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Analysis of the Subcellular Trafficking of the Glucocorticoid Receptor and Properties of the Ligand Binding Domain

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Analysis of the Subcellular Trafficking of the Glucocorticoid Receptor and Properties of the Ligand Binding Domain

By

Robyn Ewing

A thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE

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ABSTRACT

The glucocorticoid receptor (GR) is a ligand dependent transcription factor and member of the nuclear receptor superfamily. Nuclear import and export of transcription factors is accomplished through nuclear localization signals (NLS) and nuclear export signals (NES), respectively. We have determined that L687 and L690 of rat GR are necessary for the characteristically slow nuclear export of GR and may be included in the signal sequence responsible for directing post-agonist withdrawn GR from the nucleus to the cytoplasm. We also suggest that L687 and L689 of rat GR are required for efficient NL2-mediated nuclear translocation. Substitutions L687A and L689A mildly affect steroid binding and steroid off-rate, yet significantly increase the concentration of steroid required for inducing nuclear import of naïve GR. When introduced into GR_{NL1-}, these substitutions compromise the receptor's ability to transfer to the nucleus, suggesting they partially abrogate NL2 activity. We have also observed that NL1-dependent transfer does not begin until 10^{-7}M steroid, demonstrating that NL2 is the primary physiological mediator of GR nuclear import.

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Lastly, I would like to thank my friends and family for their unwavering encouragement and support. Special thanks go out to Greg, Newton, Erin and Maw for always believing in me. I dedicate this thesis to them.

ABBREVIATIONS

AF-1	transcriptional activation function 1
AF-2	transcriptional activation function 2
AR	androgen receptor
BRG-1	Brahma related gene 1
BSA	bovine serum albumin
CBP	cAMP response element binding protein-binding protein
cDNA	complementary deoxyribonucleic acid
Cort	cortisol
CRM1	chromosomal region maintenance protein 1
Cyp40	cyclophilin 40
DAPI	4'-6-Diaminidino-2-phenylindole
DBD	DNA-binding domain
Dex	dexamethasone
DMEM	Dulbeco's modified Eagle medium
DNA	deoxyribonucleic acid
DPM	dissociations per minute
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
eIF1A	translation initiation factor 1A
ER	estrogen receptor
ERR	estrogen-related receptor
FBS	fetal bovine serum

FKBP	FK-506 binding protein
FRAP	fluorescence recovery after photobleaching
GDP	guanosine diphosphate
GFP	green fluorescent protein
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRIP-1	GR interacting protein -1
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
HDAC	histone deacetylase
HIV Rev	human immunodeficiency virus regulator of virion expression protein
hnRNP A1	heterogeneous nuclear ribonucleoprotein A1
Hop	hsp70/hsp90-organizing protein
HPA	hypothalamic-pituitary-adrenal axis
HRE	hormone response element
Hsp	heat shock protein
hUBC9	human ubiquitin-conjugating enzyme 9
IBB	importin β binding domain
JNK	Jun N-terminal kinase
LB	Luria-Bertani
LBD	ligand binding domain
LMB	leptomycin B
MDM2	murine double minute 2

MMTV	mouse mammary tumour virus
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MTA1	metastatic tumour antigen 1
NE	nuclear envelope
NES	nuclear export sequence
NHR	nuclear hormone receptor
NL1	nuclear localization factor 1 of the glucocorticoid receptor
NL2	nuclear localization factor 2 of the glucocorticoid receptor
NLS	nuclear localization sequence
NP-40	nonidet-P40
NPC	nuclear pore complex
Nrf2	Nf-E2-related factor-2
NRS	nuclear retention sequence
NTF2	nuclear transport factor 2
Nups	Nucleoporins
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PKI	protein kinase A inhibitor
PR	progesterone receptor
PVDF	polyvinylidene difluoride
RanBP1	Ran binding protein 1
RanGAP	Ran guanine activating protein

RanGDP	Ran guanosine diphosphate
RanGEF	Ran guanine exchange factor
RanGTP	Ran guanosine-5'-triphosphate
RIP140	receptor interacting protein 140
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SFBS	stripped fetal bovine serum
SHR	steroid hormone receptor
siRNA	small interfering ribonucleic acid
SRC-1	steroid receptor coactivator-1
STAT	signal transducer and activator of transcription
SV40	simian virus 40
TAPS	N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TIF	transcriptional intermediary factor
TR	thyroid hormone receptor
tRNA	transfer ribonucleic acid
VDR	vitamin D receptor
WCE	whole cell extract

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INTRODUCTION

Overview of Glucocorticoid Signalling

Steroid hormones are essential for the maintenance of homeostasis in higher organisms. Glucocorticoids, a subclass of steroid hormones, play a central role in modulating physiology through numerous control mechanisms. Under control of the hypothalamic-pituitary-adrenal (HPA) axis, glucocorticoids are synthesized in the adrenal cortex and circulated systemically, enabling them to regulate a variety of cell-, tissue- and organ-specific biological functions. Well established as the primary physiological mediators of stress response, glucocorticoids have been the subject of research for more than four decades (Norgaard and Poulsen, 1991). In humans, glucocorticoid signalling is mediated primarily by cortisol. Regulated by signalling in response to physiological stressors and diurnal rhythms, cortisol exerts a wide range of physiological effects. Such effects include but are not limited to, mobilization of metabolic resources (Goodridge, 1987), increased catecholamine synthesis (Wurtman, 2002), adipogenesis (Rosen and Spiegelman, 2000), and alteration of memory consolidation (deKloet et al., 1999; Roozendaal, 2002). Clinically, synthetic glucocorticoids have proven to be effective in controlling the symptoms of a variety of chronic and acute inflammatory diseases, as well as undesirable immune responses (Buckbinder and Robinson, 2002). Prolonged stress and prolonged treatment with glucocorticoids can lead to chronic increases in cortisol levels, as seen in patients with Cushing's syndrome. Increased levels of circulating cortisol leads to immunosupression, hypertension, depression and muscle catabolism (Buckbinder and Robinson, 2002).

At the cellular level, the physiological effects of glucocorticoids are mediated primarily by a 97-kDa intracellular protein, the glucocorticoid receptor (GR). GR belongs to

the phylogenetically conserved superfamily of nuclear hormone receptors (NHRs) (Evans, 2005). NHRs are ligand-inducible transcription factors that regulate transcriptional activity through direct binding to hormone response elements (HREs) within the transcriptional regulatory regions of target genes (Beato et al., 1995; McKenna and O'Malley, 2002). All members in the NHR superfamily share a characteristic three-domain structure, which was first predicted for GR (Giguere et al., 1986). The three-domain structure consists of an N-terminal domain, which contains sequences responsible for regulation of target genes, a central DNA-binding domain (DBD) and a C-terminal or ligand binding domain (LBD). Comparison of NHR cDNA sequences reveals that members of this superfamily share a high degree of homology within their DNA binding domains (DBDs), and to a lesser extent within their ligand binding domains (LBDs). It has also been revealed that members of this superfamily have highly divergent N-terminal sequences. The NHR superfamily contains receptors for a wide array of ligands, including mineralocorticoids, androgens, progestins, estrogens, vitamin D, thyroid hormone, and retinoic acid (Bamberger et al., 1996). There are also a growing number of so called orphan-receptors, for which no specific ligand has yet been identified (McKenna and O'Malley, 2002).

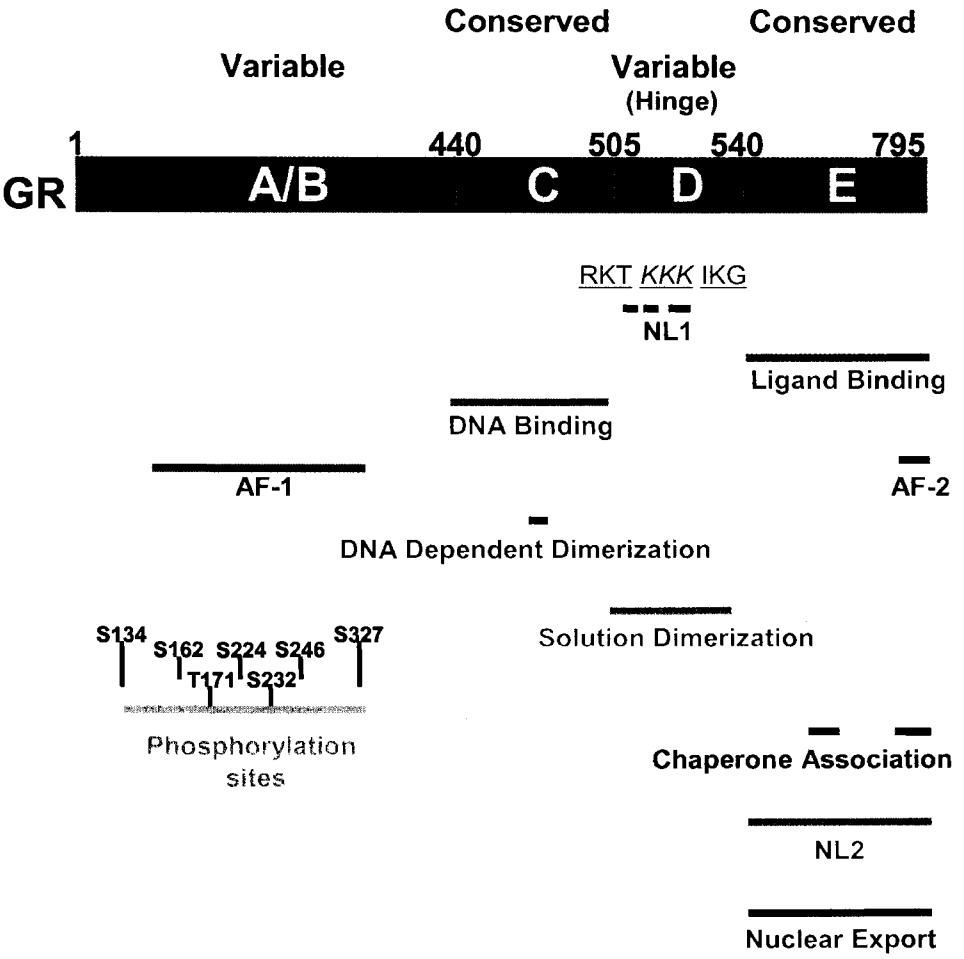
GR belongs to the steroid hormone receptor (SHR) family of the NHRs. The SHR family includes the mineralocorticoid receptor (MR), the progesterone receptor (PR), the androgen receptor (AR), the more distantly related estrogen receptor (ER) and the estrogen related receptors (ERRs). SHRs are distinguished from other NHR by the fact that, prior to ligand binding, they are associated with a molecular chaperone complex which includes hsps and immunophilins. This complex serves to maintain the receptor in a conformation that facilitates high-affinity hormone binding (Meijsing et al., 2007). The subcellular localization

of SHRs prior to ligand binding varies. Localized predominantly to the nucleus prior to ligand binding are ER (King and Greene, 1984; Welshons et al., 1984; Ylikomi et al., 1992; Htun et al., 1999) and PR (Ylikomi et al., 1992, Chandran and DeFranco, 1992). By contrast, GR is localized predominantly in the cytoplasm prior to ligand binding (Picard and Yamamoto, 1987; Wikstrom et al., 1987; Qi et al., 1989; Ogawa et al., 1995; Htun et al., 1996; Sackey et al., 1996). The localization of unliganded MR and AR remains unresolved. Naïve MR has been localized predominantly to the cytoplasm and equally distributed between the nucleus and the cytoplasm (Binart et al., 1991; Alnemri et al., 1991; Robertson et al., 1993; Lombes et al., 1994). Naïve AR is reported to be localized predominantly in the nucleus (Jenster et al., 1991; Jenster et al., 1993), cytoplasm (Simental et al., 1991; Zhou et al., 1994; Georget et al., 1997; Tyagi et al., 2000; Ozanne et al., 2000; Tomura et al., 2001), or equally distributed between both subcellular compartments (Krozowski et al., 1989; Lombes et al., 1990; Farman et al., 1991; Farman et al., 1991; Sasano et al., 1992; Fejes-Toth et al., 1998). Presently, the localization of the ERRs prior to ligand binding remains unknown.

The GR cDNA was first cloned from rat liver in 1984 (Miesfeld et al., 1984). Similar to other members of the NHR superfamily, GR is a modular protein made of domains that carry specific functions that can be confirmed outside the context of the full-length protein (Zhou and Cidlowski, 2005; Rusconi and Yamamoto, 1987; Gustafsson et al., 1986; Giguere et al., 1986; Danielsen et al., 1986; Hollenberg et al., 1987; Kumar et al., 1987). The modular structure of GR and the functional activities localized within the receptor are summarized in Figure 1.

Figure 1: Schematic outline of the modular structure of the glucocorticoid receptor

The degree of conservation of domains across the nuclear receptor superfamily is summarized above and the location of individual functional motifs is summarized below. The variable N-terminal domain contains the AF-1 activation domain and sites for receptor phosphorylation. The conserved C region contains the DNA binding region and a DNA-dependent dimerisation domain. The D region, or hinge, is variable and contains regions that mediate dimerisation of GR while in solution and NL1, a basic nuclear localization signal sequence. The E region is conserved and contains the ligand binding domain. This region also contains the AF-2 transcriptional activation function and chaperone association domains. The E region also contains sites that harbour nuclear export activity and NL2, a second nuclear localisation signal sequence.


Conserved
Conserved
Variable
Variable
(Hinge)
1
440
505
540
795
GR
A/B
C
D
E
RKT KKK IKG
NL1
Ligand Binding
DNA Binding
AF-1
AF-2
DNA Dependent Dimerization
Solution Dimerization
S134
S162
S224
S246
S327
T171
S232
Phosphorylation sites
Chaperone Association
NL2
Nuclear Export

The N-terminal A/B domain of GR is highly variable amongst receptors and contains the transcriptional activation function 1 (AF-1) (Hollenberg and Evans, 1988). AF-1 is a constitutive transcriptional activation function that can activate transcription in a ligand-independent manner. The N-terminal domain of GR also contains several serine/threonine phosphorylation sites whose phosphorylation is modulated through the cell cycle. Phosphorylation of these residues may influence receptor stability, subcellular trafficking and transcriptional regulatory potential (Hollenberg and Evans, 1988).

The central C domain is highly conserved and contains the receptor DBD. The DBD consists of two conserved Cys4 zinc fingers (Wang et al., 1999). Upon ligand binding, the DBD is exposed enabling it to recognize glucocorticoid response elements (GREs) within promoter regions of glucocorticoid-responsive genes. GREs are short palindromically arranged DNA sequences separated by three base pairs. The GRE consensus sequence, GGTACAnnnTGTTCT, acts as a DNA recognition site for GR (Scheidereit et al., 1986; Jantzen et al., 1987). Specificity of binding to this sequence is mediated through a cluster of amino acids called the P-box, found in the N-terminal zinc finger of the DBD (Umesono and Evans, 1989). The C-terminal zinc finger of the DBD contains a region called the D-box, a DNA-dependent dimerisation domain through which two GR DBDs form a homodimer when bound to DNA (Umesono and Evans, 1989).

The variable D, or hinge, region is involved in conformational changes during receptor-ligand binding (Stolte et al., 2006). Within this domain lie sites that mediate the formation of GR dimmers in solution (Savory et al., 1999) as well as the nuclear localization sequence 1 (NL1), commonly perceived as the primary NLS in most nuclear receptors. NL1 is a strong, well-characterized NLS located in the receptor hinge region (Brzozowski et al.,

1997; Xu et al., 1999; Onate et al., 1995; Spencer et al., 1997) that is comprised of three components. One component, a core basic sequence adjacent to the DBD, is required for NLS function while two smaller sequences, at the C terminus of the DBD, appear to contribute to the efficiency of the NLS (Tang et al., 1997). NL1 has been shown to bind to importin α and import through NL1 has been proposed to occur through the classical nuclear import pathway (Carey et al., 1996; Savory et al., 1999). Previous studies have revealed that a K513-515N substitution in the NL1 of GR decreases the receptor's ability to import into the nucleus and increases its rate of nuclear export both in the presence and absence of ligand (Savory et al., 1999). This suggests that GR is actively retained within the nucleus and that a nuclear retention signal (NRS) overlaps the NL1 motif (Carrigan et al., 2007).

The E domain is conserved and contains the GR LBD, which is highly homologous to other SHR LBDs. Both ligand-dependent activation and repression by GR require the intact function of the LBD (Bledsoe et al., 2002). Crystallography has revealed that the GR LBD is composed of eleven α helices which, when bound to ligand, form a binding pocket specific for glucocorticoid ligands.

Also in the E domain are sites that mediate chaperone association (Katzenellenbogen and Katzenellenbogen, 1996). Prior to ligand binding, GR is held in an inactive state as part of a heat shock protein (hsp) and immunophilins containing complex. This complex contains a dimer of hsp90, as well as an immunophilin molecule such as the FK506 binding protein (FKBP) 52 and FKBP51, Cyp40 or PP5 (DeFranco, 2000). The complex also contains chaperones including hsp70, hsp40, HOP and p23 which are required for maturation of the naïve complex (Pratt and Toft, 1997). Association of GR into these inactive complexes masks the DBD, therefore preventing GR from binding to DNA. Association between GR

and hsps also facilitates ligand binding by altering the LBD conformation in a manner that makes it more accessible to ligands. In fact, hsp association appears to be a prerequisite for GR ligand binding (Wang et al., 1999).

The E domain also contains a second activation function called AF-2. AF-2 is a ligand-dependent activation function that has been mapped to helix 12 of the GR LBD. Ligand binding induces a conformational change in the LBD of GR and as a results, AF-2 becomes exposed, allowing it to participate in interactions with various coactivator proteins. These coactivators, including steroid receptor coactivator-1 (SRC-1) and transcriptional intermediary factor (TIF), associate with the GR LBD through their LXXLL motifs (Yoshikawa et al., 2005). Recruitment of coactivator proteins leads to the formation of a large regulatory complex, eventually leading to ligand-dependent activation of target genes.

Also localised within the E domain is a second NLS called NL2. NL2 is a steroid-dependent NLS which complements NL1-mediated GR nuclear uptake (Picard and Yamamoto, 1987). Unlike NL1, the details surrounding NL2-mediated nuclear localization remain unclear. Both the sequence that comprises NL2 and identity of the karyopherins mediating NL2 nuclear import have not been elucidated. Also, while little is known about the sequence responsible for directing the nuclear export of GR, studies suggest that GR nuclear export activity is localized to the ligand binding domain of the receptor (Picard and Yamamoto, 1987).

Structure and Properties of the GR Ligand Binding Domain

The GR LBD contains many functions including ligand binding, chaperone association, nuclear localization and transcriptional activation. The ability of GR to function

efficiently requires proper folding of the receptor protein and an intact LBD. For rat GR, the LBD is comprised of the last 246 amino acids (amino acids 550-795) at the receptor C-terminus.

Recent crystallographic analysis has revealed that the GR LBD folds into a conical three-layer helical sandwich that embeds a hydrophobic pocket for binding ligand (Bledsoe et al., 2002). Similar to other NHR LBDs, the GR LBD is composed of 11 α helices, nine turns and four β sheets (Fig. 2a). The ligand binding pocket is composed of residues from helices 3, 4, 5, 6, 7, 10, the AF-2 helix (helix 12) as well as residues from β-strands 1 and 2 (Bledsoe et al., 2002). Helices 1 and 3 form one side of the helical sandwich while helices 7 and 10 form the other. Helices 4, 5, 8, and 9 are located in the top half of the protein, creating a cavity in the bottom half of the GR LBD. This bottom cavity is the ligand binding pocket. Agonist binding to the LBD induces the reorientation of the AF-2 helix which packs against helices 3, 4, and 10, adopting the so-called "agonist-bound" conformation. This conformation facilitates transactivation by exposing coactivator interaction sites (Yoshikawa et al., 2005).

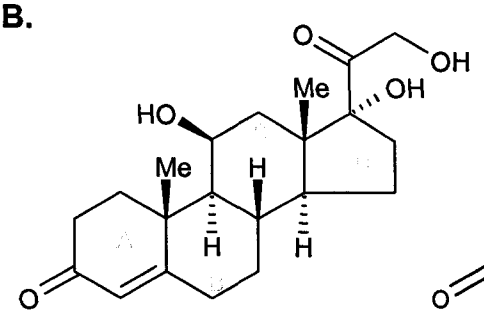
Interestingly, unlike the steroid binding pockets of PR, AR and ER, the GR pocket has an additional strand extending from helix 12. This strand forms a conserved β-sheet with a β-strand between helices 8 and 9 and is formed by structural rearrangement of helices 6 and 7. It has been suggested that this C-terminal β-strand is involved in stabilizing the AF-2 helix in an active conformation and therefore plays an important role in receptor activation (Bledsoe et al., 2002). Through crystallography, a model for the binding mode of the synthetic agonist dexamethasone (Dex) has been deduced (Bledsoe et al., 2002). GR binds to Dex via an extensive network of hydrophobic and hydrophilic interactions. In fact, it has

Figure 2: Structure of the hGR LBD complexed to RU486 and structures of steroids used in this study

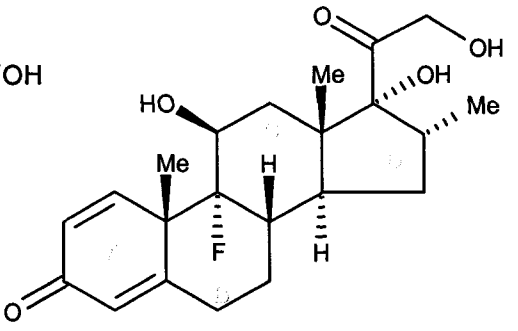
(A) Overall architecture of the hGR LBD when complexed to RU486. This image illustrates the arrangement of helices (H) in the hGR LBD. Figure made using PyMOL Molecular Graphics System (DeLano Scientific LLC). (B) Structures of steroids used in this study. All structures drawn using ISIS Draw (Elsevier MDL).

A.

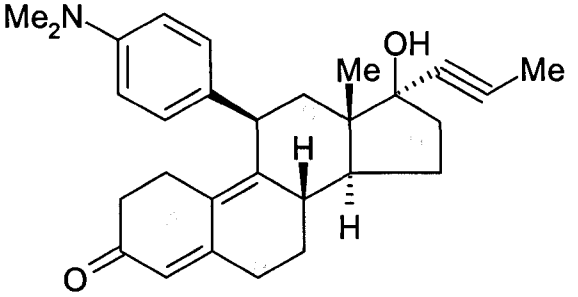
B.



Cortisol



Dexamethasone



RU486

been reported that during ligand binding almost every atom of the Dex core is associated with one or more hydrophobic residues within GR (Bledsoe et al., 2002). In addition to these hydrophobic associations, it has also been reported that all of the hydrophilic groups of Dex form hydrogen bonds with GR. Also interesting is the proposal that Dex makes direct contact with the AF-2 helix (Bledsoe et al., 2002). This could, in-turn stabilize the AF-2 helix during ligand-dependent activation of GR.

GR is capable of binding to a wide array of glucocorticoids, including the agonists cortisol and dexamethasone and the antagonist RU486. All steroids, including glucocorticoids, have a four-ring molecular structure in which the rings are designated by the letters A, B, C and D (Fig. 2b). In the LBD pocket, steroids are orientated with their A-rings toward β strands 1 and 2 and their D-rings towards the AF-2 helix. The A-ring, which has a similar structure in all classes of steroids, is anchored in the LBD via hydrogen bonding with R611 and Q570 (Bledsoe et al., 2002). The structure of the D-ring is variable amongst steroids. The defining feature of this ring in glucocorticoids is the presence of a 17β side chain containing a 20-carbonyl and a 21-OH group (Lind et al., 2000). Through site-directed mutagenesis it has been determined that interactions between GR and the D-ring of glucocorticoids are most likely mediated through residues M560, M639, Gln642 and Thr739 (Lind et al., 2000).

Functionality of the LBD relies, in-part, on the association between GR and chaperone proteins. In order for ligand to access the hydrophobic cleft in the LBD, GR must adopt a specific conformation (Pratt and Toft, 2003). Hsp90 and hsp70 are both required as chaperones for the opening of this cleft. In an ATP-dependent process, hsp70 first binds directly to the LBD, inducing a receptor conformational change resulting in partial opening

of the steroid-binding cleft. Subsequent binding of hsp90 to the GR-hsp70 complex results in the complete opening of the cleft. Once GR has adopted this labile, open conformation, hormone can enter and bind within the ligand-binding pocket (Pratt and Toft, 2003). Following ligand binding, it is commonly believed that chaperones dissociate from the GR heterocomplex, enabling GR to adopt a compact stable DNA-binding conformation (Pratt and Toft, 2003). It should be noted, however, that a variation of this theory exists where hsp90 does not dissociate from GR upon hormone binding (Kang et al., 1995; DeFranco, 2000). Presently, no common sequence required for hsp90 binding has been identified among SHRs. While several regions in the GR LBD have been implicated in the binding of hsp90 (Howard et al., 1990; Cadepond et al., 1992; Dalmon et al., 1991), the exact sequence responsible for mediating hsp90 association remains unidentified.

Nucleocytoplasmic Trafficking of Proteins

A distinguishing feature of eukaryotic cells is their separation into compartments. As most proteins are synthesized in the cytoplasm, transport systems have evolved to facilitate the trafficking of proteins between cellular compartments. In eukaryotes, the nucleus and the cytoplasm are separated by a lipid bilayer called the nuclear envelope (NE) and all macromolecular exchange between cytoplasm and nucleus takes place through elaborate multi-protein structures called nuclear pore complexes (NPCs) (Ohno et al., 1998; Rout and Aitchison, 2001).

The NPC regulates bidirectional active and passive transport between the nucleus and the cytoplasm. Active nucleocytoplasmic transport allows for the transport of large macromolecules containing a NLS through the NPC. Such molecules include proteins

involved in transcription, DNA replication, and chromatin remodelling as well as molecules synthesized in the nucleus that function in the cytoplasm like mRNAs, tRNAs and rRNAs (Komeili and O'Shea, 2001). Ions and other small molecules ($\leq$ 40 kDa) lacking NLSs can diffuse passively through the NPC channel (Kastrup et al., 2006).

In recent years, significant progress has been made on the identification of transport receptors and the specific targeting sequences they recognize on cargo molecules. Nuclear import is a process that relies on the presence of a NLS on the surface of the cargo molecule. The best characterized NLSs are composed of clusters of basic amino acid residues. NLSs can be composed of a single cluster of basic amino acids, like the simian virus 40 (SV40) large T antigen, or they can be bipartite, consisting of two independent clusters of basic amino acids separated by 10-12 residues (Taniguchi et al., 2002). An example of the later would be the NLS of the nuclear protein nucleoplasmin (Robbins et al., 1991).

NLS-mediated nucleocytoplasmic transport is accomplished by proteins belonging to the karyopherin transporter family. In general, karyopherins contain HEAT or ARM repeats that extend the entire length of the curved, elongated proteins. HEAT repeats have a hairpin-like structure comprised of two alpha helices joined by a turn, while ARM repeats are comprised of three alpha helices arranged in a more complex fashion (for review see Conti et al., 1998). Karyopherins of the importin α subfamily function exclusively in nuclear import, acting as adapter proteins that bind directly to the NLS of import substrates. In vertebrates, seven importin α homologues have been identified (Pemberton et al., 1998; Nakielny and Dreyfuss, 1999; Weis, 2002) each displaying unique affinities for various import substrates (Miyamoto et al., 1997; Kohler et al., 1999; Nadler et al., 1997; Sekimoto et al., 1997; Welch et al., 1999). In yeast, only a single protein species, SRP1, exists as an importin α

homologue (Moroianu et al., 1995; Gorlich et al., 1994; Yano et al., 1992; Walter et al., 1980). Once bound to the substrate, importin α forms trimeric complexes with another member of the karyopherins family of proteins, importin β. Therefore, importin α can be described as an adaptor protein that mediates the binding of cargo to importin β. There are 14 putative members of the importin β family in yeast, nine of which have been shown to function as importins and four as exportins (Gorlich and Kutay, 1999). Higher eukaryotes contain an even greater number of importin β family members. It has been proposed that there are more than 22 members of the importin β family in the mammalian system (Gorlich and Kutay, 1999; Kutay et al., 2000; Plafker and Macara, 2000).

Importin β adopts a superhelical structure with a large, flexible, acidic loop joining the N and C-terminal regions of the protein (Cingolani et al., 1999). Importin β binds to the IBB domain of importin α through C-terminal HEAT repeats (Cingolani et al., 1999). Docking to the nuclear pore is mediated by the N-terminal region of importin β (Bayliss et al., 2000). Dissociation of the complex occurs in the nucleus following translocation through the NPC. In the nucleus, the importin α/β-cargo complex is dissociated by the GTP-bound form of the small guanine binding protein Ran (Gorlich et al., 1996; Rexach and Blobel, 1995; Izaurralde et al., 1997; Gorlich et al., 1996). When bound to GTP, Ran has a high affinity for the N-terminal region of importin β (Vetter et al., 1999). RanGTP makes contact with the acid loop joining the N and C-terminal regions of the importin β. This destabilizes the interaction between importin β and importin α and results in the dissociation of the complex and subsequent release of the cargo into the nucleoplasm (Cingolani et al., 1999). Other adaptors that bind NLSs in cargo include snuportin, XRIPα, importin7, and RanBP8 (Holaska et al., 2001).

Mammalian NPCs consist of several copies of approximately thirty proteins called nucleoporins (Nups). Most are synthesized as soluble proteins in the cytoplasm, as only two Nups, Pom121 and gp210 are integral membrane proteins believed to be anchoring the NPC in the NE (Rabut et al., 2004). Genetic and biochemical studies with isolated Nups have demonstrated that import and export receptors interact directly with Nups. Most Nups contain phenylalanine/glycine (FG) repeats. These dipeptide repeats are believed to aid in nucleocytoplasmic transport of cargo though low-affinity association with transport receptors (Rabut et al., 2004). It is a common belief that subcomplexes and subdomains of Nups create microenvironments within the NPC that are specific for various transporters (Komeilie and O'Shea, 2001). For instance, it has been shown that in humans Nup153 contains a domain that is required for importin β nuclear import (Komeilie and O'Shea, 2001).

Nuclear transport of macromolecules is an energy-dependent process. Early studies *in vivo* demonstrated that GTP hydrolysis by Ran is necessary for the nuclear import and export of proteins displaying signal sequences (Weiss et al., 1996). Indeed, the GTPase Ran appears to be crucial for establishment of transport directionality *in vivo*. Ran's GTPase activity is stimulated by the Ran GTPase activating protein (RanGAP) which is localised in the cytoplasm. This results in the conversion of RanGTP to RanGDP. The chromatin associated Ran guanine nucleotide exchange factor (RanGEF) stimulates the release of the bound nucleotide on Ran. In the cell a RanGTP gradient exists such that levels of RanGTP in the nucleus are high and levels in the cytoplasm are comparatively low. This is a reflection of the fact that there exists an asymmetric distribution of RanGEF and RanGAP in the cell. The presence of the RanGTP gradient is thought to be essential in establishing directionality of transport. Export receptors of the importin β family require RanGTP to bind

their cargo whereas import receptors dissociate from their cargos in the presence of RanGTP. Two other components of the RanGTP cycle that participate in nuclear transport are the Ran binding protein RanBP1 and NTF2. RanBP1 aids in the dissociation of export complexes, while the small protein NTF2 is involved in the active import of RanGDP into the nucleus where it is converted to RanGTP (Komeili and O'Shea, 2001).

The study of transport machinery and the energetics behind the transport process has lead to the development of models for the nuclear import and export of protein cargo. Import receptors bind to their cargo in the cytoplasm and traverse the NPC. Once in the nucleus, the import receptor is released from the cargo molecule through binding between the import receptor and RanGTP. The nuclear export process begins in the nucleus with the formation of a trimeric complex consisting of the receptor, cargo, and RanGTP. Once formed, the complex translocates through the NPC into the cytoplasm. Subsequently, GTP hydrolysis by Ran is accomplished by the combined actions of RanBP1 and RanGAP which, in turn, leads to the dissociation of the complex.

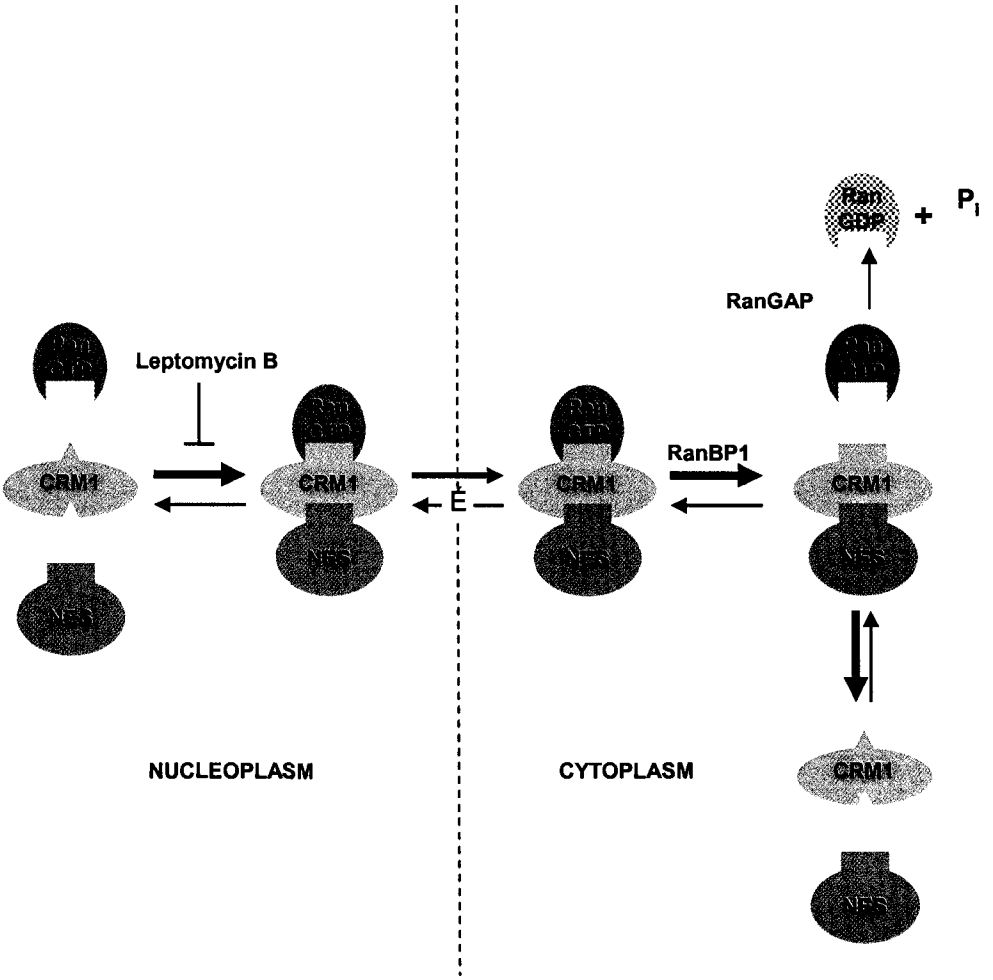
Nuclear export is accomplished through the interaction between nuclear export signals (NESs) and karyopherins of the β subclass called exportins. While no consensus sequence exist for NESs, most are comprised of hydrophobic amino acids with a repeat of leucines or isoleucines that results in an antipathic α helix that interacts with the CRM1 exportin (Fornerod et al., 1997; Askjaer et al., 1999; Ossareh-Nazari et al., 1997; Turpin et al., 1999). The leucine-rich NES was first characterized in the HIV Rev protein and the protein kinase A inhibitor (PKI) (Fisher et al., 1995; Wen et al., 1995; Fornerod et al., 1997; Stade et al., 1997).

First identified as a protein essential for maintaining chromosome structure in *Schizosaccharomyces pombe*, CRM1 (chromosomal regional maintenance protein 1) has attracted considerable attention as a nuclear export receptor as it mediates nuclear export of proteins containing leucine-rich NESs. CRM1 is the most extensively characterised nuclear export receptor and CRM1-mediated nuclear export can be distinguished from other nuclear export pathways by its selective inhibition by the potent antifungal antibiotic leptomycin B (LMB). LMB abolishes association of CRM1 with the NES by covalently and selectively binding to Cys-529 in CRM1, thereby inhibiting nuclear export of proteins (Kudo et al., 1999). CRM1 functions as an export receptor for many proteins including several STATs (McBride et al., 2000; Begitt et al., 2000), Smad1 (Xiao et al., 2001), coactivators (Amazit et al., 2003), and cyclins (Toyoshima et al., 1998; Hagting et al., 1998; Alt et al., 2000; Todier et al., 2001; Ishida et al., 2002).

Like importin β-mediated nuclear import, CRM1-mediated nuclear export also requires the participation of Ran GTPase. Figure 3 provides a model for CRM1-mediated nuclear export. In the nucleus, CRM1 recognizes and binds to leucine and/or isoleucine rich sequences in protein cargo. Direct binding of RanGTP to CRM1 stabilizes the association of the receptor with the cargo. This results in the formation of a trimeric complex which can undergo translocation through the NPC. After translocation through the NPC, the complex reaches the cytoplasmic side of the pore where RanGAP-catalyzed hydrolysis of RanGTP to RanGDP is believed to be the trigger for disassembly of the complex (Askjaer et al., 1999). Following dissociation of the complex, CRM1 and RanGDP are re-imported into the nucleus for subsequent rounds of nuclear export.

Figure 3: A model for CRM1-mediated nuclear export

In the nucleus, a trimeric complex is formed between NES cargo, RanGTP and CRM1. The formation of this complex is promoted by high concentrations of RanGTP. Complex formation can be prevented by the cyclosporine leptomycin B. Once formed, the trimeric complex crosses the NPC and enters the cytoplasm where RanBP1 or RanBP1-like domains in RanBP2-Nup358 destabilize the complex. This process is irreversible due to GTP hydrolysis on Ran stimulated by RanGAP.


Ran GDP
+
P_i
RanGAP
Leptomycin B
CRM1
NES
RanBP1
E
NUCLEOPLASM
CYTOPLASM

While the CRM1 nuclear export pathway is the most extensively characterized, alternative mechanisms are reported to be involved in protein nuclear export. Other proteins belonging to the importin β family have been shown to mediate nuclear export of proteins. CAS (Kumar et al., 2004), exportin-4 (Lipowsky et al., 2000), exportin-5 (Brownawell and Macara, 2002) and in yeast Msn5 (Kaffman et al., 1998) have all been shown to participate in nuclear export of protein cargos. Protein nuclear export can also be mediated by proteins other than those of the importin β family. It has been proposed that members of the 14-3-3 protein family play an indirect role in directing proteins to the cytoplasm. The 14-3-3 proteins bind to phosphoserine-containing motifs in a sequence specific manner (Tzivion and Avruch, 2001) and are proposed to be involved in the cytoplasmic localisation of several proteins including Cdc25 (Kumagai and Dunphy, 1999), class II deacetylases (HDACs) (McKinsey et al., 2001) and GR (Kino et al., 2003).

As mentioned earlier, NLSs and NESs can mediate transport of protein cargos in a uni-directional manner. Although rare, bi-directional transport has also been reported through trafficking signals. The most extensively characterized bi-directional transport signal is the M9 signal of the hnRNP A1 protein. This signal mediates nuclear import and export of hnRNP A1 by transportin 1 (Michael et al., 1995). Another reported case of bi-directional transport involves importin-13, which acts as an import receptor for RNA binding protein motif 8 and an export receptor for eIF1A (Mingot et al., 2001).

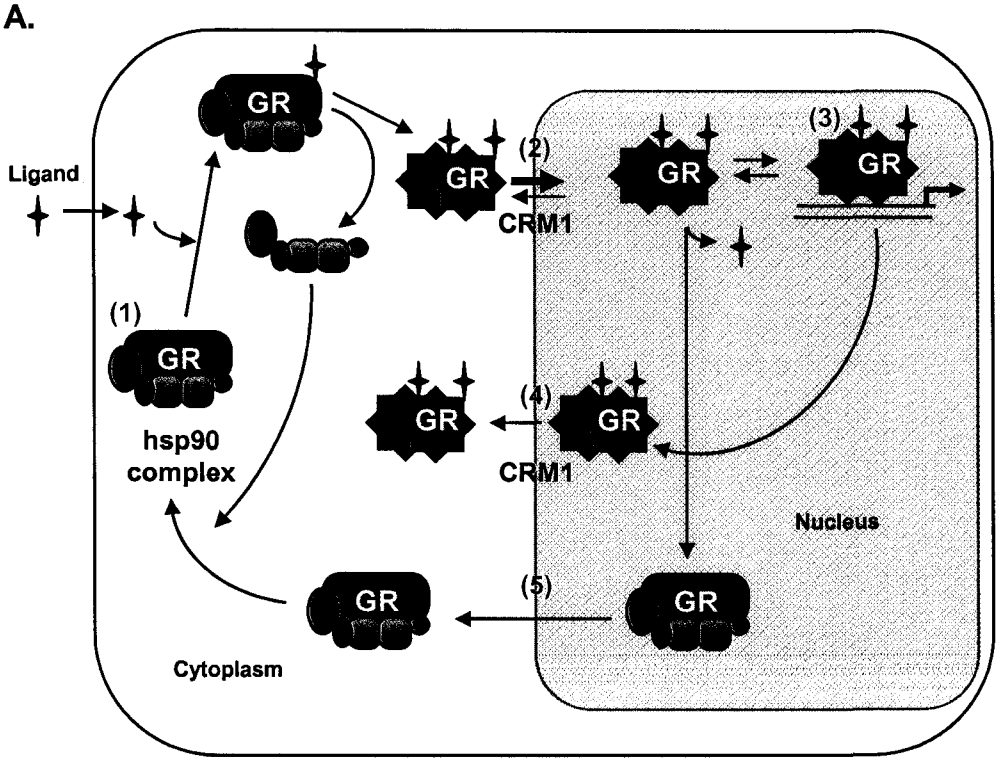
Nucleocytoplasmic Trafficking of GR

The gene-regulatory function of GR is tightly controlled by nucleocytoplasmic trafficking. Figure 4a illustrates the various mechanisms involved in regulating the

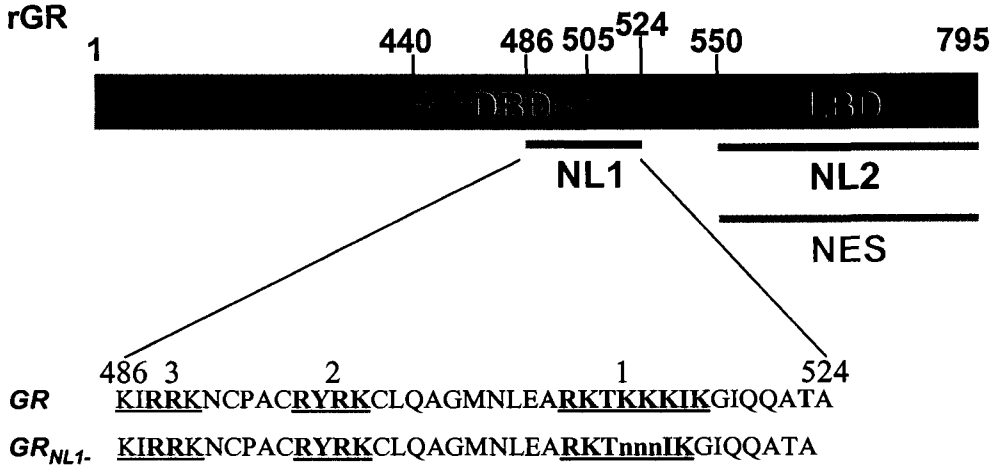
Figure 4: Various mechanisms influence the subcellular distribution of the glucocorticoid receptor

(A) Naïve GR resides predominantly within the cytoplasm in stable association with heat shock proteins and immunophilins (1). Upon steroid binding, GR dissociates from the heteromeric complex, dimmerizes and rapidly imports into the nucleus. Dissociation of GR from hsp90 prior to nuclear import remains controversial (2). In the nucleus GR binds transiently to DNA (3). Nuclear export of ligand-associated GR occurs via the CRM1-dependent nuclear export pathway (4). GR chromatin release occurs upon steroid withdrawal however redistribution of GR to the cytoplasm is a slow process. Nuclear export of ligand-withdrawn GR occurs via a non-CRM1 nuclear export pathway (5). (B) Glucocorticoid receptor signal sequences. NL1 is located in the hinge-like region of the glucocorticoid receptor and is comprised of three clusters of basic amino acids. ProtoNLS-1 is required for receptor transport, while protoNLS-2 and protoNLS-3 contribute to the efficiency of the NLS. NL2 is a steroid-dependent NLS. NL2 and the NES of GR overlap with the LBD. The motifs of these signal sequences remain unidentified.

A.


GR
Ligand
(1)
hsp90
complex
(2)
CRM1
(3)
(4)
CRM1
(5)
Nucleus
Cytoplasm

B.


rGR
1
440
486
505
524
550
795
NL1
NL2
NES
486 3 2 1 524
GR KIRRKNCPACRYRKCLQAGMNLEARKTKKKIKGIQQATA
GR$_{NL1-}$ KIRRKNCPACRYRKCLQAGMNLEARKTnnnIKGIQQATA

subcellular distribution of GR. In the absence of hormone, GR is packaged into a heteromeric complex containing a dimer of the 90 kD heat shock protein, hsp90. Upon hormone binding, GR dissociates from the complex and forms homodimers that rapidly translocate into the nucleus, with a $t_{1/2}$ of approximately 4.5 minutes, where they bind to glucocorticoid-response elements (GREs) within the promoter regions of target genes (Saporita et al., 2003).

It has been shown that dissociation from hsp90 is the rate-limiting step in GR nuclear import and that GR is imported into the nucleus approximated 30 seconds after dissociation (Cidlowski and Munck, 1980). Interestingly, an alternative theory exists where hsp90 remains associated with GR during nuclear import. Evidence to support this theory is the observation that coexpression of a NLS-hsp90 conjugate is required for nuclear accumulation of GR derivatives that lack their own NLSs (Kang et al., 1994; DeFranco, 2000). Steroid binding to GR is transient, and upon hormone dissociation, GR undergoes a conformational change and slowly redistributes to the cytoplasm from the nucleus. Once in the cytoplasm, GR is able to participate in subsequent rounds of hormone induced re-import into the nucleus (Haché et al., 1999).

As mentioned previously, GR nuclear import is mediated through two NLSs, NL1 and NL2. The sequence of NL1 has been well characterized and is located within the hinge region and overlaps with the C-terminal portion of the DBD (Picard and Yamamoto, 1987). NL1 resembles classical NLSs such as the NLS of the SV40 large T antigen and appears to mediate nuclear import of GR through the importin α-mediated nuclear import pathway (Haché et al., 1999). Figure 4b illustrates the three components of NL1. The component called protoNLS-1, a core basic sequence adjacent to the DBD, is required for NLS function

while two smaller sequences pNLS-2 and pNLS-3, at the C terminus of the DBD, appear to contribute to the efficiency of the NLS (Ylikomi et al., 1992; Tang et al., 1997). Mutation of lysine residues ^{513}KKK^{515} abrogates importin α binding and decreases the receptor's ability to import into the nucleus (Savory et al., 1999). As stated earlier, mutation of these residues also increases the rate of GR nuclear export both in the presence and absence of ligand, suggesting that GR is actively retained within the nucleus and that a nuclear retention signal (NRS) overlaps the NL1 motif (Carrigan et al., 2007).

Many transcription factors, including GR, contain additional NLSs that regulate localization under selective conditions. In GR, this second nuclear localization activity, called NL2, occurs in the LBD and was first described in 1987 (Picard and Yamamoto, 1987). As stated earlier, both the sequence that comprises NL2 and the identities of the karyopherins mediating NL2 nuclear import have not been elucidated. Previous studies have, however, localized the NL2 signal within the LBD of GR (Picard and Yamamoto, 1987) (Fig. 4b). In fact, the LBD of GR can be imported into the nucleus and is also capable of mediating nuclear transport of the characteristically cytoplasmic protein β galactosidase (Picard and Yamamoto, 1987). It has also been revealed that NL2 is agonist-dependent, as the NL1 deletion mutant GR_{NL1-} fails to import into the nucleus upon treatment with the GR antagonist RU486 (Savory et al., 1999). This potent antagonist is known to promote hsp90 dissociation upon binding to wild-type GR, ultimately leading to nuclear import of the receptor. It also appears as though NL2 mediates nuclear import of GR through a pathway other than the importin α pathway believed to be engaged by NL1. Taking into consideration that there is no obvious NLS motif in the LBD of GR and that mutation of NL1 prevents GR association with an importin α homologue *in vitro* and *in vivo* (Savory et al., 1999), it is

likely that NL2-mediated nuclear import occurs through a non-importin α nuclear import pathway. It is also worth noting that NL2 activity is poorly conserved amongst SHR. Studies suggest that ligand-dependent NLS activity similar to NL2 resides in ER, AR and GR but not in PR (Ylikomi et al., 1992).

GR nuclear export is a very slow process with a $t_{1/2}$ extending between 12 and 24 hours (Sackey et al., 1996, Haché et al., 1999, Qi et al., 1989). Using fluorescence recovery after photobleaching (FRAP), this slow export from the nucleus to the cytoplasm has been observed for both ligand-associated and steroid-withdrawn GR (Walther et al., 2003). This slow rate of GR nuclear export is not well understood and the mechanisms through which GR exports from the nucleus are only recently being discovered.

The rate of redistribution of GR to the cytoplasm from the nucleus following ligand dissociation may be influenced by several factors. While the kinetics of GR chromatin release and those of hormone dissociation are rapid, it has been shown that unliganded GR remains in the nucleus for an extended period of time (Yang and DeFranco, 1997). It has been suggested that DNA binding plays a role in altering the nuclear export-rate of GR. This is supported by the observation that the DNA binding mutant R496H has been shown to have an increased rate of nuclear export following ligand withdrawal (Sackey et al., 1996).

Amino acids 621-694 of GR have NES activity that is CRM1-independent

Studies focussing on the nuclear export of GR are somewhat limited due to its slow rate of redistribution to the cytoplasm following hormone withdrawal. As mentioned, GR is redistributed to the cytoplasm with a $t_{1/2}$ of 12-24 h (Walther, 2003). Presently, very little is known about the sequence responsible for the nuclear export of GR. Most known NESs are

comprised of hydrophobic amino acids, however the composition of these signals is highly diverse. Due to this high degree of diversity, only a loose consensus for NESs exists (Saporita et al., 2003). It has been proposed that, in its naïve state, GR shuttles freely between the nucleus and the cytoplasm and that treatment with leptomycin B (LMB) can inhibit this movement (Savory et al., 1999). Studies in our own laboratory have demonstrated that GR_{NL1-} accumulates in the nucleus upon treatment with LMB only in the presence of hormone (Walther, 2003). By contrast, nuclear export of GR_{NL1-} following hormone withdrawal is not affected by treatment with LMB (Walther, 2003). Together, these results suggest that GR exits the nucleus via the CRM1-mediated nuclear export pathway when bound to ligand and by a CRM1-independent pathway when not associated with ligand.

After having established that GR nuclear export appears to be partially regulated by CRM1, it became of great interest to identify the sequence through which GR nuclear export is mediated. Intriguingly, it has been reported that a region within the LBD of AR possesses NES activity (Saporita et al., 2003). This hypothesized NES sequence is conserved in the MR, PR, ER and GR (Saporita et al., 2003). Through homology modelling, Dr. Ella Atlas mapped this region in GR to be between amino acids 621 and 694. To study what role this region plays in nuclear export, Dr. Ella Atlas cloned amino acids 621-694 of rat GR into the GFP-C2 vector. By tagging the N-terminus of this region with GFP, the subcellular distribution of the construct could be detected via direct fluorescence. If a NES were present in this region, the GR peptide would preferentially localize in the cytoplasm. Upon transfection of GFP-GR621-694 into Cos7 cells, it was observed that the majority of cells expressing this construct displayed predominantly cytoplasmic fluorescence, indicating that

amino acids 621-694 of GR harbour NES activity (Fig. 5a). As a follow up experiment, Dr. Ella Atlas performed localization assays in the presence and absence of LMB. This was done to examine whether the cytoplasmic localization of GFP-GR621-694 is dependent upon CRM1. Results from this experiment revealed that, in the presence of LMB, GFP-GR621-694 localizes predominantly to the cytoplasm. This suggests that nuclear export through amino acids 621-694 of GR occurs in a manner that is CRM1-independent (Fig. 5a).

Amino acids 621-644 and 668-694 have NES activity that is CRM1-independent and CRM1-dependent, respectively

After establishing that amino acids 621-694 of GR have NES activity, the next intent was to define the minimal region within this peptide required for nuclear export. To do this, Dr. Ella Atlas subdivided the GFP-GR621-694 construct into three smaller ones, GFP-GR621-644, GFP-GR644-668 and GFP-GR668-694 (Fig. 5a). All three constructs have the GFP moiety fused N-terminally to the peptides which facilitates visualization of the constructs by direct fluorescence. Through localization assays, in which Dr. Ella Atlas transfected the constructs into Cos7 cells, it was determined that two of the GR peptides have NES activity. GFP-GR621-644 and GFP-GR668-694 both possess NES activity while GFP-GR644-668 does not (Fig. 5a).

To assess the LMB sensitivity of the constructs, Dr. Ella Atlas performed localization experiments in the presence and absence of LMB. After transfecting Cos7 cells with the GR peptide constructs and assessing their subcellular localization by direct fluorescence, it was

Figure 5: Amino acids 621-644 and 668-694 of GR harbour NES activity that is CRM1-independent and CRM1-dependent, respectively. Amino acids 621-644 and 668-694 have NES activity that is sensitive to point mutations

(A) Nuclear export activity and effect of LMB on nuclear export activity of GR peptides 621-694, 621-644, 644-668 and 668-694. GR peptides 621-694, 621-644 and 668-694 have NES activity, but peptide 644-668 does not. (B) Nuclear export activity and effect of LMB on nuclear export activity of GR mutant constructs GR621-644$_{LL638, 639AA}$, GR668-694$_{LL671, 674AA}$, GR668-694$_{LL687-688AA}$, GR668-694$_{LL689, 690AA}$ and GR668-694$_{SS691, 692DD}$. A and B summarize data attained through localization assays performed by Dr. Ella Atlas. N/A = not applicable.

A.

GR Peptide	Export Activity +/-	LMB Sensitivity Yes/No
621-694	++++	No
621-644	+++	No
644-668	-	N/A
668-694	++++	Yes

B.

Mutant GR Peptide	Export Activity +/-	LMB Sensitivity Yes/No
$GR621\text{-}644_{LL638,\ 639AA}$	++	No
$GR668\text{-}694_{LL671,\ 674AA}$	+++	No
$GR668\text{-}694_{LL687\text{-}688AA}$	+	Yes
$GR668\text{-}694_{LL689,\ 690AA}$	+	Yes
$GR668\text{-}694_{SS691,\ 692DD}$	-	N/A

determined that GFP-GR621-644 retains its NES activity in the presence of LMB, while GFPGR668-694 does not (Fig. 5a). This is intriguing considering full-length GR undergoes two modes of nuclear export (Walther, 2003). Naïve GR and ligand-bound GR undergo CRM1-dependent nuclear export while ligand-withdrawn GR is exported by a CRM1-independent nuclear export process (Savory et al., 1999).

Amino acids 621-644 and 668-694 have NES activity that is sensitive to point mutations

After identifying two regions in the LBD of GR that harbour NES activity, the next objective was to determine what residues are critical for mediating nuclear export of amino acids 621-644 and amino acids 668-694. As stated earlier, NES sequences are traditionally leucine and/or isoleucine rich regions (Fornerod et al., 1997; Askjaer et al., 1999; Ossareh-Nazari et al., 1997; Turpin et al., 1999). For this reason, site-directed mutagenesis was performed where leucines were specifically targeted and substituted with alanines. Also targeted for substitution were serines. It has been suggested that the phosphorylation status of GR codetermines its subcellular localization (Borro et al., 1995). In fact, it has been recognized that NES function can be regulated through phosphorylation of adjacent regions (Dominguez, 2003; Seternes, 2002; Dong et al., 2006). By substituting a serine with an alanine, the possibility of receptor phosphorylation at that particular residue is eliminated. By contrast, substituting a serine with an aspartic acid mimics receptor phosphorylation at that site. The rational behind mutagenesis was that a mutant GR peptide with a disrupted NES sequence would be equally distributed between the nucleus and the cytoplasm. Similar to what would be expected of the localization of GFP alone. By contrast, a peptide with an undisrupted NES sequence would localize more predominantly to the cytoplasm.

The subcellular localization of the mutated GFP-GR621-644 and GFP-GR668-694 constructs was investigated by Dr. Ella Atlas. While several point mutations alter the subcellular distribution of GFP-GR621-644 and GFP-GR668-694, a stretch of leucines in the 668-694 region appear to be particularly critical for mediating nuclear export. More specifically, mutations LL687, 688AA and LL689, 690AA are particularly effective at diminishing NES activity within amino acids 668-694 (Fig. 5b). Another mutation that proved interesting was substitution of serines 691 and 692 to aspartic acids. Interestingly, GFP-GR668-694$_{SS691, 692DD}$ was localized almost exclusively to the nucleus.

Project Goal

Steroidal signalling through GR is dependent on intracellular movement of the receptor. While nuclear import of GR through NL1 is fairly well understood, details surrounding nuclear export of GR and nuclear import through NL2 remain unclear. Therefore, the principal goal for my M.Sc. project was to study the intracellular movement of GR and to identify what amino acids in the receptor LBD are critical for mediating nuclear export of GR and nuclear import through NL2. To achieve my project goal, I introduced a series of point mutations over an area of the LBD thought to harbour NES activity in preliminary localization experiments and assessed what effect these mutations had on receptor localization and behaviour.

MATERIALS & METHODS

Plasmids

The pGFP-GR and pGFP-GR_{NL1-} constructs used in this study have been previously described (Savory et al., 1999). The pGFP-GR_{NL1-} construct contains the $^{513}KKK^{515}$ to $^{513}NNN^{515}$ mutation which abolishes the NL1 nuclear localization signal of GR (Savory et al., 1999). Point mutants pGFP-$GR_{LL638, 639AA}$, pGFP-$GR_{LL671, 674AA}$, pGFP-$GR_{LL687, 688AA}$, pGFP-$GR_{LL689, 689 AA}$, pGFP-$GR_{SS691, 692DD}$, pGFP-$GR_{SS691, 692AA}$, pGFP-GR_{C661S}, pGFP-GR_{L687A}, pGFP-GR_{L688A}, pGFP-GR_{L689A}, and pGFP-GR_{L690A} were derived from full-length pGFP-GR. Complementary oligonucleotides encoding desired mutations were designed for each plasmid (Appendix A). The oligonucleotides were synthesized (Invitrogen) and then used to introduce mutations using the Stratagene QuikChange™ mutagenesis kit and the Pfu proofreading polymerase (Stratagene). Similarly, the pGFP-$GR_{NL1-L687A}$, pGFP-$GR_{NL1-L688A}$, pGFP-$GR_{NL1-L689A}$, pGFP-$GR_{NL1-L690A}$ and pGFP-$GR_{NL1-C640S}$ constructs were created using the Stratagene QuikChange™ mutagenesis kit and the Pfu proofreading polymerase using full-length pGFP-GR_{NL1-} as a template. Positive clones were tested by restriction digest and mutations were confirmed by automated sequencing.

The pRL-CMV-renilla luciferase and the pMMTV-237 Luc plasmids used in the Dual-Luciferase assay have been described elsewhere (Carrigan et al., 2007). The pMMTV-237 Luc plasmid contains the mouse mammary tumor virus (MMTV) long terminal repeat sequence (-237 to +105) followed by the Luciferase reporter gene.

Plasmid Preparation

Plasmid DNA was transformed by electroporation into a competent *Escherichia coli* –DH5α strain and plated on selective LB/agar plates. Colonies were grown overnight at

37°C and single colonies were used to inoculate 5 mL of selective LB culture. These cultures were then used to inoculate 250 mL of selective LB media and allowed to grow overnight. Plasmids were prepared according to the Qiagen Maxiprep or Qiagen Miniprep protocols.

Antibodies

The BuGR antibody (Affinity BioReagents) (recognizes rat GR amino acids 408-422) was used at a dilution of 1/1000 (v/v). GFP was detected using the GFP (JL-8) primary antibody (JL-8) (Clontech) at a dilution of 1/1000 (v/v). Actin was detected using the β-actin primary antibody (Sigma) at a concentration of 1/5000 (v/v). For the coimmunoprecipitation assay, 2 μg of the GRM20 antibody (Santa Cruz Biotechnology) was used. The anti-hsp70 antibody (Stressgen) and the anti-hsp90 antibody (Stressgen) were both used at a dilution of 1/1000 (v/v). The primary antibodies BuGR, GFP (JL-8), β-actin, anti-hsp70 and GRM20 were recognized by the sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (GE Healthcare). The anti-hsp90 antibody was recognized by the goat anti-rat secondary antibody conjugated to horseradish peroxidase (GE Healthcare).

Cell Culture and Transient Transfection

Cos7 embryonic African green monkey cells (ATCC 102005) were maintained at 37°C with 5% CO_2 in high glucose Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids and sodium pyruvate. Transient transfection of Cos7 cells with cDNA expression plasmids was performed using FuGene 6™ Transfection Reagent (Roche) according to manufacturer's instructions at a ratio of 3:1 (μL and μg, respectively). Briefly, FuGene and serum-free

DMEM were combined, mixed and allowed to incubate for 5 min. Then, contents of the FuGene-DMEM tube were transferred directly to DNA, mixed and incubated for 20 min. The transfection mix was added in a dropwise fashion to cells in DMEM containing 10% charcoal-stripped FBS. The cells were incubated with the transfection mixture at 37°C for 24 h. After 24 h, media was removed from the plates by aspiration. Cells were then synchronized in G_0 by washing twice in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2PO_4 \cdot 7H_20$, 1.5 mM KH_2PO_4) and then cultured in serum-free DMEM for at least 16 h.

Preparation of Whole Cell Extracts

To prepare cell extracts, transfected cells were harvested 48 h post-transfection. Each plate was washed twice with PBS and the cells were scraped into 1 mL PBS using a rubber policeman. Cells were collected by centrifugation at 6000 g for 8 min at 4°C. Cell pellets were then resuspended in WCE buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% Glycerol, 1X Complete™ protease inhibitor cocktail (Roche)). Cells were incubated on ice for 10 min and then centrifuged at 13 000 rpm for 10 min at 4°C to remove cellular debris.

Protein Concentration Determination

Protein concentration was determined using Bradford reagent (Bio-Rad). 5 µL of the whole cell lysates was added to 795 µL ddH_2O followed by the addition of 200 µL Bio-Rad Protein Assay Reagent (Bio-Rad). The solution was mixed and allowed to develop for 10 min at RT. The absorbance of each sample was measured at 595 nm using a

spectrophotometer. The amount of protein in each sample was determined by comparison to a BSA standard curve.

SDS-PAGE and Western Blotting

Protein samples (20-50 μg) were loaded on an 8% denaturing gel and separated by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE). The SDS-PAGE gel was electrophoresed in electrode buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and then transferred to an immuno-Blot™ PVDF membrane (Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 10% Methanol) using the BioRad Mini-Protean®II Cell system (Bio-Rad). The membrane was blocked for 1 h at RT in PBST (140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2PO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, 0.05% Tween, pH 7.4) containing 5% w/v skim milk powder. Primary antibodies were diluted in PBST with 5% skim milk w/v to the optimal concentration and incubated for 1 h at RT or at 4°C overnight. Excess primary antibody was removed by washing the membrane three times (10 min each wash) with PBST. The membrane was then incubated for 1 h at RT with the suitable horseradish peroxidase-conjugated secondary antibody diluted in PBST. The membrane was then washed three times (10 min each wash) in PBST and developed using the Western Lightning™ chemiluminescence reagent (PerkinElmer) according to the manufacturer's instructions.

Coimmunoprecipitation Assay

Whole cell extracts were prepared as outlined above and were treated with 10^{-6}M Dex (Sigma) for 3 h at 4°C or were left untreated. All extracts (500 μg protein/assay) were brought up to the same volume by dilution with WCE buffer. pGFP-GR_{wt} or the relevant

mutant GFP tagged receptors, were immunoprecipitated by incubating the extracts with GRM20 antibody (2 μg) (Santa Cruz Biotechnology) overnight at 4°C. Protein A sepharose beads, which had been pre-blocked overnight at 4°C with 50 mg/mL bovine serum albumin (BSA) in IP buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% NP-40), were added to the lysates and incubated on a rotating wheel for 1 h at 4°C. The protein A sepharose beads were collected by centrifugation at 4000 g for 2 min and washed three times with 1 mL of ice-cold wash buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40). Proteins were eluted from the beads by re-suspension in 20 μL of 2xSDS sample buffer (120 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 10% betamercaptoethanol (v/v) and 0.2 mg/mL bromophenol blue) and boiling for 5 min at 95°C. The eluted proteins were subjected to SDS-PAGE and analyzed by Western blotting.

Dual-Luciferase Transcription Assay

Cos7 cells, maintained in DMEM + 10% charcoal-stripped FBS (Hyclone), were transiently transfected with 50 ng GR construct (or the derived construct as indicated), 250 ng –237 MMTV-Luciferase reporter, 25 ng CMV-renilla Luciferase as an internal control, and 175 ng pEGFP-C1 to bring the total DNA to 0.5 μg per well of a 12 well dish. All DNA was transfected using FuGene 6™ Transfection Reagent (Roche) as per manufacturer's instructions. Cells were treated with 10^{-8}M or 10^{-6}M dexamethasone (Sigma) for 16 h and lysed in Passive Lysis Buffer (Promega). A Lumistar Luminometer was used to evaluate luminescence. Transfection efficiency was normalized to the CMV-renilla luciferase control.

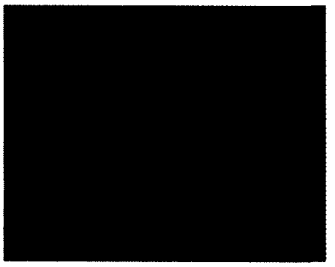
Localization and Hormone Withdrawal Assays

For direct analysis of subcellular distribution, Cos7 cells were plated on 22 mm square coverslips in 6-well dishes. 16 h after plating, the cells were transfected with 0.5 μg DNA using FuGene 6™ Transfection Reagent (Roche). 24 h post-transfection, the cells were synchronized in G_0 by incubation for 16-24 h in serum-free DMEM. For localization assays, the steroid ligand cortisol (Sigma) was used. For hormone withdrawal assays, steroid ligands cortisol (Sigma) and RU486 (Roussel-Uclaf, France) were used. The metabolically stable GR agonist dexamethasone (Sigma) was used for transcription assays. Steroid ligands were added at a final concentration of 10^{-6}M or as indicated. Steroid withdrawal was accomplished by rinsing the cells five times, for five minutes each wash, with PBS containing 5% BSA at 37°C. Following ligand withdrawal, cells were fixed overnight with 4% paraformaldehyde (EM Sciences) in PBS at 4°C. Cells were washed two times with PBS and coverslips were mounted onto microscope slides, overlaid with Vectashield DAPI-staining solution (Vector Laboratories) and sealed with nail polish.

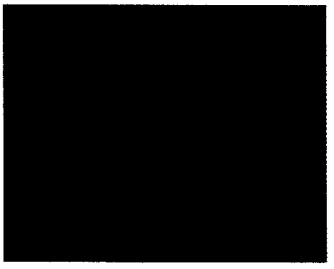
Slides were examined on a Zeiss Axiovert microscope equipped with a Xenon lamp. Digital images were captured using a cooled CCD camera (Hamamatsu Orca ER) and Simple PCI imaging software. Cells were scored into three categories based on the subcellular distribution of the protein of interest; predominantly nuclear fluorescence (N>C), fluorescence that was distributed equally between the nucleus and the cytoplasm (N=C) or predominantly cytoplasmic fluorescence (C>N). Representative images corresponding to each of the categories are shown in Figure 6. Double-blind encryption was performed for quantification and individual data points were derived from a minimum of 900 cells quantified over a minimum of three independent experiments performed in duplicate.

Figure 6: Representative images of subcellular localization categories

Categorization of nucleocytoplasmic distribution within individual cells was used to quantify subcellular distribution. Cells were scored into one of three categories. Representative images illustrate the three categories that were used; fluorescence is predominantly nuclear (N>C), fluorescence is equally distributed between the nucleus and the cytoplasm (N=C) and fluorescence is predominantly cytoplasmic (C>N).



N=C



N>C

C>N

In vitro Steroid Binding Assay

Cos7 cells were maintained in DMEM supplemented with 10% FBS. Lipofectamine™ (Invitrogen) was used (57 μL of Lipofectamine™ per 150 mm dish) to transiently transfect 3.5 μg of DNA. Prior to transfection, cells were rinsed twice in PBS and once with Opti-mem™ reduced serum media (Invitrogen). All transfection mixtures were prepared in Opti-mem™. Transfection mixtures were allowed to incubate for 45 min at RT before being dropped on the cells. Cells incubated with the transfection mixture for 16 h at 37°C. Adding an equal volume of phenol red-free DMEM supplemented with 10% stripped FBS to the cells stopped the transfection process. Cells were then incubated for an additional 8 h, washed with phenol red-free DMEM, and incubated for 24 h in phenol red-free DMEM. Cells were harvested by washing twice with PBS and then scraping the cells, using a rubber policeman, into 1.6 mL PBS. Residual cells were washed from plates with 1.6 mL of PBS. Cells were spun down at 5000 g for 5 min at 4°C. PBS was removed by aspiration and cell pellets were stored at −80°C until use.

Cells were lysed via the freeze thaw method. Cells were resuspended in 200 μL TAPS 0 (25 mM TAPS buffer pH 8.8, 10 % Glycerol, 1 mM EDTA, 2 mM Molybdate) by gently pipetting up and down and then frozen in liquid nitrogen for 30 sec. The cell suspension was then allowed to thaw on ice for 10 min. An additional 200 μL of TAPS 0 buffer was added to the suspension which was mixed again by gently pipetting. The cell suspension was then frozen and thawed a second time as described above. After mixing a final time by pipetting, the cell suspension was spun at 5000 g for 5 min at 4°C. The supernatant was removed promptly and saved. Protein yield was determined by Bradford assay (Bio-Rad).

Protein was diluted to 1 μg/mL in TAPS 0 and 60 μL of cell lysate was added to PCR tubes. A dilution series of [^{3}H] dexamethasone was made. The final concentrations (after adding to the 60 μl of cell lysate) ranged from 0.464 nM to 100 nM dexamethasone. Subsequently, 15 μL of each concentration was added to two PCR tubes and the solutions were mixed by sharp flicking. Cells were then spun down by centrifugation at 5000 g for 20 sec at 4°C and incubated for 16 h at 4°C. After incubation, 37.5 μL of 5 % dextran-coated charcoal was added to samples, which were kept on ice. Samples were then spun at 5000 g for 5 min at 4°C to precipitate the dextran-coated charcoal. 75 μL of each sample was removed and transferred to a scintillation tube containing 3 mL of scintillation fluid. Samples were mixed by inverting the tubes no less than twenty times. [^{3}H] for each sample was counted using a scintillation counter. [^{3}H] was also counted for two 10 μL samples of 2.15 nM, 1.00 nM, and 0.464 nM [^{3}H] in order to determine background counts. For data analysis see Appendix C.

In vitro Steroid Dissociation Assay

For steroid dissociation assays, cells were grown, transfected, harvested and lysed as described for the *in vitro* steroid binding assay. Protein yield was determined by Bradford assay (Bio-Rad). Protein was diluted to 1 μg/mL in TAPS 0 and 60 μL of cell lysate was added to PCR tubes. A dilution series of [^{3}H] dexamethasone was made. The final concentrations (after adding to the 60 μl of cell lysate) ranged from 0.464 nM to 100 nM dexamethasone. Subsequently, 15 μL of each concentration was added to two PCR tubes and the solutions were mixed by sharp flicking. Cells were then spun down by centrifugation at 5000 g for 20 sec at 4°C and incubated for 16 h at 4°C. Samples were treated with 200-fold excess of cold Dex plus or minus 50 μl 10% dextran coated charcoal.

Dextran coated charcoal was added to samples at various time points following treatment with 200-fold excess cold Dex (T = 0, 0.25, 0.5, 1, 1.5, 2, 3, and 4 h). Solutions were mixed by flicking no less than ten times and allowed to incubate at 25°C for 5 min. Subsequently, solutions were spun down at 5000 g for 20 sec at 25°C. Samples were mixed with 3 mL scintillation fluid and [^{3}H] for each sample was counted using a scintillation counter. For data analysis see Appendix C.

RESULTS

Identification of a Ligand-Regulated NES in the LBD of GR

Amino acids L687, L688, L689, L690, S691 and S692 are not directly associated with the Dex molecule when complexed to GR

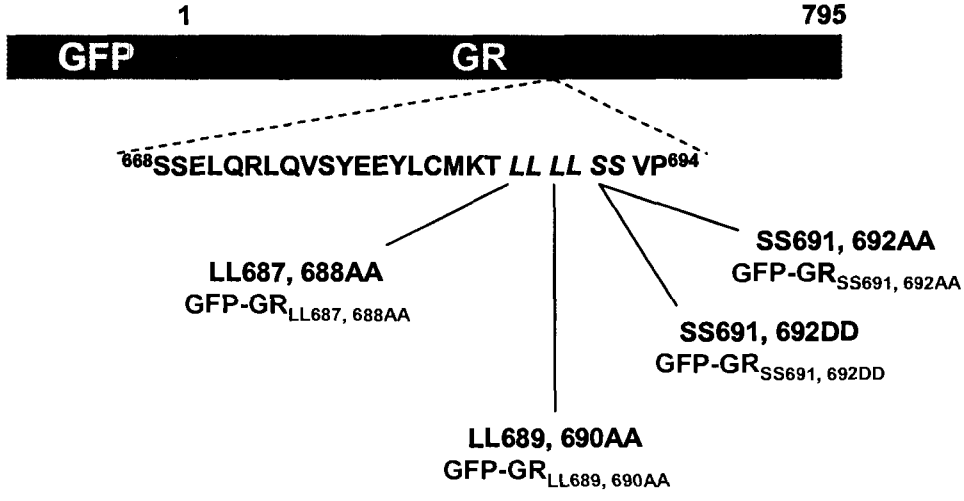
Preliminary results from the Haché lab have suggested that two regions in the LBD of GR, 621-644 and 668-694, have NES activity that is sensitive to point mutations. This was determined through GR peptide localization assays performed by Dr. Ella Atlas. While peptide based localization assays have been used to study the trafficking behaviour of proteins (Saporita et al., 2003; Kudo et al., 1999), a more in-depth understanding of the signals mediating nuclear import and export can be attained if the subcellular localization of the full-length protein is investigated. When studying the trafficking behaviour of the full-length protein, one takes into consideration the role secondary structure conformation may play in regulating protein localization. Therefore, a primary objective of this study was to identify residues within the 621-644 and 668-694 regions important for nuclear export of the full-length protein.

To investigate whether point-mutations that disrupted nuclear export of the peptides also affect localization of full-length GR, the mutations LL687, 688AA, LL689, 690AA, SS691, 692AA and SS691, 692DD were introduced into the full-length receptor. All mutations were introduced by site-directed mutagenesis (as described in Materials and Methods) into the GFP-GRC2 construct. Fusion of the GFP moiety to the constructs allows for visualization by direct fluorescence (Fig. 7a). Furthermore, by fusing GFP to the N-terminus, the possibility of disrupting AF-2 function is avoided.

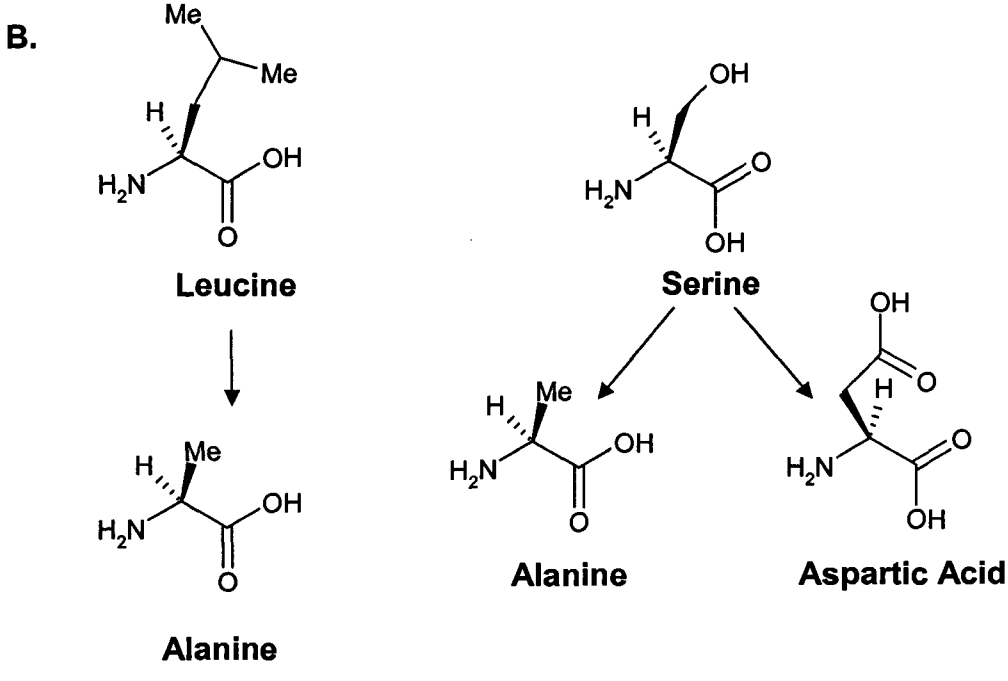
Figure 7: GR mutant constructs were generated by mutagenesis where leucines were substituted with alanines and serines were substituted with either aspartic acids or alanines

(A) Site-directed mutagenesis was used to substitute leucines with alanines and serines with either aspartic acids or alanines. Mutagenesis was performed on L687, L688 and L689, L690, giving rise to the constructs GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$. Mutagenesis was also performed on S691, S692 producing the constructs GFP-$GR_{SS691,\ 692DD}$ and GFP-$GR_{SS691,\ 692AA}$. (B) Structures of the amino acids leucine, alanine, serine and aspartic acid. All structures drawn using ISIS Draw (Elsevier MDL).

A.


1
795
GFP
GR
668SSELQRLQVSYEEYLCMKT LL LL SS VP694
LL687, 688AA
GFP-GRLL687, 688AA
SS691, 692AA
GFP-GRSS691, 692AA
SS691, 692DD
GFP-GRSS691, 692DD
LL689, 690AA
GFP-GRLL689, 690AA

B.


Leucine
Alanine
Serine
Alanine
Aspartic Acid

As mentioned earlier, NESs are typically comprised of hydrophobic amino acids with a repeat of leucines or isoleucine that direct nuclear export by the exportin CRM1 (Fornerod et al., 1997; Askjaer et al., 1999; Ossareh-Nazari et al., 1997; Turpin et al., 1999). For this reason, leucines 687, 688, 689 and 690 were targeted for mutagenesis and substituted with alanines, giving rise to the constructs GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$ (Fig. 7a). While substituting leucine with alanine has little effect on secondary structure conformation, it has been shown that this substitution diminishes the effectiveness of a NES sequence (Thyssen et al., 2006). It should be noted that both leucine and alanine are straight chain non-polar amino acids that are closely related in terms of both size and hydrophobicity (Fig. 7b). Additionally, these two amino acids have high helix forming tendencies (Dong et al., 2006). Therefore, substituting one for the other would be expected to present only minor consequences for overall conformation of the receptor.

Another amino acid that was targeted for mutagenesis was serine. As stated earlier, it has been suggested that NES function may be regulated through phosphorylation of adjacent regions (Dominguez, 2003; Seternes, 2002; Dong et al., 2006). In order to determine the potential for phosphorylation of serines 691 and 692 to play a role in regulating NES activity, site-directed mutagenesis was performed in which these adjacent serines were targeted for substitution. Substituting serines 691 and 692 with alanines (Fig. 7b) eliminates the possibility of receptor phosphorylation at those sites while substitution with aspartic acids (Fig. 7b) mimics receptor phosphorylation at S691 and S692. By mutating serines 691 and 692 to alanines and aspartic acids, two additional full-length mutant constructs were created, GFP-$GR_{SS691,\ 692AA}$ and GFP-$GR_{SS691,\ 692DD}$ (Fig. 7a).

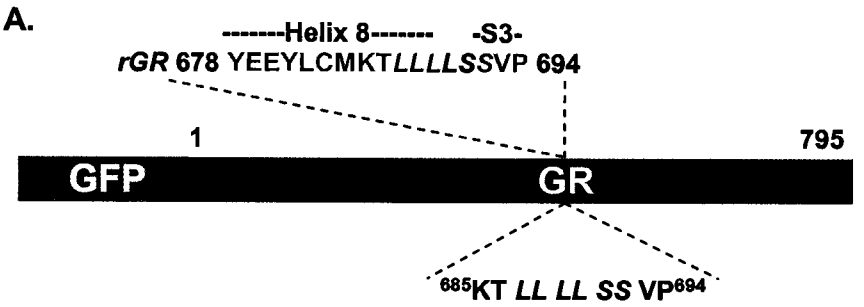
In order to verify that the amino acids targeted for site-directed mutagenesis are not directly involved in ligand binding, leucines 687-690 and serines 691 and 692 were identified in the three-dimensional molecular structure of hGR complexed with Dex. This was accomplished though homology modeling and the molecular graphics system PyMOL. Figures 8 illustrates that leucines 687-690 (Helix 8) and serines 691 and 692 (Beta Sheet 3) are not directly associated with the Dex molecule and are therefore not expected to significantly affect steroid binding.

Mutations LL687, 688AA, LL689, 690AA and SS691, 692AA have little effect on protein expression and stability, yet SS691, 692DD does

Western blot analysis was performed in order to assess the expression levels of the various GR constructs in the absence and presence of hormone. The constructs GFP-GR_{wt}, GFP-$GR_{LL687,\ 688AA}$, GFP-$GR_{LL689,\ 690AA}$, GFP-$GR_{SS691,\ 692AA}$ and GFP-$GR_{SS691,\ 692DD}$ were transiently transfected into Cos7 cells which were either left untreated or treated with 1 μM Dex for 16 h. The Cos7 cell line, an adherent kidney cell line derived from African Green monkey, is regularly used for GR expression (Doppler et al., 2001; Pujols et al., 2002) and subcellular localization (Sackey et al., 1996; Savory et al., 1999; Tazawa et al., 2003) studies as these cells lack endogenous GR. Western blot analysis revealed that the constructs GFP-$GR_{LL687,\ 688AA}$, GFP-$GR_{LL689,\ 690AA}$, GFP-$GR_{SS691,\ 692AA}$ and GFP-$GR_{SS691,\ 692DD}$ are of the same size (Fig. 9). Western blot analysis also revealed that GFP-$GR_{LL687,\ 688AA}$, GFP-$GR_{LL689,\ 690AA}$ and GFP-$GR_{SS691,\ 692AA}$ are expressed at similar levels in Cos7 cells, indicating that mutations LL687, 688AA, LL689, 690AA and SS691, 692AA have little effect on protein expression and stability, both in the presence and absence of hormone. The expression level

Figure 8: Leucines 687-690 and serines 691 and 692 of rat GR are not directly involved in ligand binding

(A) Schema highlighting the location of amino acids that were targeted for mutagenesis. Secondary structure elements are denoted above the sequence. (B) hGR complexed with Dex (yellow). Residues 687, 688, 689, 690 (highlighted in red) and 691, 692 of rat GR were identified by homology modeling. Residues were identified and labelled using the PyMOL molecular graphics system (DeLano Scientific LLC).


A.
-------Helix 8-------
-S3-
rGR 678 YEEYLCMKTLLLLSSVP 694
1
795
GFP
GR
685KT LL LL SS VP694

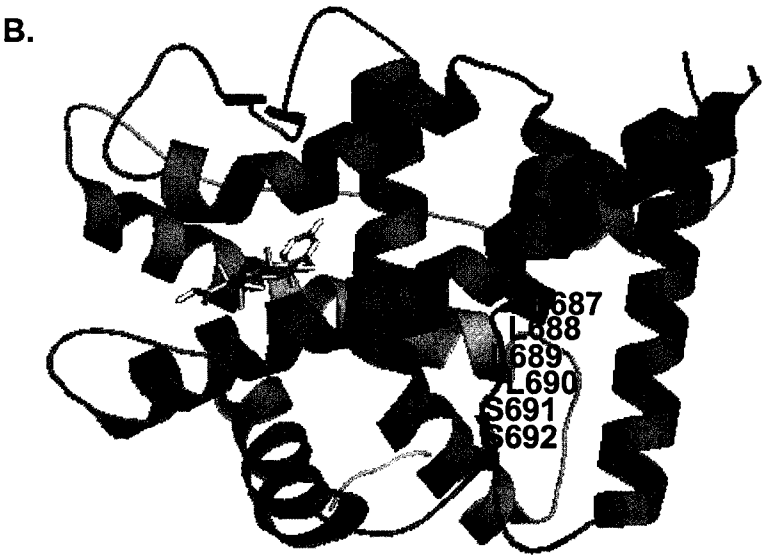
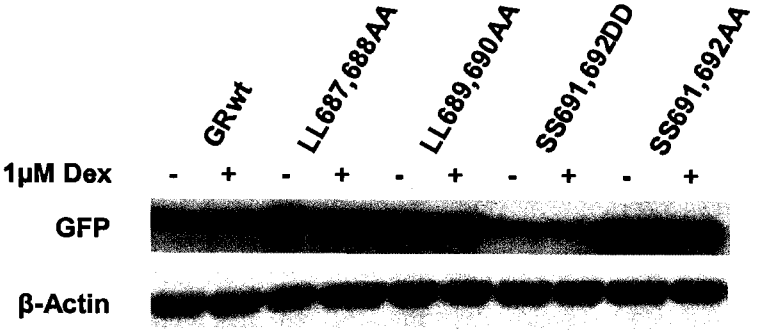

B.
687
L688
689
L690
S691
S692

Figure 9: Western blot analysis showing expression levels of the various GR constructs in the presence and absence of 1 μM Dex

Preparation of whole cell extracts and Western blot analysis were carried out as outlined in Materials and Methods. Where indicated, cells were treated with 1 μM Dex for 16 h.


GRwt
LL687,688AA
LL689,690AA
SS691,692DD
SS691,692AA
1μM Dex
- + - + - + - + - +
GFP
β-Actin

of GFP-$GR_{SS691, 692DD}$ is lower both in the presence and absence hormone than the wild-type receptor and the other GR mutant constructs. This suggests that the SS691, 692DD substitution affects protein expression and stability.

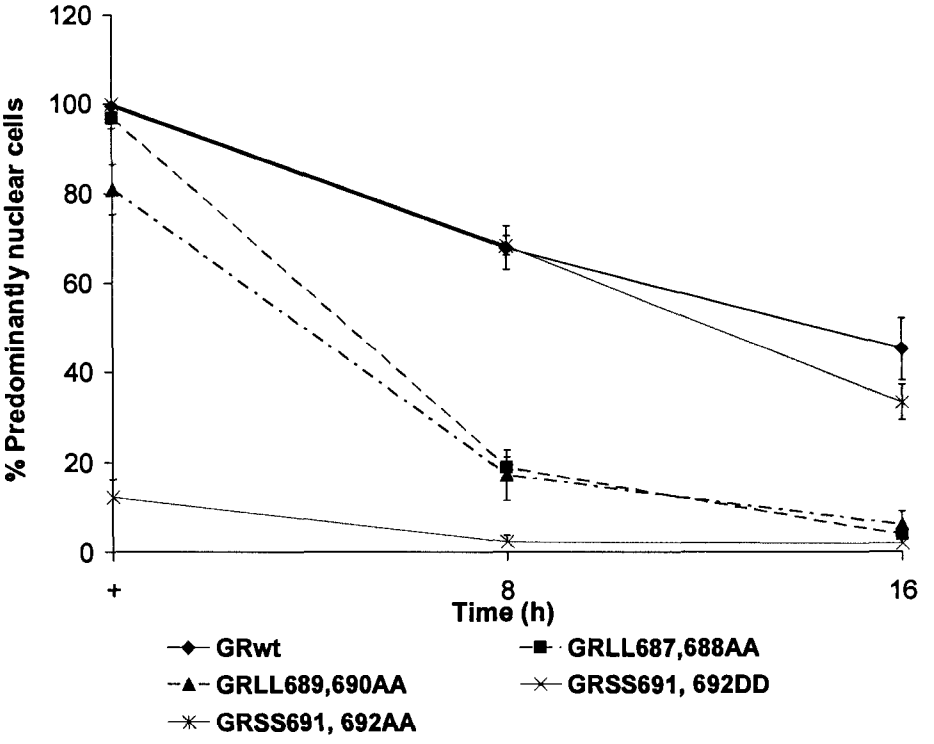
The mutant receptors GFP-$GR_{LL687, 688AA}$, GFP-$GR_{LL689, 690AA}$ and GFP-$GR_{SS691, 692AA}$ translocate into the nucleus in response to 10^{-6} M cortisol, whereas GFP-$GR_{SS691, 692DD}$ does not

To assess the steroid responsiveness of GFP-$GR_{LL687, 688AA}$, GFP-$GR_{LL689, 690AA}$, GFP-$GR_{SS691, 692DD}$ and GFP-$GR_{SS691, 692AA}$, the subcellular localization of the mutant GR constructs was assessed after treatment with 1 μM cortisol for 1 h. For these assays, Cos7 cells were transiently transfected with the aforementioned constructs and then synchronized in G_0 by withdrawing serum. Synchronizing Cos7 cells in G_0 allows for the study of nucleocytoplasmic trafficking of stably maintained pools of GR (Haché et al., 1999; Sackey et al., 1996). In Cos7 cells cultured in the absence of serum there is minimal new synthesis or degradation of GR for periods of up to 72 h following serum withdrawal (Savory et al., 1999). After synchronization, the cells were treated with 1 μM cortisol for 1 h and then fixed to facilitate visualization of the subcellular localization of the constructs by direct fluorescence.

In the absence of ligand, GFP-$GR_{LL687, 688AA}$, GFP-$GR_{LL689, 690AA}$, GFP-$GR_{SS691, 692DD}$, GFP-$GR_{SS691, 692AA}$ and GFP-GR_{wt} were observed primarily in the cytoplasm. As expected, after 1 h treatment with 1 μM cortisol GFP-GR_{wt} was localized predominantly in the nucleus of 99% ± 1% of the transfected cell (Fig. 10; Table 1). Furthermore, it was revealed that GFP-$GR_{LL687, 688AA}$ and GFP-$GR_{LL689, 690AA}$ both localized predominantly in the nucleus after

Figure 10: Substitution of leucines 687, 688 and 689, 690 to alanines in full-length GR dramatically accelerates nuclear export

Redistribution of GR to the cytoplasm following ligand withdrawal is significantly accelerated when LL687, 688AA and LL689, 690AA mutations are introduced in the LBD. Mutations were introduced using the Stratagene mutagenesis kit. Cos7 cells were plated onto glass coverslips and transiently transfected with the indicated constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum. After 16-24 h, cells were treated with 1 μM cortisol for 1 h followed by hormone withdrawal. Cells were withdrawn from hormone for the indicated time periods. Localization of constructs was assessed by direct fluorescence. Representative images are shown for each data set. Error bars represent the standard error of the means of three independent experiments done in duplicate. These experiments were performed in collaboration with Mr. Gregory Addicks.


120
100
80
60
40
20
0
% Predominantly nuclear cells
+
8
16
Time (h)
GRwt
GRLL687,688AA
GRLL689,690AA
GRSS691, 692DD
GRSS691, 692AA

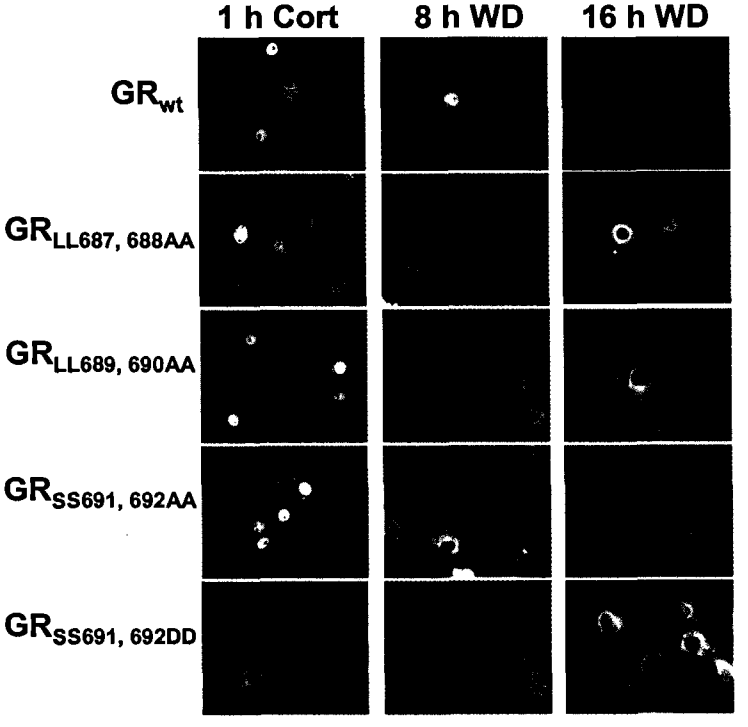

1 h Cort
8 h WD
16 h WD
GRwt
GRLL687, 688AA
GRLL689, 690AA
GRSS691, 692AA
GRSS691, 692DD

Table 1: Point mutations in the LBD accelerate nuclear export of full-length GR following withdrawal from cortisol

Redistribution of GR to the cytoplasm following ligand withdrawal is accelerated when point mutations are made in the LBD. Leucines were mutated to alanines and serines were mutated to alanines or aspartic acids. Mutations were introduced using the Stratagene mutagenesis kit. Cos7 cells were plated onto glass coverslips and transiently transfected with the indicated constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum. After 16-24 h, cells were treated with 1 μM cortisol for 1 h followed by hormone withdrawal. Cells were withdrawn from hormone for the indicated time periods. Localization of the constructs was assessed by direct fluorescence. Error represent the standard error of the means of three independent experiments done in duplicate. H^+ = hormone treatment, WD = hormone withdrawal. These experiments were performed in collaboration with Mr. Gregory Addicks.

Table 1: Point mutations in the LBD accelerate nuclear export of full-length GR following withdrawal from cortisol.

GR constructs	Treatment / Localization	
	1 h H^+ (%N>C)	1 h H^+/16 h WD (%N>C)
GR_{wt}	99 ± 1	45 ± 7
$GR_{LL687, 688AA}$	97 ± 2	4 ± 1
$GR_{LL689, 690AA}$	81 ± 6	6 ± 3
$GR_{SS691, 692DD}$	12 ± 4	2 ± 2
$GR_{SS691, 692AA}$	100 ± 0	33 ± 9

treatment with 1 μM cortisol for 1 h. In fact, GFP-$GR_{LL687, 688AA}$ and GFP-$GR_{LL689, 690AA}$ were localized predominantly in the nucleus of 97% ± 2% and 81% ± 6% of the transfected cells, respectively (Fig. 10; Table 1). This indicates that they are both able to bind hormone.

Interestingly, exchange of serines 691, 692 to aspartic acids had a very potent effect on the response of GR in the presence of 1 μM cortisol. In fact, only 12% ± 4% of cells transfected with GFP-$GR_{SS691, 692DD}$ showed predominantly nuclear fluorescence (Fig. 10; Table 1). By contrast, when serines 691 and 692 were substituted with alanines, the resulting construct GFP-$GR_{SS691, 692AA}$ was predominantly localized to the nucleus in 100% ± 0% of the transfected cells after treatment for 1 h with 1 μM cortisol (Fig. 10; Table 1). Taken together, these results indicate that substitution of serines 691 and 692 with aspartic acids compromises the receptor's ability to translocate into the nucleus upon exposure to 1 μM cortisol for 1 h.

Substitution of leucines 687, 688 and 689, 690 to alanines accelerates the nuclear export-rate of full-length GR in a CRM1-independent manner

In order to determine whether a NES sequence had been disrupted in the 687-692 region, the nuclear export-rate of each GR mutant was assessed through withdrawal assays in which the return of GR to the cytoplasm was monitored upon hormone withdrawal. If a NES sequence were disrupted by mutagenesis, the nuclear export-rate of GR would be delayed. By contrast, if a NES were not disrupted, the nuclear export-rate of GR would remain unaffected. For hormone withdrawal assays, Cos7 cells were transiently transfected with plasmids encoding for the GFP-GR mutants GFP-$GR_{LL687, 688AA}$, GFP-$GR_{LL689, 690AA}$, GFP-$GR_{SS691, 692DD}$, and GFP-$GR_{SS691, 692AA}$ as well as GFP-GR_{wt}. Following transfection, the cells

were synchronized in G_0 by withdrawing serum. After synchronization, the cells were treated with 1 μM cortisol for 1 h. Subsequently, the cells were washed extensively with PBS plus BSA to remove any unbound steroid and returned to serum-free media until they were fixed at the times indicated thereafter.

As shown in Figure 10, hormone withdrawal assays revealed that mutant constructs GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$ had markedly accelerate nuclear export-rates in comparison to wild-type GR. More specifically, at 8 h post-withdrawal GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$ remained nuclear in only 19% ± 2% and 17% ± 6% of the transfected cells, respectively, while GFP-GR_{wt} remained nuclear in 68% ± 5% of the transfected cells (Fig. 10). At 16 h post-withdrawal only 4% ± 1% of cells expressing GFP-$GR_{LL687,\ 688AA}$ displayed this construct as being predominantly nuclear. Similarly, only 6% ± 3% of cells expressing GFP-$GR_{LL689,\ 690AA}$ displayed predominantly nuclear fluorescence (Fig. 10; Table 1). By contrast, GFP-GR_{wt} remained nuclear in 45% ± 7% of the transfected cells 16 h post-withdrawal (Fig. 10; Table 1). It should be noted that the accelerated rate of nuclear export displayed by GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$ contrasted with the behaviour in the peptides where NES activity was compromised upon the introduction of the LL687, 688AA and LL689, 690AA mutations. Indeed, these results appear to exclude the possibility that mutations LL687, 688AA and LL689, 690AA diminish the effectiveness of a NES within amino acids 668-694.

As previously mentioned, GFP-$GR_{SS691,\ 692DD}$ exhibited a reduced ability to translocate into the nucleus upon incubation with 1 μM cortisol for 1 h. In fact, it localized predominantly in the nucleus of only 12% ± 4% of transfected cells while in the presence of 1 μM cortisol for 1 h (Fig. 10; Table 1). At 8 h and 16 h post-hormone withdrawal only 2%

± 1% and 2% ± 2% of cells expressing this construct displayed predominantly nuclear fluorescence (Fig. 10; Table1). By contrast, GFP-$GR_{SS691, 692AA}$, which retains the ability to efficiently translocate into the nucleus upon incubation with 1 μM cortisol for 1 h, displayed trafficking behaviour similar to GFP-GR_{wt}, localizing predominantly to the nucleus of 68% ± 2% and 33% ± 9% of the transfected cells at 8 h and 16 h post-hormone withdrawal, respectively (Fig. 10; Table 1).

After determining that mutation of leucines 687, 688, 689 and 690 to alanines accelerates nuclear export of the receptor, it became of interest to determine whether this accelerated nuclear export occurs in a CRM1-dependent manner. CRM1 directly mediates the nuclear export of many shuttling proteins and is specifically inhibited by LMB (Fornerod et al., 1997; Stade et al., 1997; Ossareh-Nazari et al., 1997; Fukuda et al., 1997). In order to examine whether a CRM1-dependent NES existed in the 687-690 region, hormone withdrawal experiments were repeated in the presence or absence of LMB. For these experiments, the subcellular localization of only GFP-$GR_{LL687, 688AA}$ was assessed. Since this construct redistributes very rapidly to the cytoplasm following ligand withdrawal (Fig. 10; Table 1), a 6 h incubation time with LMB would be sufficient to determine whether CRM1 is responsible for mediating nuclear export of ligand-withdrawn GFP-$GR_{LL687, 688AA}$. Like the previous withdrawal assays, Cos7 cells, transiently transfected with either GFP-GR_{wt} or GFP-$GR_{LL687, 688AA}$, were treated with 1 μM cortisol for 1 h and then washed extensively with PBS plus BSA to remove any unbound steroid. To assess whether nuclear export of GFP-$GR_{LL687, 688AA}$ is mediated by CRM1, LMB was present during the 6 h withdrawal period.

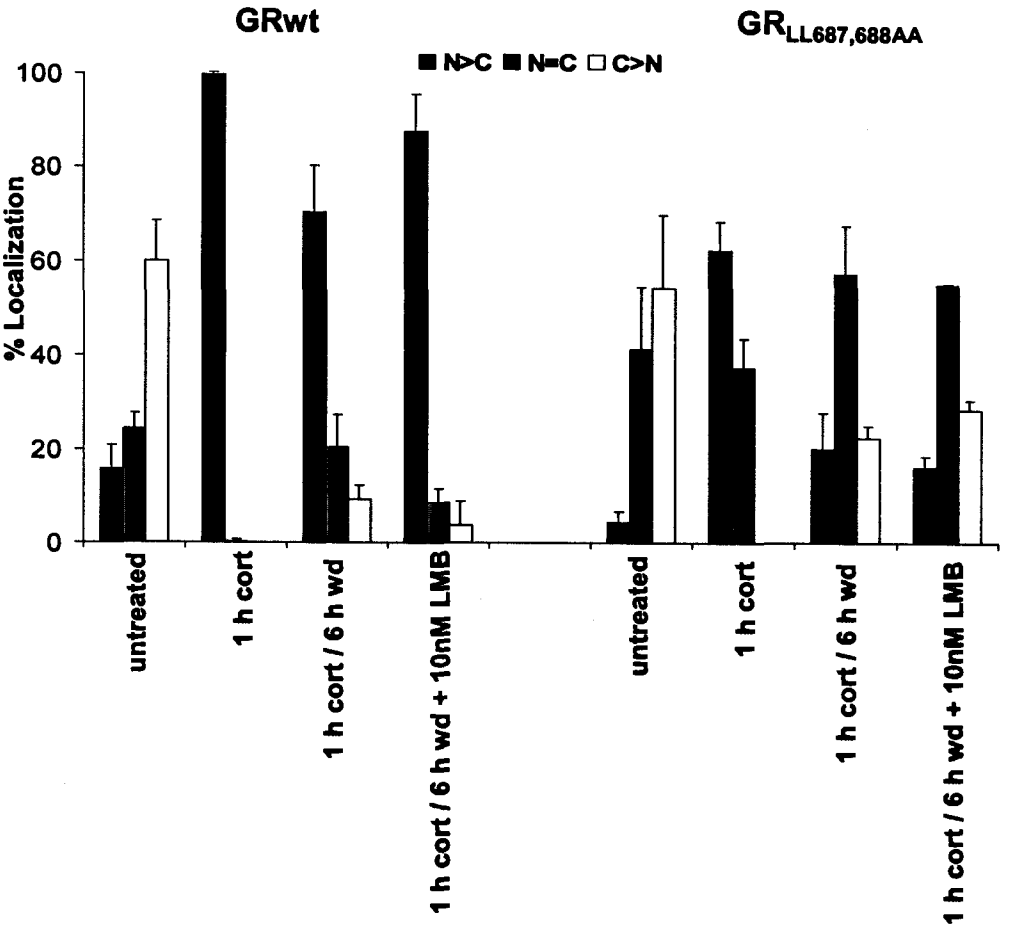
As shown in Figure 11, addition of LMB during the hormone withdrawal period does not alter the rate of redistribution of GFP-GR_{wt} to the cytoplasm following ligand withdrawal. This result indicates that GR nuclear export following ligand withdrawal is not influenced by CRM1 and is in agreement with previous observations which suggest that GR exits the nucleus via a CRM1-independent pathway when not associated with ligand (Savory et al., 1999; Walther, 2003). At 6 h post-hormone withdrawal, GFP-GR_{wt} was localized in the nucleus of 70% ± 9% and 88% ± 8% of the transfected cells in the absence and presence of LMB, respectively (Fig. 11). The difference between these two values was determined to be insignificant. At 6 h post-hormone withdrawal, GFP-$GR_{LL687, 688AA}$ was localized predominantly in the nucleus of 20% ± 8% and 16% ± 2% of the transfected cells in the absence and presence of LMB, respectively (Fig. 11). Together, these results suggest that the accelerated nuclear export of GFP-$GR_{LL687, 688AA}$ following hormone withdrawal is insensitive to LMB inhibition and, therefore, occurs in a CRM1-independent manner.

Substitution of leucines 687, 688 to alanines accelerates the nuclear export-rate of full-length GR following withdrawal from cortisol but not RU486

It has been established that the rate of redistribution of GR back to the cytoplasm following withdrawal of treatment differs greatly depending on what steroid is used to activate the receptor (Sackey et al., 1996). When withdrawn from cortisol, GR redistributes back to the cytoplasm slowly over a period of several hours (Sackey et al, 1996). When withdrawn from the synthetic antagonist RU486, GR fails to return to the cytoplasm even 48 h post-hormone withdrawal (Sackey et al., 1996). Interestingly, GR is known to become differentially hyperphosphorylated in response to RU486 (Orti et al., 1993; Hoeck et al.,

Figure 11: Accelerated nuclear export following withdrawal from hormone is CRM1-independent

LMB is unable to inhibit accelerated nuclear export of $GR_{LL687, 688AA}$ following withdrawal from hormone. Cos7 cells were plated onto glass coverslips and transiently transfected with the indicated constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum. After 16-24 h, cells were treated with 1 μM cortisol for 1 h followed by hormone withdrawal. Cells were withdrawn from hormone for 6 h in the presence and absence of 10 nM LMB. Localization of the constructs was assessed by direct fluorescence. Error bars represent the standard error of the means of three independent experiments done in duplicate. wd = hormone withdrawal.


GRwt
GR LL687,688AA
N>C N=C C>N
% Localization
100
80
60
40
20
0
untreated
1 h cort
1 h cort / 6 h wd
1 h cort / 6 h wd + 10nM LMB
untreated
1 h cort
1 h cort / 6 h wd
1 h cort / 6 h wd + 10nM LMB

1989) and it is suggested that this differential modification of GR may mediate prolonged nuclear localization following treatment with this antagonist (Haché et al., 1999).

In order to investigate whether substitution of leucines with alanines in the 687-690 region disrupts the receptor's ability to effectively translocate into the nucleus upon incubation with 1 μM RU486 for 1 h, localization assays were performed. In these assays Cos7 cells, transiently transfected with GFP-$GR_{LL687,\ 688AA}$ or GFP-$GR_{wt,}$ were treated with either 1 μM cortisol or 1 μM RU486 for 1 h prior to fixation and analysis of the subcellular distribution of the constructs. The mutant construct GFP-$GR_{LL687,\ 688AA}$ was used for these assays because the LL687, 688AA mutation appears to have no effect on the receptor's ability to translocate into the nucleus upon incubation with 1 μM cortisol for 1 h, yet it appears to be the most potent in terms of accelerating the nuclear export of GR following withdrawal from cortisol (Fig. 10).

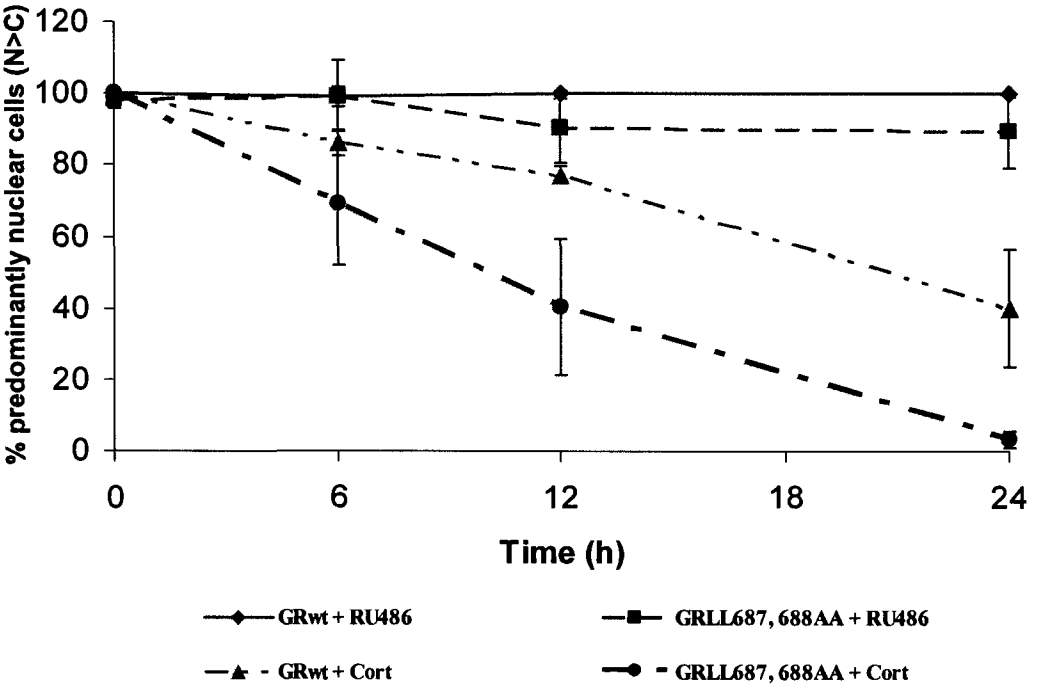
As shown in Figure 12, GFP-$GR_{LL687,\ 688AA}$ and GFP-GR_{wt} are both predominantly localized to the nucleus after treatment with 10^{-6} M cortisol for 1 h. In fact, after 1 h both GFP-GR_{wt} and $GR_{LL687,\ 688AA}$ were localized predominantly in the nucleus of 100% ± 0% of the transfected cells while in the presence of 10^{-6} M cortisol (Fig. 12a,b). Following treatment with RU486, GFP-GR_{wt} and $GR_{LL687,\ 688AA}$ were localized predominantly in the nucleus of 100% ± 0% and 98% ± 3% of transfected cells, respectively (Fig. 12a,b). This result suggests that GFP-$GR_{LL687,\ 688AA}$ is unaltered in its ability to undergo nuclear translocation in response to 1 μM RU486.

In order to determine whether substitution of leucines with alanines in the 687-690 region results in accelerated nuclear export following withdrawal from RU486, hormone withdrawal assays were performed in which Cos7 cells, transiently transfected with GFP-

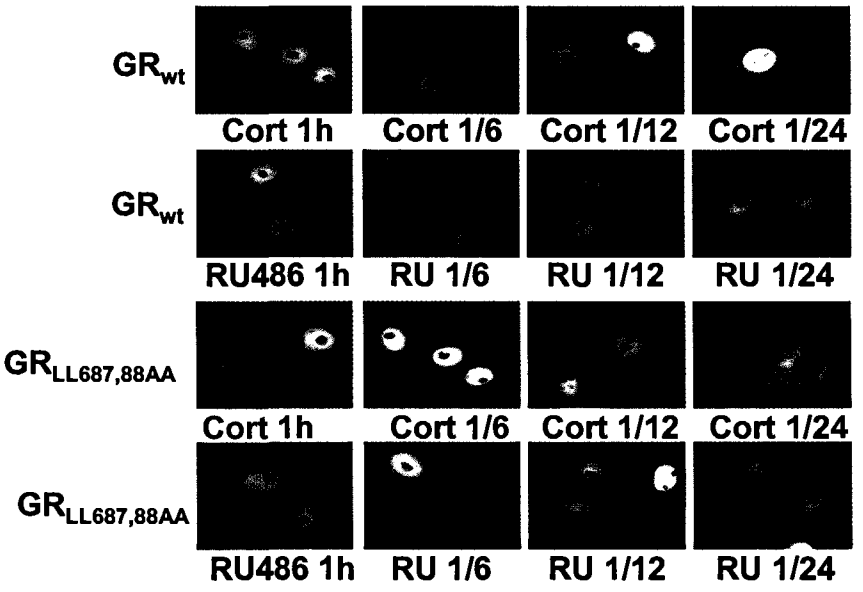
Figure 12: LL687, 688AA mutation fails to accelerate GR nuclear export following withdrawal from RU486

(A) $GR_{LL687, 688AA}$ exhibits accelerated nuclear export following withdrawal from cortisol but not RU486. Cos7 cells were plated onto glass coverslips and transiently transfected with the indicated constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum. After 16-24 h, cells were treated with 1 μM cortisol or 1 μM RU486 for 1 h followed by hormone withdrawal. Cells were withdrawn from hormone for the indicated time periods. (B) Localization of the constructs was assessed by direct fluorescence. Representative images are shown for each data set. Error bars represent the standard error of the means of three independent experiments done in duplicate. These experiments were performed in collaboration with Mr. Gregory Addicks.

A.


% predominantly nuclear cells (N>C)
120
100
80
60
40
20
0
0
6
12
18
24
Time (h)
GRwt + RU486
GRLL687, 688AA + RU486
- GRwt + Cort
- GRLL687, 688AA + Cort

B.


GRwt
Cort 1h
Cort 1/6
Cort 1/12
Cort 1/24
GRwt
RU486 1h
RU 1/6
RU 1/12
RU 1/24
GRLL687,88AA
Cort 1h
Cort 1/6
Cort 1/12
Cort 1/24
GRLL687,88AA
RU486 1h
RU 1/6
RU 1/12
RU 1/24

$GR_{LL687,\ 688AA}$ or GFP-GR_{wt} were treated with either 1 μM cortisol or 1 μM RU486 for 1 h prior to ligand withdrawal. Withdrawal from steroid was carried out by thoroughly washing the cells with PBS plus BSA. Following withdrawal, the cells were fixed to facilitate observation by direct fluorescence. As expected, GFP-$GR_{LL687,\ 688AA}$ displayed accelerated nuclear export following withdrawal from cortisol, localizing predominantly to the nucleus in only 3% ± 2% of all transfected cells 24 h post-cortisol withdrawal (Fig. 12a,b). By contrast, GFP-GR_{wt} remained localized to the nucleus in 40% ± 16% of the transfected cells 24 h post-cortisol withdrawal (Fig. 12a,b). Interestingly, like GFP-GR_{wt}, GFP-$GR_{LL687,\ 688AA}$ was unable to redistribute back to the cytoplasm 24 h post-withdrawal from RU486. In fact, 24 h post-withdrawal from RU486, GFP-GR_{wt} and GFP-$GR_{LL687,\ 688AA}$ were localized predominantly in the nucleus of 100% ± 0% and 89% ± 16% of the transfected cells, respectively (Fig. 12a,b). Taken together, these results indicate that withdrawal from RU486 does not facilitate accelerated nuclear export of GFP-$GR_{LL687,\ 688AA}$.

L687 and L690 are critical for the slow redistribution of GR to the cytoplasm following cortisol withdrawal

After determining that leucines 687-690 play a role in slowing the redistribution of GR to the cytoplasm upon withdrawal from cortisol (Fig. 10), it then became of interest to identify which leucines in this cluster convey this property to GR. As before, site-directed mutagenesis was performed, however, for this set of experiments individual leucines in the 687-690 cluster were mutated to alanines. This gave rise to the constructs GFP-GR_{L687A}, GFP-GR_{L688A}, GFP-GR_{L689A} and GFP-GR_{L690A}.

Hormone withdrawal assays were conducted in which Cos7 cells, transiently transfected with the various single mutation constructs, were treated with 1 μM cortisol for 1 h before hormone withdrawal was commenced. All four single-mutation constructs, GFP-GR_{L687A}, GFP-GR_{L688A}, GFP-GR_{L689A} and GFP-GR_{L690A} were localized predominantly in the nucleus of the transfected cells after incubation with 1 μM cortisol for 1 h, indicating that they are all responsive to hormone (Fig. 13; Table 2). Upon hormone withdrawal, it was revealed that leucines 687 and 690 are required for the characteristically slow redistribution of GR to the cytoplasm following withdrawal from cortisol. At 16 h post-hormone withdrawal, GFP-GR_{L687A} and GFP-GR_{L690A} were localized predominantly in the nucleus of 14% ± 3% and 12% ± 2% of all transfected cells, respectively (Fig. 13; Table 2). This contrasts with the slow rate at which GR redistributes to the cytoplasm following ligand withdrawal (Haché et al., 1999). As shown in Figure 10, at 16 h post-hormone withdrawal, GFP-GR_{wt} is localized predominantly in the nucleus of 45% ± 7% of all transfected cells. Like leucines 687 and 690, leucine 689 also appears to contribute to the slow redistribution of GR following withdrawal from cortisol, however, with 27% ± 3% of transfected cells expressing this construct as being predominantly nuclear at 16 h post-hormone withdrawal, it appears as though L689 does not play as vital a role as L687 and L690 in the redistribution process (Fig. 13; Table 2). Notably, at 16 h post-hormone withdrawal, GFP-GR_{L688A} remained localized predominantly in the nucleus of 38% ± 3% of all transfected cells. This finding suggests that L688 contributes very little to the GR redistribution process following withdrawal from cortisol (Fig. 13; Table 2).

Figure 13: Leucines 687 and 690 are critical for slow redistribution of GR to the cytoplasm following hormone withdrawal

Single substitution of leucines with alanines in the LBD of GR accelerates nuclear export following hormone withdrawal. Leucines 687 and 690 are the most critical residues for slow redistribution of GR to the cytoplasm following hormone withdrawal. Cos7 cells were plated onto glass coverslips and transiently transfected with the indicated constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum. After 16-24 h, cells were treated with 1 μM cortisol for 1 h followed by hormone withdrawal. Cells were withdrawn from hormone for the indicated time periods. Localization of the constructs was assessed by direct fluorescence. Representative images are shown for each data set. Error bars represent the standard error of the means of three independent experiments done in duplicate. These experiments were performed in collaboration with Mr. Gregory Addicks.

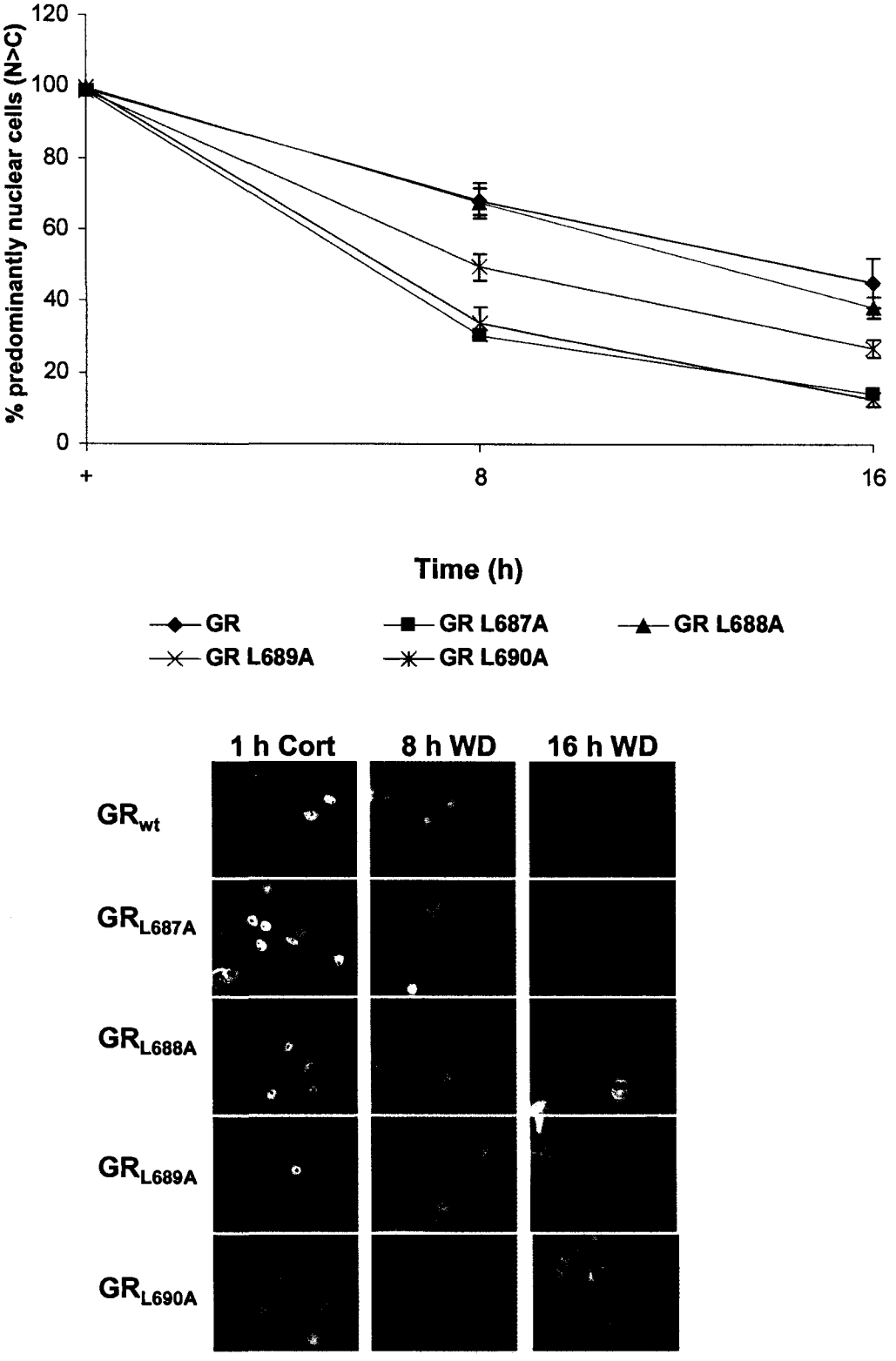

% predominantly nuclear cells (N>C)
120
100
80
60
40
20
0
+
8
16
Time (h)
GR
GR L687A
GR L688A
GR L689A
GR L690A
1 h Cort
8 h WD
16 h WD
GRwt
GRL687A
GRL688A
GRL689A
GRL690A

Table 2: Point mutations in the LBD accelerate nuclear export of full-length GR following withdrawal from cortisol, but have minor effect on steroid off-rate and K_d

Redistribution of GR to the cytoplasm following ligand withdrawal is accelerated when point mutations are made in the LBD, yet these mutations have little effect on steroid off-rate and K_d. Leucines were mutated to alanines using the Stratagene mutagenesis kit. Cos7 cells were plated onto glass coverslips and transiently transfected with the indicated constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum. After 16-24 h, cells were treated with 1 μM cortisol for 1 h followed by hormone withdrawal. Cells were withdrawn from hormone for the indicated time periods. Localization of the constructs was assessed by direct fluorescence. All steroid off-rate experiments and steroid dissociation experiments were performed by Mr. Gregory Addicks (see Materials and Methods). Error bars represent the standard error of the means of three independent experiments done in duplicate. H^+ = hormone treatment, WD = hormone withdrawal, N/A = Not Available.

Table 2: Point mutations in the LBD accelerate nuclear export of full-length GR following withdrawal from cortisol, but have minor effect on steroid off-rate and K_d.

GR construct	Treatment / Localization: 1 h H^+ (%N>C)	1 h H^+/16 h WD (%N>C)	K_d (nM)	Off-Rate $t_{1/2}$ (h)
GR_{wt}	99 ± 1	45 ± 7	3.58 ± 0.40	1 ± 0.06
GR_{L687A}	99 ± 1	14 ± 3	6.29 ± 0.36	0.6 ± 0.13
GR_{L688A}	100 ± 0	38 ± 3	2.71 ± 0.32	1 ± 0.02
GR_{L689A}	99 ± 1	27 ± 3	7.77 ± 0.92	0.5 ± 0.15
GR_{L690A}	100 ± 0	12 ± 2	3.12 ± 0.20	0.9 ± 0.14
GR_{C661S}	100 ± 0	32 ± 2	39.59 ± 12.30	N/A

Accelerated nuclear export upon mutation of L687 and L690 to alanines is not a reflection of reduced ligand affinity or increased steroid off-rate

It is well known that the interactions between a steroid and specific amino acids in the LBD of the receptor determine the receptor binding affinity of the ligand (Schaaf et al., 2005). Since leucines 687, 688, 689 and 690 reside within the receptor LBD, it had to be determined whether the accelerated nuclear export-rates displayed by GFP-GR_{L687A}, GFP-GR_{L689A}, GFP-GR_{L690A} and even GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$ were due to altered affinity for ligand.

In order to investigate whether the accelerated nuclear export-rates of GFP-$GR_{LL687,\ 688AA}$, GFP-$GR_{LL689,\ 690AA}$, GFP-GR_{L687A}, GFP-GR_{L688A}, GFP-GR_{L689A} and GFP-GR_{L690A} are due to differences in ligand affinity, hormone withdrawal assays were conducted using GFP-GR_{C661S}. This mutant GR construct displays reduced affinity for glucocorticoid steroids and a decreased relative affinity for cross-reacting steroids such as progesterone and aldosterone (Chakraborti et al., 1991). At 16 h post-hormone withdrawal, GFP-GR_{C661S} was localized predominantly in the nucleus of 32% ± 2% of all cells expressing this construct (Table 2). These results indicate that, while GFP-GR_{C661S} has a much lower affinity for ligand than GFP-GR_{wt} (39.59 ± 12.30 nM vs. 3.58 ± 0.40 nM for Dex), it undergoes nuclear export at a rate that is comparable to the wild-type receptor. This finding suggests that reduced affinity for ligand does not contribute to accelerated nuclear export.

In order to confirm that accelerated nuclear export is not a consequence of reduced ligand affinity or increased steroid off-rate, *in vitro* steroid binding assays and *in vitro* steroid dissociation experiments were performed by Mr. Gregory Addicks as outlined in Materials and Methods. Analysis revealed that the mutations of GFP-GR_{L687A} and GFP-GR_{L690A} had

only minor effects on steroid off-rate and affinity. More specifically, GFP-GR_{L687A} and GFP-GR_{L690A} have K_ds of 6.29 ± 0.36 nM and 3.12 ± 0.20 nM, respectively (Table 2). These K_ds are comparable to that of GFP-GR_{wt}, which has a K_d of 3.58 ± 0.40 nM (Table 2). Similarly, only small differences exist between the off-rates measured for GFP-GR_{wt}, GFP-GR_{L687A} and GFP-GR_{L690A}. It was determined that GFP-GR_{wt} has an off-rate of $t_{1/2}$ = 1 ± 0.06 h, while GFP-GR_{L687A} and GFP-GR_{L690A} have off-rates of $t_{1/2}$ = 0.6 ± 0.13 h and $t_{1/2}$ = 0.9 ± 0.14 h, respectively (Table 2). The other two mutant constructs, GFP-GR_{L688A} and GFP-GR_{L689A} also have K_ds and steroid off-rates that are similar to wild-type GR. It was revealed that GFP-GR_{L688A} and GFP-GR_{L689A} have K_ds of 2.71 ± 0.32 nM and 7.77 ± 0.92 nM and steroid off-rates of $t_{1/2}$ = 1 ± 0.02 h and $t_{1/2}$ = 0.5 ± 0.15 h, respectively (Table 2).

It was also revealed that the K_ds of the double mutation constructs, GFP-$GR_{LL687, 688AA}$ and GFP-$GR_{LL689, 690AA}$, were significantly higher than those attained for the single mutants and GR_{wt}. It was determined that GFP-$GR_{LL687, 688AA}$ and GFP-$GR_{LL689, 690AA}$ have K_ds of 28.50 ± 6.84 nM and 48.82 ± 5.86 nM (data not shown), respectively, suggesting that the affinities of these mutants for Dex were 8.0-fold and 13.5-fold lower than that of GR_{wt}. This result suggests that, while substitution of individual leucines with alanines has little effect on steroid binding, substitution of adjacent leucines to alanines in the 687-690 cluster drastically reduces GR's ability to bind Dex.

As a control for the *in vitro* steroid binding and *in vitro* steroid dissociation assays, the GR mutant GFP-GR_{C661S} was assessed for its ability to bind hormone and its steroid off-rate. This mutant is known to possess reduced ligand affinity (Chakraborti et al., 1991), therefore, it was of no surprise that it has a K_d value of 39.59 ± 12.30 nM (Table 2). It should be noted that off-rate was not calculated for GFP-GR_{C661S}. This mutant receptor was

poor at binding steroid and, as a result, no valid data could be attained concerning steroid off-rate. This was due to the insufficient resolution of the *in vitro* binding assay.

Based on the above results, it can be concluded that the accelerated nuclear export-rates exhibited by GFP-GR_{L687A} and GFP-GR_{L690A} cannot be attributed to reduced ligand affinity nor differences in steroid off-rates. In fact, it appears as though, despite being in the receptor LBD, substitution of individual leucines to alanines in the 687-690 cluster has only minor effects on receptor K_d and steroid off-rate. This can be concluded considering only small differences exist between the off-rates and K_ds for GFP-GR_{L687A}, GFP-GR_{L690A} and GFP-GR_{wt}.

L687 and L689 are Required for NL1-independent Nuclear Localization of GR

Leucines 687-690 are important for steroid-dependent nuclear localization of GR

Steroidal signalling through GR is dependent on the intracellular movement of the receptor. Nuclear import is mediated by NLSs, protein segments typically comprised of small clusters of basic amino acids. The primary NLS in most receptors is NL1, however, many transcription factors, including GR, contain additional NLSs that regulate receptor localization under selective conditions. In GR, this second nuclear localization activity is called NL2 and was first described in 1987 (Yamamoto et al., 1987). NL2 is a steroid-dependent NLS located in the LBD which complements NL1-mediated GR nuclear uptake. Unlike NL1, the details surrounding NL2-mediated nuclear localization remain unclear. Both the sequence that comprises NL2 and identity of the karyopherins mediating NL2 nuclear import have not been elucidated. Previous studies in our lab have revealed that NL2-

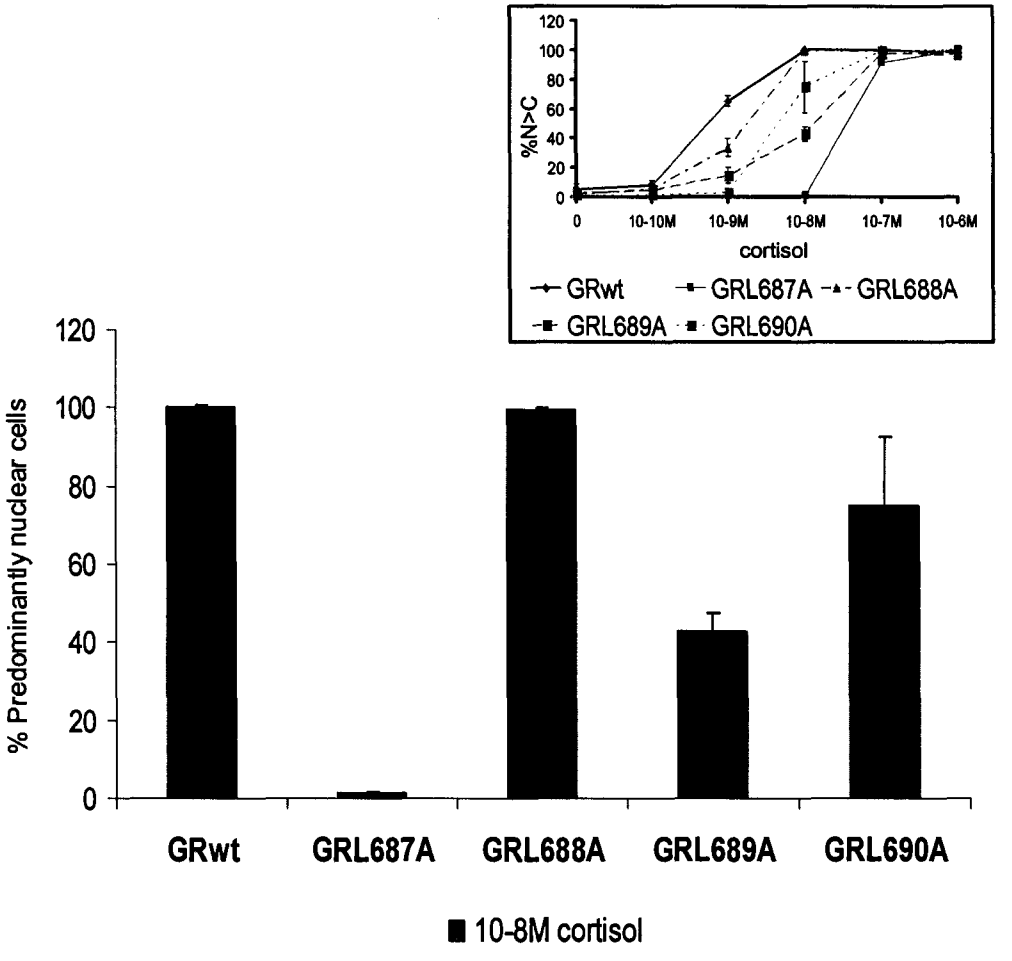
mediated transfer of GR to the nucleus is agonist specific (Savory et al., 1999), mapping NL2 to a discrete region, however, has been challenging.

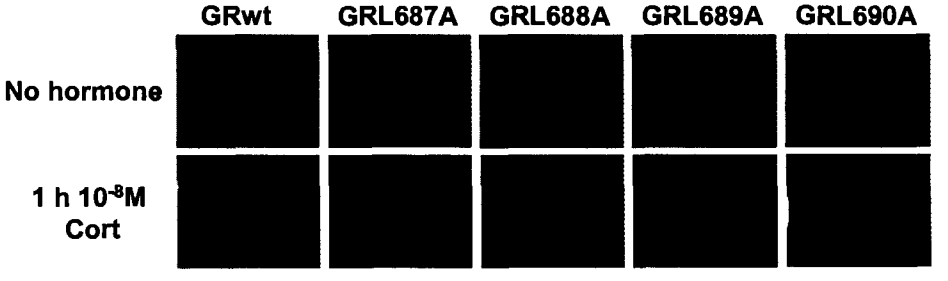
While investigating the properties of GFP-GR_{L687A}, GFP-GR_{L688A}, GFP-GR_{L689A} and GFP-GR_{L690A}, it was revealed that the mutations, L687A, L688A, L689A and L690A partially negate the ability of GR to localize to the nucleus upon treatment with hormone. This was determined via hormone titration experiments. For these experiments, Cos7 cells were transiently transfected with various constructs and then synchronized in G_0 by withdrawing serum for a period of 16-24 h. After synchronization, the cells were treated for 1 h with various concentrations of cortisol ranging from 10^{-10}M to 10^{-6}M. Following treatment, the cells were fixed using paraformaldehyde and the subcellular localization of each construct was assessed by direct fluorescence.

After analyzing the subcellular distribution of the various constructs, it was revealed that the L687A mutant, GFP-GR_{L687A}, appears to be particularly compromised in its ability to import into the nucleus upon stimulation with low levels of cortisol. In fact GFP-GR_{L687A} does not appreciably localize to the nucleus until the concentration of cortisol being delivered to the cells reaches 10^{-7}M. At this concentration, 91% ± 1% of cells expressing GFP-GR_{L687A}, display this construct as being predominantly nuclear (Fig. 14; inset). The impact this mutation has on nuclear import of GR is highlighted best when analyzing its subcellular distribution after stimulation with 10^{-8}M cortisol for 1 h. Under these saturating conditions, GFP-GR_{L687A} is localized predominantly to the nucleus in only 1% ± 0% of all transfected cells. This is significant, considering GFP-GR_{wt} reaches complete nuclear occupancy after treatment with 10^{-8}M cortisol for 1 h (Fig. 14). Another mutation that significantly negates the receptor's ability to translocate into the nucleus upon stimulation

Figure 14: Leucines 687 and 689 are important for steroid-dependent nuclear localization of GR

L687A and L689A mutations compromise GR nuclear import. Cos7 cells were plated onto glass coverslips and transiently transfected with the indicated constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum for 16-24 h. Cells were treated with 10^{-8}M cortisol as well as other concentrations of cortisol (inset) for 1 h. The localization of constructs was assessed by direct fluorescence. Representative images are shown for each data set. Error bars represent the standard error of the means of three independent experiments done in duplicate. These experiments were performed in collaboration with Mr. Gregory Addicks.


%N>C
120
100
80
60
40
20
0
0
10-10M
10-9M
10-8M
10-7M
10-6M
cortisol
GRwt
GRL687A
GRL688A
GRL689A
GRL690A
% Predominantly nuclear cells
120
100
80
60
40
20
0
GRwt
GRL687A
GRL688A
GRL689A
GRL690A
10-8M cortisol


GRwt
GRL687A
GRL688A
GRL689A
GRL690A
No hormone
1 h 10-8M Cort

with low levels of cortisol is L689A (Figure 14; inset). After treatment with 10^{-8}M cortisol for 1 h, GFP-GR_{L689A} is localized predominantly to the nucleus in 43% ± 5% of the transfected cells (Fig. 14). This demonstrates that the L689A mutation is less potent than L687A in terms of negating hormone induced nuclear translocation.

Since these mutations affect nuclear localization of the receptor in response to low concentrations of cortisol, yet have only minimal effects on steroid affinity and off-rate (Table 2), it is reasonable to suggest that the 687-690 cluster is involved in steroid-dependent nuclear localization of GR.

The L687A mutation does not disrupt hsp70 and hsp90 association with GR

In the absence of glucocorticoids, GR associates with hsp70, hsp90, a multitude of immunophilins and p23 (Meijsing et al., 2003). One possible mechanism by which the leucine to alanine mutations may alter the nuclear import of GR is that the GR mutants do not associate with hsps. It has been suggested that GR may move towards the nucleus in an hsp-dependent mechanism and that GR-hsp association is required for stable GR-steroid interaction (Galigniana et al., 1998; Pratt et al., 1996). To evaluate the possibility that the reduced nuclear import is due to a disruption in the association between the mutant receptors and hsp70/hsp90, coimmunoprecipitation assays were performed.

For the coimmunoprecipitation assays, Cos7 cells were transiently transfected with either GFP-GR_{wt} or GFP-GR_{L687A} using FuGene 6™ transfection reagent for a period of 16 h. Following transfection, the cells were synchronized in G_0 by withdrawing serum for 16-24 h. After synchronization, cells were harvested and extracts were treated with either ethanol or

10^{-6}M Dex for 3 h at 4°C. Immunoprecipitation and protein detection were subsequently performed as outline in Materials and Methods.

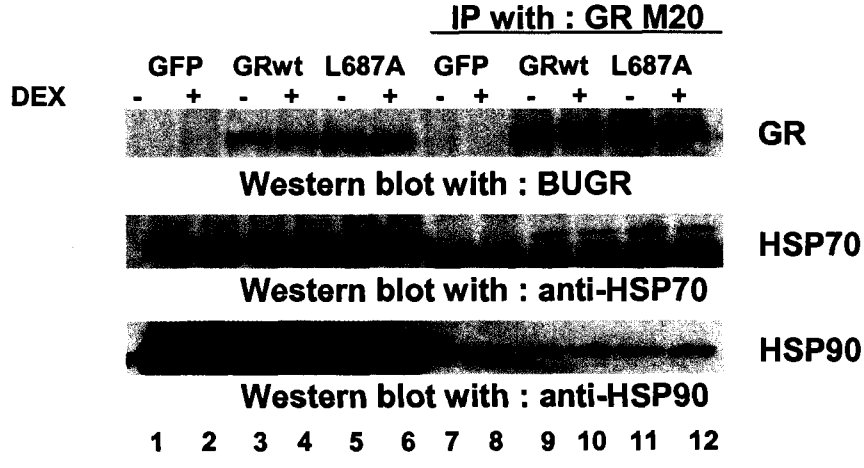
Upon analysis of the coimmunoprecipitation results, it was revealed that GFP-GR_{L687A} is associated with hsp70 and hsp90 both in the presence and absence of 10^{-6}M Dex (Fig. 15; lanes 11, 12). It should be noted that GFP-GR_{wt} was also able to associate with hsp70 and hsp90 in the presence and absence of hormone (Fig. 15; lanes 9, 10). The observation that GFP-GR_{wt} remains associated with hsp70 and hsp90 in the presence of hormone supports the suggestion that GR remains associated with hsps while in the nucleus (Galigniana et al., 1998; Galigniana et al., 2001; Galiganiana et al., 2004). It should be noted that GFP, a non-specific control for this assay, also showed evidence of being associated with hsp 70 and hsp90 (Fig. 15; lanes 7, 8). This could, however, be explained by insufficient stringency of the wash buffer. These coimmunoprecipitation results suggest that it is unlikely that reduced nuclear accumulation of the GR mutants in response to low levels of cortisol is due to a defect in hsp70 and hsp90 association.

L687 and L689 are required for efficient NL2 activity

Thus far, all of the GR single point mutants tested had intact NL1s as well as steroid affinities and steroid off-rates comparable to those of GR_{wt}. It also appears unlikely that they are affected by a defect in hsp70 and hsp90 association. Based on the aforementioned characteristics, it became of interest to investigate whether the NL2 activity of the mutated receptors had been affected. In order to examine what impact the leucine to alanine point mutations have on NL2-mediated nuclear import, these substitutions were introduced into GFP-GR_{NL1}.. As mentioned, NLSs are characteristically composed of basic amino acids,

Figure 15: GFP-GR_{L687A} associates with hsp70 and hsp90 in the presence and absence of 10^{-6}M Dex

Immunoprecipitation of GR_{wt} and GR_{L687A} with hsp70 and hsp90. Cos7 cells were transfected with either GFP-GR_{wt} or GFP-GR_{L687A} using FuGene 6™ regent for a period of 16 h. Following transfection, cells were synchronized in G_0 by withdrawing serum for 24 h. Cells were harvested and extracts were treated with either ethanol or 10^{-6}M Dex for 3 h at 4°C. Immunoprecipitation and protein detection via Western blotting were performed as outlined in Materials and Methods.


IP with : GR M20
GFP GRwt L687A GFP GRwt L687A
DEX
- + - + - + - + - + - +
GR
Western blot with : BUGR
HSP70
Western blot with : anti-HSP70
HSP90
Western blot with : anti-HSP90
1 2 3 4 5 6 7 8 9 10 11 12

such as leucine and lysine. Should a NLS be present in the 687-690 region, substitution of leucine with alanine would render the signal sequence less recognizable to transport factors. Therefore, should any of the leucines in the 687-690 cluster be involved in NL2 activity, substitution to alanines should result in decreased NL1-independent nuclear localization.

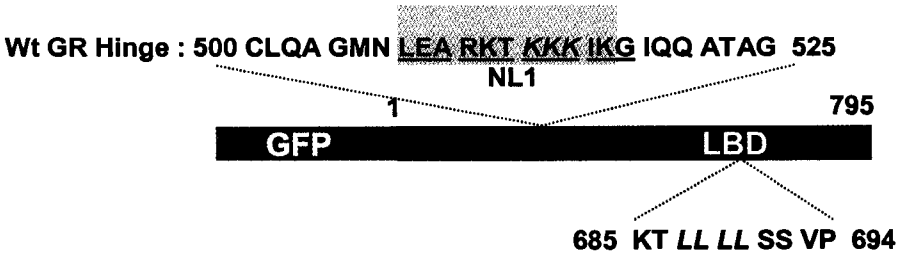
As before, leucines were mutated to alanines using site-directed mutagenesis. However, this time mutations were introduced into GFP-GR_{NL1-}. Mutation of individual leucines 687, 688, 689, 690 in GFP-GR_{NL1-} gave rise to the constructs GFP-$GR_{NL1\text{-}L687A}$, GFP-$GR_{NL1\text{-}L688A}$, GFP-$GR_{NL1\text{-}L689A}$, and GFP-$GR_{NL1\text{-}L690A}$ (Fig. 16a). To verify expression levels of the various GFP-GR_{NL1-} mutant constructs, Western blotting was performed. All of the GFP-GR_{NL1-} mutant constructs were determined to be of the expected size and were expressed at levels comparable to GFP-GR_{NL1-} (Fig. 16b). This indicates that point-mutations L687A, L688A, L689A, and L690A in GFP-GR_{NL1-} have little effect on protein expression, both in the presence and absence of hormone.

To assess what effect the leucine to alanine single mutations have on NL2-mediated nuclear localization of GR, localization assays were performed. Considering GFP-GR_{NL1-} does not appreciably become nuclear until 4 h post treatment with 10^{-6}M cortisol, initial localization assays using the GFP-GR_{NL1-} mutant constructs were performed under these conditions. As before, Cos7 cells were transiently transfected with the various GFP-GR_{NL1-} constructs for 16 h. Following transfection, the cells were synchronized in G_0 by serum starvation for 16-24 h. The cells were then treated for 4 h with 10^{-6}M cortisol. Following treatment, the cells were fixed so that the subcellular localization of the various constructs could be assessed via direct fluorescence.

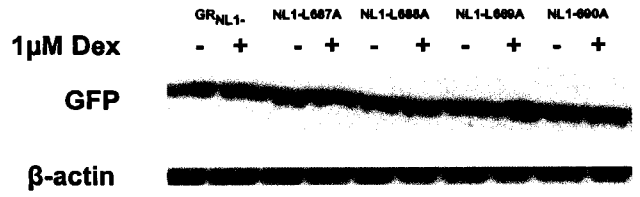
Figure 16: Western blot analysis showing expression levels of the various GR_{NL1}- constructs in the presence and absence of 1 μM Dex

(A) Representation of the GFP-GR_{NL1}- constructs used in this experiment. Point mutations made in the LBD are highlighted in italics. (B) Western blot analysis showing expression levels of the various GR_{NL1}- constructs in the presence and absence of 1 μM Dex. Preparation of whole cell extracts and Western blot analysis were carried out as outlined in Materials and Methods.

A.


Wt GR Hinge : 500 CLQA GMN LEA RKT KKK IKG IQQ ATAG 525
NL1
1
795
GFP
LBD
685 KT LL LL SS VP 694

B.


GR$_{NL1-}$
NL1-L687A
NL1-L688A
NL1-L689A
NL1-690A
1μM Dex
- + - + - + - + - +
GFP
β-actin

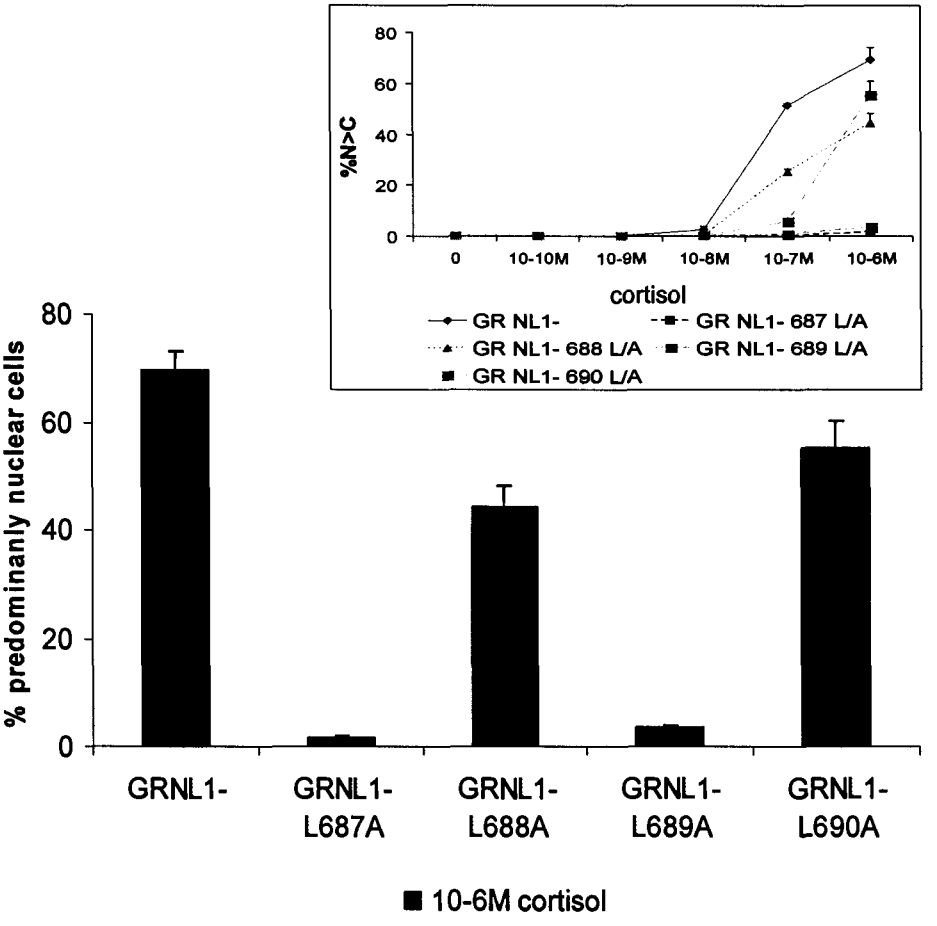
Upon analysis of the subcellular localization of the various constructs it was revealed that at 10^{-6}M cortisol two mutations, L687A and L689A, impaired nuclear import of GFP-GR_{NL1-} (Fig. 17). This indicates that GFP-$GR_{NL1\text{-}L687A}$ and GFP-$GR_{NL1\text{-}L689A}$ may display potentially strong reductions in NL2 activity. At 10^{-6}M cortisol, GFP-$GR_{NL1\text{-}L687A}$ and GFP-$GR_{NL1\text{-}L689A}$ were localized predominantly in the nucleus of 2% ± 1% and 3% ± 1% of cells expressing these constructs, respectively (Fig. 17). Two other mutations, L688A and L690A, also affected nuclear import of GFP-GR_{NL1-}, however, these mutations had only modest effects (Fig. 17). Therefore, it is unlikely that these amino acids are involved in NL2-mediated nuclear import. At 10^{-6}M cortisol, GFP-$GR_{NL1\text{-}L690A}$ and GFP-$GR_{NL1\text{-}L688A}$ were localized predominantly to the nucleus of 55% ± 5% and 44% ± 4% of the transfected cells, respectively (Fig. 17). These results indicate that GFP-$GR_{NL1\text{-}L687A}$ and GFP-$GR_{NL1\text{-}L689A}$ are severely compromised in their ability to translocate into the nucleus upon stimulation with 10^{-6}M cortisol. This suggests that L687 and L689 are particularly critical for efficient NL2-mediated nuclear localization of GR.

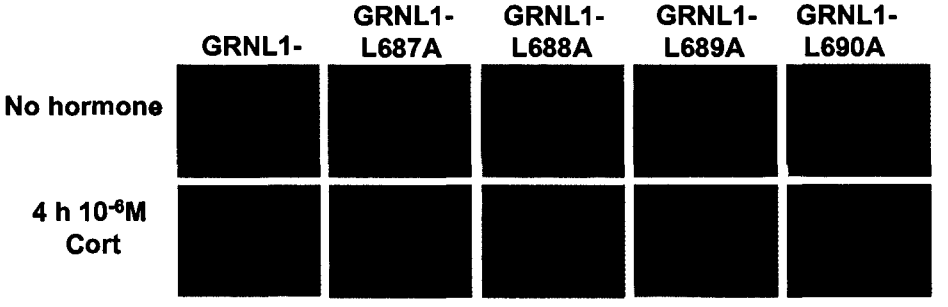
NL2 is the primary mediator of GR nuclear import in response to physiological concentrations of cortisol

After evaluating the subcellular distribution of the GFP-GR_{NL1-} mutants at high concentrations of hormone, it then became of interest to investigate what effect these mutations would have at physiological concentrations. In humans, cortisol levels exhibit a diurnal rhythm; with circulating concentrations between 1 nM and 10 nM (Bat et al., 1998; Richards et al., 2003). Therefore, in order to assess what role NL2 plays in the steroid responsiveness of GR *in vivo*, it is imperative to investigate the localization of the NL2

Figure 17: Leucines 687 and 689 are required for efficient NL2 activity

L687A and L689A mutations disrupt NL2 activity. Cos7 cells were seeded onto glass coverslips and transiently transfected with the indicated GR_{NL1}- constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum. After 16-24 h, cells were treated with 10^{-6}M cortisol for 4 h to induce partial nuclear import. Cells were also treated with various concentrations of cortisol ranging from 10^{-10}M to 10^{-6}M for 4 h (inset). Localization of the constructs was assessed by direct fluorescence. Representative images are shown for each data set. Error bars represent the standard error of the means of three independent experiments done in duplicate. These experiments were performed in collaboration with Mr. Gregory Addicks.


80
60
40
20
0
%N>C
0
10-10M
10-9M
10-8M
10-7M
10-6M
cortisol
GR NL1-
GR NL1- 687 L/A
GR NL1- 688 L/A
GR NL1- 689 L/A
GR NL1- 690 L/A
% predominanly nuclear cells
GRNL1-
GRNL1- L687A
GRNL1- L688A
GRNL1- L689A
GRNL1- L690A
10-6M cortisol


GRNL1-
GRNL1- L687A
GRNL1- L688A
GRNL1- L689A
GRNL1- L690A
No hormone
4 h 10-6M Cort

compromised receptors at 10^{-10}M to 10^{-8}M cortisol. As before, Cos7 cells were transiently transfected with the GFP-GR_{NL1}- mutants for a period of 16 h and then synchronized in G_0 by serum starvation for 16-24 h. After synchronization, the cells were treated with various concentrations of cortisol, ranging from 10^{-10}M to 10^{-6}M for 4 h. The cells were then fixed and the subcellular distribution of the various constructs was assessed by direct fluorescence.

As expected, none of the constructs were predominantly nuclear after 4 h treatment with 10^{-10}M, 10^{-9}M and 10^{-8}M cortisol. However, at 10^{-7}M cortisol GFP-GR_{NL1}-, GFP-$GR_{NL1\text{-}L688A}$, and GFP-$GR_{NL1\text{-}L690A}$ were localized predominantly to the nucleus in 51% ± 2%, 25% ± 1% and 6% ± 1% of the transfected cells, respectively (Fig. 17; inset). As seen in GFP-GR_{wt}, the L687A and L689A mutations had a very potent effect on nuclear import of the liganded receptor. Indeed, GFP-$GR_{NL1\text{-}L687A}$ and GFP-$GR_{NL1\text{-}L689A}$ were compromised in their ability to import into the nucleus upon stimulation with hormone. Unlike GFP-$GR_{NL1\text{-}L688A}$ and to a lesser extent GFP-$GR_{NL1\text{-}L690A}$, GFP-$GR_{NL1\text{-}L687A}$ and GFP-$GR_{NL1\text{-}L689A}$ were unable to significantly import into the nucleus at 10^{-7}M cortisol. In fact, GFP-$GR_{NL1\text{-}L687A}$ and GFP-$GR_{NL1\text{-}L689A}$ were localized predominantly to the nucleus in 2% ± 0% and 3% ± 0% of the transfected cells, respectively (Fig. 17; inset).

Through localization assays, it has been determined that, at physiological concentration of steroid, the nuclear import of the NL1 intact GFP-GR_{wt} is compromised upon mutation of leucines 687 and 689 to alanines (Fig. 14; inset). Results also indicate that GFP-GR_{NL1}- does not become appreciably nuclear until stimulation with 10^{-7}M cortisol for 4 h (Fig. 17; inset). Taken together, these observations may have striking implications for the role NL2 plays in GR nuclear import. These findings suggest that NL2 plays an important

role in nuclear localization of GR in response to physiological concentrations of cortisol. In other words, NL2 may be the predominant NLS *in vivo*.

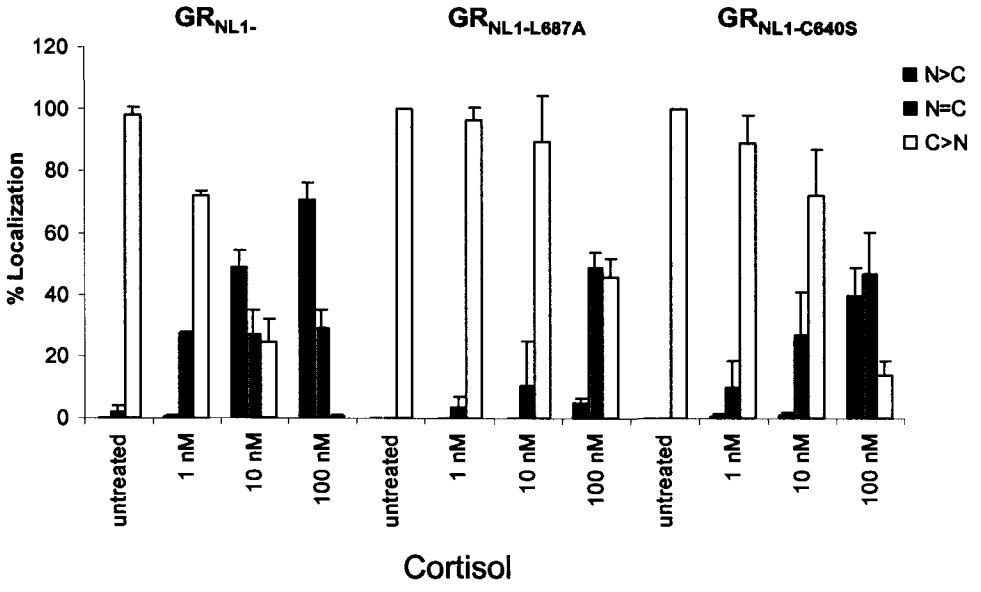
Having established that GFP-$GR_{NL1\text{-}L687A}$ is compromised in its ability to import into the nucleus at physiological concentrations of cortisol, it then became of interest to investigate whether the L687A mutation completely abrogates NL2 nuclear import activity or whether nuclear import of GFP-$GR_{NL1\text{-}L687A}$ at physiological concentrations is merely a delayed process. To investigate this, Cos7 cells transiently transfected with GFP-GR_{NL1}-, GFP-$GR_{NL1\text{-}L687A}$ and GFP-$GR_{NL1\text{-}C640S}$ were treated with concentrations of cortisol ranging from 1 nM to 100 nM for 16 h. As mentioned earlier, nuclear import of GR through NL2 is poorly understood, however it has been reported that NL2 is a weak NLS that compliments NL1 in mediating GR nuclear uptake (Yamamoto et al., 1987). GFP-$GR_{NL1\text{-}C640S}$ was used as a control for this assay. This construct has intact NL2 activity as nuclear import of GFP-GR_{C640S} is similar to that of GFP-GR_{wt} (data not shown).

Upon analysis of the subcellular distribution of the constructs it was revealed that NL2 activity remains partially intact in GFP-$GR_{NL1\text{-}L687A}$. After treatment with 100 nM cortisol for 16 h, GFP-$GR_{NL1\text{-}L687A}$ becomes partially nuclear with 49% ± 5% of all cells transfected with this construct displaying fluorescence equally distributed between the nucleus and the cytoplasm (Fig. 18a,b middle panel). While this result indicates that GFP-$GR_{NL1\text{-}L687A}$ is still able to import via NL2, it also provides evidence that the L687A substitution disrupts NL2 activity. As seen in Figure 17 (inset), after treatment with 10 nM cortisol for 4 h, GFP-GR_{NL1}- is localized predominantly to the nucleus in only 2% ± 0% of cells expressing this construct. Interestingly, after treatment with the same concentration of cortisol for 16 h, GFP-GR_{NL1}- is localized predominantly to the nucleus in 48% ± 5% of the

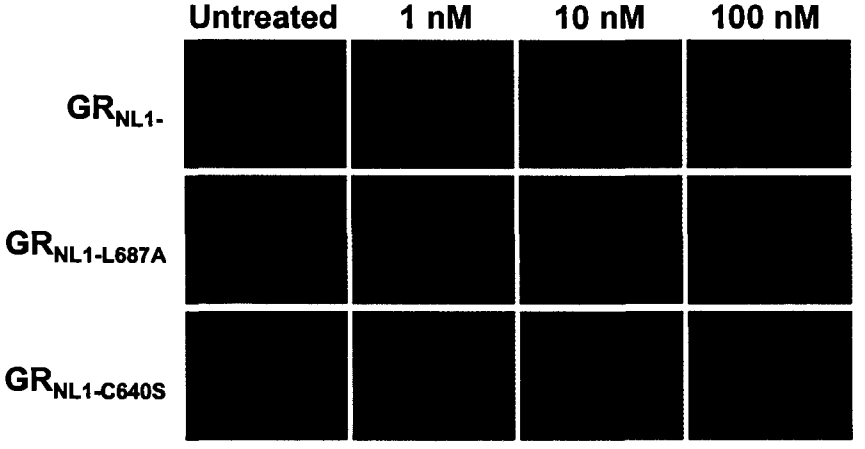
Figure 18: NL2-mediated nuclear import is a slow process

(A) GFP-GR_{L687A} is able to import into the nucleus after treatment with 100 nM cortisol for 16 h. Cos7 cells were seeded onto glass coverslips and transiently transfected with the indicated GR constructs using FuGene 6™ reagent. Cells were synchronized in G_0 by withdrawing serum. After 16-24 h, cells were treated with 1 nM, 10 nM or 100 nM cortisol for 16 h to induce nuclear import. Localization of the constructs was assessed by direct fluorescence. Error bars represent the standard error of the means of three independent experiments done in duplicate. (B) Representative images are shown for each data set.

A.


GR NL1-
GR NL1-L687A
GR NL1-C640S
N>C
N=C
C>N
% Localization
120
100
80
60
40
20
0
untreated
1 nM
10 nM
100 nM
Cortisol

B.


Untreated
1 nM
10 nM
100 nM
GR NL1-
GR NL1-L687A
GR NL1-C640S

cells expressing this construct (Fig. 18a,b top panel). This suggests that NL2-mediated nuclear import is a slow process.

After treatment with 100 nM cortisol for 16 h, GFP-$GR_{NL1\text{-}C640S}$ is appreciably nuclear. In fact, 39% ± 9% of cells transfected with GFP-$GR_{NL1\text{-}C640S}$ display this construct as being predominantly nuclear (Fig. 18a,b bottom panel). This confirms that the C640S substitution has little effect on NL2 activity.

Taken together, these results suggest that the common perception that NL1 is the dominant NLS for GR nuclear uptake may be false. These results provide evidence that NL2 is the primary contributor to nuclear import of GR in response to physiological concentrations of cortisol. Indeed, these results implicate NL2 as the primary physiological mediator of GR nuclear import.

GFP-GR_{L687A} and GFP-GR_{L689A} have intact transcriptional activity

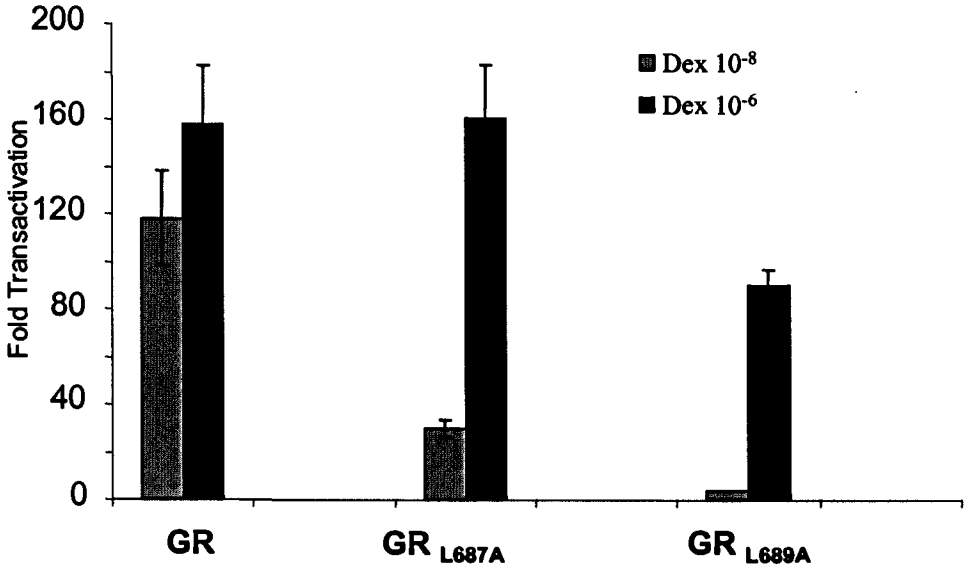
To investigate what effect the L687A and L689A mutations have on transcriptional activity, the transactivational potentials of GFP-GR_{L687A} and GFP-GR_{L689A} were assessed by Dr. Ella Atlas. For these experiments, dual-luciferase transcription assays were performed. In these assays, the various GFP-GR constructs were transiently transfected into Cos7 cells which were synchronized in G_0 by withdrawing serum for 16-24 h. Following synchronization, cells were treated with 10^{-8}M or 10^{-6}M Dex for 16 h and lysed. A Lumistar Luminometer was used to evaluate luminescence and activity was normalized to CMV-renilla Luciferase, which acted as an internal control.

Analysis of the transcription data revealed that the NL2 disrupting mutations presented no significant effect on the transactivational potential of the receptor at 10^{-6}M Dex

(Fig. 19). At 10^{-6}M Dex, a concentration of steroid that facilitates complete nuclear localization of the GR mutant constructs (Fig. 14; inset), GFP-GR_{L687A} exhibits transcriptional activity at levels similar to GFP-GR_{wt}, while GFP-GR_{L689A} is slightly less transcriptionally active (Fig. 19). At 10^{-8}M Dex, a concentration of hormone that facilitates only partial nuclear localization of GFP-GR_{L687A} and GFP-GR_{L689A} (Fig. 14), the transcriptional activities of both mutant receptors are significantly lower than that of GFP-GR_{wt} (Fig. 19). Based on the transcription assay results it can be concluded that the L687A and L689A mutations have little effect on the overall glucocorticoid inducibility of the MMTV promoter in Cos7 cells treated with 10^{-6}M Dex.

Figure 19: Transcriptional effect of the L687A and L689A point mutations

GFP-GR_{L687A} and GFP-GR_{L689A} have intact transcriptional activity. Transcriptional activity of Cos7 cells transfected with the indicated constructs from an MMTV promoter. Cells were transfected with FuGene 6™ reagent and then synchronized in G_0 by withdrawing serum for 16-24 h. Following synchronization, cells were treated with 10^{-8}M and 10^{-6}M Dex for 16 h. Activity of the constructs is quantified relative to the fold activation of the wild-type protein. Activity is normalized to a CMV-renilla internal control for each sample. Error bars represent SEM. These experiments were performed by Dr. Ella Atlas.


200
160
120
80
40
0
Fold Transactivation
Dex 10-8
Dex 10-6
GR
GR L687A
GR L689A

DISCUSSION

Glucocorticoids play a pivotal role in the maintenance of basal and stress-related homeostasis through regulation of a variety of biological processes (Clark et al., 1992). Steroidal signalling through GR is tightly controlled by trafficking of the receptor between the nucleus and the cytoplasm. Several studies have provided insight on the mechanism of GR trafficking within cells, however, a satisfactory explanation regarding the regulation of GR subcellular trafficking remains elusive. In order to fully comprehend what factors and conditions govern GR nuclear import and nuclear export, a greater understanding of the signals mediating these processes is required. The signal sequence NL1, which directs GR import into the nucleus, has been well characterized (Cadepond et al., 1992; Savory et al., 1999; Picard and Yamamoto, 1987). By contrast, the identities of the NL2 and NES of GR remain unknown. Work presented in this thesis provides new insight on the signals that mediate NL1-independent nuclear localization and nuclear export of GR.

LL687, 688AA and LL689, 690AA have an additive effect on the rate of GR nuclear export

It has been known for many years that GR undergoes slow nuclear export following ligand withdrawal, yet the mechanisms regulating the relocalization of GR to the cytoplasm remain largely unknown. Presently, it is believed that two separate pathways mediate the nuclear export of GR. While associated with ligand, GR is exported from the nucleus slowly through the CRM1 pathway as indicated by the observed increase in nuclear occupancy of GR_{NL1-} in the presence of the CRM1 inhibitor LMB (Walther, 2003). It remains unknown, however, whether nuclear export of GR is achieved through direct interaction of the receptor with CRM1 or indirectly through association with another protein bearing a CRM1-

dependent NES. Following ligand withdrawal, it appears as though GR relocalizes to the cytoplasm in a CRM1-independent manner as the addition of LMB, a potent CRM1 nuclear export inhibitor, has no apparent effect on the rate of redistribution of GR to the cytoplasm following ligand withdrawal (See Figure 11). The nature of this export pathway and the identity of the receptor involved in the nuclear export of ligand-withdrawn GR remain unknown.

Having identified CRM1 as a mediator of GR export, the primary amino acid sequence of GR was analyzed for leucine rich sequences that loosely resemble classical NES motifs. One such motif was identified in the 668-694 region of GR. Through peptide localization assays using mutated GFP-GR668-694 construct, it was revealed that mutations LL687, 688AA and LL689, 690AA are effective at diminishing NES activity within amino acids 668-694 (see Figure 5). Surprisingly, nuclear export was accelerated when these mutations, which had an inhibitory effect on nuclear export of the GR peptides, were introduced into the full-length receptor (see Figure 10).

One possible explanation for the accelerated nuclear export of GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$ is that the LL687, 688AA and LL689, 690AA mutations alter the conformation of the LBD in a manner that exposes a NES to transport machinery. While it is unlikely that large structural changes would result from leucine to alanine substitutions, it has been suggested that small structural changes can arise when substituting leucine with alanine, an amino acid that occupies a smaller space (Dong et al., 2000). For instance, Dong and colleagues suggest that conformational changes arise in the LBD of hGRα when L722 is mutated to alanine (Dong et al., 2006). It seems possible that small structural changes in the

LBD could alter the receptor's ability to be recognized by and interact with components of the nuclear export machinery.

While the rationale underlying the accelerated nuclear export of GFP-$GR_{LL687, 688AA}$ is unknown, results presented in this thesis indicate that nuclear export of this construct following ligand withdrawal occurs via a CRM1-independent pathway. This can be concluded based on the observation that LMB is unable to inhibit the accelerated nuclear export of GFP-$GR_{LL687, 688AA}$ following withdrawal from hormone (see Figure 11). Having established that GFP-GR_{wt} and GFP-$GR_{LL687, 688AA}$ are both exported by a transport receptor other than CRM1 following ligand withdrawal, determining the identity of this transport receptor will be the subject of future investigation.

Presently, the identity of the non-CRM1 receptor that mediates nuclear export of GR remains elusive. One candidate for non-CRM1 nuclear export of GR is 14-3-3. Recently, the 14-3-3σ isoform was implicated in the nuclear export of ligand-withdrawn GR and has been shown to negatively regulate GR transcription (Kino et al., 2003). Interestingly, it appears as though the binding of GR to 14-3-3σ occurs in a ligand-dependent manner. This is based on the observation that the effect of 14-3-3σ on GR nuclear export is specific to the GRα isoform and does not influence GRβ, a variant unable to bind hormone. Produced by alternative splicing of the human GR transcript, GRβ functions as a dominant negative inhibitor of GRα function (Bamberger et al., 1995) and differs from GRα at the extreme C-terminus of the LBD (Weinberger et al., 1985). While the 14-3-3σ isoform has been implicated in the nuclear export of ligand-withdrawn GR, it has also been demonstrated that nuclear export of ligand-withdrawn GR occurs in cells lacking 14-3-3σ (Kino et al., 2003). This suggests that another protein, perhaps an alternative 14-3-3 isoform, may influence GR

nuclear export. To investigate this possibility, one could assess whether an association takes place between GR and the seven known isoforms of 14-3-3. This could be determined by performing a GST-pull down assay in which isoforms of 14-3-3 would be assessed for their ability to pull down GR when precipitated with GST-beads. As a follow up experiment, potential interactions between GR and the various 14-3-3 isoforms could be confirmed through coimmunoprecipitation studies. To investigate the possibility that a 14-3-3 isoform regulates GR nuclear export, siRNA could be used to knockdown different 14-3-3 isoforms. By evaluating the subcellular localization of GR following treatment with siRNA against 14-3-3 isoforms, one could determine whether the abrogation of certain 14-3-3 isoform activities has an effect on GR subcellular localization.

Another protein recently implicated in relocalization of SHRs to the cytoplasm is the naturally occurring metastatic tumour antigen 1 (MTA1) variant. MTA1 can function as an ER co-repressor and is up-regulated in many forms of cancer (Mazumdar et al., 2001). It has been reported that liganded GR relocalizes to the cytoplasm upon expression of MTA1s, a shortened variant of MTA1 (Kumar et al., 2002). While the mechanism through which MTA1s induces cytoplasmic localization of GR is unclear, it is known that MTA1 has a very limited expression pattern. This makes it unlikely that MTA1 is responsible for modulating the subcellular localization of SHRs in the majority of cell types (Kumar et al., 2002).

Another explanation for the accelerated nuclear export of ligand withdrawn GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$ is that mutations LL687, 688AA and LL689, 690AA expose a region in the LBD through which another protein bearing a non-CRM1 NES associates with GR. An alternative possibility is that association with another protein through leucines 687-690 may activate an export signal resident within the receptor.

Theoretically, substitution of leucines with alanines in the 687-690 region could enhance an association between GR and this unknown protein. Interestingly, a consensus signature motif, LXXLL, in which L is leucine and X is any amino acid, was identified to be necessary and sufficient for ligand-dependent interactions of coactivators RIP140, SRC-1 and CBP/p300 with the nuclear receptor LBD (Heery et al., 1997). Although the LXXLL motif in coactivators has been well characterized, the role of comparable LXXLL motifs in the receptor LBD remains unclear. While the 687-690 leucine cluster does not exactly fit the LXXLL motif, its high homology to known LXXLL motifs in GR (Dong et al., 2006) and its antipathic helical structure (Bledsoe et al., 2002) make it an attractive candidate for cofactor association. It has been established that the GR-coactivator complex consists of many diverse members (reviewed in Glass, 2000), therefore, there are many proteins that could potentially assist in GR nuclear export. It is possible that a coactivator could potentially piggyback GR out of the nucleus through association with a non-CRM1 nuclear export receptor. It should be noted that the interaction between GR and coactivators is transient and that the composition of coactivators varies among different cell types (Glass, 2002). If GR nuclear export were dependent on transient interactions it is possible that GR would be associated with the protein responsible for mediating nuclear export only briefly. This could limit GR nuclear export and result in the characteristically slow redistribution of the receptor to the cytoplasm following ligand withdrawal. If the LL687, 688AA and LL689, 690AA mutations were to enhance recognition of the motif by a protein involved in mediating nuclear export, it is likely that nuclear export would appear accelerated due to prolonged or more frequent interaction between GR and the coactivator.

Closer examination of the 687-690 leucine rich region revealed that L687 and L690 are critical for the slow redistribution of GR to the cytoplasm following cortisol withdrawal (see Figure 13). The 687-690 region is α-helical and part of helix 8 of the LBD (See Figure 8) (Bledsoe et al., 2002). Therefore, as adjacent amino acids of an α-helix are 3.5 residues apart (Giannoukos et al., 1999), the mutations L687A and L690A would affect amino acid substituants that lie on the same side of the α-helix. Considering the LXXLL motif has been described to mediate protein-protein interactions (Heery et al., 1997; Torchia et al., 1997; Ding et al., 1998), it is possible that L687 and L690 are critical for binding of a protein required for nuclear export of the receptor. Also, the 687-690 region of the LBD is solvent exposed, (see Figure 8) making it easily accessible to interacting proteins.

Another potential explanation for accelerated nuclear export upon mutation of leucines to alanines in the 687-690 region is that L687 and L690 mediate interactions with a protein that masks a NES. Upon mutation of L687 and L690 to alanines a masking-protein could be released which, in turn, could expose a NES. Another possibility is that mutations L687A and L690A could prevent an association from taking place between GR and a masking protein. Masking of a transport signal through association with another protein is not a novel concept. In fact, *in vitro* experiments have shown that the addition of molybdate, a divalent metal that stabilizes the GR-hsp90 complex (Leach et al., 1979), to chaperone associated GR prevents NL1-dependent binding of GR to importin α (Savory et al., 1999). It is hypothesized that by masking NL1, the hsp90-chaperone complex sequesters unliganded GR in the cytoplasm (Savory et al., 1999).

While results suggest that L687 and L690 are involved in mediating the nucleo-cytoplasmic trafficking of GR, the functional role of the 687-690 region remains unknown.

The possibility that this region participates in protein-protein interactions involved in mediating nuclear export of GR does however, remain an attractive possibility. Interestingly, the half-time of GR relocalization to the cytoplasm following ligand withdrawal varies depending on cell type. Indeed, it has been reported that the half-time of GR relocalization to the cytoplasm following ligand withdrawal varies from 8 h (Liu and DeFranco, 2000) to between 12 and 24 h (Ottosson et al., 1994; Savory et al., 1999). If GR nuclear export following ligand withdrawal depended upon interaction with a coactivator, it would be expected that the rate of GR nuclear export would differ amongst cell types where the availability of coactivators free to interact with GR varies.

Several studies have shown that relocalization of GR to the cytoplasm occurs upon overexpression of p53 (Sengupta et al., 2000; Sengupta and Wasylyk, 2001). It should be noted, however, that MDM2-mediated degradation of p53 maintains a low level of p53 expression under resting conditions (Prives, 1998; Ashcroft and Vousden, 1999). Due to the relatively low level of p53 expression under normal cellular conditions, it is unlikely that p53 mediates the bulk of GR nuclear export in most cell types. Also proposed to stimulate GR nuclear export is the JNK signalling pathway. However, this pathway is normally inactive under resting cellular conditions (Itoh et al., 2002) and is therefore not likely to significantly influence the nuclear export of ligand-withdrawn GR.

Another explanation for the accelerated nuclear export of GFP-$GR_{LL687,\ 688AA}$ and GFP-$GR_{LL689,\ 690AA}$ is that these mutant receptors are being targeted to subdomains that hinder nuclear retention. Recently, it has been suggested that conformational changes of the LBD are major determinants of the intranuclear targeting of GR (Schaaf et al., 2005). It is known that GR forms approximately 1000-2000 focal domains consisting of 40-50 receptors

upon ligand-induced activation and translocation into the nucleus (van Steensel et al., 1995; Htun et al., 1996). Many of these focal domains are regions that display active transcription (DeFranco, 2002), however, receptors also accumulate within transcriptionally inert foci (van Steensel et al., 1995) that likely act as storage sites for receptors destined for alternative processing fates (DeFranco, 2002). It is possible that GFP-$GR_{LL687, 688AA}$ and GFP-$GR_{LL689, 690AA}$ are recruited to intranuclear subdomains where retention factors are not prevalent. This scenario could ultimately lead to accelerated nuclear export of the receptor. While recent progress has been made regarding nuclear subdomain targeting, information regarding what role it plays in GR subcellular trafficking remains limited. Therefore, whether intranuclear localization affects nucleocytoplasmic trafficking of the receptor remains speculative.

Accelerated nuclear export is not a reflection of reduced affinity for ligand or increased steroid off-rate

Studies have shown that the kinetics of GR chromatin release is closely related to the rate of hormone dissociation (Munck and Foley, 1976; Yang et al., 1997), yet GR remains localized in the nucleus for a considerable amount of time following hormone withdrawal (Haché et al., 1999; Madan and DeFranco, 1993; Sackey et al., 1996). It is also known that steroid is rapidly lost from the receptor upon ligand withdrawal (Munck and Holbrook, 1984) and that reassembly of GR into hsp-containing complexes occurs within minutes of receptor ligand dissociation (Haché et al., 1999). The rapid loss of ligand from GR has been measured at less than 10 min (Munck and Holbrook, 1984) which does not agree with the kinetics of the loss of receptor from the nucleus (>12h) (Haché et al., 1999). This suggests that nuclear export of the receptor does not depend solely on the dissociation of ligand.

Therefore, it seems unlikely that the accelerated nuclear export displayed by GFP-$GR_{LL687, 688AA}$, GFP-$GR_{LL689, 690AA}$, GFP-GR_{L687A}, GFP-GR_{L688A}, GFP-GR_{L689A} and GFP-GR_{L690A} is due to increased steroid off-rate. Furthermore, GFP-GR_{C661S} which shows reduced affinity for hormone, exports from the nucleus at a rate comparable to that of the wild-type receptor (see Table 2). Collectively, these findings suggest that accelerated nuclear export is not simply a reflection of reduced affinity for ligand.

LL687, 688AA has no effect on the rate of nuclear export of the RU486-withdrawn receptor

RU486 is a powerful type II antagonist of glucocorticoid signalling (Baulieu, 1994), better known for its anti-progestin properties and its application as a contraceptive and abortifacient agent (Spitz and Bardin, 1993). Nuclear transfer of RU486-bound GR occurs more slowly than nuclear uptake of Dex-bound GR ($t_{1/2}$ = 9-10 min vs $t_{1/2}$ = 4-5 min) (Haché et al., 1999). Interestingly, several *in vitro* studies have demonstrated that, once bound to RU486, GR dissociates from heat shock proteins with reduced efficiency (Mao et al., 1992; Moguilewsky and Philibert, 1984; Distelhorst and Howard, 1990). In fact, it is believed that the slower nuclear uptake of RU486-bound GR is a reflection of this decreased rate of dissociation rather than differences in nuclear import (Distelhorst and Howard, 1990). Intriguingly, upon withdrawal of RU486, GR redistributes inefficiently to the cytoplasm (Qi et al., 1990; Haché et al.,1999). Furthermore, prolonged nuclear occupancy of RU486-withdrawn GR does not reflect the presence of residual ligand or differences in ligand affinity as a GR mutant with a 10-fold lower affinity for ligand responds to the withdrawal of RU486 and cortisol in a manner identical to the wild-type receptor (Sackey et al., 1996).

Several scenarios have been put forth to explain the prolonged nuclear localization of GR following RU486 withdrawal. It has been suggested that the region of GR responsible for mediating nuclear export is protected when GR is bound to RU486 and is therefore, not able to efficiently bind exportin (Haché et al., 1999). Inability to bind exportin would ultimately result in permanent localization of GR within the nucleus as is observed following withdrawal from RU486. Alternatively, it is possible that RU486-withdrawn GR may associate into hsp-containing complexes whose components vary from other steroid-withdrawn GRs. Perhaps the composition of the RU486-withdrawan GR-hsp complex may vary in a manner that limits relocalization to the cytoplasm. A third possibility is that phosphorylation may play a role in the persistent localization of RU486-withdrawn GR. Several studies have shown that GR is differentially hyperphosphorylated in response to RU486 compared with cortisol (Orti et al., 1989; Hoeck et al., 1989). Interestingly, it has been shown that phosphorylation of ERα at Thr^{311} in response to estrogens by the signalling pathway mediated through the p38 MAKP blocks receptor nuclear export (Lee and Bai, 2002). It remains unclear, however, whether the effect of Thr^{311} phosphorylation on ERα nuclear export is due to an alteration in the function of a NES or whether it affects a NES-independent mechanism (Lee and Bai, 2002). Perhaps GR nuclear export is blocked through phosphorylation of the receptor in response to RU486. This would account for the prolonged nuclear localization of GR following withdrawal from RU486.

Considering GFP-$GR_{LL687,\ 688AA}$ displayed trafficking behaviour similar to that of the wild-type receptor following withdrawal from RU486, it seems as though mutation of leucines 687 and 688 to alanines has no effect on nuclear export of the RU486-withdrawn receptor. As the molecular basis for the persistent nuclear localization of RU486-withdrawn

GR remains unknown, all that can be concluded is that the mechanism(s) responsible for prolonged nuclear occupancy of RU486-withdrawn GR remain(s) unaffected by the LL687, 688AA mutation.

Nuclear retention of GR is potentially mediated through an NRS in the LBD of GR

The slow rate of GR nuclear export following ligand withdrawal is partially due to active retention (Carrigan et al., 2007). Active retention of GR within the nucleus has been proposed to occur through a transient interaction rather than sequestration within a nuclear subdomain as GR is relatively mobile within the nucleus (McNally et al., 2000; Schaaf and Cidlowski, 2003). Recently it has been determined that the NL1 motif of GR can suppress nuclear export mediated by the HIV Rev NES and, therefore functions as a nuclear retention sequence (Walther et al., 2003). Presently, the role that nuclear retention plays in GR function remains unknown. One theory is that retention of GR in the nucleus serves to protect the receptor from degradation which preferentially occurs in the cytoplasm (Liu and DeFranco, 2000). Protection from degradation, however, would not be complete as several studies have reported ligand-induced degradation of GR (Sengupta et al., 2001; Wallace and Cidlowski, 2001)

The observation that a NLS can participate in nuclear retention (Walther et al., 2003; Carrigan et al., 2007) suggests that the 687-690 region of GR, which appears to play a role in regulating the subcellular trafficking of GR, could also be involved in mediating nuclear retention. FRAP studies show that GFP-NLS-NES-556C, a construct in which the SV40 NLS and CRM1 NES are fused to the LBD of GR, is actively retained within the nucleus of cells (Walther, unpublished data). This result, although preliminary, suggests that the LBD

of GR may harbour NRS activity potent enough to overcome a strong CRM1-dependent NES. While GFP-GR_{NL1} redistributes to the cytoplasm following ligand withdrawal at a rate that is much faster than GFP-GR_{wt}, it does not become fully cytoplasmic until 4 h post-withdrawal (data not shown). This observation supports the existence of an additional NRS in GR.

At this time, it is unclear how GR is retained within the nucleus. Recently, it has been suggested that protein-protein interactions may be required for nuclear retention of SHRs (Baumann et al., 2001). As mentioned earlier, leucine rich sequences like the 687-690 region in the LBD of GR appear to be involved in protein-protein interactions (Dong et al., 2006). Several transcriptional coactivators like SRC-1 (Saitoh et al., 2002; Stenoien et al., 2001), GRIP-1 (Baumann et al., 2001), CBP (Saitoh et al., 2002; Stenoien et al., 2001) and BRG-1 (Grande et al., 1997) have been shown to colocalize with GR in the nucleus. However, whether these proteins are involved in retaining GR within the nucleus remains unknown. Results presented in this thesis show that nuclear export of GR is accelerated upon substitution of leucines with alanines in the 687-690 region (see Figures 10 and 13). If this region were involved in mediating interactions with a protein required for nuclear retention, it would be expected that upon mutation of this region nuclear retention activity would be abolished and accelerated nuclear export of the receptor would be observed.

An alternative possibility is that GR is retained within the nucleus through association with the nuclear matrix. Previous studies have shown that nuclear receptors interact with components of the nuclear matrix and the nuclear lamina in a NL1-dependent manner (Savory, Sackey and Lefevbre, unpublished results). Although the concept of a nuclear matrix remains controversial (Pederson, 2000), the existence of this relatively immobile nuclear structure is accepted by many researchers (Nickerson, 2001). GR has been

shown to be present in nuclear matrix preparations in several studies and it appears as though the DBD and LBD of GR are required for GR-nuclear matrix association (van Steensel et al., 1995; Tang et al., 1998; Tang and DeFranco, 1996). If nuclear retention of GR were mediated through interactions between the 687-690 region and the nuclear matrix, it would be expected that mutation of residues within this region would alleviate receptor retention. This could result in accelerated nuclear export of GR in a manner similar to what was observed in this thesis.

L687 and L689 are involved in steroid-dependent nuclear localization of GR

Steroidal signalling of GR is dependent on the association of the naïve receptor with molecular chaperones and trafficking between the nucleus and cytoplasm (Pratt and Toft, 2003; Pratt et al., 2004; Pratt et al., 2006). While examining the properties of the novel GR LBD mutants, it was revealed that mutations L687A, L688A, L689A and L690A partially negate the ability of GR to translocate into the nucleus upon stimulation with low levels of cortisol (see Figure 14 inset). This is highlighted best when analyzing the subcellular distribution of the constructs after stimulation with 10^{-8}M cortisol for 1 h (see Figure 14). Two mutants in particular, GFP-GR_{L687A} and GFP-GR_{L689A} are severely compromised in their ability to import into the nucleus in response to low levels of cortisol. These mutants require considerably higher concentrations of hormone to induce the same level of nuclear occupancy as the wild-type receptor. Surprisingly, GFP-GR_{L687A} and GFP-GR_{L689A} have binding affinities only 1.78 and 2.17 fold lower than that of GFP-GR_{wt} when measure *in vitro* (due to 1.78 and 2.17 fold higher dissociation constants (see Table 2)). Therefore, the L687A and L689A mutations do not affect the receptor's affinity *in vitro* for steroid to an

extent that could account for the drastic decrease in the efficiency of ligand-induced nuclear translocation. While the L687A and L689A mutations do not affect the receptor's affinity for Dex *in vitro*, the effect these mutations have on steroid binding affinities *in vivo* remains unknown. Future experiments will focus on measuring the K_ds of the various GR mutants via an *in vivo* binding assay. This will be essential, as *in vitro* results do not always reflect what occurs *in vivo*.

Reduced nuclear accumulation of GFP-GR$_{L687A}$ in response to low levels of cortisol is not due to a defect in hsp70/hsp90 association

As mentioned, unliganded, cytoplasmic GR that is competent to bind hormone exists as a heteromeric complex that contains a dimer of hsp90. *In vitro*, heterocomplex formation can be achieved with only five proteins, hsp90, hsp70, Hop, hsp40 and p23. *In vivo*, the heterocomplex contains additional factors including the immunophilins FKBP51, FKBP52 and Cyp40 (Pratt and Toft, 1997; Carello et al., 2004). These factors are likely involved in the maturation of the receptor to its hormone-binding conformation (Pratt and Toft, 1997). Important to note is the fact that the assembly of steroid receptor heteromeric complexes is a dynamic process. This was first shown for PR *in vitro*, in which association between the receptor and chaperones is transient, even in the absence of hormone (Smith, 1993). This suggests that individual components of the complex are likely to be constantly turning over. As a result, signal sequences required for nuclear import may be transiently exposed to transport proteins, even when the receptor is not bound by hormone.

Until recently, it has been accepted that, after steroid binding, the hsp90-heterocomplex bound to GR must dissociate from the receptor (a process called

"transformation"), in order to trigger nuclear translocation of the cytoplasmic receptor. Recently, evidence has been put forth that conflicts with this conventional view. Firstly, studies have shown that binding of GR to the hsp90-immunophilin heterocomplex is required for the movement of the receptor to the nucleus (Galigniana et al., 2001; Galigniana et al., 2004; Pratt et al., 2004), which appears to be powered by dyenin (Davies et al., 2002; Harell et al., 2002; Wochnik et al., 2005). Studies have shown that movement of the receptor through the cytoplasm towards the nucleus is disrupted through treatment with geldanamycin, an inhibitor of hsp90 which promotes the dissociation of the GR-hsp90-immunophilin machinery and the immunophilins-dyenin interaction (Galigniana et al., 2001; Galiganiana et al., 2004; Pratt et al., 2004; Davies et al., 2002; Harrell et al., 2002; Wochnik et al., 2005; Galigniana et al., 1998). Secondly, there is evidence that hsp90 can be cotransported with GR into the nucleus, keeping the nonliganded receptor inactive yet able to participate in transcriptional activation (Kang et al., 1995). Thirdly, hsp90 can be recovered bound to MR, a close relative of GR, immediately after nuclear translocation of the receptor, suggesting that the complex remains intact while in the nucleus (Piwien Pilipuk et al., 2007).

Studies have determined that pharmacological inhibition of hsp90 (Whitesell et al., 1994) delays nuclear translocation of ligand-bound GR (Czar et al., 1997). In order to determine whether GFP-GR_{L687A} and GFP-GR_{L689A} are unable to efficiently translocate into the nucleus upon stimulation with low levels of cortisol because of a defect in hsp association, coimmunoprecipitation assays were conducted. In these assays, GFP-GR_{L687A} and GFP-GR_{wt} were assessed for their ability to associate, *in vitro*, with hsp90 and hsp70 in the absence and presence of 10^{-6}M Dex. Upon analysis of the results, it was revealed that GFP-GR_{L687A} associates with both hsp90 and hsp70 in the presence and absence of 10^{-6}M

Dex (see Figure 15). This finding supports the hypothesis that ligand-bound GR remains associated with hsp90 and hsp70. Since GFP-GR_{L687A} is able to import into the nucleus upon stimulation with 10^{-6}M cortisol, it is not surprising that this mutant is able to bind hsp90. If the L687A mutation were to disrupt association between GR and hsp90, it would be expected that the resulting mutant construct would be unable to bind steroid. This is because association between GR and hsp90 is essential for normal hormone/receptor interaction (Bamberger et al., 1996).

The role hsp70 plays in nuclear import remains controversial. Independent studies using both *in vitro* and *in vivo* assays implicate hsp70 in nuclear import (Shi and Thomas, 1992; Imamoto et al., 1992). In fact, biochemical studies show direct binding of hsp70 to nuclear import signal sequences (Imamoto et al., 1992). It has also been determined that hsp70 stimulates NLS-directed nuclear transport in yeast (Shulga et al., 1996; Shulga et al., 1999). While various studies implicate hsp70 in nuclear import, others suggest that it does not play a role in this process (Yang and DeFranco, 1994). Either way, the observation that GFP-GR_{L687A} is able to associate with hsp70 in the presence and absence of hormone indicates that reduced nuclear accumulation of this mutant in the presence of low levels of cortisol is not due to a defect in hsp70 association.

While it has been confirmed that reduced nuclear accumulation of GFP-GR_{L687A} in response to low levels of cortisol is not due to a defect in hsp90 and hsp70 association, it remains unknown whether immunophilins, like FKBP51 and FKBP52 associate with this mutant. Several studies suggest that exchange between FKBP51 and FKBP52 is required for the import of GR into the nucleus (Harrell et al., 2004; Galigniana et al., 2002; Galigniana et al., 2001; Davies et al., 2002). FKBP52 has recently been shown to be involved in the

nuclear transport of p53 and has been shown to bind to hsp90, GR, as well as dynein through dynamitin (Galigniana et al., 2004; Pratt et al., 1999). Attempts in the Haché lab to coimmunoprecipitate GFP-GR_{L687A} and GFP-GR_{wt} with FKBP51 and FKBP52 were unsuccessful. Antibodies for these low molecular weight immunophilins are of poor quality and, as a result, their detection via Western blot is challenging.

L687 and L689 are required for efficient NL2 activity

A common feature of most members of the steroid receptor subfamily, NL1 is a strong NLS that extends beyond the C-terminus end of the DBD (LeCasse and Lefebvre, 1995; Tang et al., 1997). In addition to NL1, most steroid receptors possess a second less well characterized motif called NL2. As previously mentioned, NL2 is a steroid-dependent nuclear localization activity that complements NL1 in mediating GR nuclear uptake (Picard and Yamamoto, 1987). Interestingly, it has been determined that the NL2 activity of GR is unresponsive to treatment with the antagonist RU486 (Savory et al., 1995). NL2 activity has been described in SHRs other than GR including PR (Li et al., 2005) as well as MR (Piwien Pilipuk et al., 2007). While NL2 activity has been localized to the LBD of GR, defining the NL2 sequence has proven challenging as NL2 activity does not appear to be regulated by a basic NLS motif.

As all GR mutants tested, thus far had intact NL1, and their affinity for steroid changed only modestly, it then became of interest to determine whether the NL2 activity of these receptors was affected. This was accomplished by mutating individual leucines to alanines in the 687-690 region of GFP-GR_{NL1-}, a construct containing the NL1 abrogating substitution K513-515N. After analyzing the subcellular localization of the various GFP-

GR_{NL1}- mutant constructs, it was revealed that two mutations, L687A and L689A, severely compromise the nuclear uptake of GFP-GR_{NL1}- (see Figure 17). Interestingly, mutations L687A and L689A were also the most potent at disrupting nuclear import of GFP-GR_{wt} (see Figure 14). By contrast, the mutation L690A, which has a modest effect on the nuclear import of GFP-GR_{wt}, only modestly affected NL2 activity.

As mentioned earlier, NL2 activity does not appear to be dependent on a basic motif and the identity of the karyopherin responsible for mediating nuclear import through NL2 remains unknown. While studies have excluded importin α as the karyopherin responsible for mediating NL2 nuclear import (Savory et al., 1999), the possibility remains that NL2-mediated nuclear import of GR occurs through an importin β-related receptor. These receptors recognize cargo molecules, interact with NPCs, and circulate between the nucleus and the cytoplasm (Mingot et al., 2001). According to the direction in which they carry a cargo, importin β-related receptors can be classified as importins or exportins (Ribbeck and Gorlich, 2001). Some importin β-related receptors mediate both nuclear import and nuclear export. One such receptor is importin 13, a family member from higher eukaryotes that functions in import and export (Mingot et al., 2001). Importin 13 mediates nuclear import of the RBM8-MGN complex and of hUBC9 (Mingot et al., 2001). It also mediates the nuclear export of eIF1a (Mingot et al., 2001). As discussed earlier, the 687-690 region of GR appears to be involved in the nuclear export of GR. Through localization assays, it also appears as though residues within this region (L687 and L689) are involved in mediating NL2 activity. While NLSs have generally not been found to participate in nuclear export (Michael et al., 1995; Schmidt-Zachmann et al., 1993; Shulga et al., 1996) the possibility that a bidirectional signal resides within the 687-690 region is intriguing.

GFP-GR_{L687A} and GFP-GR_{L689A} have intact transcriptional activity

The ability of GR to induce transcription hinges on several unique but sequential steps including ligand binding, activation, nuclear translocation, binding to DNA and interaction with transcriptional machinery (Huang et al., 1999). To investigate what effect the L687A and L689A mutations have on transcriptional activity, the transactivational potentials of GFP-GR_{L687A} and GFP-GR_{L689A} were assessed at 10^{-8}M and 10^{-6}M hormone. Results presented in this thesis indicate that the NL2 disrupting mutations, L687A and L689A, present no significant effect on the transactivational potential of the receptor at 10^{-6}M Dex (see Figure 19), a concentration of hormone that facilitates complete nuclear localization of the receptors (see Figure 14 inset). As anticipated, at 10^{-8}M Dex, a concentration of hormone that facilitates only partial nuclear localization of GFP-GR_{L687A} and GFP-GR_{L689A}, both mutant receptors displayed transcriptional activity significantly lower than that of GFP-GR_{wt} (See Figure 19). This result was expected considering nuclear localization of cytoplasmic GR is a pre-requisite for transcription (Bamberger et al., 1996). Based on transcription assay results it can be concluded that the L687A and L689A mutations have little effect on the overall glucocorticoid induciblity of the MMTV promoter in Cos7 cells treated with 10^{-6}M Dex.

NL2 plays an important role in the steroid responsiveness of GR in vivo

Steroid hormones are released into the bloodstream from the adrenal cortex and gonads and transduce signals by interacting with intracellular receptors in target tissues (Meijsing et al., 2007). Serum levels of circulating glucocorticoids fluctuate about three- to fivefold over 2 h to 4 h periods throughout the day. Local availability of hormone at target

organs may display even greater and more rapid fluctuations (Meijing et al., 2007). The observation that nuclear import of GRs compromised in NL2 activity (with intact NL1 activity) is dramatically reduced at physiological concentrations of steroid (see Figure 14 inset) has striking implications for the role NL2 may play in mediating nuclear import of GR. These results suggest that NL2 may be as important, or more important, than NL1 in mediating steroid responsiveness of GR in response to physiological concentrations of steroid. Furthermore, the observation that, in the presence of low levels of hormone, GFP-GR_{NL1}- becomes increasingly nuclear over time suggests that NL2-mediated nuclear import is a slow process (see Figure 18).

Phosphorylation at S691 and S692 has the potential to dramatically reduce receptor sensitivity to steroid

The observation that GFP-$GR_{SS691,\ 692DD}$ is unable to efficiently translocate into the nucleus of cells upon stimulation with 10^{-6}M cortisol suggests that mutation of serines 691 and 692 to aspartic acids dramatically affects receptor subcellular trafficking in response to steroid. It should be noted that the binding capacity of GFP-$GR_{SS691,\ 692DD}$ for steroid was extremely low (data not shown). As a result, the dissociation constant for this construct could not be calculated due to the limited sensitivity of the *in vitro* ^{3}H Dex binding assay. The low binding capacity of GFP-$GR_{SS691,\ 692DD}$ for steroid suggests that mutation of serines 691 and 692 to aspartic acids disrupts steroid binding. Even though S691 and S692 do not directly participate in ligand binding (see Figure 8), changing the charge of these residues could indirectly result in a conformational change of the binding pocket. A change in

receptor LBD conformation could account for the reduced affinity for ligand displayed by GFP-$GR_{SS691, 692DD}$.

Interestingly, it has been suggested that dissociation of the hsp90 oligomeric complex results in a decrease of ligand-binding affinity (Bresnick et al., 1989; Nemoto et al., 1990). Also, *in vivo* experiments performed by Yang and colleagues have demonstrated that stabilization of GR/hsp90 complexes in live cells severely restricts hormone-dependent nuclear import of the receptor (Yang and DeFranco,1996). If receptor phosphorylation at S691 and S692 were to stabilize the interaction between GR and hsp90, reduced nuclear translocation in response to hormone could be expected. Alternatively, phosphorylation of S691 and S692 could destabilize the interaction between GR and hsp90. This could result in reduced affinity for hormone considering the interaction of hsp90 with the LBD of GR is required for this domain to assume an appropriate ligand-binding conformation (Bresnick et al., 1989).

SUMMARY AND CONCLUSIONS

Results presented in this thesis provide new insight on the signal-regulated processes of GR nuclear export and NL1-independent nuclear localization. It was revealed through hormone-withdrawal assays that substitution of leucines 687, 688 and 689, 690 to alanines in the LBD of GR accelerates the nuclear export-rate of the receptor in a CRM1-independent manner. This suggests that leucines in the 687-690 region may be critical for maintaining nuclear localization of GR following hormone withdrawal. It was also demonstrated through hormone withdrawal assays that accelerated nuclear export of GFP-$GR_{LL687,\ 688AA}$ occurs following withdrawal from cortisol but not RU486. This suggests that leucines in the 687-690 region may contribute to nuclear localization of GR in an agonist specific manner.

A more detailed examination of individual leucines within the 687-690 region showed that L687 and L690 are critical for the slow redistribution of GR to the cytoplasm following cortisol withdrawal. Furthermore, through *in vitro* steroid binding assays and *in vitro* steroid dissociation assays it was determined that substitution of individual leucines to alanines in the 687-690 cluster has only minor effects on receptor K_d and steroid off-rate. Therefore, it can be concluded that the accelerated nuclear export-rates exhibited by GFP-GR_{L687A} and GFP-GR_{L690A} cannot be attributed to reduced ligand affinity nor differences in steroid off-rates.

Interestingly, localization assays presented in this thesis suggest that residues within the 687-690 cluster are involved in steroid-dependent nuclear localization of GR. Coimmunoprecipitation of GFP-GR_{L687A} with hsp90 and hsp70 revealed that the reduced nuclear accumulation of the leucine to alanine substitution mutants in response to low doses of cortisol is not due to a defect in hsp association. Another observation made in this thesis

is that the L687A and L689A substitutions severely compromise GR_{NL1-} nuclear import in response to low doses of cortisol, indicating that mutations L687A and L689A partially abrogate NL2 activity. Moreover, transcription results indicate that the L687A and L689A mutations have little effect on the overall glucocorticoid induciblity of the MMTV promoter in Cos7 cells treated with 10^{-6}M Dex.

In this thesis it is shown that NL2-mediated nuclear import plays a critical role in nuclear localization of GR in response to physiological concentrations of cortisol. This statement is founded on two independent observations. Firstly, at physiological concentration of steroid, nuclear import of NL1 intact GFP-GR_{wt} is compromised upon mutation of leucines 687 and 690 to alanines. Secondly, GFP-GR_{NL1-} does not become appreciably nuclear until stimulation with 10^{-7}M cortisol for 4 h. Furthermore, the observation that GFP-GR_{NL1-} continues to accumulate in the nucleus of cells treated with low doses of cortisol over a period of 16 h suggests that NL2-mediated nuclear import is a slow process.

In this thesis it was also revealed that GFP-$GR_{SS691,\ 692DD}$ is unable to efficiently translocate into the nucleus of cells upon stimulation with 10^{-6}M cortisol for 1 h. By contrast, GFP-$GR_{SS691,\ 692AA}$ displays translocation similar to the wild-type receptor. This observation suggests that phosphorylation of GR at 691 and 692 has the potential to dramatically reduce receptor sensitivity to steroid.

It is apparent that GR subcellular localization is regulated in a complex manner and at many different levels. In order to fully understand the signal transduction capacity of GR, a more in-depth understanding of the signals regulating receptor nuclear import and nuclear export is required. It is anticipated that future studies focussing on NL2-mediated nuclear

import and nuclear export of GR will provide additional insight into the identities of the signal sequences that mediate these processes. In summary, results presented in this thesis indicate that the 687-690 region of GR plays an important role in GR nucleocytoplasmic trafficking. This discovery will in turn allow for more focused research on the mechanisms that regulate GR subcellular localization.

APPENDIX A: OLIGONUCLEOTIDE SEQUENCES

Below is a table containing all of the oligonucleotide sequences used to generate plasmids. All oligonucleotide sequences are written in the 5' to 3' orientation. Nucleotides that introduce mutations are written in bold type.

Plasmid	Primer Name	Primer Sequence
pGFP-$GR_{SS691,692DD}$	NES2 691, 2 SS-DD up	CTG TAT GAA AAC CTT ACT GCT TCT C**GA** C**GA** TGT TCC TAA GGA AGG TCT G
	NES2 691,2 SS-DD dn	CAG ACC TTC CTT AGG AAC A**TC** G**TC** GAG AAG CAG TAA GGT TTT CAT ACA G
pGFP-GR_{L626A}	NES1 626 L-A up	CTC ATG GCA TTT GCC **GC**G GGT TGG AGA TCA TAC AG
	NES1 626 L-A up	CTG TAT GAT CTC CAA CCC **GC**G GCA AAT GCC ATG AG
pGFP-GR_{L682A}	NES2 682 L-A up	GGT ATC CTA TGA AGA GTA T**GC** CTG TAT GAA AAC CTT ACT GC
	NES2 682 L-A dn	GCA GTA AGG TTT TCA TAC AG**G** **C**AT ACT CTT CAT AGG ATA CC
pGFP-GR_{M684A}	NES2 684 M-A up	CCT ATG AAG AGT ATC TCT GT**G** **G**GA AAA CCT TAC TGC TTC TCT CC
	NES2 684 M-A dn	GGA GAG AAG CAG TAA GGT TTT C**GC** ACA GAG ATA CTC TTC ATA GG
pGFP-GR_{L687A}	687 L-A up	GTA TGA AAA CC**T** **T**AC TGG CTC TCT CCT CAG TTC CTA AGG
	687 L-A dn	CTG AGG AGA GAA GCA GT**G** **C**GG TTT TCA TAC AGA GAT ACT C
pGFP-GR_{L688A}	688 L-A up	GAG TAT CTC TGT ATG AAA ACC TTA **GC**G CTT CTC TCC TCA G
	688 L-A dn	CTG AGG AGA GAA GC**G** **C**TA AGG TTT TCA TAC AGA GAT ACT C
pGFP-GR_{L689A}	689 L-A up	GTA TGA AAA CCT TAC TG**G** **C**TC TCT CCT CAG TTC CTA AGG
	689 L-A dn	CCT TAG GAA CTG AGG AGA GA**G** **C**CA GTA AGG TTT TCA TAC
pGFP-GR_{L690A}	690 L-A up	GTA TGA AAA CCT TAC TGC TT**G** **C**CT CCT CAG TTC CTA AGG
	690 L-A dn	CCT TAG GAA CTG AGG AG**G** **C**AA GCA GTA AGG TTT TCA TAC
pGFP-$GR_{LLSS689,690,691,692AAAA}$	LLSS-AAAA up	CTG TAT GAA AAC CTT ACT G**GC** T**GC** C**GC** C**GC** AGT TCC TAA GGA AGG
	LLSS-AAAA dn	CCT TCC TTA GGA ACT G**C**G G**C**G **GCA GC**C AGT AAG GTT TTC ATA CAG
pGFP-GR_{S691D}	NES2 691 S-D up	CTG TAT GAA AAC CTT ACT GCT TCT C**GA** CTC AGT TCC TAA GGA AGG TCT G

	NES2 691 S-D dn	CAG ACC TTC CTT AGG AAC TGA GTC GAG AAG CAG TAA GGT TTT CAT ACA G
pGFP-GR_{S692D}	NES2 692 S-D up	CTG TAT GAA AAC CTT ACT GCT TCT CTC C**GA T**GT TCC TAA GGA AGG TCT G
	NES2 692 S-D dn	CAG ACC TTC CTT AGG AAC **ATC** GGA GAG AAG CAG TAA GGT TTT CAT ACA G
pGFP-$GR_{SS691,692DD}$	NES2 691,2 SS-DD up	CTG TAT GAA AAC CTT ACT GCT TCT C**GA** CG**A** TGT TCC TAA GGA AGG TCT G
	NES2 691,2 SS-DD dn	CAG ACC TTC CTT AGG AAC A**TC** G**TC** GAG AAG CAG TAA GGT TTT CAT ACA G
pGFP-$GR_{LL671,\ 674AA}$	NES2 671,4 LL-AA up	CTG TTT GTC GTC TCC TCT GAA **GC**A CAA AGA **GC**G CAG GTA TCC TAT GAA GAG
	NES2 671,4 LL-AA dn	CTC TTC ATA GGA TAC CTG C**GC** TCT TTG T**GC** TTC AGA GGA GAC GAC AAA CAG
pGFP-GR_{L626A}	NES1 626 L-A up	GAT CTC TCA TGG CAT TTG CC**G** **C**GG GTT GGA GAT CAT ACA GAC AAT CAA GTG GAA ACC TGC TCT GCT TTG CTC CTG ATG GTA C
	NES1 626 L-A dn	CAT CAG GAG CAA AGC AGA GCA GGT TTC CAC TTG ATT GTC TGT ATG ATC TCC AAC CC**G** **C**GG CAA ATG CCA TGA GA
pGFP-GR_{L671A}	NES2 671 L-A up	GAT CTG TCT CCT CTG AA**G** **C**AC AAA GAT TGC AGG TAT CCT ATG AAG AGT ATC TCT GTA TGA AAA CCT TAC TGC TTC TCT CCT CAG TTC CTG GTA C
	NES2 671 L-A dn	CAG GAA CTG AGG AGA GAA GCA GTA AGG TTT TCA TAC AGA GAT ACT CTT CAT AGG ATA CCT GCA ATC TTT G**TG** **C**TT CAG AGG AGA CA
pGFP-$GR_{SS691,692AA}$	NES2 691 2 SS-AA up	GAT CTG TCT CCT CTG AAT TAC AAA GAT TGC AGG TAT CCT ATG AAG AGT ATC TCT GTA TGA AAA CCT TAC TGC TTC TC**G** CC**G** CAG TTC CTG GTA C
	NES2 691 2 SS-AA dn	CAG GAA CTG **C**GG **C**GA GAA GCA GTA AGG TTT TCA TAC AGA GAT ACT CTT CAT AGG ATA CCT GCA ATC TTT GTA ATT CAG AGG AGA CA
pGFP-$GR_{LL687,688AA}$	NES2 687,8 LL-AA up	CTG TAT GAA AAC C**GC** **CGC** **A**CT TCT CTC CGA TGT TCC TAA GGA AGG TCT G
	NES2 687,8 LL-AA dn	CAG ACC TTC CTT AGG AAC ATC GGA GAG AAG **TGC GGC** GGT TTT CAT ACA G

APPENDIX B: SOURCES OF CHEMICALS, REAGENTS, & OTHER MATERIALS

Chemical/Reagent/Material	Source
anti-hsp70 primary antibody	Stressgen, Ann Arbor, Michigan, USA
anti-hsp90 primary antibody	Stressgen, Ann Arbor, Michigan, USA
β actin primary antibody	Sigma, Missisauga, ON
betamercaptoethanol	Sigma, Missisauga, ON
Bio-Rad Protein Assay Dye Reagent	Bio-Rad, Mississauga, ON, Canada
BuGR primary antibody	Affinity BioReagents, Golden CO, USA
Charcoal Stripped Fetal Bovine Serum (SFBS)	HyClone, Logan, UT, USA
Complete™ protease inhibitor cocktail	Roche, Lavalle, QC, Canada
Cortisol	Sigma, Missisauga, ON
Dexamethasone	Sigma, Missisauga, ON
[^{3}H]Dexamethasone	GE Healthcare Limited, Buckinghamshire, UK
dextran-coated charcoal	Sigma, Missisauga, ON
DTT	Sigma, Missisauga, ON
Dulbecco's Modified Eagle's Medium (DMEM)	Invitrogen, Burlington, ON, Canada
EDTA	EMD Biosciences, Gibbstown, NJ, USA
Fetal Bovine Serum (FBS)	HyClone, Logan, UT, USA
FuGene 6™ transfection reagent	Roche, Lavalle, QC, Canada
GFP JL-8 primary antibody	Clontech, Mississauga, ON, Canada
glycerol	EMD Biosciences, Gibbstown, NJ, USA
glycine	EMD Biosciences, Gibbstown, NJ, USA

goat anti-rat secondary antibody conjugated to horseradish peroxidase	GE Healthcare Limited, Buckinghamshire, UK
GRM20 primary antibody	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Immuno-Blot PVDF membrane	Bio-Rad, Mississauga, ON, Canada
Leptomycin B (LMB)	Sigma, Missisauga, ON
Lipofectamine™ transfection reagent	Invitrogen, Burlington, ON, Canada
methanol	Fisher Scientific, Ottawa, ON, Canada
NaCl	EMD, Biosciences, Gibbstown, NJ, USA
non-essential amino acids	Invitrogen, Burlington, ON, Canada
NP-40	EMD Biosciences, Gibbstown, NJ, USA
Optimem™ reduced serum media	Invitrogen, Burlington, ON, Canada
paraformaldehyde	EMD Biosciences, Gibbstown, NJ, USA
passive lysis buffer	Promega, Madison, WC, USA
Pfu proofreading enzyme	Stratagene, La Jolla, CA, USA
phenol-red free Dulbecco's Modified Eagle's Medium (DMEM)	Invitrogen, Burlington, ON, Canada
protein A sepharose	Invitrogen, Burlington, ON, Canada
Qiagen Maxiprep kit	Qiagen, Mississauga, ON, Canada
Qiagen Miniprep kit	Qiagen, Mississauga, ON, Canada
RU486	Roussel-Uclaf, France
scintillation fluid	GE Healthcare Limited, Buckinghamshire, UK
SDS	EMD Biosciences, Gibbstown, NJ, USA

sheep anti-mouse secondary antibody conjugated to horseradish peroxidase	GE Healthcare Limited, Buckinghamshire, UK
sodium molybdate	Sigma, Missisauga, ON
sodium pyruvate	Invitrogen, Burlington, ON, Canada
Stratagen QuikChange mutagenesis kit	Stratagene, La Jolla, CA, USA
tissue culture grade BSA	Sigma, Missisauga, ON
TRIS-Base	EMD Biosciences, Gibbstown, NJ, USA
Triton X-100	EMD Biosciences, Gibbstown, NJ, USA
Tween	EMD Biosciences, Gibbstown, NJ, USA
Vectashield DAPI staining solution	Vector Laboratories, Burlington, ON, Canada
Western Lightening Chemiluminescence Reagent	PerkinElmer Life Sciences Inc, Boston, MA, USA

APPENDIX C: STEROID DISSOCIATION AND STEROID OFF-RATE DATA ANALYSIS

Steroid Dissociation

1. Eliminate non-specific counts by subtracting the sample mean obtained using untransfected cells from each sample mean.

L690A	#1 (DPM)	#2 (DPM)	Avg. (DPM)	Avg. untrans. (DPM)	Avg. – Avg. untrans. (DPM)
100 nM	9924	9888	9906	2045	7861
46.4 nM	8380	8290	8335	953	7382
21.5 nM	6711	6913	6812	508	6304
10.0 nM	4766	5378	5072	310	4762
4.64 nM	3099	3079	3089	212	2877
2.15 nM	1532	1468	1500	130	1370
1.00 nM	766	776	771	86	685
0.46 nM	350	347	349	53	296

2. Convert data from DPM to concentration of bound ^{3}H Dex.

	#1 (DPM)	#2 (DPM)	Avg. (DPM)	Avg. to 1 nM (DPM)	Avg. (DPM)
2.15 nM	8956	8961	8959	4167	
1.00 nM	5112	5052	5082	5082	4614
0.46 nM	2147	2124	2136	4592	

Total Bound [^{3}H]	Specifically Bound [^{3}H] (nM)
100 nM	1.70
46.4 nM	1.6
21.5 nM	1.37
10.0 nM	1.0
4.64 nM	0.62
2.15 nM	0.30
1.00 nM	0.15
0.46 nM	0.06

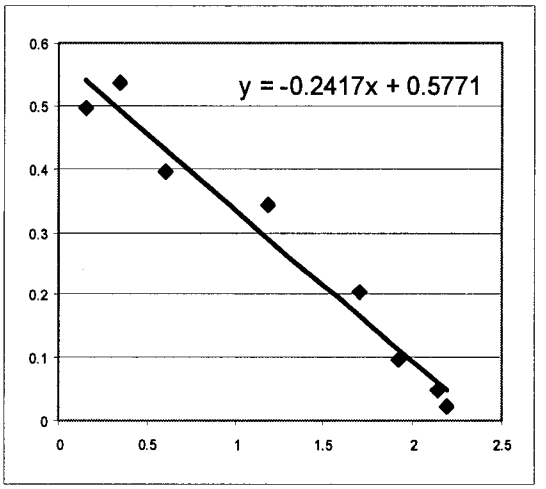
3. Determine concentration of free ^{3}H Dex by subtraction.

Total Bound [^{3}H]	Specifically Bound [^{3}H] (nM)	Free [^{3}H] (nM)
100 nM	1.70	98.3
46.4 nM	1.60	44.8
21.5 nM	1.37	20.1
10.0 nM	1.00	9.00
4.64 nM	0.62	4.02
2.15 nM	0.30	1.85
1.00 nM	0.15	0.85
0.46 nM	0.06	0.40

4. Linearize data by Scatchard Analysis.

$K_d = \frac{k\text{-}1}{k1}$

K_d = -1/slope
= -1/-0.2417
= 4.13 nM



Steroid Off-rate

1. Eliminate non-specific counts by subtracting the sample mean obtained using untransfected cells from each sample mean.

Time (h)	#1 (DPM)	#2 (DPM)	Avg. (DPM)	Avg. untrans. (DPM)	Avg. – Avg. untrans. (DPM)
0.00	537	512	525	145	380
0.25	495	527	511	147	364
0.50	441	481	461	143	318
1.00	348	294	321	158	163
1.50	257	238	248	150	98
2.00	232	191	212	169	43
3.00	180	181	181	143	38
4.00	212	234	223	201	22

2. Convert data from DPM to concentration of bound ^{3}H Dex.

	#1 (DPM)	#2 (DPM)	Avg. (DPM)	Avg. to 1 nM (DPM)	Avg. (DPM)
2.15 nM	6618	6605	6612	3075	
1.00 nM	3076	3074	3075	3075	3072
0.46 nM	1428	1426	1427	3068	

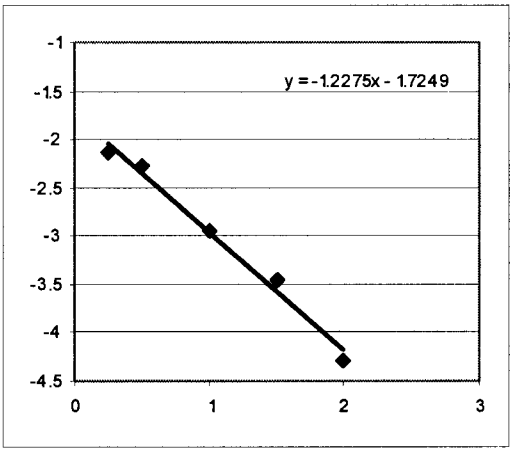
Time (h)	Specifically Bound [^{3}H] (nM)
0.0	0.124
0.25	0.118
0.50	0.104
1.00	0.053
1.50	0.032
2.00	0.014
3.00	0.012
4.00	0.007

3. Plot the natural log of the specific binding vs. time.

Time (h)	In (specifically bound)
0.0	-2.09
0.25	-2.14
0.50	-2.26
1.00	-2.94
1.50	-3.44
2.00	-4.27
3.00	-4.42
4.00	-4.96

$In[A] = In[A]_0 - kt$

$T1/2 = In2/k$
$= 0.693/1.2275$
$= 0.56$ h



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(1) Paper

Providenti, M.A., O'Brien, J.M., **Ewing, R.J.**, Paterson, E.S., and Smith, M.L. (2006). The copy-number of plasmids and other genetic elements can be determined by SYBR-Green-based quantitative real-time PCR. J. microbiol. methods. *65*, 476-487.

(2) Presentations

Ewing, R.J., Atlas, E., and Haché, R.J.G. (2005). Mechanisms of Export of the Glucocorticoid Receptor. The Ottawa Health Research Institute, 5th annual research day, Ottawa, ON.

Ewing, R.J., Addicks, G.C., Atlas, E., and Haché, R.J.G (2006). Analysis of the Subcellular Trafficking of the Glucocorticoid Receptor and Properties of the Ligand Binding Domain. The Ottawa Health Research Institute, 6th annual research day, Ottawa, ON.

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REFERENCES

Alnemri, E.S., Maksymowych, A.B., Robertson, N.M., and Litwack, G. (1991). Overexpression and characterization of the human mineralocorticoid receptor. J. Biol. Chem. *266*, 18072-18081.

Alt, J.R., Cleveland, J.L., Hannink, M., and Diehl, J.A. (2000). Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes Dev. *14*, 3102-3114.

Amazit, L., Alj, Y., Tyagi, R.K., Chauchereau, A., Loosfelt, H., Pichon, C., Pantel, J., Foulon-Guinchard, E., Leclerc, P., Milgrom, E., and Guichon-Mantel, A. (2003). Subcellular localization and mechanisms of trafficking of steroid receptor coactivator-1. J. Biol. Chem. *278*, 32195-32203.

Ashcroft, M., and Vousden, K.H. (1999). Regulation of p53 stability. Oncogene *18*, 7337-7643.

Askjaer, P., Bachi, A., Wilm, M., Bischoff, F., Weeks, D.L., Ogniewski, V., Ohno, M, Niehrs, C., Kjems, J., Mattaj, I.W., and Fornerod, M. (1999). RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase. Mol. Cell Biol. *19*, 6276-6285.

Azuma, Y., and Dasso, M. (2000). The role of Ran in nuclear function. Curr. Opin. Cell Biol. *12*, 302-307.

Bamberger, C.M., Bamberger, A.M., de Castro, M., and Chrousos, G.P. (1995). Glucocorticoid receptor β, a potential endogenous inhibitor of glucocorticoid receptor in humans. J. Clin. Invest. *95*, 2435-2441.

Bamberger, C.M., Schulte, H.M., and Chrousos, G.P. (1996). Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endo. Rev. *17*, 245-261.

Baulieu, E.E. (1994). RU486: A compound that gets itself talked about. Human Reproduction *9*, 1-6.

Baumann, C.T., Maruvada, P., Hager, G.L., and Yen, P.M. (2001). Nuclear cytoplasmic shuttling by thyroid hormone receptors. Multiple protein interactions are required for nuclear retention. J. Biol. Chem. *276*, 11237-11245.

Bayliss, R., Littlewood, T., and Stewart, M. (2000). Structural basis for the interaction between FxFG nucleoporin repeats and importin-beta in nuclear trafficking. Cell *102*, 99-108.

Beato, M., Herrlich, P., and Shutz, G. (1995). Steroid hormone receptors: many actors in search of a plot. Cell *83*, 851-857.

Beck, M., Forster, F., Ecke, M., Plitzko, J.M., Melchior, F., Gerisch, G., Baumeister, W., and Medalia, O. (2004). Nuclear pore structure and dynamics revealed by cryoelectron tomography. Science *306*, 1387-1389.

Begitt, A., Meyer, T., van Rossum, M., and Vinkemeier, U. (2000). Nucleocytoplasmic translocation of Stat1 is regulated by a leucine-rich export signal in the coiled-coil domain. Proc. Natl. Acad. Sci. USA *97*, 10418-10423.

Binart, N., Lombes, M., Rafestin-Oblin, M.E., and Baulieu, E.E. (1991). Characterization of human mineralocorticosteroid receptor expressed in the baculovirus system. Proc. Natl. Acad. Sci. USA *88*, 10681-10685.

Bledsoe, R.K., Montana, V.G., Stanley, T.B., Delves, C.J., Apolito, C.J., McKee, D.D., Consler, T.G., Parks, D.J., Stewart, E.L., Willson, T.M., Lambert, M.H., Moore, J.T., Pearce, K.H., and Xu, H.E. (2002). Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell *110*, 93-105.

Bodwell, J.E., Orti, E., Coull, J.M., Pappin, D.J., Smith L.I., and Sift, F. (1991). Identification of phosphorylated sites in the mouse glucocorticoid receptor. J. Biol. Chem. *266*, 7549-7555.

Bodwell, J.E., Hu, J.M., Orti, E., and Munck, A. (1995). Hormone induced hyperphosphorylation of specific phosphorylated sites in the mouse glucocorticoid receptor. J. Steroid Biochem. Mol. Biol. *52*, 135-140.

Bohl, C.E., Wu, Z., Miller, D.D., Bell, C.E., and Dalton, J.T. (2007). Crystal structure of the T877A humand androgen receptor ligand-binding domain complexed to cyproteone acetate provides insight for ligand-induced conformational changes and structure-based drug design. J. Biol. Chem. *288*, 13648-13655.

Borror, K.C., Garabedian, M.J., DeFranco, D.B. (1995). Glucocorticoid receptor phosphorylation in v-mos-transformed cells. Steroids *60*, 375-382.

Bresnick, E.H., Dalman, F.C., Sanchez, E.R., and Pratt, W.B. (1989). Evidence that the 90-kDa heat shock protein-binding site within the hormone-binding domain of the glucocorticoid receptor by peptide competition. J. Biol. Chem. *264*, 4992-4997.

Brownawell, A.M., and Macara, I.G. (2002). Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. J. Biol. Chem. *156*, 53-64.

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsoon, J.A., and Carlquist, M. (1997). Molecular basis for agonism and antagonism in the oestrogen receptor. Nature *389*, 753-758.

Buckbinder, L., and Robinson, R.P. (2002). The glucocorticoid receptor: molecular mechanisms and new therapeutic opportunities. Curr. Drug Targets *1*, 127-136.

Cadepond, F., Gase, J.M., Delahaye, F., Jibard, N., Schweizer-Groyet, G., Segard-Maurel, I., Evans, R., and Baulieu, E.E. (1992). Hormonal regulation of the nuclear localization signals of the human glucocorticosteroid receptor. Exp. Cell Res. *201*, 99-108.

Carello, A., Allan, R.K., Morgan, S.L., Owen, B.A., Mok, D., Minchin, R.F., Toft, D.O., and Ratajczak, T. (2004). Interaction of the hsp90 cochaperone cyclophilin 40 with hsc70. Cell Stress Chaperones *9*, 167-181.

Carey, K.L., Richards, S.A., Lounsbury, K.M., and Macara, I.G. (1996). Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates an essential function independent of nuclear protein import. J. Cell Biol. *133*, 985-996.

Carrigan, A., Walther, R.F., Salem, H.A., Wu, D., Atlas, E., Lefebvre, Y.A., Haché, R.J.G. (2007). An active nuclear retention signal in the glucocorticoid receptor functions as a strong inducer of transcriptional activity. J. Biol. Chem. *282*, 10963-10971.

Chakraborti, P.K., Garabedian, M.J., Yamamoto, K.R., and Simons, S.S.Jr. (1991). Creation of "super" glucocorticoid receptors by point mutations in the steroid binding domain. J. Biol. Chem. *266*, 22075-22078.

Chan, J.Y., Han, X., and Kan, Y.W. (1993). Proc. Natl. Acad. Sci. USA *90*, 11371-11375.

Chandran, U.R., and DeFranco, D.B. (1992). Internuclear migration of chicken progesterone receptor, but not siman virus-40 large turmor antigen, in transient heterokaryons. Mol. Endo. *6*, 837-844.

Charmandari, E., Kino, T., Ichijo, T., Zachman, K., Alatsatianos, A., and Chrousos, G.P. (2006). Functional characterization of the natural human glucocorticoid receptor (hGR) mutants hGRαR477H and hGRαG679S associated with generalized glucocorticoid resistance. J. Clin. Endo. Metab. *91*, 1535-1543.

Cidlowski, J.A., and Munck, A. (1980). Multiple forms of nuclear binding of glucocorticoid-receptor complexes in rat thymocytes. J. Steroid Biochem. *13*, 105-112.

Cingolani, G., Petosa, C., Weis, K., and Muller, C.W. (1999). Structure of importin-beta bound to the IBB domain of importin-alpha. Nature *399*, 221-229.

Clark, J.K., Schrader, W.T., and O'Malley, B.W. (1992). Mechanism of steroid hormones. Williams textbook of endocrinology, 8th Ed. (Philidephia: WB Saunders Company).

Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998). Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. Cell *94*, 193-204.

Czar, M.J., Galigniana, M.D., Silverstein, A.M., and Pratt, W.B. (1997). Geldanamycin, a heat shock protein 90-binding benzoquinone ansamysin, inhibits steroid-dependent translocation of the glucocorticoid receptor from the cytoplasm to the nucleus. Biochemistry *36*, 7776-7785.

Dalmon-Write, K., Wright, A., Gustafsson, J.A., and Carlstedt-Duke, J. (1991). Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimmer is mediated by a short segment of five amino acids. J. Biol. Chem. *266*, 3107-3112.

Danielson, M., Northrop, J.P., and Ringold, G.M. (1986). The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. EMBO J. *5*, 2513-2522.

Davies, T.H., Ning, Y.M., and Sanchez, E.R. (2002). A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J. Biol. Chem. *277*, 4597-4600.

DeFranco, D.B. (2000). Role of molecular chaperones in subnuclear trafficking of glucocorticoid receptors. Kidney Int. *57*, 1241-1249.

DeFranco, D.B. (2002). Functional implication of glucocorticoid receptor trafficking. Ernst Schering Res. Found. Workshop *40*, 91-109.

DeFranco, D.B. (2002). Navigating steroid hormone receptors through the nuclear compartment. Mol. Endo. *16*, 1449-1455.

de Kloet, E.R., Oitzl, M.S., and Joels, M. (1999). Stress and cognition: are corticosteroids good or bad guys? Trends Neurosci. *22*, 422-426.

Dey, R., Roychowdhury, P., and Mukherjee, C. (2001). Homology modeling of the ligand-binding domain of the glucocorticoid receptor: binding site interactions with cortisol and corticosterone. Pro. Eng. *14*, 565-571.

Ding, X.F., Anderson, C.M., Ma, H., Hong, H., Uht, R.M., Kushner, P.J., and Stallcup, M.R. (1998). Nuclear receptor binding-sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol. Endo. *12*, 302-313.

Distelhorst, C.W., and Howard, K.J. (1990). Evidence from pulse-chase labelling studies that the antiglucocorticoid hormone RU486 stabilizes the nonactivated form of the glucocorticoid receptor in mouse lymphoma cells. J. Steroid Biochem. Mol. Biol. *36*, 25-31.

Dittmar, K.D., Demady, D.R., Stancato, L.F., Krishna, P., and Pratt, W.B. (1997). Folding of the glucocorticoid receptor by the heat shock protein (hsp) 90-based chaperone machinery. J. Biol. Chem. *272*, 21213-21220.

Dominguez, D., Montserrat-Sentis, B., Virgos-Soler, A., Guaita, S., Grueso, J., Porta, M., Puig, I., Baulida, J., Franci, C., Garcia de Herreros A. (2003). Phosphorylation regulates the subcellular localization and activity of the snail transcriptional repressor. Mol. Cell Biol. *23*, 5078-5089.

Dong, D.D., Jewell, C.M., Bienstock, R.J., and Cidlowski, J.A. (2006). Functional analysis of the LXXLL motifs of the human glucocorticoid receptor: association with altered ligand affinity. J. Steroid Biochem. Mol. Biol. *101*, 106-117.

Doppler, W., Windeger, M., Soratroi, C., Tomasi, J., Lechner, J., Rusconi, S., Cato, A.C.B., Almlof, T., Liden, J., Okret, S., Gustafsson, J., Richard-Foy, H., Barry Star, D., Klocker, H., Edwards, D., and Geymayer, S. (2001). Expression level-dependent contribution of glucocorticoid receptor domains for functional interaction with STAT5. Mol. Cell Biol. *21*, 3266-3279.

Evans, R.M. (2005). The nuclear receptor superfamily: a rosetta stone for physilology. Mol. Endocrinol. *19*, 1429-1438.

Farman, N., Oblin, M.E., Lombes, M., Delahaye, R., Westphal, H.M., Bonvalet, J.P., and Gasc, J.M. (1991). Immunolocalization of gluco- and mineralocorticoid receptors in rabbit kidney. Am. J. Physiol. *260*, 226-233.

Fejes-Toth, G., Pearce, D., and Naray-Fejes-Toth, A. (1998). Subcellular localization of mineralocorticoid receptors in living cells: effects of receptor agonists and antagonists. Proc. Natl. Acad. Sci. USA *95*, 2973-2978.

Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W., and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell *82*, 475-483.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-1060.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intranuclear transport mediated by the nuclear export signal. Nature *390*, 308-311.

Galigniana, M.D., Scruggs, J.L., Herrington, J., Welsh, M.J., Carter-Su, C., Housley, P.R., and Pratt, W.B. (1998). Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. Mol. Endo. *12*, 1903-1913.

Galigniana, M.D., Radanyi, C., Renoir, J.M., Housley, P.R., and Pratt, W.B. (2001). Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. J. Biol. Chem. *276*, 14884-14889.

Galigniana, M.D., Harrell, J.M., Murphy, P.J.M., Chinkers, M., Radanyi, C., Renoir, J-M., Zhang, M., and Pratt, W.B. (2002). Binding of the hsp90-associated immunophilins to the cytoplasmic dynein: direct binding and *in vivo* evidence that the peptidylprolyl isomerase domain is a dynein interaction domain. Biochemistry *41*, 13602-13610.

Galigniana, M.D., Harell, J.M., O'Hagen, H.M., Ljungman, M., and Pratt, W.B. (2004). Hsp90-binding immunophilins link p53 transport to the nucleus. J. Biol. Chem. *279*, 22483-22489.

Galigniana, M.D., Piwien Pilipuk, G., Kanelakis, D.C., Burton, G., and Lantos, C.P. (2004). Molecular mechanisms of activation and nuclear translocation of the mineralocorticoid receptor upon binding pregnanesteroids. Mol. Cell Endo. *217*, 167-179.

Georget, V., Lobaccaro, J.M., Terouanne, B., Mangeat, P., Nicolas, J.C., and Sultan, C. (1997). Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor. Mol. Cell Endo. *14*, 1162-1174.

Giannoukos, G., Silverstein, A.M., Pratt, W.B., and Simons, S.S.Jr. (1999). The seven amino acids (547-553) of rat glucocorticoid receptor required for steroid and hsp90 binding contain a functionally independent LXXLL motif that is critical for steroid binding. J. Biol. Chem. *274*, 36527-36536.

Giguere, V., Hollenberg, S.M., Rosenfeld, M.G., and Evans, R.M. (1986). Functional domains of the human glucocorticoid receptor. Cell *46*, 645-652.

Glass, C.K., and Rosenfeld, M.G. (2002). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. *14*, 121-141.

Goodridge, A.G. (1987). Dietary regulation of gene expression: enzymes involved in carbohydrate and lipid metabolism. Annu. Rev. Nutr. *7*, 157-185.

Gorlich, D., Prehn, S., Laskey, R.A., Hartmann, E. (1994). Isolation of a protein that is essential for the first step of nuclear protein import. Cell *79*, 767-778.

Gorlich, D., Henklein, P., Laskey, R.A., and Hartmann, E. (1996). A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus. Embo. J. *15*, 1810-1817.

Gorlich, D. (1998). Transport into and out of the nucleus. EMBO J. *17*, 2721-2727.

Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. *15*, 607-660.

Grande, M.A., van der Kraan, I., de Jong, L., and van Driel, R. (1997). Nuclear distribution of transcription factors in relation to sites of transcription and RNA polymerase II. J. Cell Sci. *110*, 1781-1791.

Grandics, P., Miller, A., Schmidt, T.J., and Litwack, G. (1984). Phosphorylation *in vivo* of rat hepatic glucocorticoid receptor. Biochem. Biophys. Res. Commun. *120*, 59-65.

Gustafsson, J.A., Carlestedt-Duke, J., Wrange, O., Okret, S., and Wikstom, A.C. (1986). Functional analysis of the purified glucocorticoid receptor. J. Steroid Biochem. *24*, 63-68.

Haché, R.J.G., Savory, J.G.A., and Lefebvre, Y.A. (1999). Nucleocytoplasmic trafficking and glucocorticoid receptor function. Gene Therapy Mol. Biol. *4*, 99-107.

Haché, R.J.G., Tse, R., Reich, T., Savory, J.G.A. and Lefebvre, Y.A. (1999). Nucleocytoplasmic trafficking of steroid-free glucocorticoid receptor. J. Biol. Chem. *274*, 1432-1439.

Harrell, J.M., Kurek, I., Breiman, A., Radanyi, C., Renoir, J.M., Pratt, W.B., and Galigniana, M.D. (2002). All of the protein interactions that link steroid receptor-hsp90-immunophilin heterocomplexes to cytoplasmic dynein are common to plant and animal cells. Biochemistry *41*, 5581-5587.

Harrell, J.M., Murphy, P.J., Morishima, Y., Chen, H., Mansfield, J.F., Galigniana, M.D. and Pratt, W.B. (2004). Evidence for glucocorticoid receptor transport on microtubules by dyenin. J. Biol. Chem. *279*, 54647-54654.

Hagting, A., Karlsson, C., Clute, P., Jackman, M., Pines, J. (1998). MPF localization is controlled by nuclear export. EMBO J. *17*, 4127-4138.

Heery, D.M., Kalkhoven, E., Hoare, S., and Parker, M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733-736.

Hoeck, W., Rusconi, S., and Groner, B. (1989). Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment. J. Biol. Chem. *264*, 14396-14402.

Holaska, J., Blacks, B.E., Love, D.C., Hanover, J.A., Leszyk, J., and Paschal, B.M. (2001). Calreticulin is a receptor for nuclear export. J. Cell Biol. *152*, 127-140.

Hollenberg, S.M., Giguere, V., Segui, P., and Evans, R.M. (1987). Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell *49*, 39-46.

Hollenberg, S.M., and Evans, R.M. (1998). Multiple and cooperative transactivation domains of the human glucocorticoid receptor. Cell *55*, 531-541.

Housley, P.R., and Pratt, W.B. (1984). Direct demonstration of glucocorticoid receptor phosphorylation by intact 1-cells. J. Biol. Chem. *258*, 4630-4635.

Howard, K.J., Holley, S.J., Yamamoto, K.R., and Distelhorst, C.W. (1990). Mapping the hsp90 binding region of the glucocorticoid receptor. J. Biol. Chem. *265*, 11928-11935.

Htun, H., Barsony, J., Renyi, I., Gould, D.L., and Hager G.L. (1996). Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with green fluorescent protein chimera. Proc. Natl. Acad. Sci. USA *93*, 4845-4850.

Htun, H., Holth, L.T., Walker, D., Davie, J.R., and Hager, G.L. (1999). Direct visualization of the human estrogen receptor α reveals a role for ligand in the nuclear distribution of the receptor. Mol. Biol. Cell *10*, 471-486.

Hu, J.M., Bodwell, J.E., and Munck, A. (1994). Cell cycle-dependent glucocorticoid receptor phosphorylation and activity. Mol. Endo. *8*, 1709-1713.

Hu, J.M., Bodwell, J.E., and Munck, A. (1997). Control by basal phosphorylation of cell cycle-dependent, hormone-induced glucocorticoid receptor hyperphosphorylation. Mol. Endo. *11*, 305-311.

Huang, Y., and Simons, S.S. Jr. (1999). Functional analysis of R651 mutations in the putative helix 6 of rat glucocorticoid receptors. Mol. Cell Endo. *158*, 117-130.

Imamoto, N., Mastsuoka, Y., Kurihara, T., Kohno, K., Miyagi, M., Sakiyama, F., Okada, Y., Tsunasawa, S., and Yoneda, Y. (1992). Antibodies agains 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilin proteins. J. Cell Biol. *119*, 1047-1061.

Ishida, N., Hara, T., Kamura, T., Yoshida, M., Nakayama, K., Nakayama, K.I. (2002). Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. J. Biol. Chem. *277*, 14355-14358.

Ito, K., Chung, K.F., and Adcock, I.M. (2006). Update on glucocorticoid action and resistance. J. Allergy Clin. Immunol. *117*, 522-543.

Itoh, M., Adachi, M., Yaui, H., Takekawa, M., Tanaka, H., and Imai, K. (2002). Nuclear export of glucocorticoid receptor is enhanced by c-Jun N-terminal kinase-mediated phosphorylation. Mol. Endo *16*, 2382-2392.

Izaurralde, E., Kutay, U., von Kobbe, C., Mattaj, I.W., and Gorlich, D. (1996). Identification of different roles for RanGDP and RanGTP in nuclear protein import. EMBO. J. *15*, 5584-5594.

Jantzen, K., Fritton, H.P., Igo-Kemenes, T., Espel, E., Janich, S., Cato, A.C., Mugele, K., and Beato, M. (1987). Partial overlaping of binding sequences for steroid hormone receptors and DnaseI hypersensitive sites in the rabbit uteroglobin gene region. Nucleic Acids Res. *15*, 4535-4552.

Jenster, G., van der Korput, H.A., van Vroonhoven, C., van der Kwast, T.H., Trapman, J., and Brinkman, A.O. (1991). Domains of the human androgen receptor involved in steroid binding, transcriptional activation and subcellular localization. Mol. Endo. *5*, 1396-1404.

Jenster, G., Trapman, J., and Brinkman, A.O. (1993). Nuclear import of the human androgen receptor. Biochemistry *293*, 761-768.

Kaffman, A., Rank, N.M., O'Neil, E.M., Huang, L.S., O'Shea, E.K. (1998). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature *396*, 482-486.

Kang, K.I., Devin, J., Cadepond, F., Jibard, N., Guiochon-Mantel, A., Baulieu, E.E., and Catelli, M.G. (1995). *In vivo* functional protein-protein interactions: nuclear targeted hsp90 shifts cytoplasmic steroid receptor mutants into the nucleus. Proc. Natl. Acad. Sci. USA *90*, 340-344.

Kastrup, L., Oberleithneer, H., Ludwig, Y., Schafer, C., and Shahin, V. (2006). Nuclear envelope barrier leak induced by dexamethazone. J. Cell Phys. *206*, 428-434.

Katzenellenbogen, J.A., and Katzenellenbogen, B.S. (1996). Nuclear hormone receptors: ligand-activated regulators of transcription and diverse cell responses. Chem. Biol. *3*, 529-536.

Kim, S.B., Ozawa, T., and Umezawa, Y. (2005). A genetically encoded indicator for assaying bioactive chemicals that induce nuclear transport of glucocorticoid receptor. Anal. Biochemistry *347*, 213-220.

King, W.J., and Greene, G.L. (1984). Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature *307*, 745-747.

Kino, T., Souvatzoglou, E., De Martino, M.U., Tsopanomihalu, M., Wan, Y., and Chrousos, G.P. (2003). Protein 14-3-3σ interacts with and favors cytoplasmic subcellular localization of the glucocorticoid receptor, acting as a negative regulator of the glucocorticoid signaling pathway. J. Biol. Chem. *278*, 25651-25656.

Kobayashi, A., Ito, E., Toki, T., Kogame, K., Takahashi, S., Igarashi, K., Hayashi, N., and Yamamoto, M. (1999). Molecular cloning and functional characterization of a new cap'n'collar family transcription factor Nrf3. J. Biol. Chem. *247*, 6443-6452.

Kohler, M., Spec, C., Christiansen, M., Bischoff, F.R., Prehn, S., Haller, H., Gorlich, D., and Hartmann, E. (1999). Evidence for distinct substrate specificities for importin alpha family members in nuclear protein import. Mol. Cell Biol. *19*, 7782-7791.

Komeili, A., and O'Shea, E.K. (2001). New perspectives on nuclear transport. Annu. Rev. Genetics *35*, 341-364.

Krozowski, Z.S., Rundel, S.E., Wallace, C., Castell, M.J., Shen, J.H., Dowling, J., Funder, J.W., and Smith, A.I. (1989). Immunolocalization of renal mineralocorticoid receptors with an antiserum against a peptide deduced from the complementarty deoxyribonucleic acid sequence. Endocrinology, *125*, 192-198.

Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E.P., Wolff, B., Yoshida, M, and Horinouchi, S. (1999). Lemptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proc. Natl. Acad. Sci. USA *96*, 9112-9117.

Kumangai, A., and Dunphy, W.G. (1995). Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. Genes Dev. *13*, 1067-1072.

Kumar, S., Wang, R.A., Mazumdar, A., Talukder, A.H., Mandal, M., Yang, Z., Bagheri-Yarmand, R., Sahin, A., Hortobagyi, G., Adam, L., Barnes, C.J., and Vadlamudi, R.K. (2002). A naturally occurring MTA1 variant sequesters oestrogen receptor-α in the cytoplasm. Nature *418*, 654-657.

Kumar, S., Chaturvedi, N.K., Nishi, M., Kawata, M., and Tyagi, R.K. (2004). Shuttling components of import machinery involved in nuclear translocation of steroid receptors exit nucleus via exportin-1/CRM-1 independent pathway. Biochem. Biophys. Acta *1691*, 73-77.

Kumar, V., Green S., Stack, G., Berry, M., Jin, J.R., and Chambon, P. (1987). Functional domains of the human estrogen receptor. Cell *51*, 941-951.

Kurl, R.N., and Jacobs, S.T. (1984). Phosphorylation of purified glucocorticoid receptor from rat liver by an endogenous protein kinase. Biochem. Biophys. Res. Com. *119*, 700-705.

Kutay, U., Hartmann, E., Treichel, N., Calado, A., Carmo-Fonseca, M., Prehn, S., Kraft, R., Gorlich, D., and Bischoff, F.R. (2000). Identification of two novel RanGTP-binding proteins belonging to the importin β superfamily. J. Biol. Chem. *275*, 40163-40168.

Lanz, R.B., and Rusconi, S. (1994). A conserved carboxy-terminal subdomain is important for ligand interpretation and transactivation by nuclear receptors. Endo. *135*, 2183-2195.

Leach, K.L., Dahmer, M.K., Hammond, N.D., Sando, J.J., and Pratt, W.B. (1979). Molybdate inhibition of glucocorticoid receptor inactivation and transformation. J. Biol. Chem. *254*, 11884-11890.

Lee, H., and Bai, W. (2000). Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. Mol. Cell Biol. *22*, 5835-5845.

LeCasse, E., and Lefebvre, Y.A. (1995). Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. Nucleic Acids Res. *23*, 1647-1656.

Li, H., Fidler, M.L., and Lim, C.S. (2005). Effect of initial subcellular localization of progesterone receptor on import kinects and transcriptional activity. Mol. Pharma. *2*, 509-518.

Lind, Y., Greenidge, P., Gillner, M., Koehler, K.F., Wright, A., and Carlestedt-Duke, J. (2000). Functional probing of the human glucocorticoid receptor steroid-interacting surfaces by site-directed mutagenesis. J. Biol. Chem. *275*, 19041-19049.

Lipowsky, G., Bichoff, F.R., Schwarzmaier, P., Kraft, R., Kostka, S., Hartmann, E., Kutay, U., and Gorlich, D. (2000). Exportin 4: a mediator of a novel nuclear export pathway in higher eukaryotes. EMBO J. *19*, 4362-4371.

Liu, J., and DeFranco, D.B. (2000). Protrated nuclear export of glucocorticoid receptor limits its turnover and does not require the exportin 1/CRM1-directed nuclear export pathway. Mol. Endo. *14*, 40-51.

Lombes, M., Farman, N., Oblin, M.E., Baulieu, E.E., Bonvate, J.P., Erlanger, B.F., and Gasc, J.M. (1990). Immunohistochemical localization of renal mineralocorticoid receptor by using and anti-idiotypic antibody that is an internal image of aldosterone. Proc. Natl. Acad. Sci. USA *87*, 1086-1088.

Lombes, M., Binart, N., Delahaye, F., Baulieu, E.E., and Fafestin-Oblin, M.E. (1994). Differential intracellular localization of human mineralocorticosteroid receptor on binding of agonists and antagonists. Biochem. J. *302*, 191-197.

Madan, A.P., and DeFranco, D.B. (1993). Bidirectional transport of glucocorticoid receptors across the nuclear envelope. Proc. Natl. Acad. Sci. USA *90*, 3588-3592.

Mao, J., Regelson, W., and Kalimi, M. (1992). Molecular mechanism of RU486 action – a review. Mol. Cell Biochem. *109*, 1-8.

Mazumdar, A., Wang, R.A., Mishra, S.K., Adam, L., Bagheri-Yarmand, R., Mandal, M., Vadlamudi, R.K., and Dumar, R. (2001). Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. Nat. Cell Biol. *3*, 30-37.

McBride, K.M., McDonald, C., and Reich, N.C. (2000). Nuclear export signal located within the DNA-binding domain of STAT1 transcription factor. EMBO J. *19*, 6196-6120

McKenna, J.G., and O'Malley, B.W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. Cell *108*, 465-474.

McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2001). Control of muscle development by dueling HATs and HDACs. Curr. Opin. Genet. Dev. *11*, 497-504.

McNally, J.G., Muller, W.G., Walker, D., Wolford, R., and Hager, G.L. (2000). The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. Science *287*, 1262-1265.

Meijsing, S.H., Elbi, C., Luecke, H.F., Hager, G.L., and Yamamoto, K.R. (2007). The ligand binding domain controls glucocorticoid receptor dynamics independent of ligand release. Mol. Cell Biol. *27*, 2442-2451.

Meisfeld, R., Okret, S., Wikstrom, A.C., Wrange, O., Gustafsson, J.A., and Yamamoto, K.R. (1984). Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells. Nature 312, 779-781.

Michael, W.M., Choi, M., and Dreyfuss, G. (1995). A nuclear export signal in hnRNPA1: a signal-mediated, temperature-dependent nuclear protein export pathway. Cell *83*, 415-422.

Mingot, J-M., Kosta., S., Kraft, R., Hartmann, E., and Gorlich, D. (2001). Importin 13: a novel mediator of nuclear import and export. EMBO J. *20*, 3685-3694.

Miyamoto, Y., Imamoto, N., Sekimoto, T., Tachibana, T., Seki, T., Tada, S., Enomoto, T., and Yoneda, Y. (1997). Differential modes of nuclear localization signal (NLS) recognition by three distinct classes of NLS receptors. J. Biol. Chem. *272*, 26375-26381.

Moguilewsky, M.A., Philibert, D. (1984). RU38486: potent anti-glucocorticoid activity correlated with strong binding to the cytosolic glucocorticoid receptor followed by an impaired activation. J. Steroid Biochem. *20*, 271-276.

Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y.W. (1994). Nrf2 is essential for protection against acute pulmonary injury in mice. Proc. Natl. Acad. Sci. USA *91*, 9926-9930.

Moroianu, J., Hijikata, M., Bloebel, G., and Radu, A. (1995). Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide-repeat containing nucleoporins. Proc. Natl. Acad. Sci. USA. *92*, 4318-4322.

Munck, A., and Foloey, R. (1976). Kinetics of glucocorticoid-receptor complexes in rat thymus cells. J. Steroid Biochem. *7*, 1117-1122.

Munck, A., and Holbrook, N.J. (1984). Glucocorticoid-receptor complexes in rat thymus cells. J. Biol. Chem. *259*, 820-831.

Nadler, S.G., Tritchler, D., Haffar, O.K., Blake, J., Bruce, A.G., and Cleaveland, J.S. (1997). Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization signals. J. Biol. Chem. *272*, 4310-4315.

Nakielny, S., and Dreyfuss, G. (1999). Transport of proteins and RNAs in and out of the nucleus. Cell *99*, 677-690.

Nemoto, T., Ohara-Nemoto, Y., Denis, M., and Gustafsson, J.A. (1990). The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. Biochemistry *29*, 1880-1886.

Nickerson, J. (2001). Experimental observations of a nuclear matrix. J. Cell Sci. *114*, 463-474.

Norgaard, P., and Poulsen, H.S. (1991). Glucocorticoid receptors in human malignancies: a review. Annals of Onco. *2*, 541-557.

Ogawa, H., Inouye, S., Tsuji, F.I., Yasuda, K., and Umesono, K. (1995). Localization, trafficking and terperature-dependence of the Aequorea green fluorescent protein in cultured vertebrate cells. Proc. Natl. Acad. Sci. USA *92*, 11899-11903.

Ohno, M., Fornerod, M., and Mattaj, I.W. (1998). Nucleocytoplasmic transport: the last 200 nanometers. *Cell* 92, 327-336.

Onate, S.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science *270*, 1354-1357.

Orti, E., Mendel, D.B., Smith, L.I., and Munck, A. (1989). Agonist-dependent phosphorylation and nuclear dephosphorylation of glucocorticoid receptors in intact cells. J. Biol. Chem. *264*, 9728-9731.

Orti, E., Hu, L.M., and Munck, A. (1993). Kinetics of glucocorticoid receptor phosphorylation in intact cells. Evidence for hormone-induced hyperphosphorylation after activation and recycling of hyperphosphorylated receptors. J. Biol. Chem. *268*, 7779-7784.

Ossarah-Nazari, B., Bachelerie, F., and Dargemont, C. (1997). Evidence for a role for CRM1 in signal-mediated nuclear protein export. Science *278*, 141-144.

Ottosson, M., Vikmoan-Aldofsson, K., Enerback, S., Olivecrona, G., and Bjorntorp, P. (1994). The effects of cortisol on the regulation of lipoprotein lipase activity in human adipose tissue. J. Clin. Endo. Metab. *79*, 820-825.

Ozanne, D.M., Brady, M.E., Cook, S., Gaughan, L., Neal, D.E., and Robson, C.N. (2000). Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. Mol. Endo. *14*, 1618-1626.

Ozawa, H. (2005). Steroid hormones, their receptors and neuroendorine system. J. Nippon. Med. School *72*, 316-325.

Pederson, T. (2000). Half a century of "the nuclear matrix". Mol. Biol. Cell *11*, 799-805.

Pemberton, L.F., Rosenblum, J.S., and Blobel, G. (1998). A distinct and parallel pathway for the nuclear import of an mRNA-binding protein. J. Cell Biol. *139*, 1645-1653.

Picard, D., and Yamamoto, K.R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J. *6*, 3333-3340.

Piwien Pilipuk, G.P., Vinson, G.P., Sanchez, C.G., and Galigniana, M.D. (2007). Evidence for NL1-independent nuclear translocation of the mineralocorticoid receptor. Biochemistry *46*, 1389-1397.

Plafker, S.M., and Macara, I.G. (2000). Importin-11, a nuclear import receptor for the ubiquitin-conjugating enzyme, UbcM2. EMBO J. *19*, 5502-5513.

Pratt, W.B., and Toft, D.O. (1997). Steroid receptor interactions with heat shock proteins and immunophilin chaperones. Endo. Rev. *18*, 306-360.

Pratt, W.B., Silverstein, A.M., and Galigniana, M.D. (1999). A model for the cytoplasmic trafficking of signaling proteins involving the hsp90-binding immunophilins and p50cdc37. Cell Signaling *11*, 839-851.

Pratt, W.B., and Toft. D.O. (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp. Biol. Med. *228*, 111-133.

Pratt, W.B., Galigniana, M.D., Harrell, J.M., and DeFranco, D.B. (2004). Evidence for glucocorticoid receptor transport on microtubules by dynein. Cell Signaling *16*, 857-872.

Pratt, W.B., Galigniana, M.D., Morishima, Y, and Murphy, P.J. (2004). Role of molecular chaperones in steroid receptor action. Essays Biochem. *40*, 41-58.

Pratt, W.B., Morishima, Y., Murphy, M., and Harrell, M. (2006). Chaperoning of glucocorticoid receptors. Handb. Exp. Pharmacol. *172*, 111-138.

Prives, C. (1998). Signaling to p53: breaking the MDM2-p53 good cop/bad cop. Cell *95*, 5-8.

Pujols, L., Mullol, J., Roca-Ferrer, J., Torrego, A., Xaubet, A., Cidlowski, J.A., and Picado, C. (2002). Expression of glucocorticoid receptor alpha- and beta- isoforms in human cells and tissues. Am. J. Physiol. Cell Physiol. *283*, 1324-1331.

Qi, M., Hamilton, B.J., and DeFranco, D.B. (1989). v-mos oncoproteins affect the nuclear retention and reutilization of glucocorticoid receptors. Mol. Endo. *3*, 1279-1288.

Qi, M., Staasenko, L.J., and DeFranco, D.B. (1990). Recycling and desensitization of glucocorticoid receptors in v-mos transformed cells depend on the ability of nuclear receptors to modulate gene expression. Mol. Endo. *4*, 455-464.

Rabut, G., Doye, B., and Ellenberg, J. (2004). Mapping the dynamic organization of the nuclear pore complex inside living cells. Nat. Cell Biol. *6*, 1114-1121.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683-692.

Ribbeck, K., and Gorlich, D. (2001). Kinetic analysis of translocation through nuclear pore complexes. EMBO J. *20*, 1320-1330.

Rizzo, G., and Fiorucci, S. (2006). PPARs and other nuclear receptors in inflammation. Curr. Opin. Pharm. *6*, 1-7.

Robertson, N.M., Shulman, G., Karnik, S., Alnemri, E., and Litwack, G. (1993). Demonstration of nuclear translocation of the mineralocorticoid receptor (MR) using an anti-MR antibody and confocal laser scanning microscopy. Mol. Endo. *7*, 1226-1229.

Robbins, J., Dilworth, S.M., Laskey, R.A., and Dingwall, C. (1991). Two independent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell *64*, 615-623.

Robyr, D., Wolffe, A.P., and Wahli, W. (2000). Nuclear hormone coregulators in action: diversity and shared tasks. Mol. Endo. *14*, 329-346.

Roozendal, B. (2002). Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval. Neurobiol. Learn Mem. *78*, 578-595.

Rosen, E.D., and Spiegelman, B.M. (2000). Molecular regulation of adipogenesis. Annu. Rev. Cell Dev. Biol. *16*, 145-171.

Rout, M.P., and Aitchison, J.D. (2001). The nuclear pore complex as a transport machine. J. Biol. Chem. 276, 16593-16596.

Roux, S., Térouanne, B., Balaguer, P., Jausons-Loffreda, N., Pons, M., Chambon, P., Gronemeyer, H., and Nicolas, J-C. (1996). Mutations of isoleucine 747 by a threonine alters the ligand responsiveness of human glucocorticoid receptor. Mol. Endo. *10*, 1214-1226.

Rusconi, S., and Yamamoto, K.R. (1987). Functional dissection of the hormone and DNA binding activities of the purified glucocorticoid receptor. EMBO J. *6*, 1309-1315.

Sackey, F.N.A., Haché, R.J.G., Reich, T., Kwast-Welfeld, J., and Lefebvre, Y.A. (1996). Determinants of subcellular distribution of the glucocorticoid receptor. Mol. Endo. *10*, 1191-1205.

Saitoh, M., Takayanagi, R., Goto, K., Fukamizu. A., Tomura, A., Yanase, T., and Nawata, H. (2002). The presence of both the amino- and carboxyl-terminal domains in the AR is essential for the completion of a transcriptionally active form with coactivators and

intranuclear compartmentalization common to the steroid hormone receptors: a three-dimentional imaging study. Mol. Endo *16*, 694-706.

Saporita, A.J., Zhang, Q., Navai, N., Dincer, Z., Hahn, J., Cai, X., and Wang, Z. (2003). Identification and characterization of a ligand-regulated nuclear export signal in androgen receptor. J. Biol. Chem. *278*, 41998-42005.

Sasano, H., Fukushima, K., Sasaki, I., Matsuno, S., Nagura, H., and Drozowski, Z.S. (1992). Immunolocalization of mineralocorticoid receptor in human kidney pancreas, salivary, mammary, and sweat glands: a light and electron microscopic immunohistochemical study. J. Endocrinol. *132*, 305-310.

Savory, J.G., Hsu, B., Laquian, I.R., Giffin, W., Reich, T., Haché, R.J.G., and Lefebvre, Y.A. (1999). Discrimination between NL1- and NL2-mediated nuclear localization of the glucocorticoid receptor. Mol. Cell Biol. *19*, 1025-1037.

Sazer, S. (2005). Nuclear envelope: nuclear pore complexity. Curr. Biol. *15*, 23-26.

Schaaf, M.J.M., and Cidlowski, J.A. (2003). Molecular mechanisms of glucocorticoid action and resistance. J. Steroid Biochem. Mol. Biol. *83*, 37-48.

Schaaf, M.J.M., Lewis-Tuffin, L.J., and Cidlowski, J.A. (2005). Ligand-selective targeting of the glucocorticoid receptor to nuclear subdomains is associated with decreased receptor mobility. Mol. Endo. *19*, 1501-1515.

Schauer, M., Chalepakis, G., Willmann, T., and Beato, M. (1989). Binding of hormone accelerates the kinetics of glucocorticoid and progesterone receptor binding to DNA. Proc. Natl. Acad. Sci. USA *86*, 1123-1127.

Scheidereit, C., Westphal, H.M., Carlson, C., Bosshard, H., and Beato, M. (1986). Molecular model for the interaction between glucocorticoid receptor and the regulatory elements of inducible genes. DNA *5*, 383-391.

Schmidt-Zachmann, M.S., Dargemont, C., Kuhn, L.C., and Nigg, E.A. (1993). Nuclear export of proteins: the role of nuclear retention. Cell *74*, 493-504.

Sekimoto, T., Nakajima, K., Tachibana, T., Hirano, T., and Yoneda, Y. (1997). Interferon-gamma-dependent nuclear import of Stat1 is mediated by the GTPase activity of Ran/TC4. J. Biol. Chem. *271*, 31017-31020.

Sengupta, S., Vonesch, J.L., Waltzinger, C., Zheng, H., and Wasylyk, B. (2000). Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells. EMBO J. *19*, 6051-6064.

Sengupta, S., and Wasylyk, B. (2001). Ligand-dependent interaction of the glucocorticoid receptor with p53 enhances their degradation by HDM2. Genes Dev. *15*, 2367-2380.

Seternes, O.M., Johansen, B., Hegge, B., Johannessen, M., Keyes, S.M., and Moens, U. (2002). Both binding and activation of p38 mitogen-activated protein kinase (MAPK) play essential roles in regulation of the nucleocytoplasmic distribution of MAPK-activated protein kinase 5 by cellular stress. Mol. Cell Biol. *22*, 6931-6945.

Shahin, V., Ludwig, Y., Schafer, C., Nikova, D., and Oberleithner, H. (2005). Glucocorticoids remodel nuclear envelope structure and permeability. J. Cell Sci. *118*, 2881-2889.

Shi, Y., and Thomas, J.O. (1992). The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. Mol. Cell Biol. *12*, 2186-2192.

Shulga, N., Roberts, P., Gu, Z., Spitz, L., Tabb, M.M., Nomura, M., and Goldfarb, D.S. (1996). *In vivo* nuclear transport kinetics in *Saccharomyces cerevisae*: a role for heat shock protein 70 during targeting and translocation. J. Cell Biol. *135*, 329-339.

Shulga, N., James, P., Craig, E.A., and Goldfarb, D.S. (1999). A nuclear export signal prevents Saccharomyces cerevisae hsp70 Ssb1p from stimulating nuclear localization signal-directed nuclear transport. J. Biol. Chem. *274*, 16501-16507.

Simental, J.A., Sar, M., Lane, M.V., French, F.S., and Wilson, E.M. (1991). Transcriptional activation and nuclear targeting signals of the human androgen receptor. J. Biol. Chem. *266*, 510-518.

Singh, V.B., and Moudgil, V.K. (1985). Phosphorylation of rat liver glucocorticoid receptor. J. Biol. Chem. *260*, 3684-3690.

Smith, D.F. (1993). Dynamics of heat shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes. Mol. Endo. *7*, 1418-1429.

Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J., Mizzen, C.A., McKenna, N.J., Onate, S.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1997). Steroid receptor coactivator-1 is a histone acetlyltransferase. Nature *389*, 194-198.

Spitz, I.M., and Bardin, C.W. (1993). Mifepristone (RU486) – a modulator of progestin and glucocorticoid action. New Eng. J. Med. *329*, 404-412.

Sprague, B.L., Pego, R.L., Stavreva, D.A., and McNally, J.G. (2004). Analysis of binding reactions by fluorescence recovery after photobleaching. Biophys. J. *86*, 3473-3495.
Stade, K., Ford, C.S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041-1050.

Stenoien, D.L., Nye, A.C., Mancini, M.G., Pate, K., Dutertre, M., O'Malley, B.W., Smith, C.L., Beimont, A.S., and Mancini, M.A. (2001). Ligand-mediated assembly and real-time cellular dynamics of estrogen receptor α coactivator complexes in living cells. Mol. Cell Biol. *21*, 4404-4412.

Stolte, E.H., Verburg van Kemenade, B.M.L., Savelkoul, H.F.J., and Flik, G. (2006). Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. J. Endocrinol. *190*, 17-28.

Tanenbaum, D.M., Wang, Y., Williams, S.P., and Sigler, P.B. (1998). Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. Proc. Natl. Acad. Sci. USA *95*, 5998-6003.

Tang, Y., and DeFranco, D.B. (1996). ATP-dependent release of glucocorticoid receptors from the nuclear matrix. Mol. Cell Biol. *16*, 1989-2001.

Tang, Y., Ramakrishnan, C., Thomas, J., and DeFranco, D.B. (1997). A role for HDJ-2/HSDJ in correcting subnuclear trafficking, transactivation and transrepression defects of a glucocorticoid receptor zinc finger mutant. Mol. Biol. Cell *8*, 795-809.

Tang, Y., Getenberg, R.H., Vietmeier, B.M., Stallcup, M.R., Eggert, M., Renkawit, R., and DeFranco, D.B. (1998). The DNA-binding and τ2 transactivation domains of the rat glucocorticoid receptor constitute a nuclear matrix-targeting signal. Mol. Endo. *12*, 1420-1431.

Taniguchi, E., Toyoshima-Morimoto, F., and Nishida, E. (2002). Nuclear translocation of plk1 mediated by its bipartite nuclear localization signal. J. Biol. Chem. *277*, 48884-48888

Tazawa, H., Osman, W., Shoji, Y., Treuter, E., Gustafsson, J-A., and Zilliacus, J. (2003). Regulation of subcellular localization is associated with a mechanism for nuclear receptor corepresssion by RIP140. Mol. Cell Biol. *23*, 4187-4198.

Thyssen, G., Tzu-Huey, L., Lehmann, L., Ming, Z., Manju, S., and Zijie, S. (2006). LZTS2 is a novle beta-catenin-interacting protein and regulates nuclear export of beta-catenin. Mol. Cell Biol. *26*, 8857-8867.

Tomura, A., Goto, K., Morinaga, H., Nomura, M., Okabe, T., Yanse, T., Takayanagi, R., and Nawata, H. (2001). The subnuclear three-dimensional image analysis of androgen receptor fused to green fluorescence protein. J. Biol. Chem. *276*, 28395-28401,

Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K., and Rosenfeld, M.G. (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature *387*, 677-684.

Toyoshima, F., Moriguchi, T., Wade, A., Fukuda, M., and Nishida, E. (1998). Nuclear export of cyclin B1 and its possible role in DNA-damage induced G2 checkpoint. EMBO J. *17*, 2728-2735.

Turpin, P., Rodriguez, M., Thomas, D., Hay, R.T., Virelizier, J.L., and Dargemont, C. (1197). Nuclear localization of I kappa B alpha promote active transport of NF-kappa B from the nucleus to the cytoplasm. J. Cell Sci. *110*, 369-378.

Tyagi, R.K., Amazit, L., Lescop, P., Milgrom, E., and Guiochon-Mantel, A. (1998). Mechanisms of progesterone receptor export from the nuclei: role of nuclear localization signal, nuclear export signal and Ran guanosine triphosphate. Mol. Endo. *12*, 1684-1695.

Tyagi, R.K., Lavrosky, Y., Ahn, S.C., Song, C.S., Chatterjee, B., and Roy, A.K. (2000). Dynamics of intracelllular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. Mol. Endo. *14*, 1162-1174.

Tzivion, G., and Avruch, J. (2001). 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J. Biol. Chem. *277*, 3061-3064.

Umesono, K., and Evans, R.M. (1989). Determinants of target gene specificity for steroid thyroid hormone receptors. Cell *57*, 1139-1146.

van Steensel, B., Jenster, G., Damm, K., Brinkmann, A.O., and van Driel, R. (1995). Domains of the human androgen receptor and glucocorticoid receptor involved in binding to the nuclear matrix. J. Cell Biochem. *57*, 465-478.

van Steensel, B., Brink, M., van Der, M., van Binendijk, E.P., Wansink, D.G., de Jong, L., de Kloet, E.R., and van Driel, R. (1995). Localization of the glucocorticoid receptor in discrete clusters in the cell nucleus. J. Cell Sci. *108*, 3003-3011.

Vetter, I.R., Arndt, A., Kutay, U., Gorlich, D., and Wittinghofer, A. (1999). Structural view of the Ran-Importin beta interaction at 2.3 Å resolution. Cell *97*, 635-646.

Wallace, A.D., and Cidlowski, J.A. (2001). Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J. Biol. Chem. *276*, 42714-42721.

Walther, R. (2003). Mechanisms of nucleocytoplasmic trafficking of the glucocorticoid receptor, pp. 1-157.

Walther, R., Lamprecht, C., Risdale, A., Groulx, I., Lee, S., Lefebvre, Y.A., and Haché, R.J.G. (2003). Nuclear export of the glucocorticoid receptor is accelerated by cell fusion-dependent release of calreticulin. J. Biol. Chem. *278*, 37853-37864.

Wang, J.M., Préfontaine, G.G., Lemieux, M.E., Pope, L., Akimenko, M-A., and Haché, R.J.G. (1999). Developmental effects of ectopic expression of the glucocorticoid receptor DNA binding domain are alleviated by an amino acid substitution that interferes with homeodomain binding. Mol. Cell Biol. *19*, 7106-7122.

Wang, Z., Frederick, J., and Garabedian, M.J. (2002) Deciphering the phosphorylation "code" of the glucocorticoid receptor *in vivo*. J. Biol. Chem. *277*, 26573-265870.

Weinberger, C., Hollenberg, S.M., Ong, E.S., Harmon, J.M., Brower, T., Cidlowski, J., Thompson, E.B., Rosenfeld, M.G., and Evans, R.M. (1985). Identification of human glucocorticoid receptor complementary DNA clones by epitope selection. Science *228*, 740-742.

Weis, K., Dingwall, C., and Lamond, A.I. (1996). Characterization of the nuclear protein import mechanisms using Ran mutants with altered nucleotide binding specificities. EMBO J. *15*, 7120-7128.

Welch, K., Franke, J., Kohler, M., and Macara, I.G. (1999). RanBP3 contains an unusual nuclear localization signal that is imported preferentially by importin-alpha3. Mol. Cell Biol. *19*, 8400-8411.

Welshons, W.V., Lieberman, M.E., and Gorski, J. (1984). Nuclear localization of unoccupied oestrogen receptors. Nature *307*, 747-749.

Wen, W., Meinkoth, J.L., Tsien, R.Y., and Taylor, S.S. (1995). Identification of a signal for rapid export of proteins from the nucleus. Cell *82*, 463-473.

Wikstrom, A.C., Bakke, O., Okret, S., Bronnegard, M., and Gustafsson, J.A. (1987). Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. Endocrinology, *120*, 1232-1242.

Whitesell, L., Mimnaugh, E.G., De Costa, B., Meyers, C.E., and Neckers, L.M. (1994). Inhibition of heat shock HSP90-pp60v-src heteroprotein complex by benzoquinone ansamysins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. USA *91*, 8324-8328.

Wochnik, G.M., Ruegg, J., Abel, G.A., Schmidt, U., Holsboer, F., and Rein, T. (2005). FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. J. Biol. Chem. *280*, 4609-4616.

Wright, A.P.H., Zilliacus, J., McEwan, I.J., Dahlman-Wright, K., Almof, T., Carlested-Duke, J., and Gustafsson, J.A. (1993). Structure and function of the glucocorticoid receptor. J. Steroid Biochem. Mol. Biol. *47*, 11-19.

Wurtman R.J. (2002). Stress and the adrenocortical control of epinephrine synthesis. Metabolism *51*, 11-15.

Xiao, Z., Watson, N., Rodriguez, C., and Lodish, H.F. (2001). Nucleocytoplasmic shuttling of Smad1 conferred by its nuclear localization and nuclear export signals. J. Biol. Chem. *276*, 39404-39410

Xu, H.E., Lambert, M.H., Montana, V.G., Parks, D.J., Blanchard, S.G., Brown, P.J., Sternbach, D.D., Lehmann, J.M., Wisely, G.B., Willson, T.M., Kliewer, S.A., and Milburn,

M.V. (1999). Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol. Cell *3*, 397-403.

Yang, J., and DeFranco, D.B. (1994). Differential roles of heat shock protein 70 in the *in vitro* nuclear import of glucocorticoid receptor and simian virus 40 large tumor antigen. Mol Cell Biol. *14*, 5088-5098.

Yang, J., and DeFranco, D.B. (1996). Assessment of glucocorticoid receptor-heat shock protein 90 interactions *in vivo* during nucleocytoplasmic trafficking. Mol. Endo. *10*, 3-13.

Yang, J., and DeFranco, D.B. (1997). Subnuclear trafficking of glucocorticoid receptors *in vitro*: chromatic recycling and nuclear export. J. Cell Biol. *137*, 523-538.

Ylikomi, T., Bocquel, M.T., Berry, M., Gronemeyer, H., and Chambon, P. (1992). Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. EMBO J. *11*, 3681-3694.

Yoshikawa, N., Yamamoto, K., Shimizu, N., Yamada, S., Morimoto, C., and Tanaka, H. (2005). The distinct agnostic properties of the phenulpyrazolosteroid cortivazol reveal interdomain communication within the glucocorticoid receptor. Mol. Endo. *19*, 1110-1124.

Zhang, J., Tsai, F.T.F., and Geller, D.S. (2006). Differential interaction of RU486 with the progesterone and glucocorticoid receptors. J. Mol. Endo. *37*, 163-173.

Zhou, J., and Cidlowski, J.A. (2005). The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. Steroids *70*, 407-417.

Zhou, Z.X., Sar, M., Simental, J.A., Lane, M.V., and Wilson, E.M. (1994). A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences. J. Biol. Chem. *269*, 13115-13123.