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Role of molecular chaperones and TPR-domain proteins in the cytoplasmic transport of steroid receptors and their passage through the nuclear pore

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In the absence of hormone, corticosteroid receptors such as GR (glucocorticoid receptor) and MR (mineralocorticoid receptor) are primarily located in the cytoplasm. Upon steroid-binding, they rapidly accumulate in the nucleus. Regardless of their primary location, these receptors and many other nuclear factors undergo a constant and dynamic nucleocytoplasmic shuttling. All members of the steroid receptor family are known to form large oligomeric structures with the heat-shock proteins of 90-kDa (hsp90) and 70-kDa (hsp70), the small acidic protein p23, and a tetratricopeptide repeat (TPR)-domain protein such as FK506-binding proteins (FKBPs), cyclophilins (CyPs) or the serine/threonine protein phosphatase 5 (PP5). It has always been stated that the dissociation of the chaperone heterocomplex (a process normally referred to as receptor "transformation") is the first step that permits the nuclear import of steroid receptors. However the experimental evidence is consistent with a model where the chaperone machinery is required for the retrotransport of the receptor through the cytoplasm and also facilitates the passage through the nuclear pore. Recent evidence indicates that the hsp90-based chaperone system also interacts with structures of the nuclear pore such as importin β and the integral nuclear pore glycoprotein Nup62 facilitating the passage of the untransformed receptor through the nuclear pore.

Key words: immunophilins, dynein, nucleoporins, importins, shuttling, heat-shock proteins

Abbreviations: GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; hsp, heat-shock protein; IMM, high molecular weight immunophilin; GA, geldanamycin; FKBP, FK506-binding protein; TPR, tetratricopeptide repeats; PPIase, peptidyl-prolyl isomerase; NLS, nuclear localization signal; NPC, nuclear pore complex; Nup, nucleoporin; Imp-α, importin α; Imp-β, importin β

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Movement of Soluble Proteins Through the Cytoplasm

Intracellular protein transport is a fundamental mechanism for regulating both protein localization and protein function. Therefore, it is not surprising that several pathologies leading to cell death, cell proliferation or initiation and progression of cancer are related to mislocalization of a variety of soluble proteins involved in signaling cascades.[1-4] Nonetheless, it is still an unsolved problem how those soluble, non-vesicle-associated signaling molecules move within the cell to reach their sites of action.

Over the past few years, many fields of biological research have converged in the study of the subcellular distribution of signalling proteins. It is currently accepted that most soluble proteins are not confined to the cytoplasm or the nucleus in a static manner, but are capable of shuttling dynamically through the nuclear pore.[3,5-7] Protein transport across the nuclear envelope involves sequential steps: retrograde (plasma membrane to nucleus-oriented) movement through the cytoplasm, recognition by nuclear import proteins, docking to the nuclear pore, translocation across the pore, movement through the nuclear compartment, anchorage to and release from the nuclear sites of action, recognition by nuclear export proteins, anterograde (from nucleus to cell periphery directed) passage through the nuclear pore, and anterograde cytoplasmic movement. Several studies have been conducted to elucidate import and

export mechanisms through the nuclear pore of these signaling molecules, but many molecular details remain unclear.[7] Similarly, the reorganization of signaling proteins in the nucleus is an essential step for regulating their functions, but the manner by which these factors reach their sites of action is unknown.

The cytoplasmic transport of soluble proteins included into vesicles follows the mechanism of vesicular transport involving cytoskeletal tracks and molecular motors, whereas the cytoplasmic movement of soluble proteins not associated to vesicles is more difficult to explain. One possibility is that signaling cascade factors just move in the cytoplasm in a stochastic manner by simple diffusion, such that random collisions occurs between soluble proteins and/or cell structures. Consequently, after an effective collision, signaling proteins become trapped at their sites of action by protein-protein or protein-nucleic acid interactions. In this regard, there is evidence that short-range trafficking of large solutes through the cytoplasm indeed involves diffusion.[8] However, microinjection studies in axons show that soluble proteins require a microtubule-based machinery for targeted directional movement over long distances such as in the axoplasm,[9] a compartment where movement by random diffusion alone would not permit the efficient delivery of soluble proteins.[4] In neuronal cell bodies and non-neuronal cells such as fibroblasts or lymphocytes, signal proteins can also require an organized transport system to be transported more efficiently than by simple diffusion. If such mechanism were mere passive diffusion, it would be difficult to explain how proteins can exert specific effects when a given cascade is activated. In this scenario, the protein responsible for triggering the process would freely spread throughout one or more cell compartments. A priori, a mechanism of movement solely based on free diffusion also appears to collide with a basic biological concept, i.e., the fact that cellular activities are highly compartmentalized. Therefore, if proteins normally occupy the entire cell compartment when they are activated, additional mechanisms must regulate directed protein movement, most likely due to protein-protein interactions. In addition, the crowded intracellular environment may compromise the efficiency of free diffusion,[8,9] which becomes highly anomalous for large oligomeric macromolecules whose size is equivalent to, for example, the steroid receptor•hsp90-based heterocomplex. Cryoelectron tomography images show that the cytoplasm is filled with large and highly packed assembles of organized filaments and macromolecules forming interconnected functional structures rather than freely diffusing and colliding soluble complexes.[10] This architecture certainly permits the movement of soluble proteins by simple diffusion, but it also makes the delivery of signaling factors less efficient, to the point that they can even be targeted to proteasomal degradation.[11,12] For large oligomeric structures, inefficient transport by free diffusion could be the consequence of physical impairment in a crowded environment and/or due to the high viscosity of the medium among filaments, but for most soluble proteins, regardless of their size and shape, the limiting factor is the protein-protein interaction phenomenon between the soluble protein in movement and elements of the stationary phase. This can occur in a non-specific manner due to charge attraction or, more specifically for certain solutes, because they show affinity for some proteins linked to the immobile phase of the cytoskeleton. In this regard, it should be emphasized that several components of the hsp90-based heterocomplex (if not all) have been recovered bound to microtubules, microfilaments and intermediate filaments (reviewed in ref. 13).

An alternative and more direct mechanism for soluble protein movement is that in which solutes utilize a movement machinery likely involving molecular motors. This option implies the concept that specific interactions would be required to determine the direction of signal protein movement. In the case of steroid receptors, it has been proposed that a key component that directs receptor movement is the oligomeric machinery formed by hsp90, the high molecular weight immunophilin (IMM) FKBP52, and dynein/dynactin motor complex.[14] Before discussing this topic, we should turn our attention to the composition and assembly of the hsp90-based heterocomplex associated to steroid receptors.

The Steroid Receptor•hsp90•IMM Heterocomplex

Hsp90 is the most abundant of the heat-shock proteins. It is a ubiquitous and highly conserved molecular chaperone that is essential for cell survival, growth, differentiation and development.[15,16] In contrast to other chaperones, hsp90 is unique in the sense that it is not required for the biogenesis of most polypeptides and binds to prefolded or completely folded proteins. In addition, hsp90 seems to be more selective than other chaperones to interact with proteins, such that it regulates the biological function of many proteins that are signaling factors. The active conformation of these client factors depends on the chaperone. Normally, hsp90 does not function as an individual chaperone, but as a component of larger complexes containing other chaperones and a cohort of cofactors named co-chaperones, which assist hsp90 function stabilizing its active conformation and/or regulating its intrinsic ATPase activity.[15,17,18]

Steroid receptors are capable to form heterocomplexes with the chaperones hsp90 and hsp70, the co-chaperone p23, and proteins that possess sequences of 34 amino acids repeated in tandems, the TPR proteins.[16,19] Some of these hsp90-binding TPR proteins have peptidylprolyl-isomerase (PPIase) activity and are intracellular receptors for immunosuppressant drugs such as FK506, rapamycin or cyclosporine A. They are grouped into the relatively conserved family of proteins known as immunophilins (IMMs). Among the members of this family, only a few of them have been found in steroid receptor•hsp90 complexes, i.e., FKBP52, FKBP51, CyP40, and three IMM-like proteins, PP5, XAP2/ARA9 and WISp39.[20,21] Even though the biological function of these IMMs in the receptor•hsp90 heterocomplex remains poorly understood, it is accepted that they are not related to the immunosuppressant effect, a property described only for the smallest members of the family, CyP17 and FKBP12. From the structural point of view, these two IMMs differ from the high molecular weight partners in that the small immunosuppressant proteins do not possess other additional domain than the PPIase, the signature domain of the entire family.

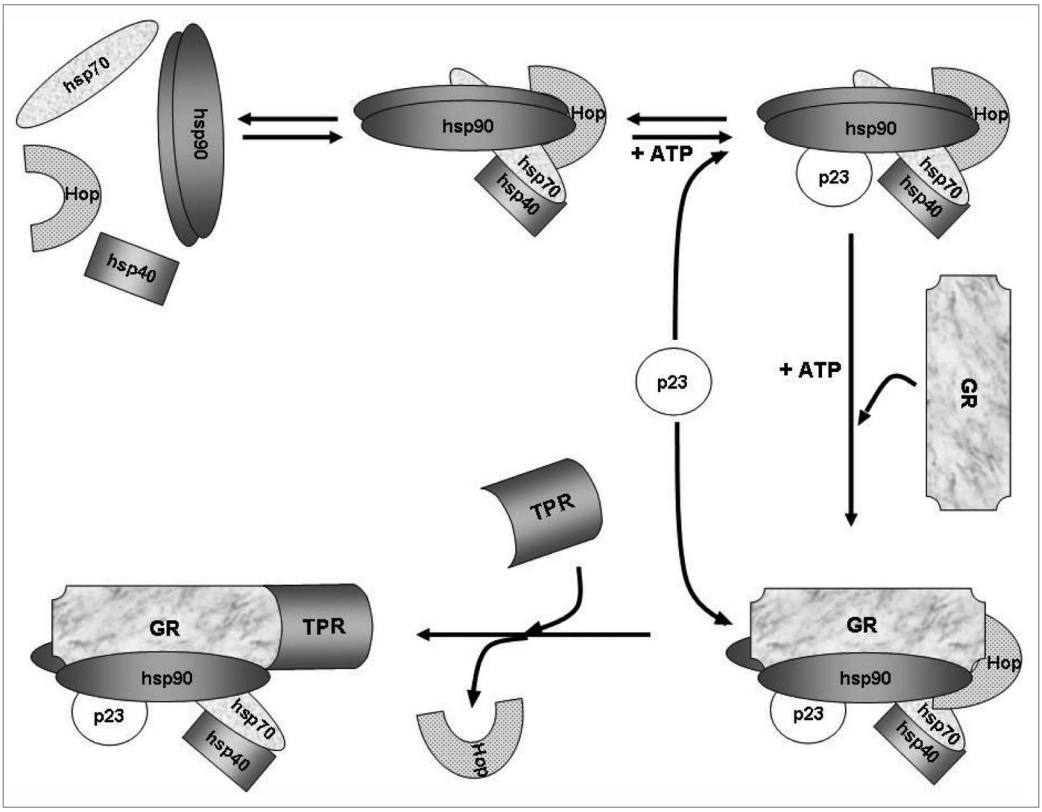

Figure 1. Maturation cycle of the GR•hsp90 heterocomplex assembly. Hsp70, hsp40, hsp90, Hop, and eventually p23, can exist preassembled in the cytosol in an ATP-dependent manner. This assembly is highly dynamic and can also take place on the client protein being primed by hsp70 and hsp40 (not drafted). The amount of p23 recruited to the complex is extremely dependent on the content of the ATP•hsp90 isoform. The chaperone complex associates to naked GR in and ATP- and K^+-dependent manner, which converts its ligand-binding domain in a high affinity site for the steroid (H). Hop is dissociated from the heterocomplex and the only one TPR-acceptor site per hsp90 dimer is occupied by other TPR-domain protein. In the presence of steroid, FKBP52 is the most abundant TPR protein recruited by GR.

During the early 1990s, it was shown that client proteins•hsp90 heterocomplexes could be assembled in vitro by incubating immunoprecipitated steroid receptors or protein kinases with rabbit reticulocyte lysate and a source of ATP.[22-24] These reconstitutions could also be achieved by using purified proteins.[25] These studies permitted the elucidation of the assembly cycle of the heterocomplex, which is summarized in **Figure 1** for the best studied hsp90 client protein, the GR. Receptor complexes are assembled in an ordered and dynamic manner, the first step being the formation of an $(hsp90)_2$•Hop•hsp70•hsp40 complex that was named *foldosome* by William Pratt.[26] This complex can be formed on the GR in a sequential manner[27] or can be transferred from pre-existing cytoplasmic complexes.[25] In all the cases, the presence of the hsp90-binding co-chaperone p23 stabilizes the complexes, although it is not essential for the receptor folding. Recent studies demonstrated that more than 90% of p23 is hsp90-bound.[28] Whereas free p23 shows a high dynamics of movement that can be approximated by a simple diffusion model, its pattern of movement becomes more restricted when it is bound to hsp90. This reflects the high level of interactions of hsp90 with several structures of the cell.[29] The stabilizing action of p23 can be mimicked by molybdate, which in turn restricts nuclear accumulation of GR. Interestingly, the overexpression of p23 also exerts the same effect.[30]

Hsp90 possesses a nucleotide-binding domain that acts as an ATP/ADP switch domain that regulates hsp90 conformation.[18,31] When bound by ADP, hsp90 shows higher affinity for hydrophobic substrates, whereas the ATP form of hsp90 is the one that binds p23 and shows better properties as chaperone for conferring biological activity to client proteins such as kinases and steroid receptors. The equilibrium between ATP•hsp90 and ADP•hsp90 states is dependent on the intrinsic ATPase activity of hsp90, which is in turn modulated by more than a dozen distinct hsp90 cofactors, a number of regulators not paralleled by other chaperone systems (reviewed in ref. 18).

Hsp70 is other key chaperone of the complex whose peptide-binding activity is coupled to the binding of ATP versus ADP, the latter showing higher affinity for hydrophobic substrates.[32] Hsp70 also possesses an intrinsic ATPase activity that is stimulated by substoiquiometric amounts of other chaperone, hsp40, which is normally associated to hsp70.[33] The initial complex between hsp90 and hsp70 is possible if, and only if, a third player is present, p60/Hop. This 60-kDa TPR-domain protein was first identified by David Smith et al.[34] and because it serves as adaptor between hsp90 and hsp70, its original

name p60 has become Hop (for hsp-organizing protein). It is interesting to point out that the *foldosome* can be formed simply by mixing its purified components in buffer. Due to didactic purposes, the scheme of **Figure 1** shows that the *foldosome* is transferred to the GR as a whole in a single ATP-dependent step. However, it can also be assembled on the receptor in a multistep pathway where hsp70•hsp40 complex primes the initiation complex bound to the ligand binding domain of the receptor (reviewed in ref. 19).

Finally, the TPR-domain co-chaperone Hop is released from the TPR-acceptor site of hsp90 in a step where BAG-1 (Bcl2-associated gene product 1) accelerates the process.[35] The TPR acceptor site is then occupied by one of various TPR-domain co-chaperones such as FKBP51, FKBP52, PP5, Cyp40, XAP2/ARA9 or WISp39, which dynamically exchange on hsp90 dimers. Even though a number of TPR proteins can potentially replace Hop, only the six proteins listed above have been recovered bound to hsp90 in receptor complexes, and all of them share the property of being IMMs or IMM-like factors. Studies of saturation binding of Hop to hsp90 dimer[36] and cross-linking of hsp90•FKBP52 complexes[37] are consistent with one TPR acceptor site per hsp90 dimer, so the relative expression of TPR proteins could determine the extent present in the complex. Thus, it has been proposed that the receptor samples its environment for co-chaperones, and its mature form is a mixture of TPR proteins determined by the relative abundance and affinity of each co-chaperone for the receptor•hsp90 complex.[16] Recent evidence suggests that TPR proteins are dynamically exchanged according to the nature of the ligand bound to the receptor.[38] Thus, aldosterone-binding to MR favors the exchange of FKBP51 for FKBP52, whereas 11,19-oxidoprogesterone binding favors the recruitment of PP5. From the functional point of view, both FKBP52 and PP5 are equally effective for receptor retrotransport because they associate dynein in similar extent.[39] On the other hand, FKBP51 fails to bind dynein efficiently.[40] In other words, there are physiologic factors that are able to regulate the composition of the hsp90•TPR-domain protein complex. It has also been suggested that immunosuppressive ligands can alter GR hormone-binding function by changing the TPR protein composition of receptor complexes.[41] In addition, IMM binding to receptor•hsp90 complexes shows selective preference for certain receptors even in the absence of any ligand. For example, Cyp40 is recovered bound to ER but not to GR or MR, FKBP52 shows preference for GR and PR, and not for AhR, which in turn recruits exclusively XAP2 and not other IMM.

It is important to emphasize that heterocomplexes are constantly assembled and disassembled, and because IMMs bind to hsp90 in a reversible manner, over time, a single receptor•hsp90 complex may be associated to several IMMs, some of them functionally redundant in their ability to bind dynein motors such as FKBP52, CyP40, WISp39 and PP5. The biological role of IMMs on transcriptional regulation is a field that still remains to be explored, but it is unlikely that they may play redundant roles as those observed for receptor movement.

Steroid Receptors as a Model for Studying the Retrograde Transport of Soluble Proteins

In the absence of ligand, some members of the steroid receptor family reside primarily in the cytoplasm whereas others are nuclear, but regardless of the primary location, receptors are not confined to any particular cell compartment and continuously shuttle between cytoplasm and nucleus.[42,43] It has always been assumed that simple diffusion is the driving force of movement for these signaling molecules. However, the observation that proteins of the dynein/dynactin motor complex co-immunoprecipitate with the hsp90•FKBP52 complex and also with GR[14,40,44] and MR[38,45] suggests that these motor proteins power the retrograde movement of steroid receptors, in which case the hsp90•FKBP52 complex should play a significant role. This was demonstrated by several approaches. Normally, the steroid-dependent nuclear accumulation of primarily cytoplasmic steroid receptors is rapid ($t_{0.5}$ = 4–5 min), but treatment of cells with the hsp90 inhibitor geldanamycin lowers the rate of translocation by an order of magnitude ($t_{0.5}$ = 40–60 min). The rapid, hsp90-dependent movement of these steroid receptors requires cytoskeletal tracts, tubulin being physically linked to the receptor•hsp90•IMM•motor protein complex. Studies of co-immunoprecipitation of dynein and subunit components of dynactin with FKBP52,[39,40,44,46] demonstrated that the motor proteins bind the N-terminal end of FKBP52 (i.e., the PPIase domain) in a manner that appears to be independent of the prolyl-isomerase activity of the IMM. Rather, the PPIase domain acts as a protein-protein interaction domain and the association of dynein/dynactin with FKBP52 is not affected by FK506.[47,48] Dynactin is a multiprotein complex required for most, if not all, cytoplasmic dynein-driven activities.[49] Because the IMM and hsp90 are part of the same functional complex, it can be envisage that the disruption of the interaction between the IMM and the motor proteins should yield the same level of inhibition of steroid receptor retrotransport as that measured in the presence of hsp90 inhibitors. That this is the case was demonstrated when the receptor was "disconnected" from the transport machinery by overexpression of the PPIase peptide (interferes with dynein binding to IMMs), the TPR peptide (blocks IMM binding to hsp90) or the p50/dynactin2 subunit of dynactin (prevents the proper assembly of dynactin and its further association with dynein).[47,48,50]

It should be emphasized that, in all these cases, the nuclear localization of the cargo was not fully inhibited but impaired, suggesting the existence of two types of transport, the rapid hsp90•FKBP52•dynein/dynactin-dependent mechanism ($t_{0.5}$ = 4–5 min), and an alternative, heterocomplex-independent and less efficient mechanism ($t_{0.5}$ = 40–60 min), which could be due to simple diffusion. Importantly, when the nuclear translocation rate of these receptors was impaired, they became highly sensitive to proteasomal degradation.[11,45] The same heterocomplex described for steroid receptors is also responsible for the cytoplasmic retrotransport of the proapototic factor p53,[47] suggesting that the hsp90-based complex may play a general role in the

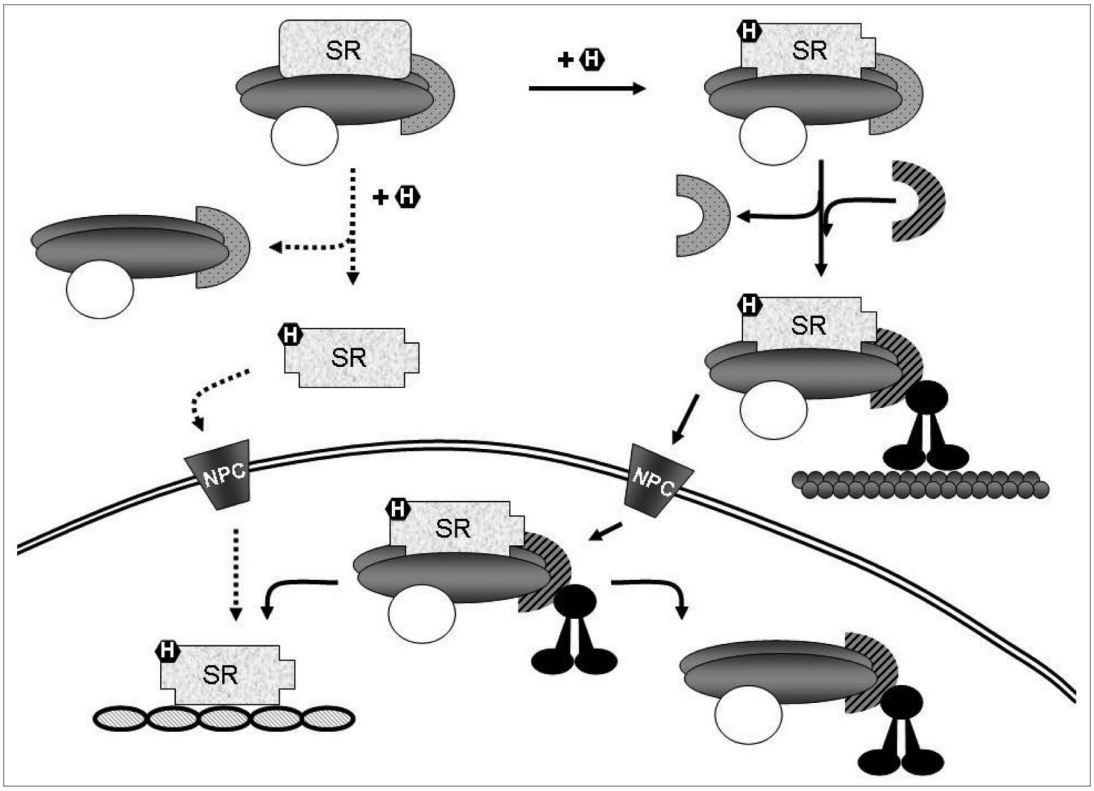

Figure 2. Models of the steroid receptor (SR)•hsp90-based heterocomplex function. The classical model for receptor activation is depicted with dashed lines. The SR-chaperone complex is dissociated immediately after hormone (H) binding. This promotes both the release of the SR from the cytoplasmic anchoring sites and its free diffusion. The passage of the transformed receptor through the NPC is facilitated because the NLS is exposed. According to the novel model (depicted with continuous lines), the SR heterocomplex recruits a TPR-domain protein (shown as a dashed crescent) able to interact with motor proteins (black). The chaperone complex serves as a traction chain for the receptor whose movement towards the nucleus is powered by dynein/dynactin on cytoskeletal tracts. The NL1 protrudes upon steroid binding and the whole SR-chaperone complex translocates through the NPC. The heterocomplex interact with structural proteins of the pore, which are also chaperoned. Receptor transformation (i.e., the dissociation of the hsp90 complex) occurs in the nucleoplasm allowing the further association of the ligand-activated receptor with its promoter sites.

retrotransport of a number of hsp90-associated factors towards the nuclear surface.

Inasmuch as the chaperone molecular bridge provides the traction chain for the nuclear factor to be transported throughout the cytoplasm via microtubules tracks, the dissociation of the hsp90-based complex from steroid receptors should not occur directly after ligand binding because the hsp90•FKBP52 complex is required for the normal mechanism of retrotransport.[14] This finding is a major advance with respect to the dogma postulated decades ago for steroid receptor activation, which sustained the principle that hsp90 "anchors" steroid receptors in the cytoplasmic compartment and only the dissociation of the chaperone permits the nuclear translocation of the receptor. A comparative scheme for both models is depicted in **Figure 2**. Note that in the new model, the IMM exchange triggered by steroid binding is accompanied by the co-recruitment of motor proteins, and the entire heterocomplex passes through the nuclear pore reaching the nucleoplasm, where transformation takes place. The exact factor or event responsible for the transformation of steroid-receptor complexes is still uncertain. In contrast to the rapid transformation observed in intact cells exposed to steroid, the dissociation of the hsp90 heterocomplex from the receptor is substantially less efficient and slower in cell-free systems, indicating that the structural and functional integrity of the cell is required.

Transport Across Nuclear Pores

In view of the fact that the hsp90•IMM chaperone system is required for receptor retrotransport, one interesting extrapolation of this novel model is the possibility that the heterocomplex could interact directly with structures of the pore. The nuclear pore complex (NPC) is a macromolecular structure of ~125-MDa that is embedded in the nuclear envelope. The basic architecture of the NPC (reviewed in ref. 51) is a cylindrical channel with a large luminal domain embedded in the nuclear envelope flanked by a ring at the cytoplasmic and nuclear surfaces, from which cytoplasmic and nuclear fibrils emanate. The barrel-like central framework is composed by eight spokes with protuberances towards the central pore. The constituent building blocks of the NPCs are called nucleoporins, which not only form the pore scaffold, but also contain FG-repeat domains that fulfill essential roles during the cargo transport. While small molecules are able to diffuse freely through this structure, molecules larger than ~40-kDa require an active

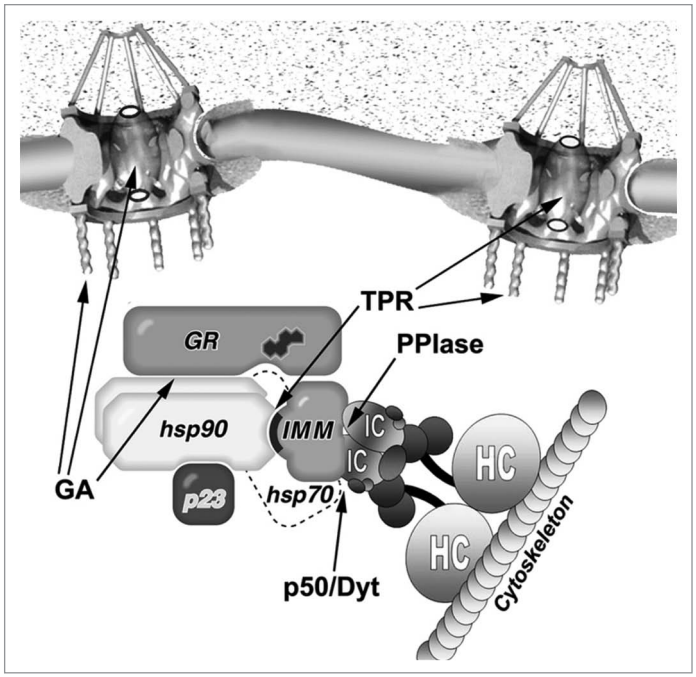

Figure 3. Points of disruption of the hsp90-based heterocomplex. The scheme shows the oligomeric structure of a mature steroid receptor. The barrel-like structure in the center of the NPC represents the integral nucleoporin Nup62, and arrows show the proved sites for the functional disruption of the system. The benzoquinone ansamycin GA disrupts hsp90 function when the chaperone is bound to GR and Nups. An excess of TPR peptide prevents the binding of the IMM to the TPR acceptor site of hsp90 and the direct association of IMMs with Nups. An excess of the PPIase peptide prevents binding of the dynein intermediate chain (IC) to the PPIase domain of the IMM, and p50/dynactin2 (p50/Dyt) disrupts the proper assembly of the dynein/dynactin complex inhibiting the motor protein function. HC: dynein heavy chain associated to microtubules.

passage mediated by adapter receptors, the importins.[52,53] Proteins possessing a classical NLS such as the SV40T antigen, nucleoplasmin and steroid receptors, utilize importin-α (Imp-α), a protein that binds the NLS of the substrate and forms a trimeric complex with importin-β (Imp-β), a factor known as the transport receptor that favors the passage of many cargoes through the NPC.[54] The FG-domains in Nups form a sieve-like hydrogel that can act as a barrier to repeal non-specific macromolecules greater than 40-kDa, but they also facilitate the translocation of the trimeric cargo complex via interactions of these FG repeats with importins (reviewed in ref. 51).

Directionality of transport for importins is accomplished via the small guanosine triphosphatase (GTPase) Ran, a Ras-related small GTPase that switches between a GDP- and a GTP-bound state. A nucleotide exchange factor in the nucleus generates Ran•GTP, and a GTPase-activating protein (GAP) that is excluded from the nucleus converts Ran•GTP to Ran•GDP at the cytoplasmic face of the nuclear pore. After the cargo-importin complex traverses the pore, Ran•GTP binds to importin and the cargo is released. The Ran•GTP•Imp-β complex passes back through the pore without cargo to the cytoplasmic face where Ran•GAP converts Ran•GTP to Ran•GDP, releasing free importins to participate in another cycle of cargo entry. Additionally, a limited number of cargoes undergo receptor-independent transport; for example, the Wnt signaling molecule β-catenin directly interacts with FG-repeats to mediate its own import.[55]

Two NLS have been identified in the GR, NL1, a strong classical bipartite signal located in the hinge region of the C-terminal end of the DNA-binding domain, and the more diffuse and uncharacterized NL2 that comprises an undefined sequence in the ligand-binding domain.[56] A third NLS domain, NL3, has recently been postulated for the MR in its C-terminal end.[57] The conventional view hitherto has always been that, in receptors primarily located in the cytoplasm such as MR or GR, the NLSs are hidden when hsp90 is in the complex. Even though hsp90 binding to GR or MR effectively overlaps the NLS, recent evidence demonstrated that antibodies against NL1 are reactive in non-liganded MR, and that the mere exposure of the NLSs in receptors transformed in the absence of ligand did not result in their automatic nuclear translocation.[58] Consequently, the mechanism for nuclear accumulation via NLS should be regulated by the ability of the receptor to change its conformation according to the stimulus that promotes its transformation and, possibly, by the recruitment of other factors that can participate in the nuclear import mechanism may be the underlying reason by which non-liganded receptors are recovered in the nucleus in the absence of steroid. We speculate with the fact that this mechanism could be the same as the one related to the slower translocation rate evidenced when the transport machinery is disrupted.

It has always been believed that receptor transformation is the first mandatory step prior to nuclear translocation. However, there is evidence that conflicts with this dogma. As discussed above, the presence of hsp90 bound to the GR is critical for its link with the cytoplasmic machinery that moves the receptor toward the nucleus. Hybrid molecules between the PR (primarily nuclear) and hsp90 constructs reveal that the receptor may be relocated in the cytoplasm in a manner that is not altered by the exposure of its NLS.[59] Hsp90 can be cotransported with the steroid receptor into the nucleus, keeping the nonliganded receptor inactive but poised for transcriptional regulation.[60] Hsp90 was recovered bound to the GR immediately after its nuclear translocation, suggesting that the complex remained intact during the process.[13] Importantly, cross-linked MR•hsp90 complexes are translocated to the nucleus in a steroid-dependent manner.[48,61] Finally, even though the GR is primarily cytoplasmic in most cell types, it is constitutively nuclear in the WCL2 cell line.[62] There is, therefore, no clear relationship between NLS availability and nuclear translocation. Instead,

it is likely that nuclear translocation is the result of a concerted mechanism between the strong NL1 sequence and perhaps the weak and diffuse NL2 signal, and/or unknown sequences remaining to be identified or characterized. The α-importin (Imp-α) independent translocation observed for a GR mutant in which NL1 was deleted agrees with the speculation that nuclear import may be NL2-dependent[63] and mediated by separate pathways. Interestingly, the nuclear translocation rate of GR in that NL1-deleted mutant is identical to the rate shown in cells where the hsp90•FKBP52•motor protein complex is disrupted. A similar observation has also been reported for a PR mutant where the active NLS is absent.[64] As expected, this PR mutant is cytoplasmic in a medium without steroid. With addition of hormone, the PR mutant translocates to the nucleus, although at a slower rate compared to that of native PR. The authors show that such slower nuclear import kinetics is due to its lack of interaction with Imp-α.

The hsp90·IMM Complex Binds to the NPC

As discussed before, the novel model of hsp90•IMM-dependent steroid receptor retrotransport implies that the chaperone machinery should interact with importins and other key factors of the NPC such as Nups. Preliminary in silico analyses of protein-protein interactions for the GR•hsp90•TPR protein heterocomplex yielded a number of potential interactors related to proteins associated with the cytoskeleton, motor proteins and factors belonging to the nuclear import/export system.[61] This in silico prediction and the experimental evidence for steroid receptor retrotransport discussed above led us to analyze the potential interaction of GR and its associated chaperone complex system with Imp-β and Nups. Proteins belonging to the untransformed receptor complex (hsp90, hsp70, p23 and TPR-domain proteins such as FKBP52 and PP5) were recovered associated with the integral nuclear pore glycoprotein Nup62 by co-immunoprecipitation assays. Interestingly Imp-β_1 was also recovered associated to GR and hsp90.[61]

Previous reports showed that the GR associates to Imp-α via its NL1 domain.[63,65,66] It appears, however, that Imp-α can bind to NL1 in the presence and absence of steroid. In addition, it has recently been reported that the GR binds to importins 7 and 8 in a steroid-independent manner,[65] all of which implies that additional factors and pathways independent of its dissociation from the heterocomplex are required for the hormonal regulation of GR localization. Importin 7 alone and Imp-α/Imp-β heterodimer were each competent to import an NL1-containing fragment of GR in an in vitro assay using permeabilized cells in the presence of Ran•GDP and ATP, whereas they failed to import purified full-length GR unless a diluted extract of cell cytosol was added. It is unlikely that such diluted extract was simply providing additional importins for the reaction since they were in great excess in the mixture. Therefore, the need of other factors is again implied.

It is possible that in the absence of hormone, the GR is tethered to the hsp90•FKBP52 heterocomplex, which may also be required for the proper function of some nuclear pore components. In a recent study[61] our laboratory showed that the GR binds to Imp-β and Nup62. Performing studies of reconstitution of the heterocomplex with purified proteins and reticulocyte lysate as source of chaperones, we demonstrated that the Nup-GR interaction is strengthened when both factors are chaperoned. This observation appears reasonable in view of our perspective that transformation of steroid receptors takes place in the nucleus. On the other hand, the discovery that Nups are chaperone-interacting proteins suggests a potential regulatory role of the chaperones for the nuclear import process, in addition to their role of acting as facilitators of protein-protein interactions during the cargo passage thorough the pore. It is known that the affinity of a protein cargo for its cognate importin adaptor influences its nucleocytoplasmic transport efficiency and represents a subtle effector of transport regulation.[67] Also, a correlation between the binding affinity of an NLS cargo for the NLS receptor, Imp-α, and the nuclear import rate for this cargo has been demonstrated. This correlation, however, is not maintained for cargoes that bind to the NLS receptor with very weak or very strong affinity.[67,68] Similarly, the interaction of the GR with Nups may also impair the efficient delivery of the receptor into the nucleus. In this sense, the strong association between GR and Nup62 (in their respective chaperoned complexes) was weakened by the presence of cytosolic factors,[61] suggesting that soluble proteins can regulate the interaction and, consequently, the import rate of cargoes.

There is evidence that Imp-α is co-internalized with the GR,[66] whereas Imp-β is not. Nonetheless, the knock-down of Imp-β significantly delayed GR nuclear import.[61] It has been reported that many importins including Imp-β do not only mediate active transport through NPCs, but they also effectively suppress the aggregation of cargoes,[69] which enhances the putative role of hsp90 associated to this protein. The anti-aggregation activity of importins involves shielding of basic patches on the cargo and predicts a precise match between cargo and receptor. However, it is hard to explain how a single factor could shield the multitude of different protein-, RNA- and DNA-binding domains in transport cargoes that are import substrates. Therefore, it may be envisioned that the presence of chaperones and cochaperones associated to importin, Nups and the cargo itself may act as a cooperative system to prevent aggregation of cargoes when a hydrophobic domain is exposed during the translocation step. This may explain why there is a more efficient interaction between Nup62 and GR when the proteins are properly folded with the hsp90 complex when compared to the "naked" proteins. It is likely that this could also favor the translocation step. On the other hand, when these complexes are disrupted by hsp90 inhibitors such as radicicol or geldanamycin, the nuclear translocation rate of GR,[14,40] MR[38,58] and AR[70] is substantially delayed.

Interestingly, it has been reported that NLS-containing proteins bound to the Imp-α/Imp-β complex dissociate slowly in the cytoplasm, whereas the release of the cargo in the nuclear basket structure facing the nucleoplasm milieu is faster. Consequently, it was postulated that the rate-limiting step in the Imp-α/Imp-β/

Nups-mediated import pathway is the dynamic assembly and disassembly of the importin•cargo complex rather than the translocation process per se.[68] Recent studies on the role of FG Nups as functional elements of the NPC permeability barrier showed that these proteins are highly flexible and devoid of an ordered secondary structure,[71] but those related to the NPC center are able to bind each other via hydrophobic interactions generating a sort of cohesive meshwork that may model the architecture of the pore.[72] If integral Nups such as Nup62 are chaperoned by hsp90, hsp70, p23 and/or TPR cochaperones, it would be entirely possible that the putative permeability barrier may be regulated by protein-protein interactions allowing (or not) the passage of certain cargoes.

Association of TPR proteins such as FKBP52 and PP5 to Nup62 seems to be hsp90-dependent, as shown by the almost complete dissociation of these IMMs from Nup62 in the presence of radicicol.[61] However, indirect immunofluorescence assays performed in intact cells treated with radicicol still show the presence of both IMMs in the perinuclear ring, suggesting that these TPR proteins may also bind in an hsp90-independent manner to other perinuclear structures, for example, other Nups. Nonetheless, competition experiments with the TPR domain overexpressed in intact cells showed that the perinuclear signal of FKBP52 was totally abolished, indicating that most, if not all type of associations of this IMM with any structure of the nuclear envelope requires the TPR domain.

The chaperone hsp70 and the cochaperone p23, both regular components of the GR•hsp90 heterocomplex, are also Nup62-associated proteins. In contrast to hsp90, this association is constitutive and suggests that both proteins are required for the proper architecture of Nup62. Hsp70 uses its ATPase cycle to control substrate binding and release,[73] likewise, substrate binding to importins is coupled to Ran•GTP cycles. However for some receptor-substrate pairs, the presence of Ran•GTP is not sufficient for cargo release; instead, an appropriate binding site for the cargo is also required.[74] Therefore, it is possible that hsp70 may be involved in substrate binding-release equilibrium in the NPC. In this sense, it is noteworthy to emphasize that hsp70 has been involved in the nuclear export mechanism of importins depending on its ATPase activity.[75]

Inasmuch as steroid receptors are always shuttling between cytoplasm and nucleus, it is tempting to speculate that GR•importins and GR•nucleoporins complexes form and disassemble constantly in a highly dynamic manner, even in the absence of hormone. Overall, there are several situations where cells broadly could alter nuclear translocation of steroid receptors and many other nuclear factors, but the mechanisms by which this is regulated are not well defined to date.

Envoy

Several signaling pathways regulate the activity of effectors in their transcriptional activity by controlling their subcellular localization. Until recently, the cytoplasmic localization of these effectors was mainly attributed to binding partners able to anchor the factor to cytoplasmic structures and/or to mask the NLSs, such that unmasking these signals or releasing the anchoring partners from the effector was sufficient to permit their nuclear localization. Among these potential effectors we have always included the steroid receptors, the molecular chaperone hsp90 being the anchoring partner responsible for both keeping the receptor in the cytoplasmic compartment and masking its NLS. A growing body of evidence has challenged this simple dogma. Actually, steroid receptors and many other signaling factors can constantly and dynamically shuttle between the cytoplasm and the nucleus, and the cytoplasmic localization of a given protein can be due to several reasons, among them, by binding of other proteins that mask the NLS (for example, IκB for NFκB), tethering the transcription factor to cytoplasmic structures (for example XAP2 for AhR), targeting the transcription factor to proteasomal degradation (for example, transformed steroid receptors), impairing the efficiency of the molecular machinery responsible for retrotransport (for example, FKBP51 for GR and MR), or simply favoring nuclear retention by attaching the client protein to nuclear structures (for example, Hic-5 for GR[76]).

The complex chaperone machinery responsible for the regulation of steroid receptor transport adds a number of checking points along the pathway of movement that may be used as potential regulatory targets. Thus, the nuclear accumulation of steroid receptors can be affected by: pharmacologic inhibition of hsp90, persistence of Hop/p60, FKBP51 or p23 bound to hsp90, disruption of the hsp90-TPR-domain protein interaction, disruption of the association of motor proteins with the PPIase-domain of certain IMMs, disassembly of dynein/dynactin complexes, interference of association of the hsp90-based complex with Nups or Imp-α, etc. Note that for reasons of simplicity, in this analysis we are not addressing the role of the chaperone system in intranuclear trafficking, attachment to nuclear matrix, nuclear speckles (where it is thought that steroid receptors mature by post-translational modifications), or the potential recruitment of FKBPs to gene promoter sites. Because the nuclear export of steroid receptors is also favored in the presence of hsp90 inhibitors,[77] the role of chaperones cannot be excluded from the general equation that balance the nucleo-cytoplasmic distribution of these receptors.

In addition to steroid receptors, there is a number of signaling proteins that shuttle dynamically between the cytoplasm and the nucleus (MAPKs, STATs, p53, NFκB, cyclins, etc.,). Ideally, the biological function of most of these nuclear factors could be regulated if we can identify the mechanism or mechanisms by which they reach their sites of action. For example, NFκB is constitutively active in many cancer cells and persistent localization in the nucleus has been implicated in tumor development. On the other hand, p53 activation promotes cell cycle arrest and apoptotic cell death, and p53 mislocalization in the cytoplasm is responsible for tumor development. Unlike NFκB, localizing p53 to the nucleus would be desirable for the control of cell survival. Based on the above-described findings suggesting a regulatory function of the hsp90•TPR-domain protein complex on some components of the NPC, it appears that the chaperone machinery first described in steroid

receptors could play a more general role on the nucleo-cytoplasmic shuttling of several soluble proteins and/or the mechanism of translocation of them through the nuclear pore. Unraveling this defy is desirable for future therapeutic studies on the regulatory scheme of nuclear transport involving cargoes, transport receptors, and the NPC, which may ultimately enable us to modulate cellular responses to internal and external stimuli according to our convenience.

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