complex. *J Biol Chem* 265:21397-21400
41. Pongratz I, Mason GGF, Poellinger L (1992) Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. *J Biol Chem* 267:13728-13734
42. Caamano CA, Morano MI, Patel PD, Watson SJ, Akil H (1993) A bacterially expressed mineralocorticoid receptor is associated *in vitro* with the 90-kilodalton heat shock protein and shows typical hormone- and DNA-binding characteristics. *Biochemistry* 32:8589-8595
43. Smith DF (1993) Dynamics of hsp90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes. *Mol Endocrinol* 7:1418-1429
44. Denis M, Gustafsson JA (1989) Translation of glucocorticoid receptor mRNA *in vitro* yields a nonactivated protein. *J Biol Chem* 264:6005-6008

45. Dalman FC, Bresnick EH, Patel PD, Perdew GH, Watson SJ, Pratt WB (1989) Direct evidence that the glucocorticoid receptor binds hsp90 at or near the termination of receptor translation *in vitro*. *J Biol Chem* 264:19815-19821
46. Smith DF, Schowalter DB, Kost SL, Toft DO (1990) Reconstitution of progesterone receptor with heat shock proteins. *Mol Endocrinol* 4:1704-1711
47. Pratt WB (1993) The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J Biol Chem* 268:21455-21458
48. Hutchison KA, Scherrer LC, Czar MJ, Ning Y, Sanchez ER, Leach KL, Deibel MR, Pratt WB (1993) FK506 binding to the 56-kilodalton immunophilin (hsp56) in the glucocorticoid receptor heterocomplex has no effect on receptor folding or function. *Biochemistry* 32:3953-3957
49. Dalman FC, Scherrer LC, Taylor LP, Akil H, Pratt WB (1991) Localization of the 90-kDa heat shock protein-binding site within the hormone-binding domain of the glucocorticoid receptor by peptide competition. *J Biol Chem* 266:3482-3490
50. Viitanen PV, Lubben TH, Reed J, Goloubinoff P, O'Keefe DP, Lorimer GH (1990) Chaperonin-facilitated refolding of ribulosebiphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K<sup>+</sup> dependent. *Biochemistry* 29:5665-5671
51. Schlossman DM, Schmid SL, Braell WA, Rothman JE (1984) An enzyme that removes clathrin coats: purification of an uncoating ATPase. *J Cell Biol* 99:723-733
52. Scherrer LC, Hutchison KA, Sanchez ER, Randall SK, Pratt WB (1992) A heat shock protein complex isolated from rabbit reticulocyte lysate can reconstitute a functional glucocorticoid receptor-hsp90 complex. *Biochemistry* 31:7325-7329
53. Brugge JS (1986) Interaction of the Rous sarcoma virus protein pp60<sup>src</sup> with cellular proteins pp50 and pp90. *Curr Top Microbiol Immunol* 123:1-22
54. Hutchison KA, Brott BK, De Leon JH, Perdew GH, Jove R, Pratt WB (1992) Reconstitution of the multiprotein complex of pp60<sup>src</sup>, hsp90 and p50 in a cell-free system. *J Biol Chem* 267:2902-2908
55. Hutchison KA, Stancato LF, Jove R, Pratt WB (1992) The protein-protein complex between p60<sup>v-src</sup> and hsp90 is stabilized by molybdate, vanadate, tungstate and an endogenous cytosolic metal. *J Biol Chem* 267:13952-13957
56. Stancato LF, Chow YH, Hutchinson KA, Perdew GH, Jove R, Pratt WB (1993) Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. *J Biol Chem* 268:21711-21716
57. Gething MJ, Sambrook K (1992) Protein folding in the cell. *Nature* 355:33-45
58. Guichon-Mantel A, Lescop P, Christin-Maitre S, Loosfelt H, Perrot-Appianat M, Milgrom E (1991) Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO J* 10:3851-3859
59. Chandran UR, DeFranco DB (1992) Internuclear migration of chicken progesterone receptor, but not simian virus-40 large tumor antigen, in transient heterokaryons. *Mol Endocrinol* 6:837-844
60. Pratt WB (1992) Control of steroid receptor function and cytoplasmic-nuclear transport by heat shock proteins. *BioEssays* 14:841-848
61. Hsu S, Qi M, DeFranco DB (1992) Cell cycle regulation of glucocorticoid receptor function. *EMBO J* 11:3457-3468
62. Martins VR, Pratt WB, Terracio L, Hirst MA, Ringold GM, Housley PR (1991) Demonstration by confocal microscopy that unliganded overexpressed glucocorticoid receptors are distributed in a nonrandom manner throughout all planes of the nucleus. *Mol Endocrinol* 5:217-225
63. Vale RD (1987) Intracellular transport using microtubule-based motors. *Annu Rev Cell Biol* 3:347-378
64. Akner G, Sundqvist KG, Denis M, Wikstrom AC, Gustafsson JA (1990) Immunocytochemical localization of glucocorticoid receptor in human gingival fibroblasts and evidence for a colocalization of glucocorticoid receptor with cytoplasmic microtubules. *Eur J Cell Biol* 53:390-401
65. Akner G, Mossberg K, Wikstrom AC, Sundqvist KG, Gustafsson JA (1991) Evidence for colocalization of glucocorticoid receptor with cytoplasmic microtubules in human gingival fibroblasts, using two different monoclonal anti-GR antibodies, confocal laser scanning microscopy and image analysis. *J Steroid Biochem Mol Biol* 39:419-432
66. Sanchez ER, Redmond T, Scherrer LC, Bresnick EH, Welsh MJ, Pratt WB (1988) Evidence that the 90-kilodalton heat shock protein is associated with tubulin-containing complexes in L cell cytosol and in intact PtK cells. *Mol Endocrinol* 2:756-760
67. Redmond T, Sanchez ER, Bresnick EH, Schlesinger MJ, Toft DO, Pratt WB, Welsh MJ (1989) Immunofluorescence colocalization of the 90-kDa heat shock protein and microtubules in interphase and mitotic mammalian cells. *Eur J Cell Biol* 50:66-75
68. Barsony J, Pike JW, DeLuca HF, Marx SJ (1990) Immunocytology with microwave-fixed fibroblasts shows 1 $\alpha$ , 25-dihydroxy vitamin D<sub>3</sub>-dependent rapid and estrogen-dependent slow reorganization of vitamin D receptors. *J Cell Biol* 111:2385-2395
69. Barsony J, McKoy W (1992) Molybdate increases intracellular 3',5'-guanosine cyclic monophosphate and stabilizes vitamin D receptor association with tubulin-containing filaments. *J Biol Chem* 267:24457-24465
70. Ruff VA, Yem AW, Munns PL, Adams LD, Reardon IM, Deibel MR, Leach KL (1992) Tissue distribution and cellular localization of hsp56, an FK506-binding protein. *J Biol Chem* 267:21285-21288
71. Gibbons IR (1988) Dynein ATPases as microtubule motors. *J Biol Chem* 263:15837-15840
72. Silver PA (1991) How proteins enter the nucleus. *Cell* 64:489-497
73. Picard D, Yamamoto KR (1987) Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J* 6:3333-3340
74. Qi M, Hamilton BJ, DeFranco D (1989) *v-mos* oncoproteins affect nuclear retention and reutilization of glucocorticoid receptors. *Mol Endocrinol* 3:1279-1288