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1085-9195/99/30/1–24/$16.00

Regulation of Steroid Receptor Subcellular Trafficking

Donald B. DeFranco

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, E-mail: dod1@vms.cis.pitt.edu

ABSTRACT

Cellular responses to external signals often reflect alterations in gene expression. The activation of cell surface hormone or growth factor receptors upon the binding of appropriate ligands mobilizes signal transduction cascades that can ultimately impact the activity of defined sets of transcription factors. The interpretation of hormonal signals can also be initiated intracellularly, as is the case for steroid hormone receptors. In addition to recognizing specific hormones, steroid hormone receptors also function as transcription factors and directly transduce hormonal signals to activation or repression of unique target genes. The delivery of activated steroid receptors to high-affinity genomic sites must be efficient to account for the rapidity and selectivity of many transcriptional responses to steroid hormones. Thus, the signal transduction capacity of steroid hormone receptors will be affected by the efficiency of receptor trafficking both between different subcellular compartments (i.e., the cytoplasm and nucleus) and within a specific compartment (i.e., the nucleus). This article will highlight the recent advances in our understanding of subcellular and subnuclear trafficking of steroid receptors.

Index Entries: Glucocorticoid receptor; nuclear import; nuclear export; nucleocytoplasmic shuttling; nuclear matrix; heat shock proteins.

SUBCELLULAR LOCALIZATION OF UNLIGANDED STEROID RECEPTORS

The pioneering work of the Jensen and Gorski *(1,2)* laboratories established that ligand-bound steroid receptors were tightly associated with nuclei, and led to the provocative hypothesis that steroid hormone receptors might be direct regulators of gene transcription. Despite the considerable progress made in subsequent years in understanding the mechanisms of steroid receptor-regulated transcription, fundamental issues regarding receptor subcellular trafficking remained unresolved. In which subcellular compartment do these signal transduction proteins first encounter ligand? Is ligand binding required for the accumulation of steroid receptors within the nucleus? As disparate results were obtained regarding steroid receptor trafficking, the possibility was considered that distinct subcellular trafficking pathways exist for different receptors. For example, unoccupied progesterone receptors (PRs) and estrogen receptors (ERs) were found by most investigators to localize predominately within nuclei *(3,4)*, whereas in most studies unliganded glucocorticoid receptors (GRs) *(5–7)*, mineralocorticoid receptors (MRs) *(8)*, and androgen receptors (ARs) *(9,10)* were localized within the cytoplasm. Although the experimental paradigms utilized in these studies were sound, they were limited in their ability to discern dynamic aspects of steroid receptor trafficking.

Using sophisticated cell biological approaches, Milgrom and co-workers first established that nuclear transport of steroid receptors (in particular rabbit PR and human ER) is bi-directional *(11)*. Nucleocytoplasmic shuttling of other steroid receptors was later confirmed *(12–14)*. Thus, steroid receptors are not statically confined to either the cytoplasmic or nuclear compartment, but establish an equilibrium distribution between these compartments based on the relationship between nuclear import vs nuclear export rates. Receptors will accumulate within the cytoplasm if nuclear import is rate limiting, whereas a limitation in the rate of nuclear export would lead to the preferential accumulation of receptors within nuclei *(15)*. The overall rate of receptor import most likely reflects the summa-

tion of multiple, kinetically distinct steps *(15)*, any of which could serve to regulate the efficiency of receptor nuclear import or export.

SIGNAL SEQUENCES FOR NUCLEAR IMPORT OF STEROID RECEPTORS

One of the first questions that arose from the recognition that nuclear transport of steroid receptors is bi-directional concerned the relationship between signal sequences that direct receptor nuclear import vs export. A constitutive nuclear localization signal sequence (NLS) has been mapped within the DNA-binding domain (DBD) of various steroid receptors *(6,10,16–18)* and shown to be comprised of multiple proto-signals. These proto-signals, which are characterized by a prevalence of basic amino acids, are so defined because they only function when present in unique pairs *(17,18)*. As such, steroid receptor NLSs resemble the prototype bipartite NLS first identified within the nucleoplasmin protein *(19)*.

A separate hormone-dependent NLS appears to be located within the ligand-binding domain (LBD) of rat GR *(6)*. The rat GR LBD does not possess sequences homologous to the bipartite, basic NLS found in the receptor's DBD implicating the existence of a distinct NLS. However, there has been some speculation that the hormone-dependent NLS may only serve an accessory role in nuclear transport *(16)*. A number of proteins that lack NLSs can be transported into nuclei in association with an NLS-containing protein *(20,21)*. Thus, it is conceivable that the hormone-dependent NLS activity associated with some steroid receptor LBDs is imparted by LBD-associated proteins that possess bona fide NLSs, and not by a unique LBD-encoded NLS. If this is indeed the case, the LBD co-transporting partner might remain associated with ligand-bound—and presumably activated receptors, during their passage through the nuclear pore complex (NPC). Although there are some attractive—candidates for such LBD-co-transporting substrates (e.g., hsp70 and hsp90) *(22,23)*, definitive proof that this co-transport operates in physiologically relevant contexts has not been provided.

CYTOPLASMIC RETENTION OF STEROID RECEPTORS

Unliganded, cytoplasmic GRs exist as heteromeric complexes that minimally contain a dimer of the 90 kDa heat shock protein,

hsp90, and particular immunophilin proteins such as the FK506-binding protein FKBP52 *(24)*. Although the constitutive nuclear localization of LBD-deleted GRs suggested that nuclear import of the receptor is restricted by their association within heteromeric complexes *(6,25)*, recent studies have raised questions about the simplicity of this model. For example, steroid receptor homodimers *(26)*, or receptor heteromeric complexes possessing hsp90 *(23)*, have the capacity to efficiently import into nuclei. In the later case, receptors, that were deleted of their own NLS were found to import into nuclei when co-expressed with an hsp90 chimera possessing a linked heterologous NLS *(23)*. These results imply that receptors are not restricted in their movement through the NPC when associated with molecular chaperones. However, it is unclear whether this "piggy-backing" of receptors with NLS-containing proteins is a mechanism that normally operates to direct receptors to the nucleus.

A different view of the impact of molecular chaperones on steroid receptor nuclear import has emerged when pharmacological approaches were used to manipulate receptor-chaperone associations. For example, sodium molybdate inhibits hormone-dependent in vivo nuclear import of GR and both hormone-dependent and -independent import of PR *(27)*. Because this treatment stabilized hsp90-containing heteromeric complexes *(27)*, these results suggest that the association of steroid-receptors with molecular chaperones might restrict receptor nuclear import. In vivo nuclear export of GR was not inhibited by molybdate *(27)*, suggesting that GR heteromeric complexes either are not formed within the nucleus, or have little impact on receptor nuclear export. In a related study, in vivo nuclear import of GR was found to be inhibited by geldanomycin *(28)*, an benzoquinone ansamycin that interacts with hsp90 *(29)* and affects its interactions with other molecular chaperones *(30)*.

The resolution of conflicting hypotheses concerning steroid receptor/molecular chaperone co-transport must take into account the fact that steroid receptor heteromeric complexes are not static entities. Thus, it may be inappropriate to consider unliganded receptors as stable, heteromeric complexes from which associated chaperones are quantitatively released upon hormone binding. The elegant reconstitution studies of Smith and co-workers *(31)* established that hsp90 associated with PR rapidly turns over in vitro, readily exchanging with a "soluble" pool of receptor-free hsp90. Furthermore, it appears that an ordered exchange of multi-subunit

molecular chaperone complexes of varying composition is required for steroid receptors to acquire the capacity to bind hormone in vitro. *(30,32)*. It is presumed that multiple associations with defined heteromeric molecular chaperone complexes are required for the LBD to attain an active conformation. If a particular exchange reaction is blocked, receptor heteromeric complexes may preferentially accumulate that represent transient intermediates in the receptor-folding pathway *(30)*.

Given this context, I hypothesize that receptor heteromeric complexes must be dynamic for receptors to be directed to appropriate subcellular trafficking pathways (*see* Fig. 1). If this is indeed the case, then steroid-receptor nuclear import would be disrupted by any experimental manipulation that alters the dynamic exchange of molecular chaperones with receptors. The inhibition of receptor nuclear import that accompanies sodium molybdate *(27)* or geldanomycin *(28)* treatment could result from the stabilization of intermediate receptor heteromeric complexes that are incapable of productively interacting with the nuclear import machinery. This notion is consistent with the observed co-transport of steroid receptors complexed with NLS-conjugated hsp90 since in this context receptor-hsp90 exchange may not be dramatically altered.

Additional support for this model can be inferred from the results of studies by Pratt and co-workers *(33)*, who examined the role of GR-associated FKBP52 in nuclear import. Specifically, microinjection of FKBP52 antibodies was found to inhibit hormone-dependent nuclear import of GR in vivo. Although an alternative explanation was offered *(33)*, I hypothesize that inhibition of GR nuclear import in this case could be owing to stabilization of GR-heteromeric complexes that possessed FKBP52. To summarize, the "maturation" of steroid receptors brought about by the ordered, dynamic exchange with various chaperone complexes may not only be required for appropriate folding of the LBD, but for receptors to acquire the capacity to interact productively with the nuclear import machinery.

In Fig. 1, a simple model is presented to illustrate the impact of receptor heteromeric complex exchange on nuclear import. Nuclear import is comprised of separate docking and translocation steps that differ in their adenosine triphosphate (ATP) requirements *(34,35)*. The overall rate of receptor import will be governed not only by the rate of receptor docking to, and translocation through, the NPC, but

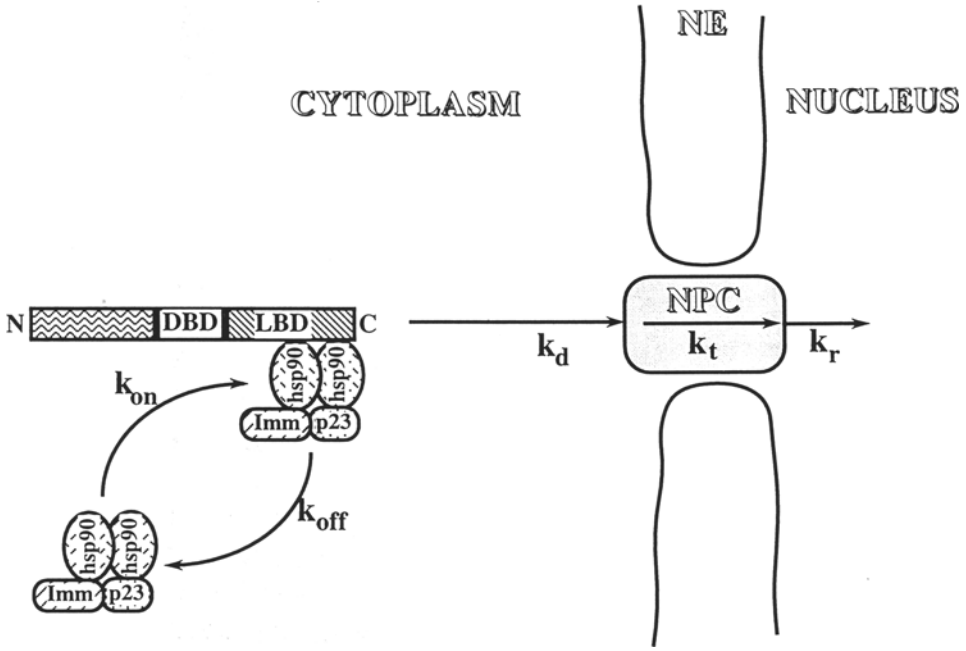

Fig. 1. Model of steroid-receptor nuclear import. Unliganded steroid receptor protein, with its DNA-binding domain (DBD) and ligand-binding domain (LBD) indicated, is depicted in the cytoplasm. This form of the receptor is associated with an hsp90 dimer, an immunophilin protein (Imm) and a 23 kDa protein, p23. The kinetics of exchange of the receptor-associated hsp90, Imm, and p23 complex are governed by k_{on} and k_{off}. Translocation of receptors from the cytoplasm to the nucleus proceeds via a minimum of three distinct steps. Rate constants for receptor docking to the cytoplasmic face of the NPC (k_d), translocation through the NPC (k_t), and release from the nucleoplasmic face of the NPC (k_r) are shown. See text for further details.

also by the rate of receptor exchange from heteromeric complexes (Fig. 1). An idealized complex is shown in Fig. 1 that possesses an hsp90 dimer, an immunophilin, and p23. The composition of the receptor heteromeric complex that presents receptors to the nuclear import machinery is unknown. If one assumes that the rate of receptor docking to the NPC, translocation through the NPC and release from the NPC are not rate limiting, then the rate of receptor import could be governed strictly by k_{off}, the rate of receptor release from heteromeric complexes. Importantly, if the rate of this exchange varies for different steroid receptors (e.g., *see* ref. *36*), the rate of receptor nuclear import would be affected. Irrespective of the nuclear

export capacity of steroid receptors, the equilibrium distribution of individual steroid receptors would differ as a result of an inherent difference in the kinetics of heteromeric complex turnover.

An essential aspect of steroid receptor trafficking that is obviously lacking in the model presented in Fig. 1 concerns the targeting of cytoplasmic receptors to the NPC. How do receptors, once associated with nuclear import factors, locate the NPC? There have been speculations that perhaps specific molecular motors are involved in guiding cytoplasmic receptors along tracks to the NPC *(25)*. Short filaments that extend from the cytoplasmic face of the NPC appear to serve as docking sites for importing substrates *(34)*, but additional cytoskeletal elements that extend greater distances from the NPC have not been found. Furthermore, docking of NLS-conjugated substrates to the NPC both in vivo and in vitro does not require ATP *(34)*. If a molecular "tracking" system is not used to move NLS-linked substrates through the cytoplasm, it is unclear how rapid efficient docking of import-competent complexes to the NPC ensues. Despite the advances in our understanding of NPC structure and function, it is surprising that this fundamental aspect of subcellular trafficking remains elusive.

SIGNAL SEQUENCES FOR NUCLEAR EXPORT OF STEROID RECEPTORS

Does a specific signal sequence direct the nuclear export of steroid receptors? Currently, two distinct nuclear export signal sequences (NESs) have been characterized. A leucine-rich NES is responsible for the rapid, energy-dependent export of the protein kinase A inhibitor peptide (PKI) *(37)*, the HIV-1 Rev protein *(38)*, and IκBα *(39)*. This type of NES interacts with a novel NPC protein that may participate in some aspect of the nuclear export process *(40)*. However, not all shuttling proteins possess a leucine-rich NES. For example, the hnRNP A1 protein possesses an unrelated NES that is interdigitated with its NLS *(41)*. This unique bi-functional NLS/NES is recognized by a 90 kDa protein termed transportin, which has been shown using in vitro assays to function in nuclear protein import *(42)*. Steroid receptors do not appear to utilize either of these established NESs for nuclear export since their NLS is not homologous to the hnRNP A1 NLS/NES *(41)*. Likewise, truncated receptors that lack any apparent leucine-rich sequence export from nuclei in

transient heterokaryon assays with an efficiency indistinguishable from wild-type receptors *(14)*.

NUCLEAR RETENTION OF STEROID RECEPTORS

Although the existence of steroid receptor NESs remains controversial (e.g., *see* ref. *43*), it is formally possible that steroid-receptor nuclear export utilizes a signal sequence-independent nuclear export pathway *(44)*. In this case, nuclear-export may be regulated strictly by the efficiency of receptor retention within nuclei. What factors might regulate steroid receptor nuclear retention? It has recently been proposed that nuclear retention of GR is regulated primarily by its DNA-binding capacity *(45)*. However, this hypothesis is not supported by the fact that GR DNA-binding mutants are not distinguished in their nuclear-export properties from wild-type GR, as assessed by a transient heterokaryon assay *(14)*. Given the differences in methodology utilized in these two studies, and the inherent limitations of both assays, the role of steroid receptor DNA binding in nuclear retention remains unresolved. In addition, mutations within steroid receptor DBDs may not only impact its DNA binding capacity, but also alter receptor interactions with proteins that influence nuclear retention. For example, the DBDs of AR and GR appear to possess important determinants for nuclear matrix binding *(46,47)*. If distinct segments of steroid receptor DBDs comprise binding sites for chromatin- or nuclear matrix-associated proteins, such mutations may have an effect on nuclear retention of receptors irrespective of their influence on receptor DNA binding.

We have utilized an in vitro approach to examine the relationship between GR nuclear retention and export *(48)*. Our results indicate that the dissociation of ligand from occupied nuclear GRs is accompanied by rapid release of receptors from high-affinity chromatin-binding sites *(48)*. Interestingly, GR release from chromatin was not associated with a correspondingly rapid export from nuclei. Thus, nuclear retention of GRs does not appear to be strictly governed by tight association of receptors with chromatin and the nuclear matrix *(48)*. Rather, receptors may be retained within a unique nuclear-export staging area following their release from chromatin. The transfer of receptors from this staging area to the NPC appears to be the rate-limiting step in GR nuclear export. GRs that release from the chromatin do not appear to collect at the nucleo-

plasmic face of the NPC *(48)*, implying that export staging areas may not be physically linked to the NPC. Frationation experiments established that these receptors are also not associated with the nuclear matrix *(48)*. It remains to be established whether this putative nuclear-export staging area represents a novel subnuclear compartment or is a component of chromatin that is distinguished simply by its relatively low affinity for GR. Irrespective of the identity of this nuclear export staging area, it will be of interest to reveal whether nuclear-export of other proteins is also limited by analogous interactions with this putative, novel subnuclear compartment.

Additional mechanistic details of steroid-receptor nuclear export are currently very limited. Steroid-receptor nuclear export has been postulated to be energy-independent, based primarily on the efflux of nuclear receptors from cells subjected to prolonged ATP depletion *(11)*. However, when ATP-depletion conditions were more limited, GR nuclear retention was actually increased *(49–51)*. More recently, we have extended these studies and found a dramatic increase in nuclear matrix-binding of GR (and PR) in ATP-depleted cells *(47)*. The increased association of GRs with the nuclear matrix of ATP-depleted cells is reversed upon restoration of cellular ATP levels *(47)*. These studies provided an explanation for effects of metabolic inhibitors on GR nuclear retention observed as early as 1972 *(51)*. As the binding of steroid receptors to the nuclear matrix clearly limits their nuclear-export capacity, at least one step in the overall nuclear export of steroid receptors appears to be ATP-dependent, i.e., their release from the nuclear matrix.

SUBNUCLEAR TRAFFICKING OF STEROID RECEPTORS

What is the fate of steroid receptors that enter the nucleus following passage through the NPC? In vivo footprinting analyses have established that steroid receptors can rapidly locate specific target sites within the genome *(52,53)*. What mechanism is utilized by steroid receptors to traffic within the nucleus? When sophisticated cell imaging techniques were applied to visualize steroid receptors within the nucleus, receptors were found to be localized within discrete subnuclear regions (i.e., speckles or foci) and not randomly distributed *(54)*. More recently Van Steensel and co-workers found that endogenous GRs in a variety of cultured cell lines collected into 1000–2000 small nuclear speckles in hormone-treated cells *(55)*.

Importantly, this staining pattern was not dependent on the method of cell fixation and permeabilization. In cells treated with hormone antagonist, an indistinguishable speckling pattern was noted, implying that receptors in nuclear speckles are not actively engaging the transcription machinery *(55)*. In support of this conclusion, newly synthesized RNA or RNA polymerase II did not co-localize with GR speckles *(55)*. In direct contrast to these results, different nuclear staining patterns of agonist- vs antagonist-bound GR were observed by Htun and co-workers in living cells using a green fluorescent protein-GR fusion protein *(56)*. Because Htun and co-workers did not visualize sites of active transcription *(56)*, it is not known what proportion, if any, of agonist-bound GRs visualized in their studies, are actively involved in transcriptional regulation.

If discrete steroid-receptor nuclear foci visualized under light microscopy do not represent receptors actively engaged in transcription, what is their physiological relevance? RNA splicing factors have also been found to localize within discrete foci or "speckles" *(57)*. These speckles may be associated with discrete nuclear structures designated as interchromatin granule clusters (ICGCs) *(57)* and represent storage sites for splicing factors that are not actively participating in RNA processing *(58)*. RNA splicing factors are not confined to the ICGCs and can be recruited to sites of active transcription *(58)* that are associated with another nuclear structure, the perichromatin fibrils (PCFs) *(57)*. Given the close association between PCFs and ICGCs, it may be difficult to make precise assignments of subnuclear compartmentalization based strictly on the appearance of speckles at the light microscope level. Thus, functionally distinct speckles may exist that could represent various intermediate stages of subnuclear trafficking. Steroid-receptor nuclear speckles are distinct from speckles that possess splicing factors such as SC-35 *(55)*, suggesting that there may be discrete subsets of storage sites within the nucleus that differ in their composition and function.

Nuclear steroid receptors have also been examined at high resolution using electron microscopy *(58,59)*. However, even in this case conflicting results were generated concerning the effects of hormone on the precise subnuclear localization of receptors. It is difficult to make meaningful comparisons between earlier electron microscopic studies, because the technology to assess ultrastructure of the nucleus has advanced dramatically in recent years *(57,61)*. Advances in both sample preparation and imaging have permitted a much

more refined view of the compartmentalization of transcription domains within the nucleus *(57)*. Once these sophisticated techniques are applied to the analysis of steroid-receptor localization within the nucleus, controversies surrounding the identity and functional significance of receptor foci may finally be resolved.

STEROID–RECEPTOR INTERACTIONS WITH THE NUCLEAR MATRIX

The nucleus is highly organized into discrete structural and functional domains that serve to compartmentalize the processes of DNA replication, transcription, and RNA splicing *(57)*. The molecular framework for such subnuclear organization is supplied by the nuclear matrix, a ribonuclear protein network of interconnected filaments *(62)*. The composition of the nuclear matrix varies between different cell and tissue types and can even change within an individual cell type in response to external signals *(63,64)*. Many transcription factors that associate with the nuclear matrix can also partition to soluble compartments of the nucleus *(65–67)*, further exemplifying the dynamic nature of protein association with the nuclear matrix. Thus, there is some plasticity in nuclear-matrix composition that may be relevant to either the establishment of cell type-specific gene expression during development, or the regulation of established genetic programs by environmental factors.

Steroid receptors were the first transcription factors found to bind to the nuclear matrix *(68)*, owing mainly to the availability of high specific activity radiolabeled steroids. The interaction between steroid receptors and the nuclear matrix was shown to be hormone-dependent, and appeared to involve saturable, high-affinity interactions *(68)*. Recently, discrete domains of steroid receptors required for nuclear-matrix binding have been identified. For AR and GR, the DBD and LBD contribute to nuclear-matrix binding, although the relative contributions of these domains differ between these two highly related proteins *(46)*. The relative proportion of steroid receptors associated with the nuclear matrix varies in different target tissues, particularly for sex steroid receptors *(69)*. It has been proposed that this cell type- or tissue-specific binding of steroid receptors to the nuclear matrix may be mediated by specific acceptor proteins. A candidate steroid-receptor, nuclear-matrix acceptor protein has been isolated from chick oviduct *(70)*, but the role this

protein plays in steroid-receptor regulation of transcription has yet to be established.

Despite the recognition nearly 20 years ago of steroid-receptor binding to the nuclear matrix, the factors and mechanisms that regulate this association remain enigmatic. However, recent work from our laboratory has revealed some novel aspects of receptor binding to the nuclear matrix that could have important biological implications. In particular, we found that GR interactions with the nuclear matrix are dynamic in intact cells and regulated by an ATP-driven process *(47)*. This dynamic process is disrupted upon depletion of cellular ATP pools, leading to a dramatic increase in the amount of GR associated with the nuclear matrix *(47)*. The fact that ATP depletion might disrupt sub-nuclear compartmentalizaion of GR was suggested from early studies of both Ishii *(52)*, and Munck and co-workers *(47)*. In our studies, the nuclear-matrix binding of other nuclear proteins, such as the SV40 large tumor antigen, was found not to be affected by ATP-depletion, arguing that a nonspecific collapse of nuclear proteins unto the nuclear matrix was not occurring under these conditions *(47)*. In addition, receptors that collected on the nuclear matrix of ATP-depleted cells were not permanently trapped there and could be released upon restoration of cellular ATP pools *(47)*. Based on these results, we hypothesized that, although GR binding to the nuclear matrix is ATP-independent, receptor release from the matrix is ATP-dependent *(47)*.

What is the potential impact of dynamic interactions between steroid receptors and the nuclear matrix? It is tempting to speculate that this subnuclear trafficking pathway may play a role in receptor localization of specific, high-affinity binding sites. Thus, the rapid binding and ATP-dependent release of steroid receptors from the nuclear matrix may allow more effective scanning of the genome as receptors search of specific target sites. In this way, the receptor's capacity for movement within the nucleus would not be restricted by its association with nontarget sites, which would be in vast excess. Likewise, receptors would not be limited to a "linear" scan of the genome once DNA-bound. Because either cell-type specific transcription factors *(71)* or active genes *(64,72)* can be associated with specific regions of the matrix, both receptor-DNA and receptor-protein interactions could be involved in this matrix scanning. As suggested previously *(71)*, receptor targeting to the specific nuclear-matrix sites may be influenced by specific matrix-associated acceptor pro-

teins *(72)*. As such, our model is an elaboration of the previously proposed nuclear matrix acceptor hypothesis, but adds the notion that the search for such sites is a dynamic one that utilizes the energy of ATP hydrolysis.

INFLUENCE OF MOLECULAR CHAPERONES ON STEROID RECEPTOR SUBNUCLEAR TRAFFICKING

How is subnuclear trafficking of steroid receptors regulated? A number of molecular chaperones use ATP binding and/or hydrolysis to regulate their ability to deliver proteins to and from distinct compartments within the cytoplasm *(73,74)*. Perhaps these proteins serve analogous roles in the nucleus to direct proteins, such as steroid receptors, to distinct subnuclear compartments. As discussed in a recent review *(75)*, a number of chaperones are responsible for maintaining structural and functional integrity of the nucleus. These include nucleoplasmin, which is involved in histone-DNA assembly and fibrilarin, which functions in ribosome assembly in the nucleolus *(75)*. Clearly, complex multi-subunit assemblies are constantly forming and turning over within the nucleus and it is not too surprising that nuclear chaperones exist to facilitate these processes.

Although many heat-shock proteins were initially thought to reside exclusively within the cytoplasm, significant levels of these molecular chaperones also accumulate within the nucleus *(76,77)*. What is the functional significance of nuclear heat-shock proteins? The accumulation of hsp70 within nucleoli of heat-shocked cells serves an important protective function particularly with regard to maintaining ribosome structure and biogenesis *(78)*. Recent results obtained in yeast also suggest that nucleolar hsp70 may protect the mRNA export pathway from irreversible damage under conditions of thermal stress *(78)*. In addition to these protective functions, the chaperoning activity of heat-shock proteins in the nucleus may extend to biochemical processes operating in nonstressed cells. For example, hsp90 has been shown to affect the in vitro DNA-binding activity of the basic-loop-helix transcription factors MyoD and E12 *(80,81)*. Likewise, hsp70 was found to stimulate specific DNA-binding activity of ER *(82)*. The stimulatory effect of hsp70 on in vitro DNA-binding does not appear to extend to other members of the steroid-receptor super-family *(83)*.

The possibility that heat-shock proteins have an impact on nuclear functions of steroid receptors has also been suggested by in vivo experiments. GR mediated transactivation is potentiated in transfected cells that are subjected to chemical or thermal stress *(84,85)*. The mechanism responsible for this "heat-shock potentiation effect" (HSPE) has not been established, although it appears to involve some factor(s) that is(are) induced upon heat shock *(86)*. The possibility that some heat-shock proteins are involved in HSPE has not been ruled out. In yeast, mutations of an hsp70 partner, the Ydj-1 protein, were found to alter GR *(87)* and AR *(88)* transactivation activity. Effects of Ydj-1 mutations on AR and GR transactivation were only revealed in the presence of the receptor's carboxyl-terminal LBD *(87,88)*, implicating a role for the Ydj-1 protein in some aspect of hormone-dependent signaling. This is consistent with the functioning of chaperones in assembly and maintenance of an active hormone-binding conformation of the LBD *(31,89)*. However, steroid-receptor LBDs encode transactivation *(90)* and nuclear matrix binding domains *(46,47)* whose activities are both manifested in the nucleus. Thus, the possibility that the hsp70 and one of its partners (i.e., homologs of DnaJ; *91*) affect nuclear functions of steroid receptors remains viable.

In our recent analysis of a rat GR zinc-finger point mutant, we have made a number of surprising observations that support the view that molecular chaperone effects on steroid receptor function indeed extends to the nuclear compartment. An LBD-truncated form of rat GR that possesses a point mutation at a conserved arginine in the second zinc finger (i.e., R496) exhibited aberrant subnuclear trafficking *(18)*. Specifically, LBD-truncated receptors possessing R496 mutations were found not to distribute in a diffuse, uniform pattern characteristic of wild-type receptors, but accumulated within a few large foci *(18)*. Confocal microscopy revealed that these foci are distributed throughout all planes of the nucleus and vary in number from 6–10 foci/cell *(18)*. Importantly, none of the other mutations within the GR DBD generated this mistargeting phenotype, demonstrating that this is not merely a property associated with GRs defective in DNA-binding. Because mutations of this arginine to either another basic amino acid (i.e., lysine), or an acidic (i.e., aspartic acid), noncharged polar (i.e., serine) or nonpolar (i.e., leucine) amino acid confers the identical mistargeting defect *(18)*, it is unlikely that a fortuitous alternative subnuclear targeting signal was generated by the

R496 mutations. Rather, we hypothesize that an arginine at position 496 of rat GR is absolutely required to insure appropriate subnuclear trafficking of LBD-deleted receptors.

The nuclear mistargeting associated with the R496 mutant receptors was not autonomous, because hsp70 was found to co-localize within nuclear foci containing mutant receptors *(18)*. This result implied that a stress response may have been mobilized in cells expressing mistargeted nuclear receptors, but not to an extent sufficient to correct the mistargeting defect. What factors might be limiting the ability of a molecular chaperone (i.e., hsp70) to manage mistargeted nuclear receptors? Members of the DnaJ family of molecular chaperones stimulate the ATPase activity of hsp70 in vitro *(92,93)* and are considered co-chaperones of hsp70 analogous to the physiological functioning of bacterial DnaJ and DnaK (i.e., hsp70 homolog) proteins. Could some member of the DnaJ family be limiting, and thereby impairing, the ability of the cellular chaperone machinery to correct R496 mutant rat GR mistargeting? This hypothesis, in fact, was supported by the observation that overexpression of a human homolog of DnaJ (i.e., HSDJ-2) corrected mistargeting of LBD-truncated R496 mutant rat GRs *(18)*. This is the first example of a nuclear function for the hsp70/DnaJ chaperone pair. The fact that a DnaJ mutant, which did not correct R496 mutant rat GR mistargeting *(18)*, coincidentally localized with receptor foci suggests that the recognition of mistargeted nuclear proteins (such as the R496 mutant rat GRs) can be functionally separated from its correction activity.

Why are LBD-truncated R496 mutant GRs uniquely mistargeted? Interestingly, the position that this arginine occupies within the rat GR DBD (i.e., immediately following the final cysteine of the second zinc finger) is absolutely conserved in all members of the nuclear-receptor super-family *(94)*. In addition, for members of the nuclear-receptor super-family whose structures have been determined (i.e., GR, ER, and thyroid hormone receptor), this arginine has been found to make both specific and nonspecific phosphate backbone contacts *(95–97)*. Becuase mutations at other amino acids within the rat GR DBD that make phosphate contacts do not lead to mistargeting defects *(18)*, the loss of a single phosphate contact is not sufficient to generate nuclear mistargeting or rat GR. Therefore, the phosphate contact made by the arginine at position 496 of rat GR is unique and may be essential for appropriate subnuclear trafficking of the receptor. R496 in rat GR falls within one of three α helical moieties within

the DBD, and is the only amino acid within this particular α helix that makes direct contact to DNA *(95–97)*.

Inherent in the model previously invoked to explain mistargeting of R496 mutant rat GRs is an involvement of DNA binding in receptor trafficking within the nucleus. R496 mutant rat GRs do not form cytoplasmic foci and maintain their capacity to import into nuclei. Because the rat GR NLS includes amino acids immediately surrounding R496 *(18)*, this mutation does not disrupt receptor structure to such an extent that interferes with its ability to interact with the nuclear-transport machinery. Only upon entry into the nucleus is the defect in R496 function first apparent. It is possible that R496 mistargeting is not initiated following its initial contact with DNA, but only following its release from the nuclear-import machinery. The fact that foci containing R496 mutant rat GRs are distributed throughout all planes of the nucleus makes it unlikely that the mistargeting defect is expressed immediately upon release from the NPC. However, R496 mutant receptors may initially target to GR-specific storage sites, which do not represent high-affinity chromatin-binding sites, and become unable to release from this compartment. Regardless of the actual physical location of R496 mutant rat GR foci, it is intriguing that these foci are limited in number and typically uniform in size and shape *(18)*. Additional experiments with second-site mutations within R496 mutant receptors may be informative in defining the mechanistic basis for foci formation.

Because hsp70/DnaJ chaperone pair can correct the nuclear mistargeting defect of R496 mutant rat GRs, does this implicate a role for hsp70 and/or DnaJ in the movement of wild-type receptors within the nucleus? This is unclear and must await additional in vivo and in vitro analyses of steroid-receptor subnuclear trafficking. However, the results obtained with the R496 mutant are instructive in drawing attention to potential regulators of steroid receptor subnuclear trafficking. Irrespective of whether hsp70 and/or DnaJ chaperones participate in trafficking of nuclear receptors, our results suggest that they can function to survey the nucleus for mistargeted or misfolded proteins, and under certain conditions, correct such defects. Given the multitude of macromolecular assemblies within the nucleus, and conformational transitions that result from many protein–nucleic acid interactions, it may be time to expand our view of the physiological functions of hsp70 and DnaJ, and consider the nucleus as an additional realm for these chaperones.

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