

Review article

Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement

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Abstract

The ubiquitous protein chaperone hsp90 has been shown to regulate more than 100 proteins involved in cellular signalling. These proteins are called ‘client proteins’ for hsp90, and a multiprotein hsp90/hsp70-based chaperone machinery forms client protein•hsp90 heterocomplexes in the cytoplasm and the nucleus. In the case of signalling proteins that act as transcription factors, the client protein•hsp90 complexes also contain one of several TPR domain immunophilins or immunophilin homologs that bind to a TPR domain binding site on hsp90. Using several intracellular receptors and the tumor suppressor p53 as examples, we review evidence that dynamic assembly of heterocomplexes with hsp90 is required for rapid movement through the cytoplasm to the nucleus along microtubular tracks. The role of the immunophilin in this system is to connect the client protein•hsp90 complex to cytoplasmic dynein, the motor protein for retrograde movement toward the nucleus. Upon arrival at the nuclear pores, the receptor•hsp90•immunophilin complexes are transferred to the nuclear interior by importin-dependent facilitated diffusion. The unliganded receptors then distribute within the nucleus to diffuse patches from which they proceed in a ligand-dependent manner to discrete nuclear foci where chromatin binding occurs. We review evidence that dynamic assembly of heterocomplexes with hsp90 is required for movement to these foci and for the dynamic exchange of transcription factors between chromatin and the nucleoplasm.

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1. Introduction

After ligand-dependent activation through receptors in the plasma membrane or after direct receptor activation in the cytoplasm, signalling proteins that affect gene transcription must move to their sites of action within the nucleus. This movement can be divided into four general steps: (1) movement through the cytoplasm to the nuclear pores, (2)

transport across the nuclear pore complex, (3) movement within the nucleus to loci for transcriptional activation, and (4) subsequent dynamic exchange of transcription factors between chromatin and the nucleoplasmic compartment. The steroid receptors have proven to be useful models for studying the movement of transcription factors in each of these movement phases. These receptors form complexes with the ubiquitous and essential chaperone hsp90, and these complexes also contain tetratricopeptide repeat (TPR) domain immunophilins. There is a growing body of evidence that both hsp90 and the hsp90-binding immunophilins participate in various phases of receptor movement.

Since 1990, over 100 protein kinases and transcription factors involved in cellular signalling have been reported to be regulated by hsp90 [1]. Regulation is achieved in multiple ways. In some cases, hsp90 is required for signalling protein function. For example, binding of hsp90 to glucocorticoid (GR), mineralocorticoid and aryl hydrocarbon (AHR) receptors is required for ligand binding activity. In several cases to

Abbreviations: hsp, heat shock protein; SR, steroid receptor; GR, glucocorticoid receptor; AR, androgen receptor; AHR, aryl hydrocarbon receptor; GFP, green fluorescent protein; HBD, hormone binding domain; FKBP, FK506-binding protein; CyP, cyclosporine A-binding protein; PPIase, peptidylprolyl isomerase; TPR, tetratricopeptide repeat; ARA9, AHR-associated protein 9; XAP2, hepatitis B virus X-activating protein 2; AIP, AHR-interacting protein; Arnt, AHR nuclear translocator; PPAR, peroxisome proliferator-activated receptor; NLS, nuclear localization signal; NES, nuclear export signal; Hop, hsp organizing protein.

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be reviewed here, dynamic binding to hsp90 is required for rapid signalling protein movement. There is some evidence that binding of hsp90 to transcription factors may play a role in the termination of transcriptional activation. Finally, in many and perhaps all cases where there is a persistent complex with hsp90, the signalling protein is stabilized to rapid degradation by the ubiquitin-proteasome pathway. In this way, hsp90 serves to maintain the abundance of a key signalling protein, usually a protein kinase, such that normal signal transduction through a pathway occurs.

In this review, we will focus on the roles of hsp90 and the hsp90-binding immunophilins in signalling protein movement, emphasizing the considerable recent progress in understanding cytoplasmic–nuclear shuttling of three transcription factors—the GR, the AHR, and the tumor suppressor p53. We also review studies indicating that dynamic assembly of heterocomplexes with hsp90 is required for the high mobility of transcription factors within the nucleus. The trafficking of signalling proteins was addressed in a 1999 review in *Cellular Signalling* [2], and this review will emphasize work published in the past five years.

2. Assembly of signalling protein • hsp90 • immunophilin complexes

Signalling protein • hsp90 complexes are formed by a multiprotein hsp90/hsp70-based chaperone machinery, and the assembly of these so-called ‘client protein’ • hsp90 complexes has been the subject of a recent detailed review [1]. Briefly, the common pathway for complex assembly involves an initial ATP-dependent interaction of the client protein with the essential chaperone hsp70 and its nonessential cochaperone hsp40 to form a client protein • hsp70 complex that is now ‘primed’ to bind hsp90 and the nonessential cochaperone Hop. A second ATP-dependent reaction then occurs, producing a client protein • hsp90 complex in which the bound hsp90 is converted to its ATP-dependent conformation. The small, ubiquitous cochaperone p23 then binds dynamically to the bound hsp90 to maintain it in the ATP-dependent conformation, thus stabilizing the client protein • hsp90 complex. This assembly machinery is ubiquitous and conserved among animal and plant cells, indicating that it performs essential housekeeping functions [3].

After their formation, the client protein • hsp90 • p23 complexes diverge from the common pathway in that protein kinase • hsp90 complexes quite selectively bind p50^{cdc37} whereas transcription factor • hsp90 complexes bind primarily TPR domain immunophilins and immunophilin homologs (reviewed in Ref. [2]). Both p50^{cdc37} and the TPR domain immunophilins bind directly to hsp90 but at different sites, and the protein kinase complexes select for the presence of p50^{cdc37} because it also binds directly to the catalytic domain of the kinase [4,5]. Similarly, transcription factors, such as the steroid receptors (SR) and the aryl hydrocarbon receptor, form hsp90 heterocomplexes that

contain a TPR domain immunophilin because the immunophilin binds directly to the transcription factor [6,7].

2.1. The TPR domain immunophilins

Several TPR domain proteins (Table 1) bind to a common TPR acceptor site located at the C-terminus of hsp90 [15–19]. Tetratricopeptide repeats are degenerate sequences of 34 amino acids that are involved in a variety of protein–protein interactions [20]. The core of the TPR binding site on hsp90 is the MEEVD sequence [21,22], and although the

Table 1
Mammalian TPR proteins that bind to hsp90

Protein	Proposed role in SR action
Hop (Hsp Organizing Protein)	Binds via independent TPR domains to form the hsp70 • Hop • hsp90 machinery for client protein • hsp90 heterocomplex assembly; Hop promotes rate of assembly but is not essential for assembly
<i>Immunophilins</i> FKBP52	Found in SR • hsp90 heterocomplexes: Targets retrograde SR movement by binding via PPlase domain to cytoplasmic dynein; effect is independent of PPlase activity Increases GR hormone binding affinity in vivo; effect requires both hsp90 binding and PPlase activity [8]
FKBP51	Found in SR • hsp90 heterocomplexes; FKBP51 expression blocks effect of FKBP52 on GR steroid binding activity [8]; dynein binding status unknown
CyP-40	Found in SR • hsp90 heterocomplexes and binds to cytoplasmic dynein
<i>Immunophilin homologs</i> PP5	An okadaic acid-sensitive protein-serine phosphatase with a TPR domain and a PPlase homology domain [9]; PP5 is found in SR • hsp90 heterocomplexes, and it binds to cytoplasmic dynein; its phosphatase activity may be important for cytoplasmic–nuclear trafficking
ARA9 (XAP2, AIP)	Found in aryl hydrocarbon receptor (AHR) • hsp90 heterocomplexes; mediates the cytoplasmic localization of the AHR; has a PPlase homology domain that does not interact with, or interacts only very weakly with, cytoplasmic dynein [56]
CHIP (C-terminus of Hsc70 Interacting Protein)	Binds via TPR domain to hsc70, hsp70, or hsp90 [10]; CHIP interaction with hsc70/hsp70 causes proteasome-dependent degradation of many hsp90 client proteins; CHIP is an E3 ubiquitin ligase for the GR [11]
UNC-45	Binds via N-terminal TPR to hsp90 and via C-terminal regions to myosin [12]
Tom 70	Mitochondrial import receptor with a TPR domain that binds hsp90 [13]
Tpr2	A TPR domain protein that recognizes hsp90 and hsp70; it contains a J domain and stimulates ATP hydrolysis by hsp70; its expression affects GR activity negatively [14]

TPR proteins listed in Table 1 compete with each other for binding to this site, there are some differences in their binding determinants [23–25] (reviewed in Ref. [26]). Hsp90 forms a homodimer, the TPR binding site lies within the dimerization domain, and the number of TPR acceptor sites per dimer has been controversial. Studies of saturation binding of Hop to hsp90 dimer [16] and cross-linking of hsp90•FKBP52 complexes [7] are consistent with one TPR binding site per dimer. In contrast, isothermal titration calorimetry studies are consistent with binding of two molecules of TPR protein to an hsp90 dimer [27,28]. In cross-linking studies, Gehring and his colleagues determined a heteromeric structure of 1 receptor:2 hsp90:1 immunophilin for several steroid receptor heterocomplexes (reviewed in Ref. [29]), and that is the stoichiometry that we will assume in this review.

The immunophilins are conserved proteins that bind immunosuppressant drugs, such as FK506, rapamycin and cyclosporine A. All members of the immunophilin family have peptidylprolyl isomerase (PPIase) activity, and they are divided into two classes: the FKBP5s bind FK506 and rapamycin, and the cyclophilins (CyPs) bind cyclosporine A. The immunosuppressant drugs occupy the PPIase site on the immunophilin, blocking its ability to direct *cis*–*trans* isomerization of peptidyl–prolyl bonds. Three high molecular weight immunophilins with TPR domains—FKBP52, FKBP51, CyP40—have been found in steroid receptor•hsp90 complexes (Fig. 1) [1]. A fourth SR•hsp90 complex protein, protein phosphatase 5 (PP5), is a protein-serine phosphatase with three TPRs and a PPIase homology domain with weak FK506 binding activity but no isomerase activity [9]. Because these TPR proteins can exchange for binding to hsp90, any single SR•hsp90 complex can theoretically be associated over time with more than one

immunophilin. However, it has been shown that, at any point in time, the immunophilins exist in separate GR•hsp90 hsp90 heterocomplexes [30,31]. The relative amounts of FKBP52, FKBP51, CyP40 and PP5 may vary somewhat among the different steroid receptor heterocomplexes [26] according to immunophilin interaction with the receptor itself. There is a clear difference between the steroid receptor complexes, which do not contain ARA9, and the AHR, which appears to be bound exclusively to ARA9 as a result of direct ARA9 binding to the AHR itself [32]. The TPR domain immunophilins are distributed widely among animal and plant cells and TPR domain binding to hsp90 is conserved [33–35]. This suggests that immunophilin binding to hsp90 is essential for both the action of the TPR domain immunophilins and for major functions of hsp90.

2.2. The immunophilin PPIase activity

The broad distribution of the TPR domain immunophilins and the presence of more than one member of the family in most cells suggest that their function(s) is (are) important for cell homeostasis and that there may be redundancy in their action(s). The presence of the PPIase domain leads naturally to the proposal that the action of the hsp90-binding immunophilins is due to isomerization of prolyl peptide bonds. Early experiments in intact cells demonstrated that the isomerase inhibitors FK506 [36,37] and cyclosporine A [31] could enhance dexamethasone-induced expression from a reporter plasmid. Subsequent experiments in cytosol preparations showed that addition FK506 at 25 °C stabilized both progesterone [181] and glucocorticoid [182] receptor•hsp90 complexes, and heterocomplex stabilization was accompanied by a twofold decrease in the K_D for ligand binding. It is unclear whether these direct effects reflect inhibition of the PPIase activity of hsp90-binding immunophilins or a physical stabilization. In addition, there are indirect effects. It is known that both dexamethasone and the immunosuppressant drugs are transported out of the cell by the multidrug transporter Mdr1 (reviewed in Ref. [29]), and Kralli and Yamamoto [38] showed that FK506 potentiates dexamethasone responsiveness in L cells by increasing dexamethasone accumulation without altering the hormone binding properties of the GR.

Subsequent studies with squirrel monkey cells suggested that immunophilins may be responsible for the relative glucocorticoid insensitivity of these New World primates. Squirrel monkeys have very high levels of circulating corticosteroid and require much higher levels of hormone for GR-dependent transactivation. However, the cloned Bolivian squirrel monkey GR expressed *in vitro* was found to bind dexamethasone with the same affinity as the human GR [39]. Interest then focused on the high ratio of FKBP51 to FKBP52 found in squirrel monkey cells [40–42]. Human FKBP51 and squirrel monkey FKBP51 are 94% identical and have similar X-ray structures [43], but at similar levels of expression, squirrel monkey FKBP51 is much more

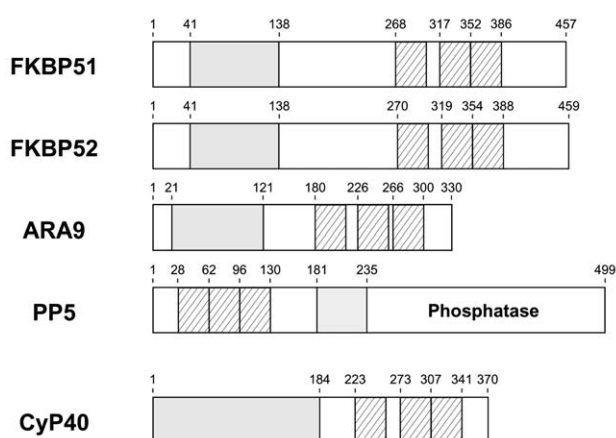


Fig. 1. Functional domain structure of the hsp90-binding, TPR domain immunophilins and immunophilin homologs. FKBP51 and FKBP52 have an FK506-binding type of PPIase domain with isomerase activity. ARA9 and PP5 have domains homologous to the FK506-binding domain but have no isomerase activity. CyP40 has a cyclosporine A-binding type of PPIase domain with isomerase activity. PPIase and PPIase homology domains are shaded, TPRs are slashed boxes. The numbering is for the human proteins.

effective at reducing GR hormone binding affinity and reporter gene expression. In contrast to these observations, Patel et al. [44] found that the human GR has a normal dose response for transactivation regardless of whether it is expressed in New World or Old World primate cells. They cloned the Guyanese squirrel monkey GR, and showed that it had the same high affinity binding activity as the human GR when expressed in COS-1 cells, but it had an order of magnitude higher EC50 at transactivation than the human GR in both squirrel monkey (New World) and COS-1 (Old World) cells. In this case, the conclusion was that glucocorticoid resistance in the Guyanese squirrel monkey is at least partly attributable to a naturally occurring mutation in the GR gene that impairs GR transactivating activity.

To determine directly whether the hsp90-binding FKBP5s affect GR steroid binding activity, Riggs et al. [8] expressed human FKBP51 and FKBP52 in *Saccharomyces cerevisiae*, which does not contain any TPR domain FKBP5s of its own, and they showed that FKBP52 selectively potentiates GR-dependent reporter gene activation. The potentiation was due to an increase in GR hormone binding affinity that required both the hsp90 binding activity and the PPIase activity of FKBP52. Co-expression of FKBP51 with FKBP52 blocked the potentiation but coexpression of PP5 did not affect the potentiation [8]. This work provides the first evidence that an hsp90-binding immunophilin can affect a client protein function (i.e. steroid binding) through its peptidylprolyl isomerase activity. Presumably, the folding change due to isomerization occurs in the client protein itself, although that remains to be demonstrated. It should be noted that GR that has been assembled into GR•hsp90 heterocomplexes by the purified heterocomplex assembly system in the absence of immunophilins has normal high affinity steroid binding activity. However, that does not in any way argue against this model, because the GR that is the client protein in those assays is immunopurified from cell lysates where it was properly folded and in high affinity binding state before it was stripped of its associated chaperones.

The work of Riggs et al. [8] in yeast has raised the important notion that a major function of the hsp90-bound immunophilins is to modify the folding, and thus the structure and function, of hsp90-bound client proteins. In the future, it will be important to see if this model applies to a variety of hsp90 client proteins and to other immunophilins besides FKBP52. At present, the observation is specific to the GR, as opposed to the estrogen receptor (also a client protein), and is specific to FKBP52 versus other hsp90-bound immunophilins [8]. It is curious that FKBP51 and FKBP52 have highly conserved domain structures (Fig. 1) and possess active isomerase domains, yet FKBP52 increases GR steroid binding activity and FKBP51 counteracts this effect. Such a yin and yang action is not consistent with the notion of redundancy in action of TPR domain immunophilins, but it could be of considerable regulatory importance. In subsequent sections of this review, we will

not be concerned with the isomerase activity of the hsp90-bound immunophilins. Rather, we will focus on the PPIase domains as protein–protein interaction domains that serve to link transcription factor•hsp90 complexes to cytoplasmic dynein, the motor protein responsible for retrograde movement toward the nucleus along microtubular tracks.

3. Involvement of hsp90, immunophilins, and dynein in receptor movement through the cytoplasm

The GR is an excellent model for studying the cytoplasmic–nuclear movement of a transcription factor, because it is normally located in the cytoplasm of hormone-free cells and its rapid movement to the nucleus is hormone-dependent, placing movement under the control of the investigator. It is well established that the steroid receptors constantly shuttle between the cytoplasm and the nucleus, with ligand-dependent transformation of the GR•hsp90 complex favoring a change in this flux to a nuclear accumulation (reviewed in Ref. [45]). On the basis of biochemical observations, it was thought for many years that ligand-dependent transformation of the GR resulted in release from hsp90, leaving the chaperone-free receptor to move to the nucleus. However, it is now clear that GR translocation occurs in association with hsp90 [46,47], and it is thought that ligand-dependent transformation converts the receptor from a state that forms ‘persistent’ complexes with hsp90 to a state that is in a much more dynamic receptor•hsp90 complex assembly/disassembly cycle. The proposal that dynamic GR•hsp90 complex assembly is required for rapid receptor movement is supported by a variety of observations that have been previously reviewed [2,48].

The model for retrograde GR movement by dynein motors along cytoskeletal tracks that is diagrammed in Fig. 2 was first proposed in 1993 [49]. By that time, it was well established that vesicles moved in both neurites and cell bodies along cytoskeletal tracks in a process requiring molecular motors [50], with cytoplasmic dynein being the motor protein responsible for movement in the retrograde direction toward the nucleus (reviewed in Refs. [51,52]). We reasoned that protein solutes that were not associated with vesicles might move in a similar manner. In 1993, the only immunophilin known to be in GR•hsp90 complexes was FKBP52, which, although it localizes predominantly to the nucleus, has a cytoplasmic component that localizes to microtubules [53–55]. Accordingly, we asked if cytosolic FKBP52 is in complexes with cytoplasmic dynein and we showed by coimmunoadsorption that it is [54]. Thus, we proposed that one function of FKBP52 was to link the receptor•hsp90 complex to the motor protein.

This model has been advanced considerably in the past few years. It is now known that several of the hsp90-binding immunophilins are linked via their PPIase domains to cytoplasmic dynein [56], and the entire complex shown in Fig. 2 has been isolated from cells and has been recon-

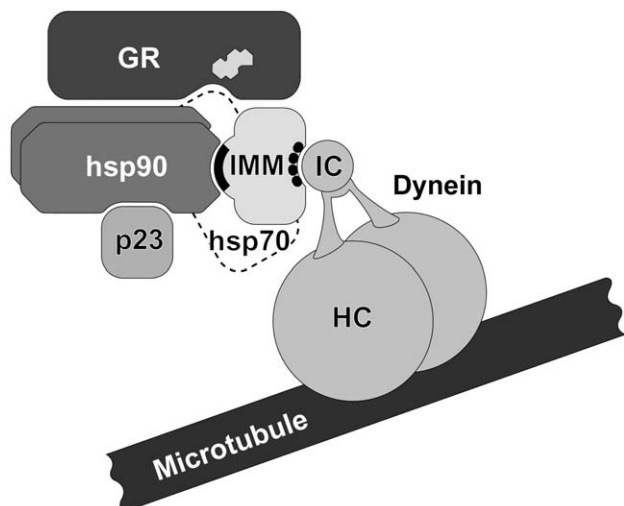


Fig. 2. TPR domain immunophilins link the GR•hsp90 heterocomplex to dynein for retrograde movement along microtubules. Cytoplasmic dynein is the motor protein that processes along microtubules in a retrograde movement to the nucleus. Dynein is a large multisubunit complex (~ 1.2 MDa) comprised of two heavy chains (HC) that have the processive motor activity, three intermediate chains (IC), and some light chains that are not shown. Also not shown is the dynein-associated dynactin complex, of which dynamitin is a component. The immunophilin (IMM) links to GR-bound hsp90 via its TPR domain (solid black crescent) and it links to dynein or a component of the dynactin complex via its PPIase domain (dotted crescent).

structured in a cell-free system [57–59]. The cytoplasmic–nuclear movement of the GR and some other transcription factors has been impeded or blocked by inhibitors of hsp90, by coexpression of a PPIase domain fragment that blocks immunophilin binding to dynein, and by coexpression of dynamitin, which dissociates dynein from its cargoes. Thus, there is strong *in vivo* evidence for the validity of the GR movement model of Fig. 2.

3.1. Inhibition of signalling protein movement with hsp90 inhibitors

Hsp90 is a member of a small family of proteins, the GHKL (*G*yrase, *H*sp90, *H*istidine *K*inase, *M*ut *L*) family, which possess a unique binding pocket for ATP [60]. This nucleotide binding site near the N-terminus of hsp90 is the site of action for the hsp90 inhibitors geldanamycin and radicicol. In binding to this site, geldanamycin and radicicol prevent hsp90 from assuming its ATP-dependent conformation, thus blocking client protein•hsp90 assembly and hsp90 action. The discovery by the Neckers laboratory, in 1994, that geldanamycin acts as a quite specific inhibitor of hsp90 [61] provided a rapid means of screening for hsp90-regulated targets, initiating a rapid escalation in the identification of hsp90-regulated signalling pathways [1].

As shown first with the endogenous GR [62] and then with a transfected green fluorescent protein fusion with the GR (GFP-GR) [63], treatment of cells with geldanamycin slows the rate of receptor translocation to the nucleus by an

order of magnitude (from $t_{1/2} \sim 4.5$ min to $t_{1/2} \sim 45$ min). The rapid hsp90-dependent movement requires intact cytoskeleton [63], and when hsp90 is inhibited there is slow movement that appears to occur by diffusion (Fig. 3). Axons and dendrites are specialized cytoplasmic extensions where proteins cannot move by random diffusion alone, and ligand-dependent retrograde movement of the GFP-GR in neurites is blocked by geldanamycin [64], indicating that the hsp90-dependent movement machinery is obligatory in these structures. Geldanamycin and radicicol also impede ligand-dependent movement of the androgen receptor to the nucleus [65,66], suggesting that all of the members of the nuclear receptor family that form persistent complexes with

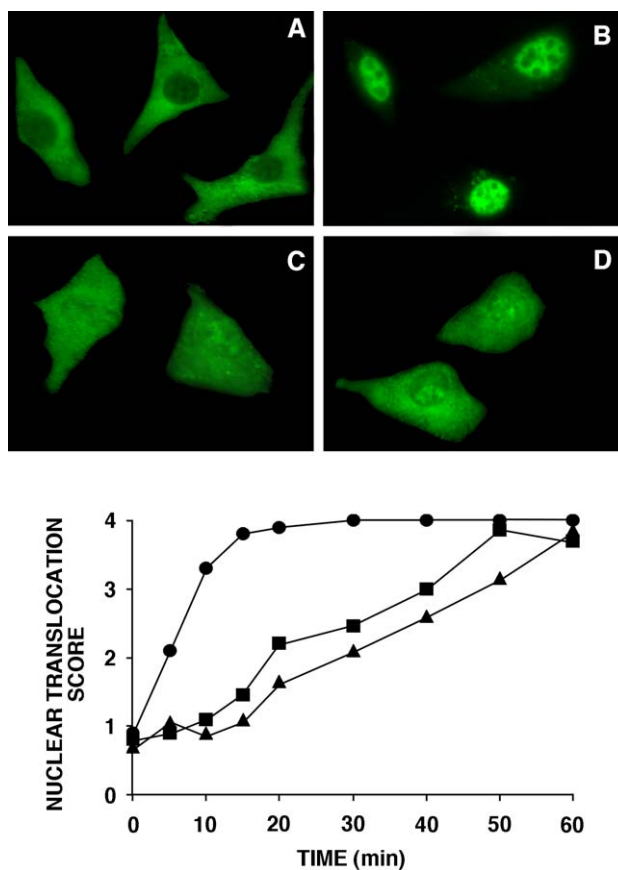


Fig. 3. Geldanamycin treatment or cotransfection of the PPIase domain fragment of FKBP52 inhibits steroid-dependent GFP-GR translocation to the nucleus. 3T3 cells were transfected with a plasmid expressing GFP-GR and in the case of D cotransfected with a plasmid expressing the PPIase domain fragment of FKBP52. Two days after transfection, $1 \mu\text{M}$ dexamethasone or ethanol was added, the incubation was continued for 20 min, and cells were fixed and fluorescence was visualized. (A), Untreated cells; (B), cells treated with dexamethasone; (C), cells treated with geldanamycin and dexamethasone; (D), cells cotransfected with PPIase domain treated with dexamethasone. The graph shows data compiled from >100 cells scored for nuclear translocation on a scale from 0 for nuclear fluorescence much less than cytoplasmic fluorescence to 4 for nuclear fluorescence much greater than cytoplasmic fluorescence. See Ref. [58] for details of methods. (●) Cells treated with dexamethasone; (▲) cells treated with dexamethasone and geldanamycin; (■) cells cotransfected with the PPIase domain and treated with dexamethasone.

hsp90 and are localized to the cytoplasm of hormone-free cells may require dynamic heterocomplex assembly with hsp90 for rapid movement through the cytoplasm to nuclear pores.

The AHR is different from steroid receptors in that its DNA binding domain is a basic helix-loop-helix rather than a double zinc finger structure, and rather than forming homodimers in the nucleus it forms heterodimers with the Arnt (*Aryl hydrocarbon receptor nuclear translocator*) protein. However, like the steroid receptors, the AHR shuttles in and out of the nucleus, it forms persistent complexes with hsp90, and ligand-induced nuclear accumulation of the AHR is inhibited by geldanamycin [67,68].

The tumor suppressor protein p53 is a transcription factor that can induce cell growth arrest, apoptosis, cell differentiation and DNA repair in response to DNA strand breakage and other types of cell stress (reviewed in Ref. [69]). P53 mutations occur in more than half of all human tumors and inactivation of p53 is the most common alteration found in human cancer. One mechanism of inactivation is exclusion from the nucleus, and some p53 mutants retained in the cytoplasm are in complex with hsp90 [70,71]. Using a temperature-sensitive mutant of p53 where cytoplasmic–nuclear movement occurs upon shift to permissive temperature, we have shown that p53 movement is impeded by radicicol [72].

Although almost all of the work to date has examined the role of hsp90 in trafficking of signalling proteins that are protein solutes in the cytoplasm, hsp90 interacts with the cytoplasmic portions of a number of plasma membrane receptors and ion channels. Recent evidence suggests that hsp90 is involved in the trafficking as well as the turnover of some of these membrane associated signalling proteins. Thus, in addition to accelerating their proteasomal degradation, geldanamycin has been found to inhibit the intracellular trafficking of two receptor tyrosine kinases, epidermal growth factor [73] and ErbB2 [74], and it inhibits the maturation and targeting of two ion channels, the CFTR chloride channel [75] and the hERG cardiac potassium channel [76]. Radicicol treatment of brain slices inhibits the constitutive trafficking of AMPA-type glutamate receptors back into synapses during their continuous cycling between synaptic and non-synaptic sites [77]. Interestingly, the synaptic cycling of AMPA receptors was also impeded by expression of the hsp90-binding TPR domain fragment of PP5 but not by a TPR domain mutant that does not bind hsp90, suggesting that the cycling may be mediated by an hsp90-binding TPR domain protein. As another example, hsp90 is required for signalling by the α subunit of the heteromeric G₁₂ protein [78], and geldanamycin inhibits the targeting of G α_{12} into lipid rafts, thus inhibiting its movement into discrete membrane domains [79].

At this time, we are clearly at an early stage of understanding the role of hsp90 in signalling protein trafficking through the cytoplasm to the nucleus, from the Golgi to the plasma membrane, and signalling protein movement at the

inner surface of the plasma membrane. Whether hsp90 plays a general role in the targeted movement of proteins or a more specific role in the movement of a limited number of signalling proteins is not known. What we propose is that one function of the hsp90/hsp70-based chaperone machinery in forming client protein•hsp90 complexes is to ‘capture’ proteins into multichaperone complexes that, through the hsp90-bound immunophilins, can link them to motor systems for their movement along cytoskeleton. An important concept is that the chaperone machinery can interact with proteins in their native, least energy state without regard to a protein’s size, shape, amino acid sequence or function [1]. This ability to interact with a wide variety of client proteins combined with the diversity that arises from the various TPR-domain proteins that associate with the client protein and hsp90 may provide an integrated system for targeted movement of proteins to diverse sites of action within the cell.

3.2. The immunophilin role in movement

Steroid receptor•hsp90 complexes were first formed *in vitro* by incubating immunoadsorbed receptors stripped of their associated chaperones with rabbit reticulocyte lysate. In addition to containing the hsp90 heterocomplex assembly machinery (hsp90, hsp70, Hop, hsp40, p23), reticulocyte lysate contains immunophilins and cytoplasmic dynein. GR•hsp90 heterocomplexes prepared in this manner contain TPR domain immunophilins and cytoplasmic dynein [58]. The linkages shown in Fig. 2 are known because addition of a TPR fragment of PP5 to reticulocyte lysate yields GR•hsp90 complexes without immunophilins or dynein, and addition of an expressed PPIase domain fragment of FKBP52 yields GR•hsp90•immunophilin complexes without dynein [58]. The fragment of FKBP52 comprising the segment between the PPIase domain and the first TPR (Fig. 1) has less homology with FKBP12 (28%) than the PPIase domain fragment (49%) [80], and neither it nor FKBP12 compete for immunophilin binding to dynein [57]. Also, the presence of FK506 has no effect on the immunophilin association with dynein [57]. Immunoadsorption of immunophilins from brain cytosol showed that FKBP52, Cyp40 and PP5 were in native complexes with dynein that could be disrupted by incubation with the PPIase domain fragment of FKBP52 [56]. In contrast to these three immunophilins (FKBP51 was not tested), ARA9 was not recovered in cytosolic complexes with dynein [56].

Thus, the PPIase domains of FKBP52, Cyp40 and PP5 determine immunophilin binding in a heterocomplex with cytoplasmic dynein. All PPIase or PPIase homology domains do not bind dynein (e.g. FKBP12, ARA9), and binding occurs even when isomerase activity is blocked with FK506. It is unclear whether it is dynein itself or perhaps a component of the dynein-associated dynactin complex (e.g. dynamitin) that interacts with the PPIase domain. We have identified a weak binding of purified

FKBP52, PP5 and a PPIase domain fragment with purified, expressed mouse cytoplasmic dynein intermediate chain [56]. But, it should be noted that similar weak interactions occur with peptidyl prolines [81], and direct binding of PPIase to dynein intermediate chain could be nonspecific in this way. Cytoplasmic dynein is thought to link to vesicles and organelles indirectly through dynactin (reviewed in Ref. [51]), and immunophilin interaction with cytoplasmic dynein could reflect such an indirect linkage.

Davies et al. [59] have developed an intriguing model in which steroid binding to the LBD of the GR induces an exchange of immunophilins and increased amounts of dynein in the GR•hsp90•immunophilin complexes. Focusing on just the hsp90-binding FKBP52, they note that prior to introducing dexamethasone, cytosolic GR•hsp90 complexes contain primarily FKBP51, but after cytosol is incubated with dexamethasone at 4 °C, the complexes contain primarily FKBP52. This ligand-induced exchange of FKBP51 for FKBP52 is accompanied by a threefold increase in receptor-associated cytoplasmic dynein. The same switching of FKBP51 and increased dynein was seen when receptors were exposed to dexamethasone in intact cells at 4 °C, and this switching was accompanied by receptor movement to the nucleus. Here again, we see the notion of FKBP51 and FKBP52 having opposing yin and yang actions, much as was seen in the data of Riggs et al. [8] for the effect of these two immunophilins on steroid binding affinity.

It is known that the NL1 nuclear localization signal is occluded in the unliganded GR and becomes accessible to NL1-specific antibody upon steroid-dependent transformation [82], and it is clear that the NL1 site is blocked or conformationally altered by hsp90 and opened by its removal [83]. Binding of steroid deep within the ligand binding cleft promotes closing of the cleft with concomitant loss of the GR's ability to form 'persistent' complexes with hsp90. This change in hsp90 binding by the LBD may open the positively charged NL1 as a potential binding site for FKBP52, which possesses a short negatively charged hinge segment that lies just C-terminal to its PPIase domain. This negatively charged segment of FKBP52 is electrostatically complementary to the NL1 region of the GR (reviewed in Ref. [84]), which is required for direct binding of the FKBP52 to hsp90-free receptor [7]. Opening up the NL1 upon steroid binding to the LBD may thus favor the binding of FKBP52 over FKBP51 and account for the ligand-induced change. Because the presence of dynein in the GR•hsp90•immunophilin complex increases with the exchange for FKBP52, it is inherent to the model that FKBP51 does not bind dynein or binds it very poorly compared to FKBP52. Although this would be predicted, the dynein binding status of FKBP51 is unknown.

Like the PPIase domains themselves, the interactions of hsp90-binding immunophilins with dynein are conserved. It has been shown, for example, that the wheat TPR domain

immunophilins wFKBP73 and wFKBP77 bind through their PPIase domains to mammalian cytoplasmic dynein [35]. Indeed, the entire assembly system is conserved, in that immunopurified mouse GR incubated with wheat germ lysate forms GR•wheat hsp90•wheat immunophilin complexes that bind rabbit cytoplasmic dynein [35]. This suggests that that ability to form the 'transportosome' complex [49] shown in Fig. 2 is fundamental in the biology of the eukaryotic cell.

Like FKBP52, the portion of PP5 that is cytoplasmic colocalizes with microtubules, and in cells transfected with a plasmid encoding the PPIase domain fragment of FKBP52, the microtubular localization of PP5 is disrupted [56]. This is consistent with the microtubular localization of PP5 being determined by binding through its PPIase domain to the dynein/dynactin complex. In cells where the PPIase domain fragment is coexpressed with the GFP-GR, steroid-dependent translocation of the receptor to the nucleus is slowed in the same manner as it is when cells are treated with geldanamycin (Fig. 3) [58]. The tumor suppressor p53 is in hsp90 heterocomplexes that contain the same immunophilins as the GR, and expression of the PPIase domain also inhibits translocation of a temperature-sensitive mutant of p53 to the nucleus [72]. Coexpression of FKBP12, which does not compete for immunophilin binding to dynein *in vitro*, does not affect GFP-GR translocation *in vivo*. Thus, like hsp90 itself, the hsp90-binding immunophilins are required for rapid movement to the nucleus, consistent with their role in linking the GR•hsp90 complex to the dynein motor for such retrograde movement (Fig. 2).

The immunophilin homolog PP5 is of particular interest with regard to another role in movement relating to its phosphatase domain. PP5 (reviewed in Ref. [85]) is an okadaic acid-sensitive protein-serine phosphatase that in its basal state has a low activity because of autoinhibition by its TPR domain. When the TPR domain is removed by partial proteolysis, the phosphatase activity increases severalfold [86,87]. The interaction of PP5 through its TPR domain with the TPR acceptor site on hsp90 also relieves the autoinhibition, increasing its phosphatase activity [88]. PP5 is of special interest because an okadaic acid-sensitive phosphatase activity is required for GR shuttling [47,89]. However, suppression of PP5 expression was found to cause both nuclear accumulation of GFP-GR [90] and to activate GR-dependent expression from a reporter in the absence of hormone [91]. These studies led to the conclusion that PP5 affects the cytoplasmic–nuclear shuttling of the GR by suppressing nuclear accumulation [91]. This is precisely the opposite of what would be predicted if PP5 were the target of the okadaic acid effect in cytoplasmic–nuclear shuttling experiments [47,89]. Despite the current confusion, given that PP5 is the only TPR domain, okadaic acid-sensitive protein phosphatase found in GR•hsp90 heterocomplexes, it is likely that its phosphatase activity will be found to play an important role in receptor translocation.

3.3. Roles of other hsp90-binding TPR domain proteins in movement

In addition to a role in targeting protein movement through the cytoplasm by linking hsp90 client proteins to dynein, it has been proposed that some hsp90-binding TPR domain proteins may act at targeted organelles to accept protein•hsp90 complexes [92]. For proteins with mitochondrial localization signals, the Tom70 component of the mitochondrial import receptor may serve this purpose. The association of the hsp90/hsp70-based chaperone machinery with mitochondrial import started with the partial purification of a large mass complex (200–250 kDa) containing hsp70 that maintained proteins to be imported into mitochondria in an import competent state [93]. This complex was shown to contain hsp90 as well as hsp70 and to assemble GR•hsp90 heterocomplexes in which the receptor had steroid binding affinity [94]. Indeed, this was the first demonstration that hsp90 and hsp70 worked together in a multi-chaperone assembly machinery. In a subsequent study of TPR proteins binding to hsp90, it was shown that Tom70 (known then as Mas70p) bound to hsp90 in a manner that was competed by Hop [92]. It was proposed at that time that proteins being moved to the mitochondria might undergo a “hand off” from the movement system to Tom70. In a recent definitive study, Young et al. [13] demonstrated that hsp90 and hsp70 dock onto a Tom70 TPR domain at the outer mitochondrial membrane to deliver a set of preproteins through the membrane.

A similar system may have evolved for import of peroxisomal proteins. A variety of proteins termed peroxins (Pex) are required for peroxisome assembly and protein import. Peroxisomal proteins destined for the peroxisomal matrix are targeted to the organelle by peroxisomal targeting signals (PTSs). Most peroxisomal matrix proteins are targeted by PTS1 and their import is determined by Pex5p [95], which may not only bind PTS1-containing proteins but participate in their entry into the matrix and export into cytosol [96]. Pex5p contains seven TPRs in the C-terminus, and an intact TPR domain is necessary but not sufficient for interactions with PTS1-containing proteins [97,98]. Both hsp70 and ATP are involved in the binding of Pex5p to PTS1 protein [99], and import of isocitrate lyase into peroxisomes is inhibited by antibodies against wheat germ hsp70 and *Escherichia coli* hsp90 [100]. Pex5p has also been reported to coimmunoabsorb with hsp90 [101]. Pex5p may be involved in moving chaperone-bound PTS1 proteins to the peroxisome where it interacts with other peroxins to effect their entry.

ARA9 (also called XAP2 and AIP) was isolated in yeast two-hybrid screens for proteins interacting with the AHR [102–104]. ARA9 contains three TPRs in its C-terminus and a PPIase homology domain (50% similarity and 27% identity with human FKBP52 PPIase domain) without PPIase activity in its N-terminus [102–104]. The TPR domain binds hsp90 and the N-terminal region is essential

for ARA9 to regulate intracellular localization of the AHR [105]. By immunofluorescence, the unliganded AHR is located in both the cytoplasm and nucleus, but when ARA9 is overexpressed, the AHR is redistributed to the cytoplasm [67,106–108]. This redistribution of AHR to the cytoplasm is inhibited by geldanamycin [67], suggesting that both hsp90 and ARA9 are required for anterograde movement from the nucleus or for trapping of the AHR in the cytoplasm. The ARA9-mediated cytoplasmic localization of the AHR is inhibited by cytochalasin B, which inhibits polymerization of actin filaments [109]. Thus, it is currently thought that ARA9/XAP2/AIP anchors the ligand-free AHR to actin filaments to maintain its cytoplasmic localization [109]. Although ARA9/XAP2/AIP has not been found in steroid receptor•hsp90 heterocomplexes, it has been found in complex with PPAR α , which is also a member of the nuclear receptor superfamily [110]. PPAR α mediates the carcinogenic effects of peroxisome proliferators in rodents and has been recovered from cytosols as a PPAR α •hsp90•ARA9 complex [110].

3.4. Role of cytoplasmic dynein in movement

The tumor suppressor p53 was the first transcription factor shown to be moved to the nucleus by cytoplasmic dynein [111]. p53 was found to colocalize with microtubules in several human carcinoma cell lines and to be in cytosolic heterocomplexes with microtubules. Both overexpression of dynamitin and microinjection of anti-dynein antibody before DNA damage abrogated subsequent nuclear accumulation of p53 [111]. Dynamitin is a 50-kDa subunit of the dynein-associated dynactin complex, and its overexpression blocks dynein function by dissociating the motor protein from its cargoes [51,112]. Inhibition of movement through expression of dynamitin is powerful evidence that movement occurs through attachment to cytoplasmic dynein. Microtubule-perturbing drugs also inhibit nuclear accumulation of p53 [112], consistent with dynein-dependent movement along microtubular tracks.

Although the linkage between p53 and dynein was not worked out when these experiments were performed, it is now clear that p53•hsp90 complexes are linked to cytoplasmic dynein by immunophilins in the same manner as shown for the GR in Fig. 2 [72]. In DLD-1 human colorectal adenocarcinoma cells, which contain a mutant p53 that is located in the cytoplasm in complex with hsp90, both the GR and p53 are in complexes with the same hsp90-binding immunophilins, which are present in the similar relative amounts in each complex. Thus, it is not surprising that p53 movement to the nucleus in human colorectal carcinoma cells expressing a mouse p53 temperature-sensitive mutant is inhibited by geldanamycin treatment and by expression of a PPIase domain fragment in the same manner as the GR [72].

In contrast to p53, the hormone-free GR has usually been found to be diffusely located throughout the cytoplasm by

immunofluorescence, although there are some reports of colocalization with microtubules (reviewed in Ref. [113]). Like p53, however, GR•hsp90•immunophilin•dynein heterocomplexes immunoadsorbed from cytosol of taxol-treated cells contain tubulin, and GR translocation to the nucleus is inhibited by coexpression of dynamitin (JM Harrell, work in progress). Now that techniques for implicating dynein in movement have been worked out, it is likely that other transcription factors will be found to move rapidly through the cytoplasm in a dynein-dependent manner.

4. Transport across the nuclear pores

Once they arrive at the nuclear membrane, signalling proteins undergo a facilitated diffusion through nuclear pores, which allow the selective inward and outward passage of transport receptors called importins and exportins (reviewed in Refs. [114–116]). In the case of proteins with a classical nuclear localization signal (NLS), such as SV40 T antigen, nucleoplasmin and steroid receptors, two importin proteins are involved in nuclear entry. Importin- α is the NLS receptor, and it also binds importin- β , which is the unit of the complex that interacts with motifs in the nucleoporins to facilitate passage through the pore. Passage is very rapid, and it is unidirectional because of cycling of Ran. Ran is a Ras-related small GTPase that switches between a GDP- and a GTP-bound state. A nucleotide exchange factor in the nucleus generates RanGTP, and a GTPase-activating protein (GAP) that is excluded from the nucleus converts RanGTP to RanGDP at the cytoplasmic face of the nuclear pore. After the cargo-importin complex traverses the pore, RanGTP binds to importin and the cargo is released. The RanGTP•importin- β complex passes back through the pore without cargo to the cytoplasmic face where RanGAP converts RanGTP to RanGDP, releasing free importin to participate in another cycle of cargo entry. To shuttle in and out of the nucleus, signalling proteins that are transcription factors (e.g., steroid receptors, AHR, p53) possess nuclear export signals (NES) that determine movement in the reverse direction through the pores to the cytoplasm. NES proteins bind exportins, and passage is unidirectional in the same Ran-regulated manner. CRM1 is an exportin for NES proteins that is inhibited by the drug leptomycin B, which has been a useful tool for studying signalling protein movement.

Unlike the passage of proteins into mitochondria and other organelles where proteins must be unfolded to pass, proteins pass through the nuclear pores intact, and the pores are large enough to permit passage of multimolecular complexes up to $1-3 \times 10^6$ Da [114]. Given the dynamic nature of the client protein•hsp90 heterocomplex assembly/disassembly cycle, it is possible that heterocomplexes could be disassembled before passage and reassembled on the nuclear side of the pore. It is clear, however, that the GR can pass both into [117] and out of [118] the nucleus as a GR•hsp90 heterocomplex; thus, there is no requirement for

heterocomplex disassembly prior to pore passage. At this time, it seems likely that the dominant mode of passage through nuclear pores is as receptor•hsp90•immunophilin heterocomplexes (see discussion in Ref. [118]). Indeed, because neither hsp90 nor the hsp90-binding immunophilins have NLSs of their own, it seems likely that their presence in the nucleus must be due to the fact that they are carried in by multiple NLS-containing client proteins.

The factors that determine the cytoplasmic versus nuclear localization of ligand-free steroid receptors are not known. Some hormone-free receptors (e.g. glucocorticoid and mineralocorticoid receptors) are predominantly localized to the cytoplasm while others (e.g. estrogen and progesterone receptors) are located in the nucleus. This difference in localization exists despite the fact that all of the receptors are shuttling in and out of the nucleus and all are in receptor•hsp90•immunophilin complexes [29,45]. One contribution to the localization might be that the NLS in the case of the receptors that are in the cytoplasm is repressed in the unliganded-receptor•hsp90•immunophilin complex whereas the NLS is accessible in unliganded-receptor complexes that are nuclear in localization. In that case, the localization should be entirely receptor-specific, but that is not the case. The unliganded mouse GR in mouse L cells, for example, is cytoplasmic, whereas the mouse receptor expressed in Chinese hamster ovary cells is nuclear, and in both cases it is present as GR•hsp90 heterocomplexes and the receptors react equivalently with antibody directed against the NL1 [119]. Preferential accumulation of steroid receptors in cytoplasm or nucleus would result if one direction of passage through the nuclear pore were favored over the other (see Ref. [48] for discussion), but differences in receptor localization due to factors affecting the intrinsic rate of receptor passage through the nuclear pore in either direction have not been established. One can of course change localization by deletion or addition of an NLS or NES, but the factors determining the localization of the ligand-free wild-type receptors remain unclear.

The involvement of importins in receptor entry into the nucleus is buttressed by several observations. For example, the unliganded GR•hsp90 complex has been shown to bind importin- α *in vitro*, with the NL1 being critical for binding [46], and fluorescence resonance energy transfer data indicate that the GR interacts directly with importin- α *in vivo* [120]. The importance of Ran in GR import is supported by the observation that expression of some Ran mutants markedly reduces GFP-GR accumulation in the nucleus [121]. The AHR has an NLS in its N-terminus that binds importin- α , which targets the receptor to the nuclear rim [122]. Binding of the AHR to importin- α is ligand-dependent and inhibited by geldanamycin, implying a role for hsp90 [68]. One study focused on binding of AHR to importin- β , and coexpression of ARA9 was found to decrease recovery of importin- β with the receptor [123], an effect that could account for redistribution of the AHR to the cytoplasm upon ARA9 expression [67,106–108,123]. The steroid and xeno-

biotic receptor (SXR) is an orphan nuclear receptor that activates the expression of some drug metabolizing enzymes (e.g., CYP3A4) and the drug efflux transporter ABCB1. Import of GFP-SXR was promoted by addition of importin- α in an in vitro nuclear transport assay [124].

In contrast to the consistent data for a role for importins in receptor import into nuclei, conflicting data have been published regarding a requirement for CRM1 in nuclear export. GR export from nuclei of Cos7 cells was inhibited by leptomycin B [46], and leptomycin B promoted nuclear accumulation of the progesterone B receptor in T47D human breast cancer cells [125], implying a role for CRM1 in export of these receptors. Leptomycin B induces nuclear accumulation of AHR, and immunoprecipitation of the AHR is accompanied by coimmunoprecipitation of CRM1 [109]. On the other hand, nuclear export of the GR in BHK cells was found to be insensitive to leptomycin B and to be regulated by calreticulin instead of CRM1 [126]. Calreticulin is normally considered to be a chaperone protein of the endoplasmic reticulum, but in this case it mediated Ran-dependent GR nuclear export, with the DNA binding domain of the receptor functioning as an NES [126]. Liu and DeFranco [127] found that slow nuclear export of wild-type GR following hormone withdrawal in Cos1 cells was not inhibited by leptomycin B, but rapid export of a GR chimera containing the NES from I κ B proceeds through the CRM1 pathway. Interestingly, a leptomycin B-insensitive NES has been identified in the ligand binding domain of the androgen receptor [128]. This CRM1-independent NES is active in the absence of androgen and repressed upon ligand-binding, leading the authors to suggest this provides a mechanism by which androgen regulates the nuclear-cytoplasmic shuttling of its receptor [128]. Lee and Bai [129] showed that mutation of a phosphorylated threonine in the NES of estrogen receptor α to alanine converts the receptor from leptomycin B-insensitivity to leptomycin B-sensitivity. Thus, it is conceivable that nuclear receptors have the capacity to utilize multiple distinct pathways for nuclear export. This could generate an additional level of control for receptor trafficking as each distinct nuclear export pathway could be subjected to unique regulatory influences.

In addition to importins and exportins, the chaperone hsc70/hsp70 has been reported to play a role in passage of some classical NLS proteins (SV40 T antigen, nucleoplasm) through nuclear pores [130,131]. It has been suggested that hsp70 is somehow involved in NLS recognition [131], but no clear and consistent role for hsp70 has been defined. Yang and DeFranco [132] directly compared the nuclear import of SV40 T antigen and the GR in permeabilized cells. They demonstrated that depletion of hsp70 did not affect GR import under the same conditions where SV40 T antigen import was inhibited. If hsp70 acts to facilitate NLS interactions with transport receptors, its role in nuclear import may be restricted to substrates whose NLSs are relatively inaccessible or not configured appropriately.

Many signalling responses cause the nuclear localization of transcription factors or of protein kinases that are translocated and then activate transcription factors located in the nucleus. Here, we have touched on factors regulating nuclear localization only of hsp90 client proteins. The factors regulating nuclear localization of several families of signalling proteins that are not client proteins for hsp90 have been reviewed by Cyr [133].

5. Movement within the nucleus

The early studies of steroid receptor localization in nuclei by indirect immunofluorescence showed them to be dispersed throughout the nucleus and to be excluded from nucleoli. Subsequent examination of endogenous glucocorticoid [134,135] and mineralocorticoid [136] receptors by confocal laser scanning microscopy revealed that they were present in multiple discrete foci located throughout the nonnucleolar space. The development of receptor fusions with jellyfish fluorescent proteins permitted examination of receptor localization and movement in nuclei of living cells. Within a 4-year period, multiple papers were published showing that glucocorticoid [137,138], mineralocorticoid [138,139], progesterone [140], estrogen [141], androgen [142,143], thyroid hormone [144], vitamin D [145] and retinoid X [145] receptor fusion proteins accumulated in punctuate foci throughout the nucleus excluding nucleoli (reviewed in Ref. [146]).

In several of the receptor fusion protein papers, it is noted that in the absence of ligand, the localization of nuclear fluorescence is less defined and formation of the discrete foci is agonist-dependent [137,139–141,143,145]. Thus, there is the impression that receptors that have passed through the nuclear pores move to ‘staging areas’ from which they can proceed to discrete foci if they have undergone ligand-dependent transformation [146]. There is some evidence that the cycling of receptor into receptor•hsp90 complexes may be involved in the ligand-dependent movement of receptor to the discrete nuclear foci where chromatin binding is thought to occur. Georget et al. [65] constructed a GFP-NLS-AR, which contained three nuclear localization signals between GFP and the AR and was localized diffusely in the nucleus in the absence of hormone. When these receptors were bound with steroid at 4 °C and then the temperature was jumped to 37 °C in the presence of geldanamycin, the ligand-dependent formation of discrete nuclear foci was inhibited [65].

Virtually nothing is known about how molecules move within the nucleus. Protein mobility within the nucleus can be diffusion-limited [183], but this may apply to a limited fraction of resident nuclear proteins. Furthermore, interactions of nuclear proteins with either soluble or solid state partners clearly retard their mobility [183]. As nuclear protein function is often directed to specific subnuclear compartments, a movement system may exist to facilitate

such targeted delivery. For example, movement of proteins from nucleoli along tracks through the nucleus to the nuclear pores has been demonstrated [147], but the nature of the filaments (nuclear actin?) and potential motor proteins (nuclear myosin?) is unknown. However, it is possible that movement of steroid receptors within the nucleus may occur on a movement system with some of the features of movement through the cytoplasm as depicted in Fig. 2. It should be emphasized again here that unliganded receptors that are nuclear are recovered in the cytosol after cell rupture where they are present as receptor•hsp90•immunophilin heterocomplexes [29]. Indeed, hsp90-binding immunophilins may be involved in targeting movement within the nucleus. It has been shown, for example, that the nuclear FKBP52 in Chinese hamster ovary cells expressing mouse GR is located in the same nonrandom loci throughout the nucleus as the hormone-free GR [54]. Such a localization is consistent with the notion that FKBP52 might target receptor movement, at least to the proposed ‘staging areas’ within the nucleus.

5.1. Hsp90 and receptor cycling within the nucleus

In 1976, Munk and Foley [148] observed that steroid dissociated from nuclear GRs upon hormone withdrawal much faster than receptors returned to the cytoplasm and

that these nuclear GRs could rebind hormone. Given the subsequent demonstration that GR•hsp90 complex is necessary for hormone binding [29], this suggested that the receptor could recycle to the GR•hsp90 complex within the nucleus. In several important experiments, the DeFranco laboratory has demonstrated such a trafficking cycle within the nucleus (reviewed in Ref. [48,149–152]). Using digitonin-permeabilized cells to examine in vitro nuclear export of the GR, they showed that GR released from chromatin could recycle to chromatin upon rebinding hormone without exiting the nucleus [153]. Geldanamycin inhibits recycling of these hormone-withdrawn GRs to the hormone binding state, and it inhibits GR release from chromatin during hormone withdrawal [154]. This is consistent with a role for the hsp90/hsp70-based chaperone machinery in the termination of transcriptional activation as free hormone levels decline. Furthermore, in recent experiments using a novel in situ system to assess GR nuclear mobility, receptor movement within the nucleus was reconstituted with the purified hsp90/hsp70-based chaperone machinery [180]. Thus, as illustrated in Fig. 4, molecular chaperones may participate in various stages of GR trafficking within the nucleus and facilitate both receptor exchange from specific binding sites within chromatin and overall mobility as the receptor navigates the nuclear space in search of high affinity target sites.

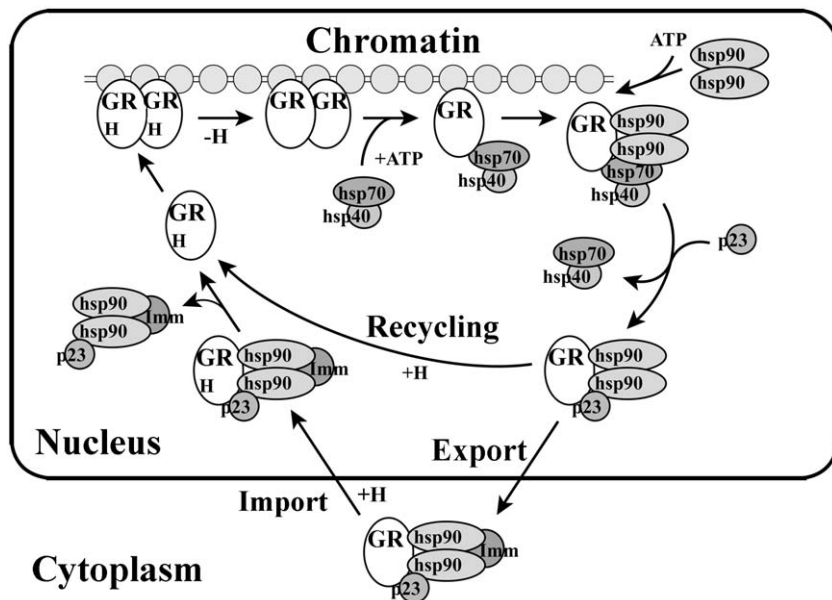


Fig. 4. Model for the recycling of GRs in the nucleus by the hsp90/hsp70-based chaperone machinery. After dissociation of hormone (H), the GRs are released from high-affinity chromatin binding sites by the chaperone machinery. The chaperone machinery is depicted here as acting in two ATP-dependent steps, the first involving hsp70 and the second hsp90, as discussed by Pratt and Toft [1]. This is the sequence of events determined for GR•hsp90 heterocomplex assembly by reticulocyte lysate and by the purified chaperone machinery. Although it has not been demonstrated that hsp70/hsp40 is the first component of the chaperone machinery to interact with chromatin-bound GR, it is likely that the same mechanism applies. It is also not known whether GR dimer to monomer conversion occurs while the receptor is bound to chromatin or while it is assembling into a complex with hsp90, but the stoichiometry of the final heterocomplex is one molecule of GR bound to a dimer of hsp90 [29]. The nonessential cochaperone Hop, which brings the chaperones together into an hsp90•Hop•hsp70hsp40 complex, has been omitted to simplify presentation. When the GR-bound hsp90 achieves the ATP-dependent conformation, the GR has returned to its hormone binding state and p23 can bind to hsp90 to stabilize the complex. Nuclear GR that has been recycled into GR•hsp90•p23 complexes can bind hormone without exiting the nucleus and be recycled to the chromatin-bound state.

5.2. Effect of p23 on transcription complex disassembly

The p23 component of the heterocomplex assembly system is not essential for receptor•hsp90 heterocomplex assembly *in vitro* [155] or *in vivo* [156,157], but it has become an important tool for examining the role of hsp90 in transcription complex disassembly and receptor recycling in the nucleus. In studies *in vitro*, two mechanisms of p23 action have been demonstrated. In GR•hsp90 assembly experiments, p23 has been shown to act as an hsp90 cochaperone and bind to GR•hsp90 complexes once they are formed, stabilizing them to disassembly [158]. p23 also has a direct chaperone action of its own in that it inhibits aggregation of denatured proteins, maintaining them in a folding-competent state [159,160]. Both of these mechanisms have been invoked to explain *in vivo* effects of p23 on nuclear receptor cycling.

The first *in vivo* observations were made by Knoblauch and Garabedian [161] who performed a screen in yeast to identify factors that would improve function of a mutant estrogen receptor with decreased hormone binding capacity. The yeast homolog of p23 (yhp23) was isolated, and its overexpression was shown to increase estrogen receptor-dependent transcriptional activation by increasing estradiol binding *in vivo*. When the estrogen receptor and GFP-yhp23 were coexpressed, the GFP-yhp23 moved with the receptor to the nucleus and was released back into the cytoplasm when yeast were treated with estradiol. Expression of human p23 in MCF-7 cells also increased transcriptional activation by the estrogen receptor [161]. These observations were interpreted in terms of p23 acting as an hsp90 cochaperone to support a role for the hsp90/hsp70-based chaperone machinery in estrogen receptor signal transduction [161].

Freeman et al. [162] examined the effect of p23 on several intracellular receptors in yeast and found that glucocorticoid and progesterone receptor-dependent transcription was increased, whereas transcription from mineralocorticoid, estrogen, androgen, thyroid hormone, and retinoic acid receptors was decreased. It was determined that p23 interacted preferentially with thyroid hormone-receptor-response element ternary complexes *in vitro* to stimulate receptor dissociation from DNA. p23 appeared to compete with the coactivator GRIP1 in that a fragment of GRIP1 inhibited p23-dependent dissociation of thyroid hormone receptor from DNA. The interpretation here was that p23 effects are different from those of hsp90, with p23 directly interacting with the receptor at a late step in receptor-mediated signal transduction [162]. Freeman and Yamamoto [163] targeted p23 by fusion to the Gal4 DNA binding domain to localize *in vivo* at GAL4 binding sites neighboring thyroid hormone or glucocorticoid response elements in a reporter. Expression of Gal4-p23 reduced thyroid hormone receptor-dependent transcriptional activation by 100-fold and GR-dependent activation by 35-fold. In contrast, Gal4-hsp90 produced only a slight inhibition

(twofold). Less extensive p23 inhibition was observed with the non-receptor transcription factors NF- κ B and AP1 expressing from similar linked promoters after their activation by tumor necrosis factor α and phorbol myristate acetate, respectively. Importantly, it was shown in chromatin immunoprecipitation assays that both p23 and hsp90 localized to glucocorticoid response elements in a hormone-dependent manner. In experiments *in vitro*, p23 inhibited transcriptional activation by preformed regulatory complexes, consistent with a role of p23 in promoting disassembly of the complexes [163].

Because transcriptional inhibition by forced localization of p23 to a hormone response element abrogated receptor-induced activation and hsp90 inhibited activation less, the notion that p23 causes disassembly of transcriptional regulatory complexes via a direct chaperone effect has been seriously considered [163,164]. However, as the authors note, it was not determined whether disassembly of intact complexes requires energy [163]. Although it was demonstrated under very different conditions, GR release from nuclear matrix has been found to be ATP-dependent [165], and it is likely that p23 is exerting its action through hsp90. It has been shown that p23 is the limiting component of the multiprotein hsp90/hsp70-based chaperone system *in vivo*, and that it acts *in vivo* to stabilize client protein•hsp90 complexes [166]. The Garabedian laboratory has shown that the ability of yeast p23 mutants to increase or decrease estrogen receptor-dependent signal transduction correlates with their ability to bind hsp90 [167]. Taken together, these observations argue rather strongly that p23 effects *in vivo* do not reflect a direct chaperoning interaction with the client protein of hsp90. Rather, they reflect a direct interaction with hsp90 as a cochaperone to stabilize its association with the client protein, thus increasing the efficiency of the hsp90/hsp70-based chaperone machinery [166].

5.3. Rapid exchange of receptors with regulatory sites

The availability of GFP-receptor fusion proteins has permitted the study of real-time movement of receptors in subregions of the nucleus using photobleaching techniques, such as fluorescence recovery after photobleaching (FRAP). The observations have led to the realization that a number of transcription factors are in highly dynamic interactions with their regulatory sites (reviewed in Ref. [168]). The Hager laboratory examined the binding of GFP-GR to an artificial amplified array of mouse mammary tumor virus reporter elements on chromosome 4 in a mouse cell line [169]. This array includes 800 to 1200 binding sites for the GR, and it displays as a single patch of bright fluorescence in cells expressing GFP-GR treated with dexamethasone. Photobleaching experiments showed that the hormone-bound GR exchanges rapidly with the chromosomal regulatory sites with a half maximal time for fluorescence recovery of ~ 5 s [169]. The work of Hager and his colleagues is consistent with a dynamic “hit and run” model in which the

ligand-activated GR binds to chromatin, recruits a remodeling activity, facilitates transcription factor binding and is then lost from the template [169–171]. The GR coactivator GRIP-1 undergoes the same rapid exchange as the GR [172].

Stenoien et al. [173] examined the nuclear localization of bioluminescent derivatives of estrogen receptor α and steroid receptor coactivator 1 (SRC-1). Estradiol caused the receptor to change from a diffuse nucleoplasmic pattern to localize in discrete foci and the coactivator colocalized to the same foci in an estradiol-dependent manner. Subsequent photobleaching experiments [174] revealed a particularly high mobility for the unliganded estrogen receptor chimera in the nucleus (fluorescence recovery $t_{1/2} < 1$ s) whereas the mobility of the hormone-bound receptor was somewhat slower ($t_{1/2} \sim 5$ – 6 s), with SRC-1 showing a similar mobility. Thus, the agonist-bound estrogen receptor proceeds to intranuclear foci where it is matrix-bound, but the receptors within the foci undergo rapid exchange with the nucleoplasm. Under conditions of ATP depletion, the unliganded ER was immobilized, whereas there was little effect on receptor mobility in estradiol-treated cells [174]. The authors note that the ATP dependency of the mobility of the unliganded receptor could reflect ATP-dependent hsp90 heterocomplex assembly.

At this time, the mechanism of signalling protein movement within the nucleus is unknown. Strong arguments have been advanced that protein movement within the nucleus is diffusional and that stochastic mechanisms are involved in the regulation of gene expression [175,176]. While there may be some subset of nuclear proteins whose mobility is strictly limited by diffusion, it seems likely that the successful orchestration of complex regulated biochemical pathways in the nucleus would require some mechanism for direct delivery of transcription factors and assembly of regulatory complexes. Here, we have summarized evidence that the hsp90/hsp70-based assembly machinery may be involved in movement of steroid receptors to discrete foci within the nucleus where transcriptional regulation is thought to occur as well as in disassembly of the regulatory complexes. However, other chaperones and cochaperones as well as a potential karyoskeleton may be involved in this process in addition to the components of the hsp90/hsp70-based assembly machinery. The demonstration of the high mobility of receptors in the nucleus and the effects of geldanamycin and p23 on receptor release from chromatin have stimulated a number of perspective and opinion pieces [150,152,164,168,177–179] that attest to the broad interest in this topic.

6. Summary

Inherent to an understanding of cellular signalling is the ultimate understanding of how signalling proteins travel to their sites of action in various cell compartments. Initially,

interest focused on the nature of the signals (e.g. NLSs, NESs, MLSs) that target the movement of protein solutes to specific cellular compartments. Recently, several laboratories in the signal transduction field have begun to focus on the mechanisms of signalling protein movement within the cytoplasm and nucleus. Although there is considerable evidence that the dynamic assembly of heterocomplexes with hsp90 is involved in the movement of transcription factors that are hsp90 client proteins within the cytoplasm and within the nucleus, the notion that hsp90 is involved in signalling protein movement is at an early stage of development. We do not yet know whether dynamic hsp90 heterocomplex assembly is involved in the movement of just a few transcription factors, or whether the hsp90/hsp70-based chaperone machinery is involved in the long-range movement and local mobility of a wide range of signalling protein solutes, including some of the many protein kinases whose activity and/or turnover are regulated by hsp90 [1].

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