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Nucleocytoplasmic trafficking and glucocorticoid receptor function

Review Article

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Abbreviations: **GR**, glucocorticoid receptor; **DBD**, DNA-binding domain; **LBD**, ligand-binding domain; **NL** or **NLS**, nuclear localization signal; **hsp**, heat shock protein; **WT**, wild type.

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Summary

The glucocorticoid receptor (GR) is a ligand activated transcription factor that redistributes between nucleus and cytoplasm in response to the addition and withdrawal of steroidal ligands. Localization of the receptor in the cell is dynamic and changes in GR localization reflect the shifting of equilibria between several competing cellular pathways. Since the naïve receptor is transformed from a transcriptionally inert cytoplasmic factor to a potent sequence-specific, DNA-bound transcriptional regulator, delimiting the controls on receptor localization is seminal to understanding how receptor activity may be manipulated or controlled within the cell. A number of recent reports have begun to reveal that the controls on GR trafficking are more sophisticated than previously expected and point to an important role for trafficking controls in the regulation of the steroid response.

I. Introduction

The glucocorticoid receptor (GR) is encoded by one of six genes for steroid hormone receptors. Steroid receptors are ligand-activated transcription factors of the nuclear hormone receptor superfamily with highly similar cys-4 type zinc fingered DNA binding domains. Receptor ligand binding domains are less well conserved and determine specific responses to steroid hormone agonists and antagonists. Steroid receptors are distinguished from other members of the nuclear receptor family by their association in the absence of ligand with a chaperone complex anchored to the receptors by hsp90, but also including a number of other heat shock proteins and immunophilins (Pratt and Toft, 1997). Association of unliganded receptors with the chaperone complex is dynamic and assembly of the mature complexes follows an ordered pathway (Pratt and Toft, 1997; Smith, 1998). For GR, hsp association appears to be a prerequisite for steroid binding and thus functions as an important control point for steroid signaling (Bresnick et al., 1989; Picard et al., 1990).

The modular structure of GR and the functional activities localized within the receptor are summarized in **Figure 1**. The central DNA binding domain is preceded by an extended amino terminus that contains a ligand-independent transcriptional activation function (Bocquel et al., 1989; Hollenberg and Evans, 1988). The amino terminus of GR also contains several serine/threonine phosphorylation sites whose phosphorylation is modulated through the cell cycle and which may have some effects on the transcriptional regulatory potential of GR as well as on receptor stability and subcellular trafficking (Bodwell et al., 1998; Hsu and DeFranco, 1995; Hsu et al., 1992; Hu et al., 1997; Munck and Holbrook, 1984; Webster et al., 1997). A second, steroid-dependent transcriptional activation function is located within the C-terminal ligand binding domain of

the receptor (Bocquel et al., 1989; Danielian et al., 1992; Hollenberg and Evans, 1988). The hsp90 binding surface of GR is also localized to the C-terminal region of the receptor (Dalman et al., 1991; Howard et al., 1990). Ligand binding induces receptor transformation, characterized by the dissociation of GR from the chaperone complex and, in the presence of hormone agonist, leads to the free transcriptionally active form of the receptor (Beato et al., 1996).

Function of GR involves the cyclic redistribution of the receptor between the nucleus and cytoplasm. This proposed cycle is shown in its simplest form in **Figure 2**. In the absence of steroidal stimulus, naïve GR is localized predominantly to the cytoplasm under most circumstances and is complexed with the chaperone complex (Picard and Yamamoto, 1987; Sackey et al., 1996). Upon ligand binding, the chaperone complex is dissociated and the receptor dimerizes and is rapidly transferred to the nucleus (Cidlowski et al., 1990; Picard and Yamamoto, 1987; Sackey et al., 1996; Wikstrom et al., 1987). The precise sequence of these events remains to be completely elucidated. Upon arrival in the nucleus the activated receptors bind to specific response elements and regulate gene transcription through the recruitment of transcriptional coregulatory proteins that promote changes in chromatin structure and stimulate the basal transcriptional machinery (Beato and Sanchez-Pacheco, 1996; Beato et al., 1996; Glass et al., 1997; Wolffe, 1997). Upon loss of ligand or withdrawal of stimulus, GR becomes reassociated with the chaperone complex and becomes slowly redistributed to the cytoplasm (Haché et al., 1999; Qi et al., 1989; Sackey et al., 1996).

A series of recent studies have shown that this simple visual model for the cycling and recycling of GR likely occurs above a complex regulatory network in which constitutive receptor trafficking is subject to the push and pull of multiple regulatory signals.

II. Nuclear-cytoplasmic transport signals within GR

Despite intensive study into the regulatory control of nucleo-cytoplasmic trafficking of GR and other steroid receptors, our knowledge of the signals that mediate the transfer of GR across the nuclear membrane is relatively modest. GR has been shown to have two independent nuclear localization signals, NL1 in the hinge region of the receptor between the DBD and LBD and NL2 within the LBD itself (Picard and Yamamoto, 1987). NL2 is a unique feature of GR, as the other steroid receptors lack similar activities in their LBDs.

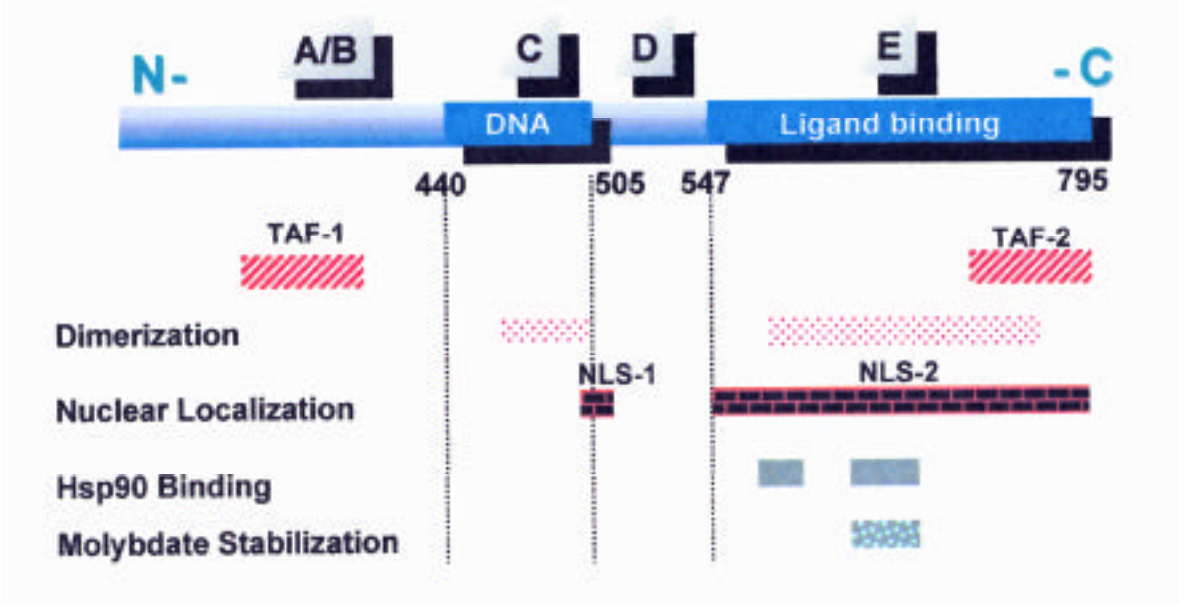

Figure 1. Schematic depiction of functional motifs within the glucocorticoid receptor. The position of the DNA and ligand binding domains of rat GR are shown integrated into the schema depicting the A-E domain organization employed for nuclear hormone receptors, while location of transcriptional activation functions (TAF's), nuclear localization signals (NLS's) dimerization motifs and the hsp90 binding determinants of GR are summarized below.

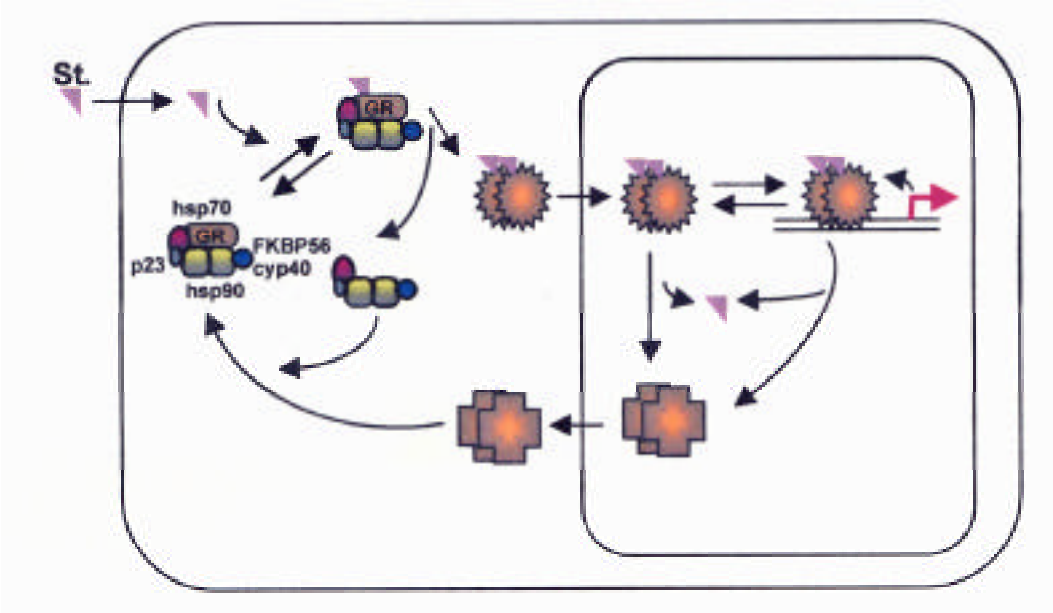

Figure 2. Simplified model for the response of glucocorticoid receptor to steroid hormone. Prior to steroid treatment, naïve GR is localized to the cytoplasm in a complex with heat shock proteins and immunophilins that is anchored by hsp90. Upon steroid treatment, liganded GR dissociates from the hsp-immunophilin complex, transfers to the nucleus and activates transcription as a dimer from specific DNA response elements. Steroid binding is a transient event. Upon loss of steroid, the receptor becomes recycled into a similar or identical hsp complex that is able to respond to subsequent hormonal challenge.

The core of the nuclear localization sequence that is NL1 is a series of basic amino acids (rGR 510-RKTKKKIK-517) that resemble classical basic NLSs such as that of SV40 T antigen and which appear to mediate the binding of GR to the importin α NLS binding proteins (Savory et al., 1999). Several amino acids N-terminal to this NL1 core are two additional small groups of basic amino acids that may also contribute to the import of GR into the nucleus (Tang et al., 1997). Mutations within these two additional basic motifs decrease the level of nuclear occupancy of GR (Tang et al., 1997). However the mutations examined to date have also compromised DNA binding by GR. This may be significant as a separate study showed that other mutations in the GR DBD that impair DNA binding without affecting these basic motifs led to a similar decrease in the nuclear occupancy of the liganded receptor (Sackey et al., 1996). In addition, substitutions within the NL1 core were sufficient to abrogate the nuclear import of GR fragments also lacking NL2 (Savory et al., 1999). Thus the significance of the contribution of these two additional basic motifs to the nuclear import of GR remains to be confirmed.

Study of the NL2 nuclear import signal in the ligand binding domain of GR has been slowed by it's overlap with the chaperone binding region of GR and an apparent strict dependence on bound steroid (Dalman et al., 1991; Howard et al., 1990; Picard and Yamamoto, 1987; Savory et al., 1999). Indeed, the minimal NL2 signal described to date overlaps completely with the minimal GR ligand binding domain (rGR aa 540-795) (Picard and Yamamoto, 1987). However, the minimal LBD of GR is sufficient for transfer of the receptor to the nucleus and also mediates the nuclear transport of normally cytoplasmic proteins such as β-galactosidase in hybrid experiments (Picard and Yamamoto, 1987). NL2 appears to be strictly dependent upon the binding of steroid agonists to GR (Savory et al., 1999). The treatment of cells with the GR antagonist RU486, which binds to GR and induces chaperone dissociation and the transfer of WT receptor to the nucleus, has been shown to be unable to promote the nuclear transfer of GRs in which NL1 has been inactivated (Savory et al., 1999). These results suggest that NL2 is highly dependent upon the three dimensional structure of the GR LBD bound to steroid agonist and is likely to overlap with the positioning of the C-terminal α-helix of GR that is also crucial for the AF-2 activity of receptor. Withdrawal of steroid agonist leads to the inactivation of NL2 and the rapid return of $NL1^-$ GR to the cytoplasm (Savory et al., 1999).

NL2 appears to mediate the nuclear uptake of GR through a pathway that is different from the importin α-dependent pathway that is likely to be employed by NL1. The GR LBD lacks an obvious basic motif that might become exposed upon steroid binding. In addition, mutation of the GR NL1 has been shown to prevent the binding of agonist-bound receptor to an importin α homologue in vitro and in vivo (Savory et al., 1999). Third, a recent kinetic analysis of NL2-mediated nuclear import of GR identified clear differences in the kinetics of nuclear import and the levels of nuclear occupancy of WT GR and GR with a mutation inactivating NL1 (Savory et al., 1999). While no

experimental information presently exists to substantiate hypothetical rationales for requirement for NL2 in the function of GR, it may be expected that NL2 imparts a selected capacity for the nuclear import of GR under particular or peculiar physiological circumstances that meets a requirement for GR function that is lacking for the other steroid receptors.

By contrast to the picture emerging for the nuclear import of GR, there is little information on the nature or position of signals that mediate the nuclear export of GR. The export of GR from the nucleus however, appears to be an active process that is ATP-dependent and is likely to be signal-mediated. In the only study performed to date, separate deletion of the N-terminus and LBD had no apparent effect on the export of GR from the nucleus (Yang et al., 1997). This suggests either that a nuclear export signal for GR is encoded within the receptor DBD or that the N-terminus and LBD contain distinct export signals.

The export of many and perhaps even most proteins from the nucleus has been shown to be accomplished through a protein family called exportins, that are related to the importin β nuclear importers (Ullman et al., 1997). GR contains at least 5 hydrophobic motifs that exhibit similarity to the motifs recognized by the exportins and thus may be potential export signals (R. Haché, unpublished observation). Further, nuclear localization of GR is promoted by the treatment of cells with leptomycin B, a specific inhibitor of CRM1-mediated nuclear import, suggesting that the export of GR from the nucleus may involve the exportin CRM1 (Savory et al., 1999).

III. Nuclear import of GR in response to steroid treatment

Interestingly, the import of GR into the nucleus may be mediated by two separate pathways that are distinguished by a dependence upon hsp90 and association of the receptor with the cytoskeleton. Several recent studies have shown that inhibition of the nuclear transport of GR by pharmacological agents and the divalent anion molybdate can be reversed by treatment of cells with agents that induce the depolarization of the cytoskeletal network.

In the first instance it was shown that geldanamycin, a compound that inhibits ATP hydrolysis by hsp90 and which blocks the maturation of steroid receptor complexes inhibited the transfer of GR to the nucleus when added to cells following steroid treatment, but prior to the dissociation of GR from the chaperone complex (Czar et al., 1997; Galigniana et al., 1999; Galigniana et al., 1998). However, in cells in which the cytoskeleton had been disrupted by pretreatment with colcemid or cytochalasin D, geldanamycin no longer inhibited the transfer of GR to the nucleus (Galigniana et al., 1998). The implications of this work have been that GR may reach the nucleus primarily by tracking along the cytoskeleton to the nuclear pore in a manner that involves the continued contact of the receptor with hsp90, whereas in the absence of the cytoskeleton or hsp90 the GR is free to diffuse through the cytoplasm until it encounters the nuclear import machinery. Similarly, other studies have suggested that other components of the GR chaperone complex, such as hsp56/FKBP56 may also be required for the nuclear import of GR following ligand binding (Czar et al., 1995). However, while provocative, these studies are based mainly on using agents targeting the chaperone complex to block nuclear import of GR following steroid treatment. Therefore, to date, the alternative possibility that these agents extend the contact between GR and the chaperone complex or otherwise alter the complex in a manner that leads to the promotion or stabilization of an interaction between GR and the cytoskeleton that inhibits nuclear import, can not yet be completely excluded.

IV. Localization and trafficking of the unliganded receptor

GR exchanges rapidly between inactive, chaperone-associated forms and the freed, transcriptionally active state. Further, the liganded receptor shuttles rapidly between nucleus and cytoplasm despite the near complete visualization of liganded GR in the nucleus (Madan and DeFranco, 1993). These results are similar to those obtained with other steroid receptors (Dauvois et al., 1993; Guiochon-Mantel et al., 1991), and reflect the rapid trafficking of liganded GR between heterokaryon nuclei, which could only happen if the liganded receptor were rapidly shuttling between nucleus and cytoplasm. By contrast it has long been hypothesized that unliganded GR is localized to the cytoplasm because the association of GR into the chaperone complex masks the nuclear localization signals on GR in much the same way that IκB has been shown to retain NFκB in the cytoplasm by blocking NLS exposure (Beg et al., 1992; Hutchison et al., 1993; Pratt, 1993).

Recent studies have provided results that indicate that chaperone association is not equivalent to IκB association, and that the trafficking of unliganded, chaperone-associated GR may be constitutive. The first indication that the association of GR into the chaperone complex is not sufficient to trap GR in the cytoplasm was obtained in studies examining the movement of the receptor in the cell following the withdrawal of ligand treatment. For example, while steroid is rapidly lost from GR upon hormone withdrawal (Munck and Holbrook, 1984) and reassembly into hsp90-containing chaperone complexes occurs within minutes (Haché et al., 1999), the relocalization or redistribution of GR to the cytoplasm upon the withdrawal of steroid treatment occurs only over a period of many hours (Haché et al., 1999; Madan and DeFranco, 1993; Sackey et al., 1996). Moreover, we have demonstrated in cells withdrawn from the steroid antagonist RU486, that the GR

remains entirely localized to the nucleus for periods of up to 48 h following the withdrawal of the antagonist (Sackey et al., 1996). However, chaperone association, steroid binding capacity and transcriptional activation potential of these GRs was recovered within minutes of withdrawal of the antagonist (Haché et al., 1999).

But do the ligand-withdrawn, chaperone associated GRs continue to shuttle or traffic across the nuclear membrane? Apparently so, as both RU486 withdrawn GRs and the almost completely nuclear GRs withdrawn from cortisol treatment for only 1 h were found to continue to transfer efficiently between heterokaryon nuclei in cell fusion experiments (Haché et al., 1999). Thus, reassembly of GR into the chaperone complex following ligand withdrawal does not appear to be a barrier to the re-import of GR from the cytoplasm into the nucleus. Further, it has been shown that GRs can reassemble into the chaperone complex without first exiting the nucleus (Liu and DeFranco, 1999). Thus is appears likely that bi-directional transport of GR across the nuclear membrane is not markedly impeded by chaperone association.

Not unexpectedly, given the dependence of NL2 on steroidal ligand, maintenance of ligand withdrawn GRs in the nucleus appeared to be entirely dependent upon NL1 and correlated with the binding of the GR-chaperone complex with importin α (Savory et al., 1999). Molybdate is a divalent metal ion that binds to hsp90 and which is known to artificially stabilize the GR-chaperone complex (Leach et al., 1979). In vitro, addition of molybdate to chaperone associated GR prevented the NL1-dependent binding of GR to importin α (Savory et al., 1999), while micro-injection of molybdate in to tissue culture cells blocked the re-import of hormone-withdrawn GR into the nucleus (Yang and DeFranco, 1996). Unlike WT GR, GRs directed to the nucleus entirely under the control of NL2 redistributed rapidly to the cytoplasm following withdrawal of steroid while reassociating indistinguishably with the chaperone complex (Savory et al., 1999).

If the GR that reassociates into a chaperone complex continues to traffic extensively between nucleus and cytoplasm, is it tenable to continue to suggest that the naïve GR-chaperone complex is statically localized to the cytoplasm? It has been established for several years now that although PR and ERα are constitutively nuclear proteins, the naïve, chaperone associated forms of both receptors exchange continuously between nucleus and cytoplasm. A number of studies provide experimental support for the notion that it is also likely that naïve GR traffics continuously between nucleus and cytoplasm despite its primary localization to the cytoplasm.

Nonetheless, the evidence favoring trafficking of naïve GR remains indirect. For example, it has been noticed in several cell lines, that overexpression of GR leads to increased accumulation of the naïve, hsp-associated receptor to the nucleus (Martins et al., 1991; Sanchez et al., 1990). Indeed, the nuclear accumulation of such GRs appears to be NL1-dependent (Savory et al., 1999). Addition of leptomycin B to cells cultured in the absence of steroidal ligands, which is expected to decrease GR export from the nucleus promoted the nuclear accumulation of previously cytoplasmic naïve GRs (Savory et al., 1999). It has also been reported that addition of a nuclear retention signal to the N-terminus of GR, that is unable to promote the nuclear import of heterologous proteins, shifts otherwise cytoplasmic naïve receptor almost complete to the nucleus (Haché et al., 1999). While a direct demonstration of the entry of naïve WT GR to the nucleus under normal culture conditions remains to be accomplished, experiments designed to conclusively demonstrate trafficking of the naïve cytoplasmic GR now seem likely to yield a positive result.

V. An expanded model for the nucleo-cytoplasmic exchange of GR: A role for retention mechanisms and the control transfer rates

When brought together, recent developments in our understanding of the movement of GR in the cell suggest a more dynamic movement of GR about the cell than has been previously appreciated (detailed in **Figure 3**). In the first instance, it now appears that chaperone associated GRs are not statically localized in the cells, but are exchanged continuously between nucleus and the cytoplasm. At physiological levels of expression in most cells, naïve GR is seen as being predominantly cytoplasmic. However, this localization is likely overlying the continuous exchange of the receptor between nucleus and cytoplasm. That increasing the level of expression of GR is sufficient to promote the accumulation of the naïve receptor in the nucleus suggests that nuclear accumulation may occur through a saturation of retention mechanisms that otherwise promote the maintenance of GR in the cytoplasm. What these retention mechanism might be is not clear, but they may possibly include interactions between the GR-chaperone complex and the cytoskeleton.

At the far end the steroid response, the interaction of chaperone-associated, steroid-withdrawn GRs with the nuclear matrix, that correlates directly with the slow redistribution of these GRs to the cytoplasm in the face of continuous nucleocytoplasmic exchange supports active nuclear retention as being important for slowing or preventing the redistribution of shuttling receptors to the cytoplasm. However, it remains possible that direct regulation of the rates of nuclear import and export may contribute in important ways to the localization of naïve and ligand withdrawn GR in the cell.

Upon ligand binding, GR dramatically changes its interactions with the chaperone complex through a process

that results in a receptor that may only remain loosely associated with hsps including hsp90 and hsp56. This liganded GR dimerizes and accumulates rapidly on DNA in the nucleus. DNA binding appears to be a strong determinant for nuclear localization of GR, as mutations in the receptor that impair DNA binding lead to a form of liganded GR that becomes only modestly more nuclear than cytoplasmic (Sackey et al., 1996). In this context, that the rapid reassociation of GRs with hsps upon loss of ligand does not result in immediate effects on the nuclear localization of GR, reinforces the apparent importance of the transfer of the GR from DNA to the nuclear matrix upon release of ligand from the receptor. With time however, the interactions that maintain the nuclear occupancy of the steroid-withdrawn GRs appear to be slowly reversed, with a concomitant slow redistribution of the receptor to the cytoplasm.

One intriguing open question is why GRs withdrawn from RU486 are apparently unable to relocalize to the cytoplasm for extended periods or time (Sackey et al., 1996). Thus it would seem that RU486-withdrawn GRs may associate indefinitely with the nuclear matrix or some other subnuclear compartment, while the association of agonist-withdrawn GRs with the same or alternative compartments becomes slowly reversed over a period of several hours. One possibility is that differences in phosphorylation patterns within the N-terminus of GR may promote the long term interaction of the GR-chaperone complexes with the nuclear matrix. Certainly it has been reported that GR becomes differentially phosphorylated in response to steroid agonists and RU486 (Hsu et al., 1992). Further, both the differential phosphorylation and long term nuclear retention of RU486-withdrawn GRs may have something to do with differences that have been observed in the subnuclear targeting of the liganded receptors (Htun et al., 1996). However, as the GRs in cells withdrawn from the cell cycle and receptors in actively growing cells return to the cytoplasm with similar kinetics following the withdrawal of agonists, it would seem unlikely that the redistribution of the shuttling, unliganded hsp-reassociated GRs is dependent of specific effects of the cell cycle on the receptor (Hsu et al., 1992).

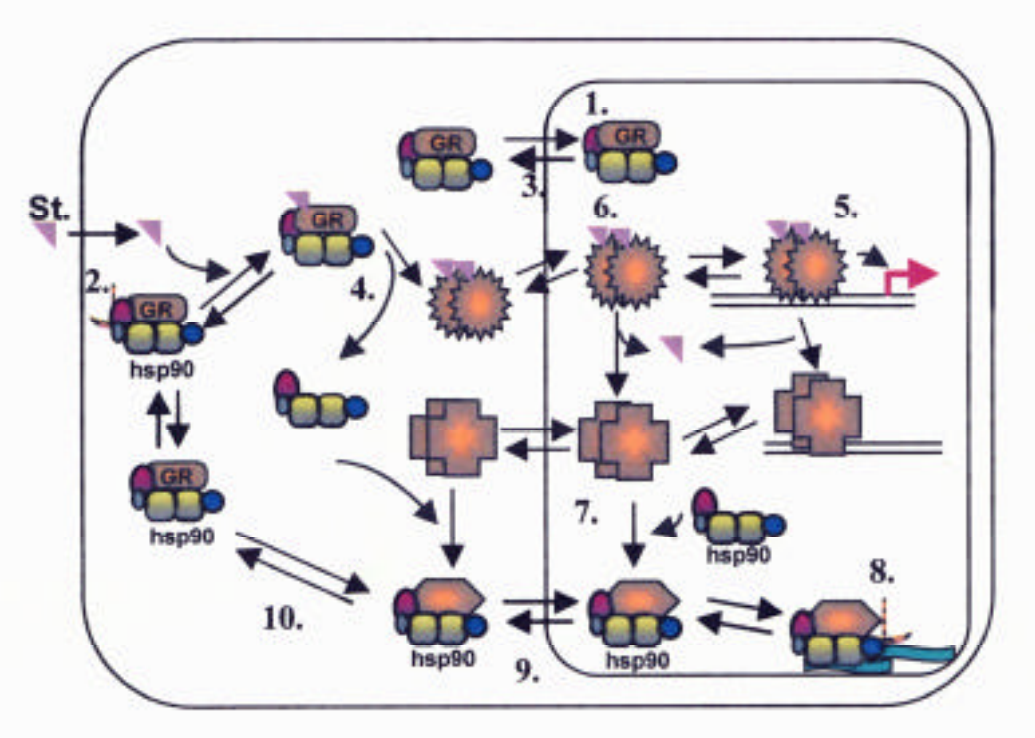

Figure 3. Expanded model for the subcellular distribution and nucleocytoplasmic trafficking of GR. Schematic presentation of the events identified in the localization and trafficking of GR prior to, during and following steroid treatment. Naïve, hsp associated receptor is hypothesized to traffic continuously between nucleus and cytoplasm (1.), but may be preferentially retained in the cytoplasm through active retention (2.) or an imbalance in nuclear import and export rates (3.). Upon steroid treatment, liganded GRs dissociate from the chaperone complex (4.), dimerize and accumulate on DNA in the cell nucleus to regulate transcription (5.), all the while continuing to traffic rapidly between nucleus and cytoplasm (6.) Following loss of ligand, the shuttling free GRs reassociate with the chaperone complex (7.) and localize to the nuclear matrix (8.) while continuing to shuttle between nucleus and cytoplasm (9.). Over an extended period of time, the receptor-chaperone complex reorients in some way and relocalizes to the cytoplasm (8.).

In addition to changes in the subcytoplasmic and subnuclear retention, changes in the actual rates of nuclear import and export of liganded and unliganded GRs could also influence receptor localization in response to the binding and release of ligand. Thus while association of GR with molecular chaperones does not appear to block binding to importin α, it remains possible that access to the NLS and binding to importin α may be reduced relative to free GR. Decreased affinity for importin α upon chaperone association would be expected to result in a slower rate of nuclear import. If the rate of nuclear export were maintained, or even increased at the same time, the long-term result would be localization or redistribution of the shuttling receptor to the cytoplasm prior to hormone treatment and following hormone withdrawal.

There is also evidence that is beginning to emerge that localization of GR may be regulated through the control of export rates. For example, using digitonin permeabilized cells it has been observed that the nuclear export of GR, hsp90, hsp70, but not other proteins is specifically inhibited by incubation with tyrosine kinase inhibitors (DeFranco et al., 1991; Yang et al., 1997). These results suggest that some component of the GR export pathway may be dependent upon tyrosine phosphorylation. To date however, there is little evidence that GR itself may be phosphorylated on tyrosine. Conversely the treatment of cells with the serine-threonine phosphatase inhibitor okadaic acid or appears to prevent the re-transfer of steroid-withdrawn GRs to the nucleus (Galigniana et al., 1999). This inhibition is lifted upon co-treatment with agents that depolarize the cytoskeleton. A similar inhibition of the maintenance of GR in the nucleus was observed earlier upon over expression of the serine-threonine kinase v-*mos* (Qi et al., 1989). These data suggest that nuclear import or the cytoplasmic retention of GR may be directly regulated by serine-threonine kinases and phosphatases, an interesting parallel to the potential dependence of GR export and/ or nuclear retention by tyrosine kinases.

VII. Concluding remarks

The trafficking and localization of GR as it has been described in this review reflects the situation observed in asynchronously growing cells and cells that have be withdrawn from the cell cycle by serum starvation. Further progress in understanding how localization and trafficking of GR in the cell affects the responses of the receptor to steroid will require more precise delimitation of the molecular events that control subcellular movements of the receptor including a description of the mechanisms mediating the nuclear export of GR, factors influencing movement of GR across the nuclear membrane, and the continued identification of factors that determine the specific retention of GR in subcellular compartments.

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