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Inquiries into the Subcellular Localization of the Glucocorticoid Receptor

© Ian R. Laquian

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University of Ottawa
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Abstract

In the absence of hormone, the glucocorticoid receptor (GR) resides in the cytoplasm existing in an inactive form complexed to heat shock proteins and immunophilins. Upon ligand addition, GR dissociates from the protein complex and transforms into an active free form that rapidly and completely translocates to the nucleus to regulate transcription of specific genes. Upon ligand withdrawal, GR is re-packaged into its multiprotein heterocomplex and is slowly redistributed to the cytoplasm. In this study an indirect immunofluorescence assay was employed to characterize the subcellular localization of a series of GR mutants effectively truncating, deleting or mutating specific regions of the receptor N-terminus. In the first part of my study, I delimited a specific region of GR (amino acids 100-200 of the N-terminus) necessary for both its initial cytoplasmic localization prior to ligand addition and return to the cytoplasm upon subsequent hormone withdrawal. Although found necessary, the sequence was not sufficient in imparting cytoplasmic localization to a ubiquitously localized protein in the absence of hormone.

Nuclear import of liganded GR is mediated through a well characterized sequence called NL1, which is adjacent to the receptor DNA binding domain and a second uncharacterized motif NL2, located in the ligand binding domain. Not much has been known about the NL2 mediated nuclear import of GR. The second part of my study was devoted to further characterizing the kinetics of an NL1 deficient GR mutant. Consistent with previous findings in our laboratory, NL2-mediated nuclear transfer was shown to occur more slowly than wild type receptor. I found that the withdrawal of hormone resulted in a more rapid redistribution to the cytoplasm of the NL1 mutant than that seen with wild type GR. Upon secondary stimulation, I observed a return to the nucleus of the NL1 mutant GR at a rate comparable to its nuclear import after a primary stimulation. Finally, I have assessed the effects of the export inhibitor Leptomycin B (LMB) on the subcellular trafficking of GR and the NL1 deficient mutant. I showed that in the absence of ligand, GR is capable of first entering, and secondly, of accumulating in the nucleus, presumably due to a block in export. Export inhibition by LMB provides the first indirect evidence to suggest that the export of GR is mediated in a CRM1 dependent manner.

Dedication

Anyone who really knows me, knows that I feel I lead a charmed life. They would also know that the thing I hold most dear is family. This thesis is dedicated to them. You will never know how much I cherish our love and openness. To my parents, Rodolfo and Josephine, who have worked so hard and sacrificed so much, so that I would have the freedom to follow my dreams, I thank you. To my sister Haydee, whose support and humor have eased the rough rides, I appreciate all the advice and look forward to your many calls. Finally, to my sister Joy, who has demonstrated true strength in the face of adversity. We have always laughed at the fact that you differ most amongst the children in our family. I can only hope that I am moving towards becoming more like you. You have shown me that problems are relative and one must endure to succeed. We will all look back at this time and only see how much closer you have brought the family together. Is it any reason I consider my life to be so charmed.

Acknowledgment

I would like to acknowledge my supervisor Dr. Yvonne Lefebvre for allowing me the opportunity to practice science in her lab. Her experience and insight have taught me to think carefully and critically, valuable traits to possess in any field one pursues.

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I would like to thank the members of the Haché/Lefebvre labs for making my stay enjoyable and full of laughs. A special mention to Drs. Joanne Savory, Ted Zerucha, and Gratien Prefontaine for their advice and senior presence in the lab, as well as Terry Reich for his technical support.

Finally, a special thanks to Ms. Jennifer Wilson, without whose help this thesis could not have been written. I mean this figuratively and literally. Her care and friendship were present when most needed and for this I owe the deepest of thanks. I will always remember and treasure our time together.

Table of Contents

List of Figures and Tables

Abbreviations

AF	activation function
AR	androgen receptor
Arnt	aryl hydrocarbon nuclear translocator
ATP	adenosine triphosphate
bHLH	basic Helix-Loop-Helix
BSA	bovine serum albumin
CAS	cellular apoptosis susceptibility gene
CBP	CREB Binding Protein
CDC	cell division control
CK	casein kinase
CRD	cytoplasmic retention domains
CRM	chromosome region maintenance
DBD	DNA binding domain
D-box	distal box
Dex	dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ER	estrogen receptor
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GR	glucocorticoid receptor
GREs	glucocorticoid response elements
GR_{NL1^-}	GR with a mutation in NL1
GR_{WT}	wild type GR
GTP	guanidine triphosphate
h.	hour
HAT	histone acetyltransferase
HIV	human immunodeficiency virus
HREs	hormone response elements
hsp	heat shock protein
IIF	indirect immunofluorescence
K	lysine
kDa	kilodalton
LBD	ligand binding domain

LMB	leptomycin B
min	minutes
mM	millimolar
MR	mineralocorticoid receptor
N	asparagine or nuclear
NE	nuclear envelope
NES	nuclear export signal
NF-κB	nuclear factor κB
NGS	normal goat serum
NL1	nuclear localization signal-1
NL2	nuclear localization signal-2
NLS	nuclear localization signal
NPC	nuclear pore complex
NPI	nuclear protein interactor
NTF	nuclear transport factor
P-box	proximal box
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKI	protein kinase inhibitor
PR	progesterone receptor
PTAC	pore targeting complex
Ran	Ras related nuclear protein
RAR	retinoic acid receptor
RPM	rotations per minute
RT	room temperature
RU486	RU38486
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophroresis
SRC-1	steroid receptor coactivator-1
SRP	suppression of RNA polymerase
STAT	Signal Tranducers and Activators of Transcription
SV 40	simian virus 40
TAF	transactivation function
TBP	tata box binding protein
TF	transcription factor
TR	thyroid receptor
VDR	vitamin D receptor

w/v	weight per volume
w/w	weight per weight
xnf7	xenopus factor 7
xmyoD	xenopus myogenic factor

Introduction

Steroid Hormone Receptors

Classical steroid hormone molecules were isolated based on their abilities to affect development, cell differentiation, and organ physiology. Their lipophilic nature, which enables them to diffuse from a source and permeate to a target, brought forth the idea that steroid hormones were potent regulators of gene expression. The development of radiolabeled ligands allowed for the identification of binding proteins that were shown to translocate from the cytoplasm to the nucleus, implying a link between transcriptional control and physiology. Providing support for this theory was the observation that ecdysteroids, the metamorphic hormones of insects, induced chromosomal puffing at specific sites in the Drosophila polytene chromosome (1-3).

In the mid-1970's, steroids were shown to be targeted to their responsive tissues by the presence of specific high affinity receptor proteins. Together with the subsequent identification of hormonally responsive target genes within these tissues, these biochemical findings led to a proposed model for steroid hormone action [reviewed in (4)]. In this model, it was postulated that the binding of hormone to its cytoplasmically localized receptor induces an allosteric change that enables the hormone-receptor complex to enter the nucleus, bind to high affinity sites in chromatin and modulate transcription. The cloning of steroid receptors in the mid-1980's provided a critical enhancement in understanding the molecular basis for this model.

The glucocorticoid receptor was the first of the steroid receptors to be cloned (5). The use of receptor specific antibodies and low stringency hybridization techniques allowed for the subsequent identification of other hormone receptors, as the genes for estrogen (ER) (6), androgen (AR) (7), progesterone (PR) (8), and mineralocorticoid receptors (MR) (9) were eventually cloned. By 1990, a total of 15 proteins were identified as receptors for all the lipophilic ligands known. To date, over 150 receptors have been classified, including thyroid (TR) (10), vitamin D_3 (VDR) (11), and retinoic acid receptor (RAR) (12). Moreover, molecular cloning techniques have allowed for the identification of receptors with no apparent ligand, appropriately named orphan receptors. As the number of characterized receptors increased, the term 'superfamily' was eventually implemented to encompass all of the known nuclear hormone receptors.

DNA sequence analysis revealed regions of close homology between receptors, confirming the proposed modular structure of nuclear hormone receptors. Nuclear receptors are composed of three modular domains, generally characterized by a variable N-terminal domain, a highly conserved DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD). The DBD is responsible for targeting the receptor to specific DNA sequences, while the LBD can be thought of as a molecular switch that, upon selectively binding ligand, shifts the receptor to a transcriptionally active state.

Mechanism of Action of Hormone Receptors

Steroid receptors are a subset of an increasing number of nuclear receptors, which act as transcription factors to regulate expression of specific genes. They exist in at least two forms, identified by their sedimentation positions on a sucrose gradient. In the absence of hormone, steroid receptors sediment at 8-10S, and are found to be associated in a heterocomplex of proteins comprised of heat shock proteins (hsps 90, 70 and 60), high molecular weight immunophilins (p56/59 and Cyp-40), and other various proteins (p60, and p23) (13-16). Upon exposure to hormone, liganded receptors dissociate from the multi-protein heterocomplex and are found to sediment at 4S on sucrose density gradients (17, 18).

The molecular chaperone hsp 90 is a ubiquitous, highly conserved abundant protein that is found associated with steroid receptors (19). For GR and MR, hsp 90 associates with the LBD and is necessary to maintain the receptor in a high affinity ligand binding conformation. Once hormone binding has occurred however, the ligand binding ability of GR and MR can be sustained in the absence of hsps (18, 20, 21). This characteristic is not universal for all steroid receptors, as ER (22), PR (23), and AR (24) do not require hsp 90 to bind ligand. Several potential functions for hsp 90 have been suggested, such as its involvement in kinase activity (25-28) and protein folding or assembly (29). In steroid receptors, hsp 90 association is thought to mask functional domains, repressing receptor functions such as DNA binding, transcriptional activation, and dimerization (13-15). Further interest lies in the suggested involvement of hsp 90 in receptor trafficking, particularly since it has been shown that blocking assembly of the

hsp 90:GR complex with geldanamycin, effectively inhibits the steroid dependent transfer of GR to the nucleus (30).

Hsp 70 is also conserved, ubiquitous, and abundant, and binds reversibly to hydrophobic regions of unfolded proteins in an ATP-dependent manner (31, 32). Like hsp 90, hsp 70 appears to have several functions in the cell related to protein folding, unfolding, disassembly of complexes, and import of proteins through membranes (33, 34).

Immunophilins are ubiquitous proteins that bind immunosuppressant drugs with high affinity and specificity (35). The actual biological activities of these proteins remain unknown, however members of the immunophilin family possess a peptidylprolyl isomerase activity, suggesting a role in the chaperoning and folding of proteins in the cell (36-38). Immunophilins have also been shown to be involved in receptor trafficking to the nucleus, although their roles to date are still undefined (39).

Inactive heterocomplexed receptors are unable to bind to DNA or activate transcription (13). Steroid hormone receptor activity begins with the addition of hormone. Upon ligand binding, the dormant receptor is immediately activated, undergoing a conformational transformation that enables its dissociation from the hsp-complex followed by its subsequent entry into the nucleus. Regulation of specific target genes occurs through receptor binding of specific DNA sequences known as hormone response elements (HREs). These sequences, located in the enhancer-promoter regions of target genes consist of two hexameric half sites separated by three nucleotides (40-44).

Steroid receptors have been shown to interact directly with components of the basal transcriptional machinery such as TF-IIB, TBP, and TAF_{II} 30 (45-48). Transcriptional co-activators such as steroid/nuclear coactivator –1 (SRC-1) (49), and CREB Binding Protein (p300/CBP) (50) have also been shown to associate with steroid receptors. It has therefore been suggested that these proteins, which contain intrinsic histone acetyltransferase (HAT) activity, serve to enhance the transcriptional activity of steroid receptors by increasing accessibility to DNA (51).

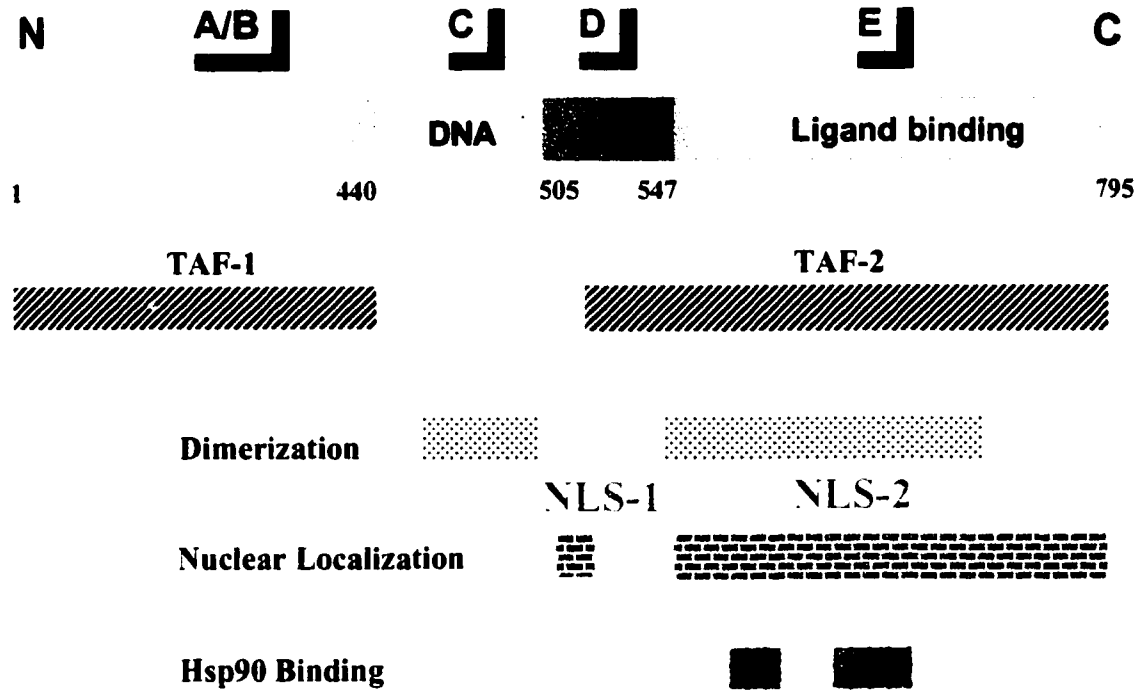
Structure and Function of the Rat Glucocorticoid Receptor

GR was the first nuclear steroid receptor to be cloned (5). GR, like other members of the nuclear receptor superfamily, has a primary structure organized into six homologous domains, A-F (summarized in Fig. 1).

The N-terminal domain or A/B domain spans amino acids 1-439. Deletion experiments uncovered a hormone independent transcriptional activation domain termed TAF-1 (transactivation function-1) that mapped to amino acids 98-292 (52-54). The N-terminal domain also contains a number of phosphorylation sites, and in terms of sequence and size, is the domain with the least amount of similarity amongst members of the steroid receptor family (55, 56).

Figure 1. Schematic depiction of rat GR and its functional domains.

Schema of rat GR that highlights the modular structure, particularly the variable N-terminal domain, the centrally located DNA binding domain and the C-terminal ligand binding domain. Labels A-F represent functional domains common to all nuclear receptors. Transactivation functions TAF-1 and TAF-2, are shown to map to the N- and C-terminal regions of GR (52-54, 73-75). Nuclear localization signals NL1 and NL2 are depicted to map to the hinge region and the ligand binding domain respectively. Hsp 90 binds to multiple sites in the ligand binding domain (68-71).


N
A/B
C
D
E
C
DNA
Ligand binding
1
440
505
547
795
TAF-1
TAF-2
Dimerization
NLS-1
NLS-2
Nuclear Localization
Hsp90 Binding

In contrast, the centrally localized DNA binding domain (DBD) or C domain, spanning amino acids 440-504, is the most highly conserved region amongst steroid receptors. The DBD is characterized by two zinc fingers, each formed by the tetrahedral coordination of four cysteine residues around a zinc atom (57-59). Sequence-specific DNA binding involves a subregion of the DBD located at the base of the first N-terminal zinc finger. This region is termed the P box and is responsible for the base specific recognition and contact of glucocorticoid response elements (GRE) (60, 61). A second subregion termed the D box forms the base of the second C-terminal zinc finger. This region is responsible for DNA dependent dimerization (60, 62, 63) and specific protein-protein interactions with a variety of transcription factors including Oct 1 and 2, (64), NF-κB (65), and Stat 5 (66).

The hinge region or D domain lies between the DNA binding domain and the C-terminal ligand binding domain. Spanning amino acids 505-546, the hinge region contains a well characterized nuclear localization sequence termed NL1. This tri-partite sequence, which is similar to that found in ER and PR, shares homology to the classic type nuclear localization signal (NLS) typified in the SV40 large T antigen (67).

Finally, the ligand binding domain (LBD) or E/F domain, is appropriately named for its ability to bind ligand, consequently regulating receptor activity. In addition, this domain which encompasses the final amino acids 547-795, is also involved in other functions including hsp 90 binding (68-71), nuclear localization, and transcriptional activation. Due to the complexity of the LBD, precisely mapping the second nuclear localization signal (NL2), responsible for the hormone dependent nuclear targeting of the

GR LBD has so far been difficult (72). In contrast, the transactivation function (TAF 2 or AF-2), originally mapped to the entire LBD, has been further deciphered to contain an autonomous activating domain (AF-2 AD) located in the last 14 amino acids of the GR LBD. AF-2 AD is conserved amongst all known transcriptionally active members of the nuclear receptor superfamily (73-75), and is responsible for the interaction of the LBD with transcriptional co-activators like SRC-1, GRIP-1, and CBP/p300 (49, 76).

Molecular Trafficking Across the Nuclear Membrane

The Nuclear Pore

In eukaryotic cells, the nuclear envelope (NE) serves as a physical barrier to separate the nucleoplasm from the cytoplasm (reviewed in (77)). The NE is composed of three components: i) a lipid bilayer; ii) lamins; and iii) nucleoporins. Lamins are a matrix of proteins that are located directly underneath the lipid bilayer. They provide the structural support to the nucleus as well as sites of attachment for chromosomal DNA (77). Nucleoporins are proteins that together compose the nuclear pore complex (NPC), creating an aqueous channel across the nuclear envelope. The NPC provides the sole avenue for macromolecular transport between the nucleus and cytoplasm.

Roughly cylindrical in shape, the NPC spans the inner and outer membranes of the NE with a diameter of about ~125 nm in the same plane. Extensive structural studies (reviewed in (78, 79)) have revealed that the NPC consists of a 52000 kDa basic framework which is sandwiched between a 21000 kDa nuclear ring and a 22000 kDa

cytoplasmic ring. The central framework of the NPC is composed of eight multidomain 'spokes' embracing a central pore which harbors a 12 Mda gated channel whose definitive structure and exact involvement in mediated transport remain to be firmly established. The cytoplasmic ring is decorated with eight 30-50 nm long kinked filaments, whereas the nuclear ring is capped with a basket-like assembly built from eight thin, 50-100 nm long filaments joined distally by a 30-50 nm diameter terminal ring. Approximately 100 or more nucleoporins are estimated to constitute the NPC, (reviewed in (80, 81)), of these only about 15% of the entire NPC mass have been molecularly characterized. Further dissecting the molecular composition of the NPC will contribute largely to deciphering the involvement of specific nucleoporins in nucleocytoplasmic trafficking of macromolecules.

Passive Diffusion vs. Signal Mediated Import

Entry into the nucleus can occur in one of two ways, passive diffusion or signal-mediated protein import (reviewed in (82)). The NPC allows for the free movement of small molecules and ions through channels in its structure spanning approximately 10 nm in diameter. Passive diffusion is non-saturable and requires no energy. It has been shown however, that nuclear stores of calcium may regulate the conformational state of the NPC, thereby affecting the free passage of molecules between the cytoplasm and the nucleus (reviewed (83)).

Proteins larger than 40-60 kDa are unable to passively diffuse into the nucleus. For these proteins, import into the nucleus is an active process that requires a signal, in the form of nuclear localization sequences (NLSs), and energy, in the form of nucleoside

triphosphates. Thus far, signal mediated import is believed to proceed in at least three defined steps: binding of an NLS containing protein by the import machinery, adherence to the nuclear envelope followed by its subsequent energy-dependent translocation through the NPC (84-90).

Nuclear Localization Signals (NLSs)

NLSs are responsible for targeting proteins to the nucleus. They are typically defined by systematic deletion and transfer experiments, which assess both necessity and sufficiency of the signal. There are many different types of NLSs, however, they are generally characterized by short stretches (~6) of basic amino acids (usually lysine or arginine residues). Traditionally they are classified into two major types. The first, contains a single cluster of basic residues within one polypeptide chain, exemplified by the simian virus 40 (SV40) large T antigen, whose specific NLS was identified to be PKKKRKV (91-94). NLSs identified in other proteins are shown to be very similar to the SV40 NLS (95, 96). Signals of the second class are more complex, consisting of two basic structures separated by a 10 amino acid spacer. Accordingly, these NLSs are referred to as bipartite, and were first discovered in the *Xenopus* nucleoplasmin protein. The specific sequence is as follows, KRPAATKKAGQAKKK (97). Another type of NLS is the M9 domain contained within the heterogeneous ribonucleoprotein A1 (hnRNP A1) protein. The M9 domain, NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPR NQGGY, is notably different from the basic mono- or bipartite NLSs. It is rich in glycine residues, does not exhibit the typical basic cluster motif, and has also been shown to have a role in nuclear export (98).

Transport Factors Involved in Nuclear Protein Import

Uncovering the players involved in the NLS-dependent import of proteins was greatly aided by the development of an *in vitro* system that closely reproduces the import process (80). In this system, the nuclear accumulation of a fluorescent substrate is monitored upon introduction into digitonin-permeabilized cells. A major consequence of plasma membrane permeabilization is the depletion of the soluble contents of the cells. The observation that active import depends on the readdition of cytosolic fractions allowed for the purification and subsequent molecular characterization of four soluble factors required for import, namely importin α, importin β, the GTPase Ran/TC4, and NTF2 (nuclear transport factor 2) (85-87, 90, 99-103).

Importin α was the first of these cytosolic factors to be cloned and sequenced. Purified from Xenopus oocytes, its molecular weight varies between 54 and 60 kDa, and has since been isolated and cloned in a number of species (80, 101). Yeast homologues and synonyms for importin α include karyopherin α, PTAC (pore targeting complex) 58, pendulin, NPI-1 (nucleoprotein interactor 1) and Srp1p/Kap60p/Rsl1p (suppressor of RNA polymerase I) (104). Importin α contains two functional domains: an importin-β binding domain (IBB domain) at the amino terminus, and an NLS binding site consisting of eight so-called arm repeats, each primarily composed of ~42 hydrophobic amino acids. This motif originally identified in Armadillo/plakoglobin/β-catenin proteins has been speculated to mediate protein-protein interactions between importin α and NLS containing proteins, as well as with other cytosolic factors (103, 105-107). To date,

evidence suggests that importin α is capable of binding to only those NLSs of the basic or bipartite motif (101, 108). Immunofluorescence analysis depicts importin α localized to the cytoplasm, the NE, and the nucleus of mammalian cells (103). More recently the mechanisms regarding the export of importin α from the nucleus have been uncovered, further demonstrating the likelihood that importin α is recycled to participate in multiple rounds of transport (109).

Importin β, originally identified and purified from bovine erythrocytes, has also been molecularly characterized in various other species (88, 99, 110, 111). It is a ~97 kDa protein that is responsible for the initial docking to the cytoplasmic filaments of the NPC (88, 89). Yeast homologues and synonyms for importin β include karyopherin β, PTAC 97, and Kap95p. The structure of importin β can be divided into three domains, an N-terminal domain capable of binding RanGTP, an internal region that binds to specific nucleoporins, and a C-terminal region that binds specifically to importin α (112, 113). Importin β is localized within the cytoplasm and the NE, but is excluded from the nucleus of intact cells (89, 100). The search of importin-β related proteins revealed two additional relatives, transportin/karyopherin β2/Kap104p and Kap123/Pse1p/karyopherin β3. These transporters are involved in alternative import pathways, the first responsible for the nuclear import of mRNA-binding proteins that contain the M9 domain (hnRNPA1), the second for the nuclear targeting of specific ribosomal proteins (108, 114, 115). It is expected that other genes related to importin-β will encode carriers for as yet unidentified cargoes.

The GTPase Ran (or TC4) is an abundant nuclear protein that has been implicated in multiple reactions following the initial docking of the import substrate to the cytoplasmic periphery of the NPC (86, 87, 116, 117). Like all G proteins, Ran acts as a 'molecular switch' cycling between an active form, Ran-GTP, and an inactive form, Ran-GDP. GTP hydrolysis by Ran is essential for passage of proteins through the nuclear pore (86, 87). The spontaneous conversion between these two states is very slow and must be facilitated by specific factors. Three regulators of Ran have been identified so far: RCC1 (regulator of chromosome condensation 1), Ran's major guanine nucleotide exchange factor (GEF) generates Ran-GTP (118); second, RanGAP1, a cytoplasmic Ran GTPase-activating protein, converts Ran-GTP into Ran-GDP (119); and third, RanBP1, a cytoplasmic protein which preferentially binds to Ran-GTP, facilitates the GTPase activation by RanGAP1 (120-122). In the absence of RanGAP1, RanBP1 also inhibits nucleotide exchange on Ran-GTP (122).

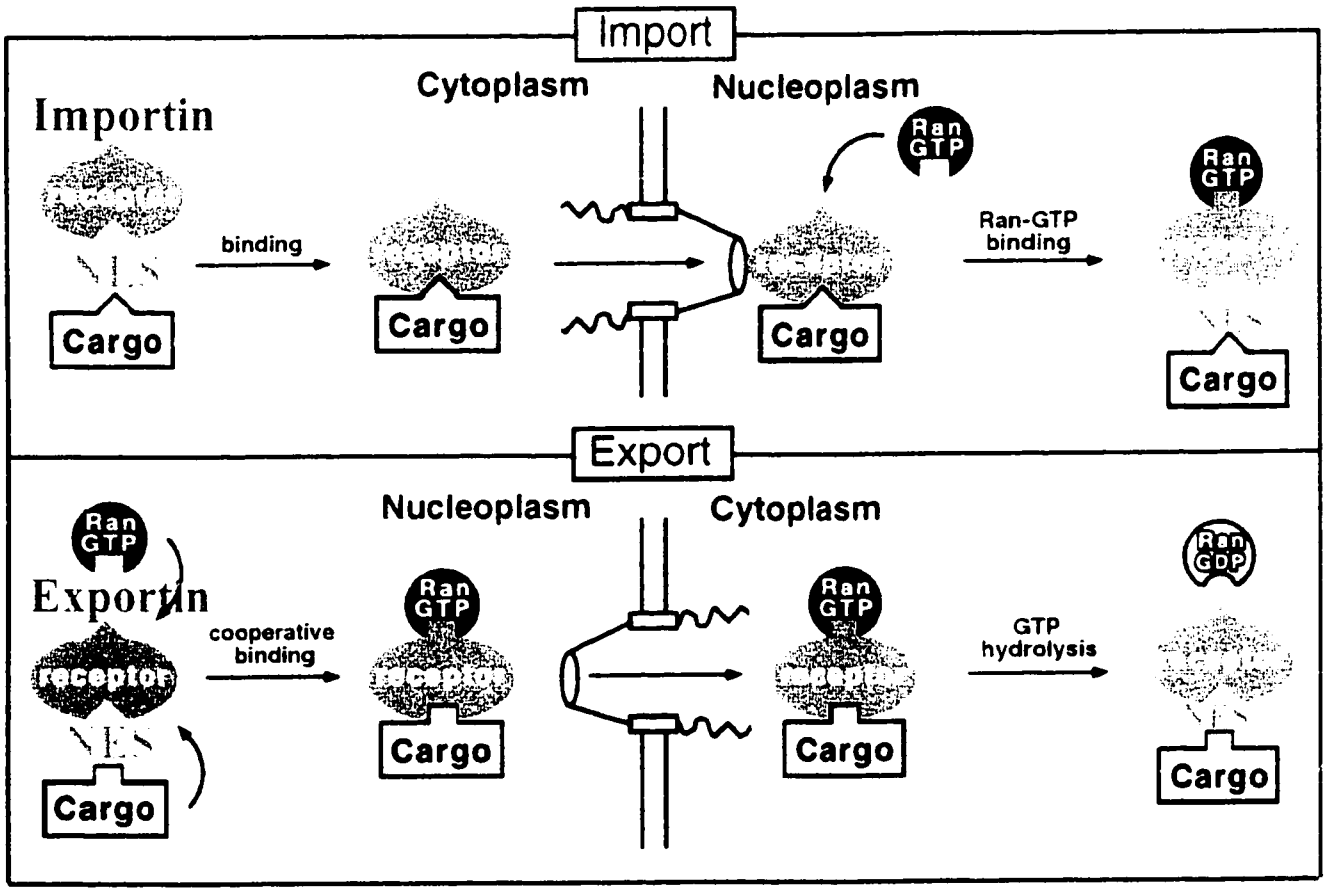
NTF2 was identified by its depletion from HeLa cell cytosol (90). Together with Ran, NTF2 is able to translocate the import ligand complex across the central gated channel after it has been docked to the NPC. It subsequently was shown to interact with NPCs, isolated nucleoporins, Ran-GDP, and importin β *in vitro* (123-125). It has been proposed that NTF2 may mediate the delivery of the import ligand from the initial docking site to the central gated channel acting as a regulator of the RanGTPase cycle when Ran is confined to the NPC (126).

NLS-dependent Protein Import

The mechanisms involved in the nuclear import of NLS-containing proteins have been identified experimentally, and are believed to occur in the following manner (reviewed in (104)) (summarized in Fig.2). The initial cytoplasmic event is the binding of the import substrate via its NLS to importin α, followed by recognition and subsequent binding of importin-α by importin-β (102, 111). Importin α binds the NLS protein, while importin β strengthens the affinity of the complex for the NLS. Importin β mediates docking of the cargo-carrier complex to the cytoplasmic filaments of the NPC, from where it is transferred to the cytoplasmic entry of the central channel and subsequently translocated through the NPC (84, 88, 89). This energy-dependent transfer through the NPC requires GTP hydrolysis by Ran and appears to be facilitated by NTF2 (85-87, 90). The translocation ends approximately at the terminal end of the nuclear basket where the binding of importin β by RanGTP disassembles the importin heterodimer and releases the import substrate into the nucleoplasm (105, 117). Finally, the importin subunits are returned to the cytoplasm through distinct export pathways (105, 106, 113).

Figure 2. A representative model for signal mediated nuclear import and export.

Import of proteins begins with NLS recognition and binding by the importin heterodimer, followed by translocation through the NPC. In the nucleus, NLS containing cargo is released through complex binding by RanGTP (104). Similarly, the export of proteins begins with the formation of a trimeric complex. Exportin recognizes and binds to NES containing proteins, while co-operative binding by RanGTP enables facilitated passage through the NPC. In the cytoplasm, GTP hydrolysis releases the NES from the export receptor complex (109, 148).


Import
Cytoplasm
Nucleoplasm
Importin
binding
Cargo
Cargo
Ran GTP
Ran-GTP binding
Ran GTP
Cargo
Export
Nucleoplasm
Cytoplasm
Ran GTP
Exportin
receptor
cooperative binding
Cargo
Ran GTP
Cargo
Ran GTP
Cargo
GTP hydrolysis
Ran GDP
Cargo

Export of importin α occurs through two distinct pathways. The first involves CAS (Cellular Apoptosis Susceptibility gene), a recently identified export receptor for importin α. CAS preferentially binds importin α that is free from NLS containing substrate, forming a trimeric complex with Ran-GTP. The complex is exported and subsequently dissociated in the cytoplasm through the combined action of RanGAP1 and RanBP1 (109). The second mechanism involves the formation of another trimeric complex whose partners include importin α, the nuclear cap-binding protein complex (CBC), and capped U snRNAs. This complex is also exported, however dissociation in this case is carried out by importin β (127).

The export mechanism for importin β is unknown, however upon dissociation of the NLS-importin α/β ternary complex by Ran-GTP, importin β remains docked on the nuclear side of the pore, and may still be bound to Ran-GTP (88, 128). This association is released by the binding of importin α and the nucleoporin C-Nup1, which inevitably reconstructs the importin α/β heterodimer, allowing for successive rounds of nuclear import (128).

Nuclear Export

Much less is known regarding the mechanisms involved in the export of proteins from the nucleus. Nuclear export, like nuclear import, is also initiated by a specific signal. Kinetic competition experiments in *Xenopus* oocytes have shown that different classes of RNA, including mRNA, snRNA, tRNA, and rRNA, are exported through

distinct pathways (129-132). Since most, if not all, RNAs associate with proteins in the nucleus, it was postulated that RNA export events are mediated by proteins containing appropriate export signals (104).

Elegant techniques such as heterokaryon analyses, and nuclear injection assays allowed for the search of protein nuclear export signals (NESs) that promote the export of heterologous proteins that would otherwise be restricted to the nucleus. The search has so far yielded three types of NESs. The first NESs were identified in PKI (protein kinase inhibitor) and the HIV-1 transactivating protein Rev (133, 134). These hydrophobic sequences, LALK-LAGLDI and LPPLERLTLD, respectively, are characterized by critical leucine residues; removal of one of these residues inactivates this sequence and prevents export (135). Other export signals include the aforementioned M9 sequence of hnRNP A1, a proposed exporter of mRNA, and a 24 amino acid signal found in hnRNP K that is termed KNS for hnRNP K nuclear shuttling domain (98, 136). When fused to a nuclear restricted protein, these NESs are sufficient to initiate export in a rapid, temperature-dependent manner. Recently it was shown that the leucine-rich NES also interacts with phenylalanine-glycine (FG) repeats of several nucleoporins, suggesting that translocation through the NPC requires a sequential binding of the NES to the FG repeats (137, 138). Similar NESs have now been identified in quite a number of other proteins including TFIIIA, and IκBα (133, 139-143). Although these sequences may function as NESs when isolated, functionality must be assessed in their native protein context.

NES-containing proteins are exported in an active and saturable manner, indicating that the process is receptor mediated (134) (summarized in Fig. 2). CRM1

(chromosomal region maintenance 1) was identified to be responsible for the intracellular transport mediated by NESs (144). In yeast, CRM1 is necessary for the export of protein and mRNA from the nucleus (145). A nuclear protein capable of shuttling between the nucleus and cytoplasm, CRM1 shares significant homology with importin β-like transport factors (145). Experiments using the Streptomyces metabolite Leptomycin B (LMB) further solidified the role of CRM1 as an export receptor for NES containing proteins. LMB, originally shown to inhibit the nuclear export of the HIV-1 Rev protein (146), has recently been shown to directly bind CRM1, specifically disrupting the association of CRM1 to NESs (147). Based on its newly identified role as an export receptor, CRM1 was renamed exportin1.

Similar to the process of signal mediated protein import, Ran-GTP is an essential component of the export receptor complex. Exportin1 binds cooperatively to Ran-GTP in the presence of an NES-containing substrate, however, the formation of this complex in the presence of Ran-GDP does not occur (148). CAS, the aforementioned nuclear exporter of importin α also cooperatively binds importin α and Ran-GTP, demonstrating a role for Ran as a regulator in the import and export of proteins from the nucleus (109).

Regulation of Protein Subcellular Localization

Nuclear proteins, which are synthesized in the cytoplasm, have to be imported into the nucleus in order to exert their activity. Import of some proteins into the nucleus, in particular, transcription factors, is not a constitutive process; instead it appears to be modulated in response to external stimuli, cell cycle progression, and developmental cues. These findings lead to a concept in which the nuclear transport of proteins constitutes an important regulatory checkpoint in the control of cellular growth and differentiation. Examples of such regulation include direct phosphorylation of the transported protein, masking of NLSs, cytoplasmic retention by binding to an anchoring protein and modulation of the import machinery itself.

Regulation of nuclear translocation by direct phosphorylation of an NLS-containing protein was first studied in the SV40-NLS. The SV40-NLS is shown to flank a site that can greatly enhance the rate of nuclear import. This effect seems to be mediated by phosphorylation at the site targeted by casein kinase II (CKII) (149). In contrast to the effects of CKII, phosphorylation by the cell-division-control kinase $p34^{cdc2}$ at a second site near the SV40-NLS substantially reduces the extent of nuclear import (150). Subcellular localization of the yeast transcription factor SWI5 is similarly modulated by phosphorylation, however localization varies with cell cycle progression. SWI5, cytoplasmic in S, G2, and M phase, accumulates in the nucleus upon G1 phase. Nuclear entry of SWI5 is inhibited through phosphorylation by CDC28 (the yeast homologue of $p34^{cdc2}$). Dephosphorylation at the end of mitosis triggers nuclear entry of SWI5 coinciding with the inactivation of CDC28 (151). Various other shuttling proteins

have been shown to contain potential CKII or p34 ^{cdc2} sites flanking their NLSs. These include nucleoplasmin, mouse c-Abl, c-Myc, polyoma T antigen, and human p53, whose subcellular localization is also cell-cycle dependent (150, 152). In proteins characterized so far, the phosphorylation by CKII promotes nuclear uptake, while p34 ^{cdc2} phosphorylation inhibits entry, leaving a provocative theory involving the dual regulation of nuclear import by these two kinases. Other kinases have been implicated in the regulation of protein nuclear import. Of particular interest is the cAMP-dependent protein kinase (PKA), whose activity directly or indirectly affects the subcellular localization of various proteins (153).

Nuclear translocation can also be affected through the interaction with an inhibitor protein. The Rel family of proteins, including NF-κB, c-Rel, and v-Rel provide one of the better documented examples of transcriptional regulation through NLS masking and subsequent cytoplasmic retention. NF-κB is sequestered in the cytoplasm through an NLS masking interaction with its inhibitor protein I-κB. Stimulation by mitogens or cytokines induce the phosphorylation of I-κB by PKA, allowing for the rapid nuclear transport of NF-κB upon release from I-κB. By contrast to the effects of anchoring inhibitors, many transcription factors active in the form of multi-protein complexes, are transported into the nucleus through dimerization with other nuclear factors (154, 155).

Directly masking NLSs is not the only manner by which proteins are sequestered in the cytoplasm. The existence of cytoplasmic retention domains (CRDs), best exemplified in the *Xenopus* factor xnf7, provides for an anchoring mechanism

independent of NLS transport inhibition. Like their NLS counterparts, CRDs are characterized through deletion and transfer analyses. Strong evidence for the anchoring function of CRDs comes from the demonstration that xnf7 remains cytoplasmically localized, even upon the addition of a second NLS to its carboxy-terminal end (156). The cytoplasmic retention of *Xenopus* myogenic factor XMyoD also appears to be mediated in a similar manner (157).

Unlike the examples above which consider only the regulatory affects on the transport protein or its interacting partner, the active control of import by components of the import machinery is also a valuable area of interest. Comparing the uptake of different sized nucleoplasmin-coated gold particles in serum-depleted cells confirmed that the nuclear uptake of particles is higher in proliferating cells, possibly as a result of higher pore permeability and number (158). This coincides well with the earlier findings that the gated channel of the NPC undergoes a nearly twofold decrease in diameter during quiescence, than when compared to proliferating cells (159). Once again combining the importance of cell-cycle progression on nuclear uptake, other proteins involved in gene expression, including c-Fos, and c-Myc, have also shown their subcellular localization to be dependent upon specific requirements at precise times in the cell cycle (160).

Nucleocytoplasmic Trafficking of GR

Glucocorticoids are homeostatic steroids that play key roles in a wide variety of vital functions including inflammatory reactions, regulating stress response, producing surfactants in the lung, and controlling the immune system. Glucocorticoids influence these many biological processes by signaling through a high affinity intracellular receptor that is itself a transcription factor whose subcellular localization is regulated (summarized in Fig.3). Prior to hormone treatment, unliganded, inactive GR exists in the cytoplasm, bound in a multiprotein heterocomplex (67, 161-163). Upon ligand addition, GR dissociates from the protein complex and transforms into an active free form that rapidly and completely translocates to the nucleus ($t_{1/2}$ = 4-5 min). GR acts as a homodimer capable of regulating transcription, primarily by binding to palindromic DNA binding sites in the transcriptional regulatory regions of target genes (164). DNA binding has been shown to be a significant determinant for GR nuclear retention in addition to its ability to bind to the nuclear envelope (165, 166), the nuclear matrix (167), and other nuclear proteins (168-170). Upon loss of ligand, GR redistributes slowly to the cytoplasm ($t_{1/2}$ = 12-24 h) and is found once again re-associated in the multiprotein complex, capable of responding to additional treatment with hormone (163). The mechanism by which GR returns to the cytoplasm has yet to be uncovered.

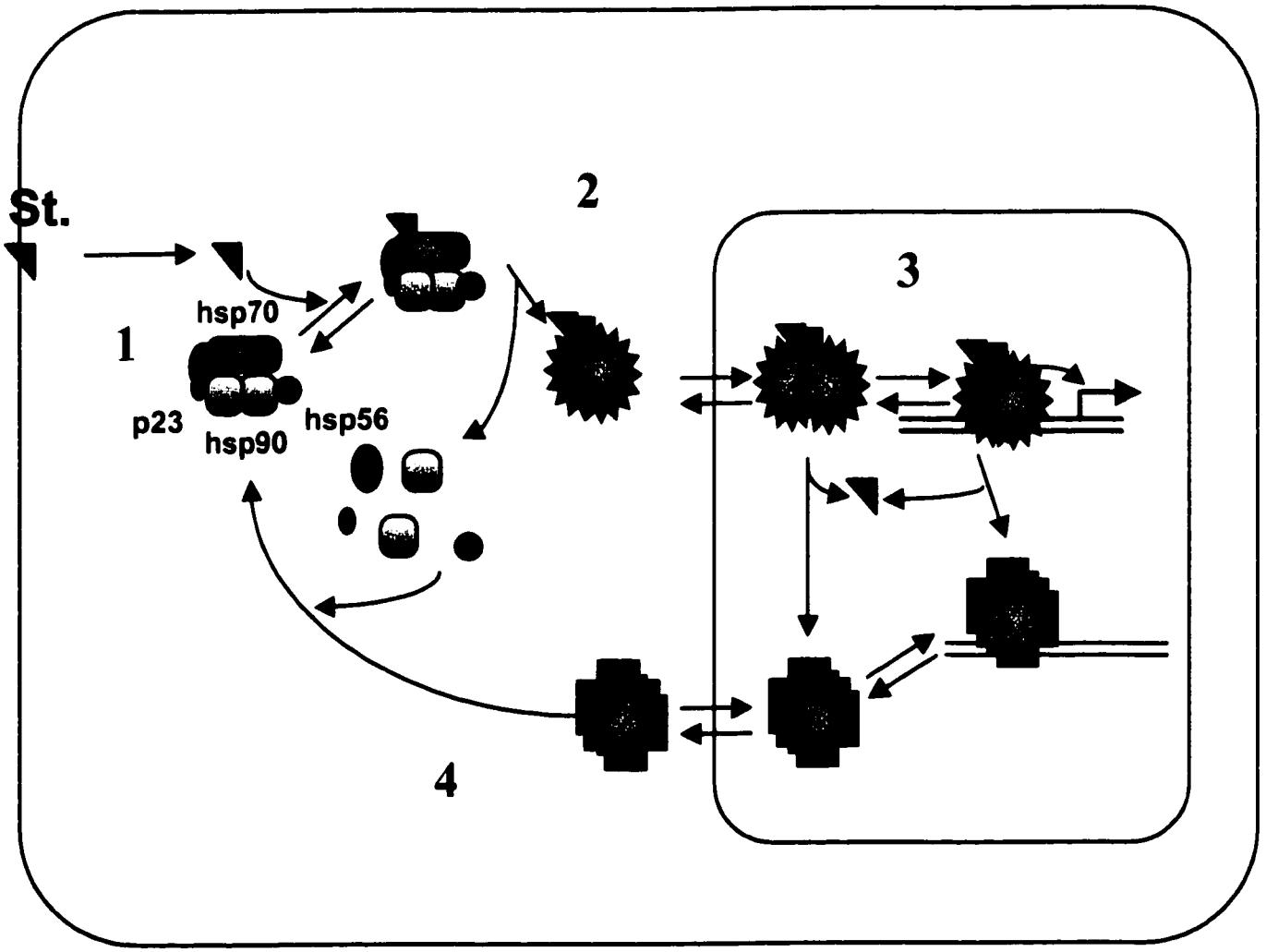
Nuclear import of liganded GR is mediated through a well-characterized nuclear localization sequence, NL1, located in the hinge region of the receptor, and a second unidentified motif, NL2, situated in the LBD (67, 72). Studies effectively knocking out the NL1 activity of GR have uncovered that nuclear uptake mediated by NL2 is agonist

specific, acts independent of binding to importin-α, and occurs more slowly than NL1 mediated nuclear translocation (162).

With regard to nucleocytoplasmic trafficking, GR shares many similarities with the other steroid hormone receptors. They all contain similar NLSs that direct nuclear import (67). They associate into comparable multi-protein heterocomplexes in their naïve states (171), and when liganded, they all possess the ability to shuttle continuously between the nucleus and cytoplasm (171-173).

Figure 3. Schematic representation of the nucleocytoplasmic trafficking of GR.

1. In the absence of hormone GR is localized to the cytoplasm, bound in a multiprotein heterocomplex. 2. Upon hormone addition, GR dissociates from the hsp-complex and is transformed into its active form (161-163). 3. GR then enters the nucleus where it regulates transcription of target genes by binding to its specific response elements (164). 4. Upon loss of ligand, GR is redistributed to the cytoplasm where it is found once again bound to the multiprotein heterocomplex (163).


St.
2
3
hsp70
1
p23
hsp90
hsp56
4

In the absence of hormone however, the differences in their subcellular localizations are unquestionable. Unliganded PR (174) and ER (175) are located in the nucleus, while GR, MR, and possibly AR are found in the cytoplasm (67, 163, 176, 177). Historically, the observed cytoplasmic localization of GR was believed to be a consequence of NL1 masking by hsps, preventing GR access to the nuclear import machinery (15, 39, 178). Very recently however, it has been shown that even unliganded hsp-complexed GR is capable of transferring efficiently between heterokaryon nuclei, and that an N-terminally fused nuclear retention signal is able to induce the nuclear accumulation of naïve GR in the absence of steroid (161). These results suggest that the cytoplasmic localization of unliganded GR may represent a regulated skewed equilibrium distribution between the rates of transfer across the nuclear membrane. Alternatively, an argument also exists whereby the localization of GR to the cytoplasm reflects the activity of an active retention mechanism.

Project Goals

The molecular basis for the differential cytoplasmic localization of unliganded GR is not well understood. The major part of my M.Sc. research work focused on investigating if there exists within GR, a determinant, such as an amino acid sequence, responsible for its initial cytoplasmic localization in the absence of hormone and/or for its return to the cytoplasm after hormone treatment and subsequent withdrawal. The experimental approach employed to achieve this goal involved the construction of GR truncation mutants followed by the use of an indirect immunofluorescence assay to assess the subcellular localization of individual mutants in comparison to wtGR. These mutants will provide a region for investigation, and upon construction of further truncations, will be used to aid in further delimiting a specific amino acid sequence necessary for cytoplasmic localization of GR in the absence of steroid. Upon discovery of a specific determinant, sufficiency to impart cytoplasmic localization will be assessed by fusing this sequence to a protein otherwise found ubiquitously localized in the cell.

In addition, as there were other aspects related to the subcellular localization of the glucocorticoid receptor uncovered in previous studies conducted in the laboratory characterizating the NL2 function of GR, I completed these previous studies, by investigating: 1) the effect of withdrawal of hormone on the redistribution of a mutant GR whose NL1 function had been destroyed, 2) the kinetics of NL2 mediated nuclear uptake upon hormone treatment, withdrawal, and subsequent restimulation, and 3) the effect of the newly reported drug leptomycin B, which inhibits CRM1 mediated export as described above, on the redistribution of the wild type glucocorticoid receptor and this

NL1 mutant GR. The approach employed to achieve these goals once again made use of the indirect immunofluorescence assay to determine the effects these various treatments imparted on the site-directed NL1 mutated GR construct (GR_{NL1}), which was created and kindly donated by Mr. Brian Hsu.

Materials and Methods

Enzymes, Chemicals and Ligands

All restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs (NEB) (Mississauga, ON, Canada). Fetal Bovine Serum, stripped and untreated, were purchased from Hyclone USA (Logan, UT, USA), and tissue culture reagents were obtained from GIBCO BRL (Burlington, ON, Canada). The GR ligand cortisol was obtained from Steroloids Inc. (Wilton, NH, USA), and all other chemicals were purchased from BDH (Quebec, Canada).

Expression Constructs

GR truncation mutants and WT constructs were expressed from plasmids containing fragments of rat GR, donated from Yamamoto et al. (4) fused C-terminally to a 6x repeat of the myc tag epitope. To generate $GR_{407\text{-}795}$, amino acids 407-795 were amplified by polymerase chain reaction (PCR) from plasmid 6RGR (containing the full length GR sequence) using forward primer (5'-GCCCCGCTCGAGTCAGTGTTTTCTAATGGG TACTCAAGCC-3'), and reverse primer (5'-TCCCCCGGGATCCTCATTTTTGAT GAAACAGAAGCT-3'). The PCR product was digested with BamHI and XhoI (New England Biolabs), and subcloned in frame into pTlmyc (a pSG5 derivative containing 6 copies of a myc tag epitope) digested with BamHI and XhoI. All successive truncation mutants were generated in the same manner, utilizing the same restriction enzyme sites

and the same reverse primer, differing only in the use of the forward primers selected for PCR amplification. $GR_{300-795}$ was amplified using the forward primer (5'-TCCCCCCC TCGAGAAAGATGATTTCATTGAACTTTGCAC-3'). $GR_{200-795}$ was amplified using the forward primer (5'-GAAACCGCTCGAGGGCAGTGTGAAATTGTATCCCA-3'). $GR_{175-795}$ was amplified using the forward primer (5'-CCGGAATTCGCGGCCGCA AGGAGTTTCCCAAAACTCAC-3'). $GR_{125-795}$ was amplified using the forward primer (5'-GAACCGCTCGAGTACCCACAGCAGGGCC-3'), and $GR_{100-795}$ was amplified using the forward primer (5'-ACCCGCTCGAGTCCAAAGCCGTTTCACTGTC-3').

To generate the construct GFP-$GR_{200-795}$, the enhanced green fluorescent protein (eGFP) was PCR amplified from pEGFP-C1 (Clonetech) using the forward primer (5'-CCG GAATTCGCGGCCGCATGGTGAGCAAGGGC-3'), and reverse primer (5'-CCGG AATTCTCGAGCTTGTACAGCTCGTCCAT-3'). The PCR product was digested with NotI and XhoI and ligated into $GR_{300-795}$ digested with NotI and XhoI. GFP was subcloned in frame, C-terminally to the myc tag repeat, and N-terminally to the GR sequence spanning amino acids 300-795.

Similarly, generating the deletion mutant $GR_{100-200/407-795}$, amino acids 100-200 were PCR amplified from plasmid 6RGR using forward primer (5'-CCGGAATTCGCGGCCGC TCCAAAGCCGTTTCACTGTC-3'), and reverse primer (5'-CCGGAATTCTCGAGG CCTCCGTTGGTGCCGGTC-3'). The PCR product was digested with NotI and XhoI and was subconed into $GR_{407-795}$, again C-terminally to the myc tag repeats, and N-terminally to the GR sequence spanning amino acids 407-795.

Oligonucleotides for PCR were synthesized using a Beckman Oligo 1000 DNA synthesizer, and all PCR reactions were carried out using VENT DNA polymerase, which contains a DNA sequence proof reading ability, allowing for large fragment amplification. PCR conditions for a single cycle of amplification were as follows: 1) Denaturation, 95°C for 1 min., 2) Annealing, 55°C for 1min., and 3) Extension, 72°C for 2 min. Annealing temperatures were periodically changed depending on the primer used, and conditions were repeated for 25 cycles of amplification.

Ligations were carried out at room temperature for 30 min. using 1 ul of T4 DNA ligase in a final volume of 20 uL consisting of (1x) ligase buffer, dH_2O, and restriction enzyme digested PCR product and vector. Transformation of DH5α cells was performed by heat shocking cells at 42°C for 2 min. Cultures were plated on solid LB containing ampicillin and incubated overnight at 37°C. Individual colonies were selected and used to seed 5 mL cultures for miniprep analysis and restriction enzyme mapping. Upon isolating the desired construct, large-scale amounts of DNA were prepared via double cesium-chloride centrifugation and isolation.

Cell Lines and Maintenance

COS7 cells (ATCC), originating from a monkey kidney cell line were selected for experiments due to the absence of endogenous glucocorticoid receptor. Cells were grown in 15 mL flasks (Falcon) containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Cells were passaged every 3 to 4 days and

discarded upon reaching passage number 12. Incubator conditions were set to a temperature of 37°C and a 5% CO_2 level.

Transient Transfections

COS7 cells were seeded into 60 mm plates and left overnight to reach confluent levels of 60-80%. Cells were transiently transfected using the Lipofectamine (GIBCO) method of transfection. Selected constructs of DNA (0.5-1.0 ug) were combined with 10 uL of Lipofectamine reagent in 500 uL of serum free OptiMEM media (GIBCO). The mixture was vortexed and left to sit for 45 minutes. Cells were pre-washed twice with 1x phosphate buffered saline (PBS), then placed in OptiMEM media for 5 minutes. Lipofectamine/DNA mixtures were topped up to 2 mL with OptiMEM and added directly to the plates, which were incubated for no less than 5 hours. Transfections were stopped by adding an equal 2 mL volume of DMEM supplemented with 20 % charcoal stripped fetal bovine serum. Cells were incubated overnight to allow expression of the desired proteins.

Sixteen hours following the termination of transfection, the media was replaced with DMEM supplemented with 10% charcoal stripped FBS. Eight hours later, cells were replated onto poly-L-lysine (SIGMA) coated glass coverslips and incubated in 35mm 6 well tissue culture dishes for no less than 8 hours in DMEM supplemented with 10% charcoal stripped FBS. Cells were synchronized to G_0 by washing twice with 1x PBS, followed by incubation in serum-free DMEM for a further 18 hours prior to initiation of hormone treatment.

Preparation of Poly-L Lysine Glass Coverslips

Glass coverslips were flame sterilized in 95% ethanol and coated with a 1x solution of poly-L lysine. Upon air drying, coverslips were subsequently washed with pyrogen-free dH_2O and left standing upright to dry prior to usage. All steps were performed under a sterile biological fume hood.

Hormone Treatment and Withdrawal

Cortisol was added to a final concentration of 10^{-6} M in serum-free DMEM for one hour. For subsequent cortisol withdrawal, cells were washed three times with 1xPBS, once with serum-free DMEM, and incubated in serum-free DMEM containing 5% bovine serum albumin (BSA) for 24 hours. Cells treated with the export inhibitor leptomycin B (LMB) were cultured in the same manner prior to hormone addition, and LMB was used to a final concentration of 200nM in serum-free DMEM.

Semi-Quantitative Indirect Immunofluorescence Assay (IFF)

Following experimental treatment, cells on coverslips were fixed at room temperature (RT) in 3% (w/v) paraformaldehyde in PBS for 30 min. Intrinsic fluorescence due to fixation was quenched by washing with a solution of 0.1 M glycine in PBS for 10 min at RT. Cells were permeabilized with 0.5 % Triton X-100 in PBS for 30 min at RT, followed by incubation with 5% normal goat serum (NGS) which blocks non specific

binding of antibody. After aspiration of NGS, 0.5 mL of a 5 ug/mL solution of BuGR-2 (Affinitiy Bioreagents), (a monoclonal anti-GR antibody) was added to the coverslips, followed by an overnight incubation at 4°C. The following day, cells were extensively washed (5x in 2 mL of PBS for 2 min) and incubated in a 0.6 ug/mL sheep anti-mouse fluorescein-isothiocyanate-conjugated (FITC) IgG (Boehringer Mannheim) secondary antibody. Incubation was carried out at RT under darkness with gentle shaking for 45 min. Washing was repeated (4x in 2 mL of PBS for 2 min), and coverslips were mounted in glycerol/PBS (1:1 v/v) onto glass slides. Cells were examined for the subcellular localization of GR constructs on a Zeiss Axiophot photomicroscope. Quantification was carried out under blinded encryption and cells were classified into one of five categories ranging from exclusively nuclear (N), nuclear greater than cytoplasmic (N>C), nuclear equals cytoplasmic (N=C), cytoplasmic greater than nuclear (C>N), and exclusively cytoplasmic (C) (163). To simplify further, the two categories of nuclear staining were combined (N+N>C), as well as the two categories for cytoplasmic staining (C+C>N), essentially leaving three classification types. For each experiment, a minimum of 400 cells was counted for each specific treatment type. Experiments were repeated 3-5 times.

Results Part I

1. A Semi-Quantitative Indirect Immunofluorescence Assay (IFF) for the analysis of GR Subcellular Localization

To investigate the subcellular localization of GR, a semi-quantitative indirect immunofluorescence assay was employed to directly visualize the localization of specific constructs after various treatments. In these experiments, COS-7 cells which lack endogenous GR, were transiently transfected with a variety of GR constructs. The COS-7 cells were then synchronized to G_0, treated with agonist according to experimental conditions described, then fixed and stained for visualization. Since the nucleocytoplasmic trafficking of GR is cell cycle dependent, G_0 was chosen to ensure a uniform response, as well as to mimic physiological conditions, since most glucocorticoid target tissues are not actively proliferating (179, 180). Furthermore, in G_0, a stable population of GR is provided for visualization as protein degradation and *de novo* synthesis are inhibited (180). In the analyses, cells were classified into one of five categories ranging from exclusively nuclear (N), nuclear greater than cytoplasmic (N>C), nuclear equals cytoplasmic (N=C), cytoplasmic greater than nuclear (C>N), and exclusively cytoplasmic (C) (See Fig. 4). Results for each category are presented as a percentage of the total cell population counted. This system was first described in early experiments which uncovered the signals responsible for mediating nuclear localization of GR (67), and has more recently been utilized to assess the nucleocytoplasmic trafficking of GR DNA binding mutants (163). To simplify results in my investigations, the categories for exclusively nuclear (N) and nuclear greater than cytoplasmic (N>C)

were combined (N+N>C), as well as the categories for exclusively cytoplasmic and cytoplasmic greater than nuclear (C+C>N). Therefore, three classifications are represented in the bar graphs of figures 5-16 error bars reflecting the standard deviations among at least three separate experiments. Refer to table 1 for a summary of results of the subcellular localization of the various GR truncation and deletion mutants.

2. A determinant in the N-terminus of GR is responsible for cytoplasmic localization in the absence of hormone.

The search for a specific determinant within GR responsible for cytoplasmic localization in the absence of hormone began with a strategy to create N- and C-terminal truncation mutants to assess the subcellular localization of separate modular domains of GR. In these studies, a wtGR construct lacking the SV40 origin of replication was employed as a control for subcellular localization (pTlmyc-GR). This construct was then used as the backbone for all truncation mutants eventually constructed. I began by creating a truncation that effectively removed the N-terminus of GR ($GR_{407-795}$) (Fig. 5A). To ensure that there would be no passive diffusion through the NPC, all mutants were fused C-terminally to a myc tag, increasing the size of each construct to near or above the 60 kDa limit that restricts passive diffusion (See Table 1).

Assessing the subcellular localization of the wtGR construct (pTlmyc-GR), we found that the levels of nuclear and cytoplasmically stained cells (~7% and ~75% respectively) are comparable to those found in previous experiments for wtGR when

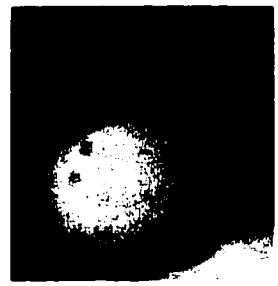
Figure 4. Visualizing the subcellular localization of GR.

Representative micrographs of cells demonstrating (i) exclusively cytoplasmic staining, (ii) cytoplasmic greater than nuclear staining, (iii) cytoplasmic equals nuclear staining, (iv) nuclear greater than cytoplasmic staining, and (v) exclusively nuclear staining.

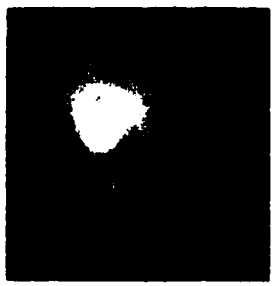
i

ii

iii



iv



v

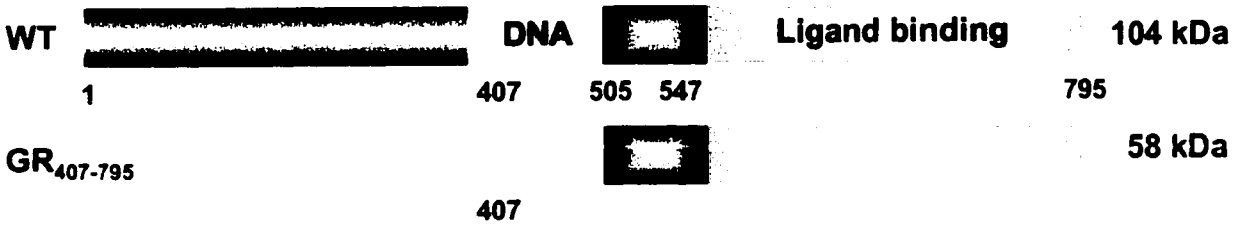
studying the subcellular localization of GR DNA binding mutants (163) (Fig. 5B). Comparing the subcellular localization of the full length GR to that of the N-terminally truncated construct before exposure to hormone showed that the level of nuclear stained cells is appreciably higher with the truncated GR mutant (~42%) than with the wtGR (~7%) (Fig. 5B). In comparison to wtGR which remains predominantly cytoplasmic in the absence of steroid (~76%), the level of cytoplasmically stained cells (~40%) was observed to decrease for the N-terminally truncated mutant. This result suggests that detaching the N-terminus of GR removes a sequence responsible, at least in part, for the favored cytoplasmic compartmentalization, usually observed in the absence of steroid.

Endeavoring to further delimit the determinant for cytoplasmic localization within the N-terminus, a series of additional mutants was constructed in which incremental portions of the GR N-terminal domain were truncated. Constructs $GR_{400-795}$ and $GR_{200-795}$, which contain approximately the C-terminal 100 and 200 amino acids of the N-terminus respectively, were created and tested for their individual subcellular localizations before exposure to hormone (Fig. 6). To determine whether size would affect the localization, a green fluorescent protein (GFP) was fused N-terminally to $GR_{200-795}$ and C-terminally to the myc tag. The construct GFP-$GR_{200-795}$ essentially added another 28 kDa to the original $GR_{200-795}$. Similar to the N-terminal truncation mutant in the absence of steroid, the levels of nuclear staining observed for these three new constructs were appreciably higher than that for wtGR in the absence of steroid. The percentage of nuclear and cytoplasmically stained cells for the $GR_{300-795}$ construct were

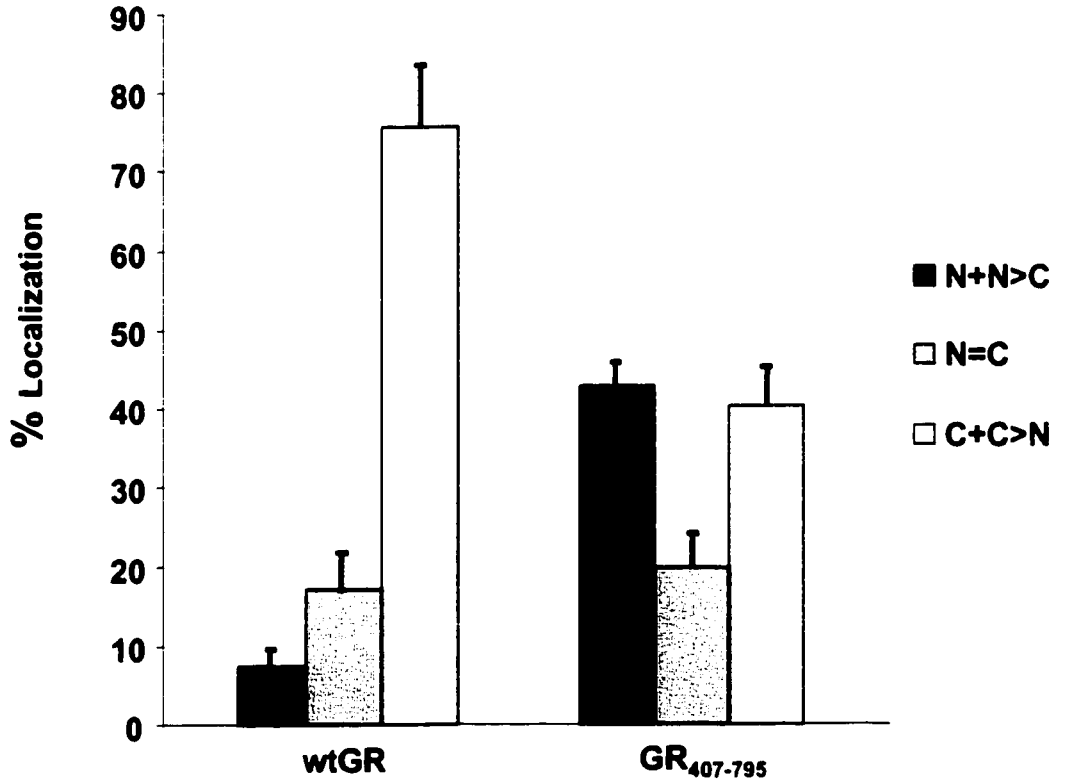
Figure 5. Removal of the GR N-terminus abrogates cytoplasmic localization in the absence of steroid.

A. Schematic representation of full length rat wtGR (top) and the N-terminally truncated mutant $GR_{407-795}$ (below). **B.** Graphical representation of the subcellular localization of wtGR and $GR_{407-795}$ in the absence of steroid. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.

A


WT
DNA
Ligand binding
104 kDa
1
407
505
547
795
GR407-795
58 kDa
407

B


% Localization
90
80
70
60
50
40
30
20
10
0
wtGR
GR407-795
N+N>C
N=C
C+C>N

~48% and ~32% respectively, while those for the $GR_{200-795}$ construct were approximately ~32% and 39% respectively. These results, although differing in their relative levels of nuclear occupancy, still demonstrate a loss in favored cytoplasmic compartmentalization as displayed by the wtGR prior to hormone treatment. GFP-$GR_{200-795}$ showed no appreciable difference in nuclear and cytoplasmic staining when compared to its counterpart lacking GFP (~36% and 25% respectively) confirming that size was not a factor between the subcellular localizations of GR truncation mutants.

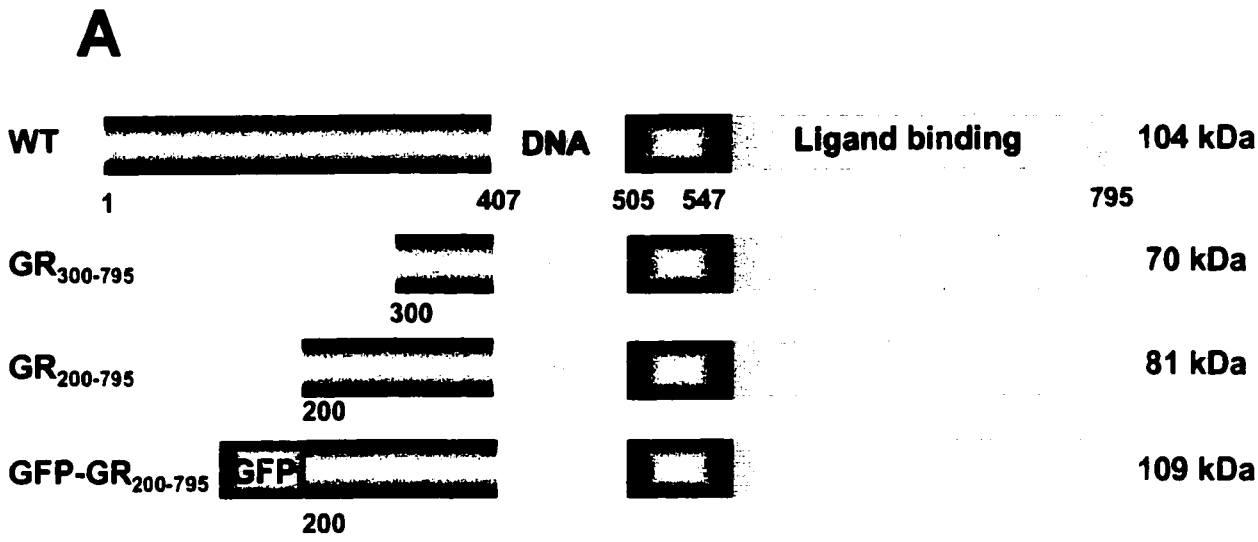
3. The addition of amino acids 100-200 re-establishes cytoplasmic localization of GR in the absence of hormone.

Unable to distinguish any appreciable difference between the aforementioned truncation mutants with regard to subcellular compartmentalization, another construct was created that included yet another hundred amino acids of the N-terminus. The mutant $GR_{100-795}$ excludes the first 100 amino acids of the GR peptide sequence. Comparing the subcellular localization of this mutant to wtGR, we found that in the absence of steroid, the profiles for subcellular occupancy for both constructs were nearly identical (Fig. 7). This result demonstrates that the cytoplasmic localization of GR is re-established with the addition of amino acids 100-200 to $GR_{200-795}$.

In attempts to further delimit a boundary within the newly added hundred amino acids, additional mutants were created that truncated GR between amino acids 100 and 200 ($GR_{125-795}$ and $GR_{175-795}$). These constructs did not retain the ability to remain

Figure 6. Additional truncations to the N-terminus of GR abrogates cytoplasmic localization in the absence of steroid.

A. Schematic representation of full length rat wtGR and the incremental N-terminal truncation mutants $GR_{300\text{-}795}$, $GR_{200\text{-}795}$, and GFP-$GR_{200\text{-}795}$. **B.** Graphical representation of the subcellular localization of wtGR and the N-terminally truncated mutants $GR_{300\text{-}795}$, $GR_{200\text{-}795}$, and GFP-$GR_{200\text{-}795}$ in the absence of steroid. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.


A
WT
DNA
Ligand binding
104 kDa
1
407
505
547
795
GR300-795
70 kDa
300
GR200-795
81 kDa
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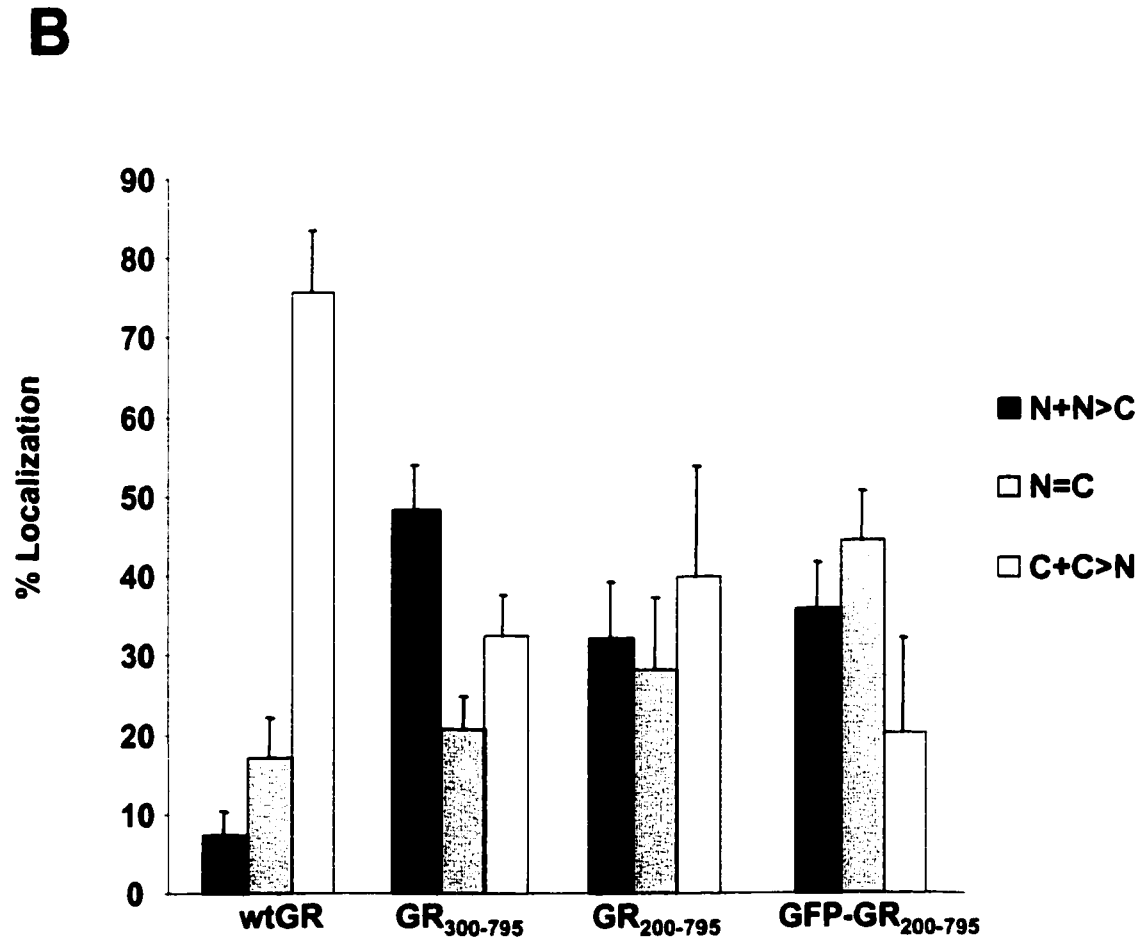
GFP-GR200-795
GFP
109 kDa
200


B
% Localization
90
80
70
60
50
40
30
20
10
0
wtGR
GR300-795
GR200-795
GFP-GR200-795
N+N>C
N=C
C+C>N

cytoplasmically localized in the absence of steroid (Fig. 8), suggesting that the boundary for a determinant capable of imparting cytoplasmic localization to GR exists around amino acid 100 of the N-terminus. Levels of cytoplasmically occupied differed somewhat for $GR_{125-795}$ (55%) compared to that of $GR_{175-795}$ (35%). However they were still not comparable to the near 75% levels observed from wtGR and $GR_{100-795}$. Differences between the two constructs with regard to the levels of nuclear staining were also apparent, with $GR_{175-795}$ displaying a slightly higher amount (~30%), in comparison to $GR_{125-795}$ (~20%). Due to the low level of nuclear staining portrayed by $GR_{125-795}$, it was tempting to extend the N-terminal boundary to amino acid 125. However in comparing the levels of nuclear occupancy to wtGR, and $GR_{100-795}$, the difference between the constructs was still clearly visible. For this reason it was decided that among our mutants, amino acid 100 would represent the N-terminal boundary.

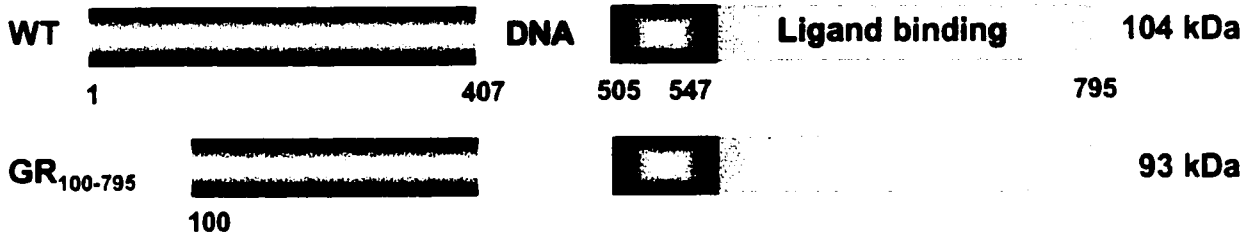
4. GR truncation mutants are capable of entering the nucleus upon hormone treatment.

In the absence of hormone, GR resides predominantly in the cytoplasm. It was important to determine whether the N-terminal truncations affected any other characteristics of GR in order to establish the specificity of the localization defects observed. To do this we assessed 1) hormone binding and 2) the ability of hormone to shift GR into the nucleus. Knowing that the addition of hormone to cells in which wtGR is present in the cytoplasm causes complete translocation of GR to the nucleus, we

Figure 7. Addition of amino acids 100-200 re-establishes cytoplasmic localization of GR in the absence of steroid.

A. Schematic representation of full length rat wtGR (top) and the N-terminal truncation mutant $GR_{100\text{-}795}$. **B.** Graphical representation of the subcellular localization of wtGR and mutant $GR_{100\text{-}795}$ in the absence of steroid. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.

A


WT
DNA
Ligand binding
104 kDa
1
407
505
547
795
GR100-795
93 kDa
100

B

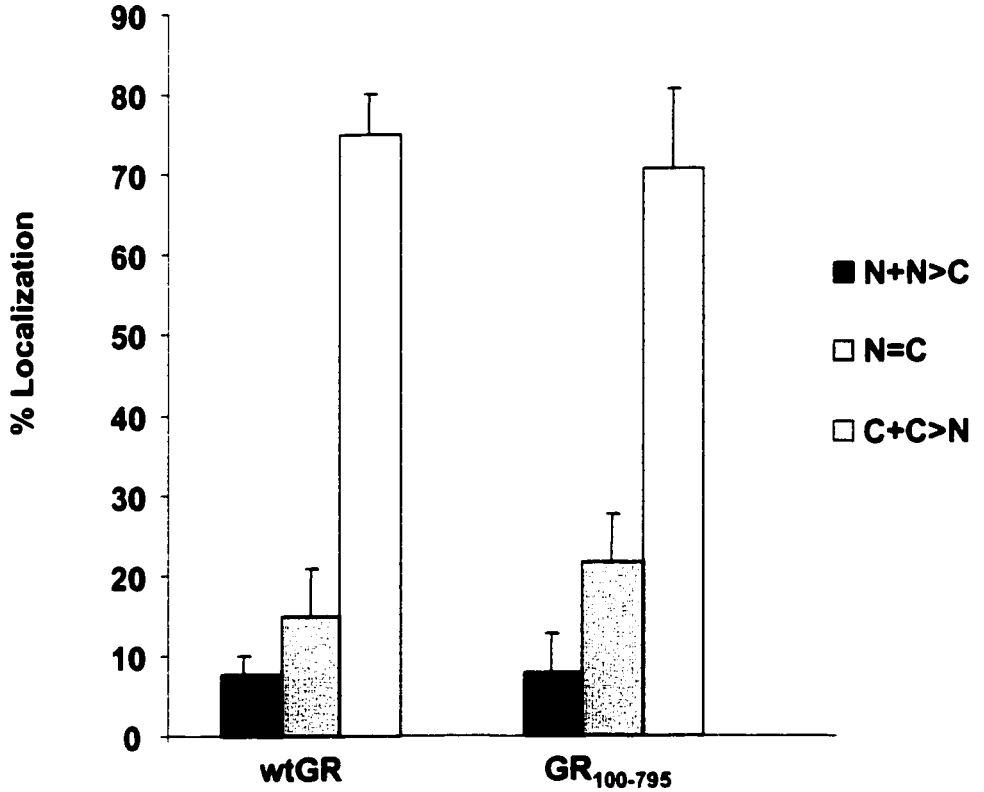
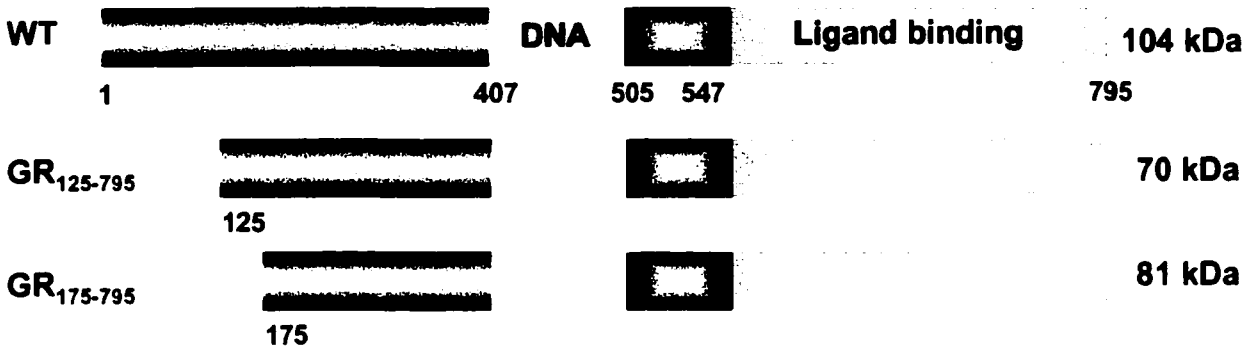

% Localization
90
80
70
60
50
40
30
20
10
0
wtGR
GR100-795
N+N>C
N=C
C+C>N

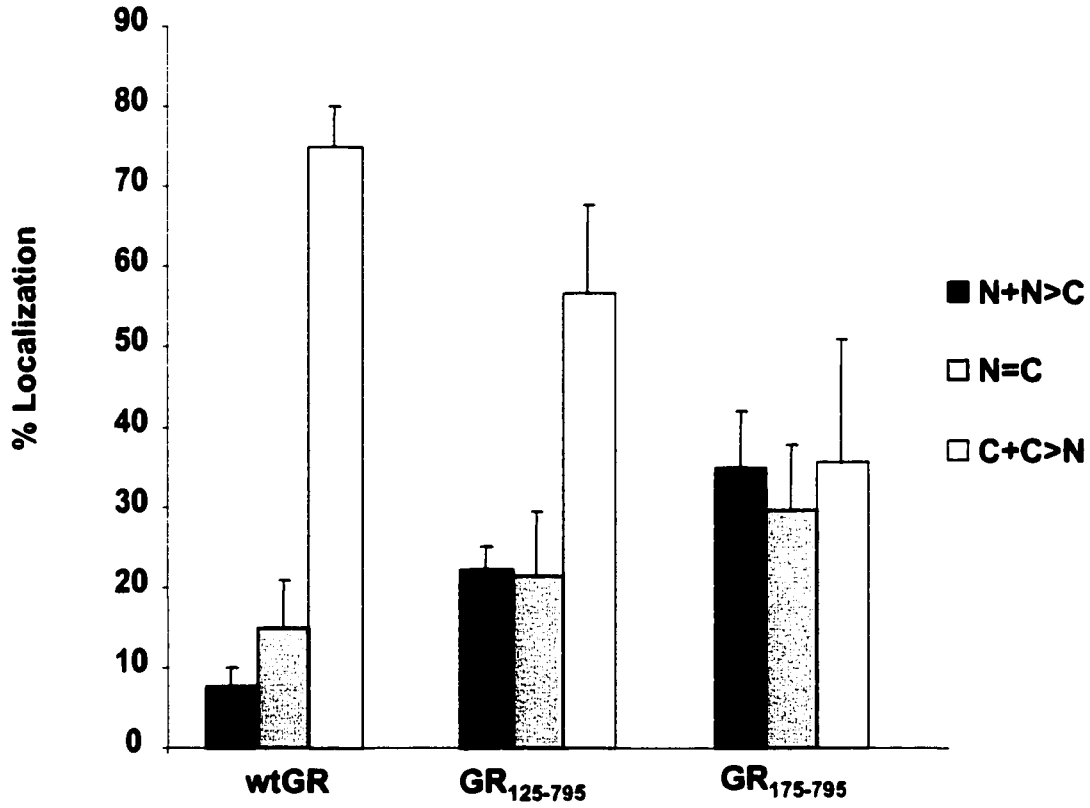
Figure 8. Truncations within amino acids 100-200 revoke cytoplasmic localization in the absence of steroid.

A. Schematic representation of full length rat wtGR (top), and the N-terminal truncation mutants $GR_{125\text{-}795}$ (middle), and $GR_{175\text{-}795}$ (bottom). **B.** Graphical representation of the subcellular localization of wtGR and the N-terminal truncation mutants $GR_{125\text{-}795}$, and $GR_{175\text{-}795}$ in the absence of steroid. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.

A


WT
DNA
Ligand binding
104 kDa
1
407
505
547
795
GR125-795
70 kDa
125
GR175-795
81 kDa
175

B


% Localization
90
80
70
60
50
40
30
20
10
0
wtGR
GR125-795
GR175-795
N+N>C
N=C
C+C>N

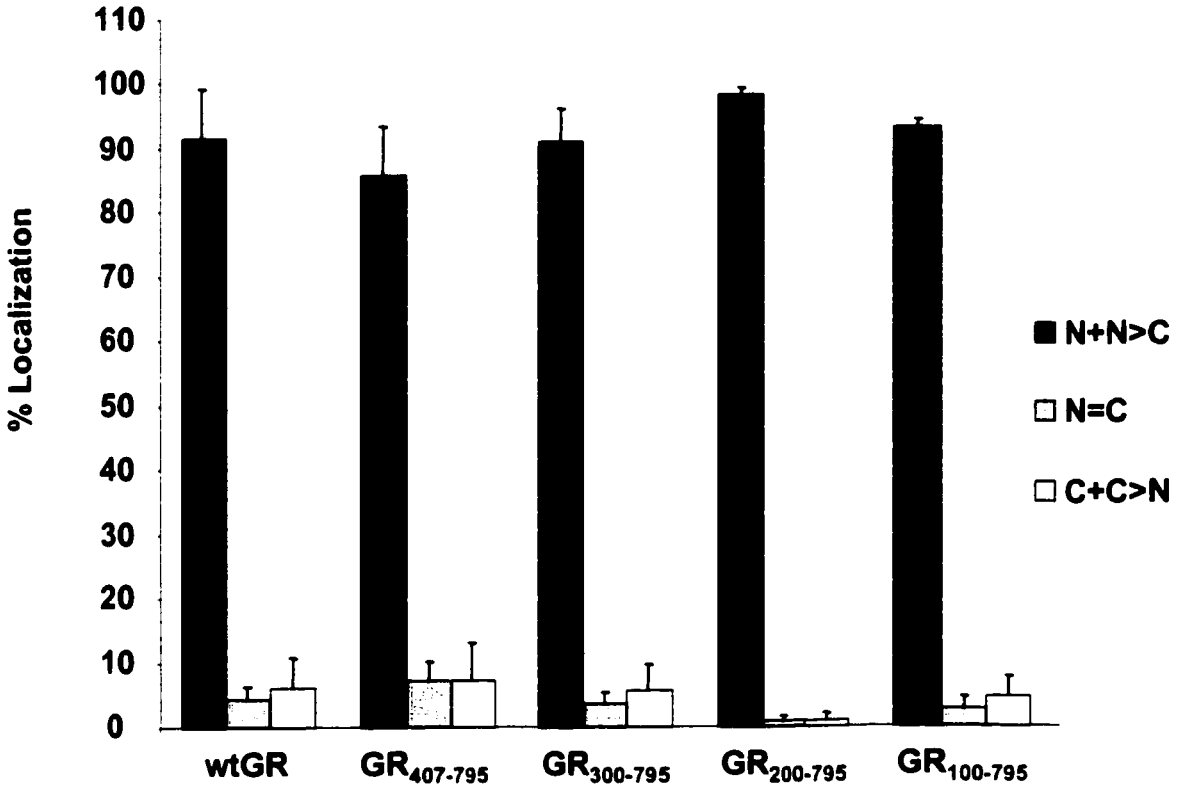
reasoned that an assay could be used in which the outcome measured would be nuclear translocation in response to hormone. Therefore cells transfected with the individual truncation mutants alongside wtGR were treated with hormone and nuclear translocation was determined. The result of treating cells containing mutant GRs with cortisol for one hour was that all constructs were able to enter the nucleus to levels comparable to wtGR (Fig. 9). This result shows that functional characteristics of GR such as nuclear import are not affected by truncations to the N-terminus at least as measured by this assay.

5. The addition of amino acids 100-200 re-establishes cytoplasmic redistribution of GR upon hormone treatment and subsequent withdrawal.

GR entry into the nucleus occurs in a rapid and complete manner ($t_{1/2}$ = 4-5 min) (163). Upon withdrawal of hormone, GR is found redistributed to the cytoplasm. This occurs by an undefined, appreciably slower process ($t_{1/2}$ =12-24 hrs) (163). Presumably the redistribution to the cytoplasm after hormone treatment is also dependent upon a determinant within the sequence of GR. Since, prior to hormone treatment, wtGR and the mutant pTImyc-$GR_{100-795}$ behave similarly in terms of subcellular localization, and further truncations were shown to cause a loss in cytoplasmic localization, the question regarding the subcellular localization of mutant GRs after hormone addition and subsequent withdrawal was addressed. Cells separately transfected with wtGR and the N-terminal truncation mutants ($GR_{300-795}$, $GR_{200-795}$, and $GR_{100-795}$) were treated with cortisol for one hour, washed, then left in steroid free medium for 24 hours prior to fixing and staining. Cortisol was chosen over the synthetic glucocorticoid dexamethasone, since

Figure 9. GR truncation mutants are capable of entering the nucleus upon exposure to hormone.

Graphical representation of the subcellular localization of wtGR and the N-terminally truncated mutants $GR_{407-795}$, $GR_{300-795}$, $GR_{200-795}$, and $GR_{100-795}$ upon one hour treatment with the synthetic glucocorticoid dexamethasone. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.


% Localization
110
100
90
80
70
60
50
40
30
20
10
0
wtGR
GR407-795
GR300-795
GR200-795
GR100-795
N+N>C
N=C
C+C>N

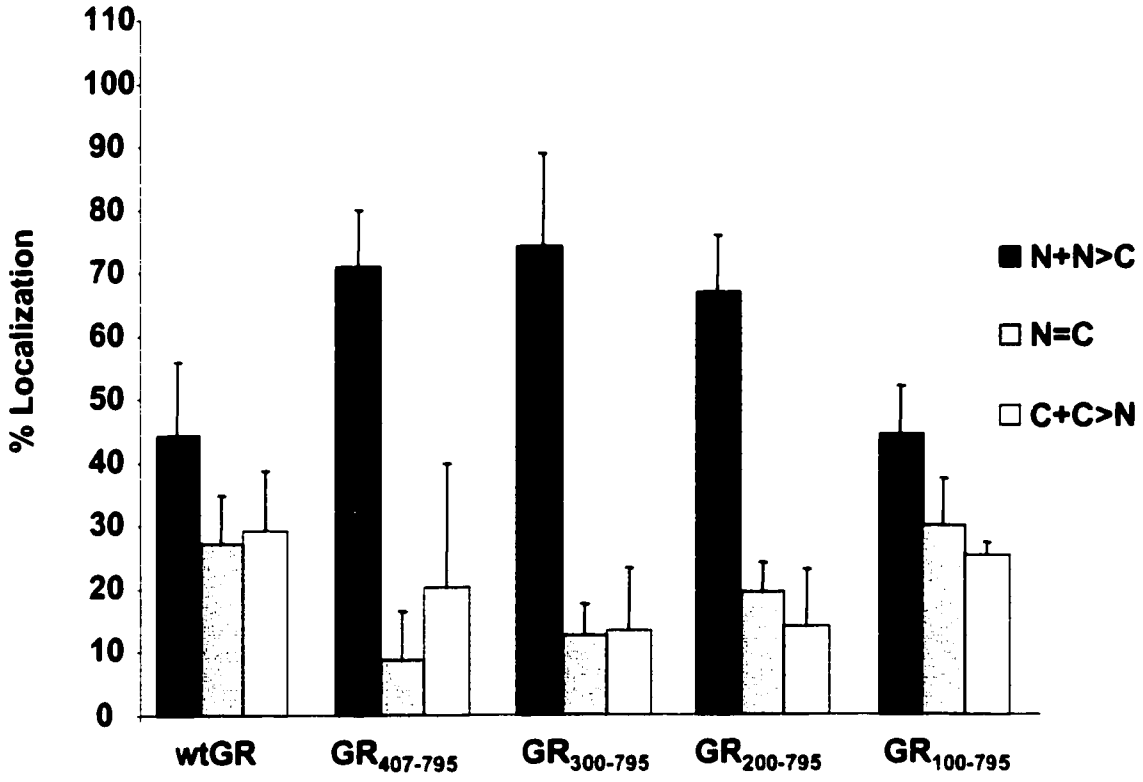
cortisol is more easily metabolized by cells, while dex is shown to remain present upon multiple washing and withdrawal. After 24 hours of steroid withdrawal we observe that cells containing wtGR appear to have a lower percentage of nuclear stained cells (~44%) when compared to $GR_{407-795}$ (~71%), $GR_{300-795}$ (~74%), and $GR_{200-795}$ (~67%). For these three mutants, cytoplasmic redistribution appears to be inhibited (Fig. 10). Interestingly, the percentage of cells transfected with $GR_{100-795}$ showing nuclear staining was once again nearly identical to wtGR (~44%). This result demonstrates that the addition of amino acids 100-200 not only serves to retain cytoplasmic localization in the absence of steroid, but also re-establishes cytoplasmic redistribution upon hormone treatment and subsequent withdrawal.

6. Amino acids 100-200 of GR are not sufficient to impart cytoplasmic localization.

The truncation of the first 99 amino acids of GR resulted in a subcellular distribution identical to the wtGR, while the deletion of the second 100 amino acids caused a noticeable shift to the nucleus. This observation may indicate that the specific signal or peptide sequence responsible for the cytoplasmic localization of GR in the absence of hormone possibly begins at amino acid 100. Thus far, deletion constructs had indicated that amino acids 100-200 were found to be necessary for cytoplasmic localization. However it had not been demonstrated that this one hundred amino acid sequence was sufficient to impart this activity on an otherwise non-cytoplasmically

Figure 10. Addition of amino acids 100-200 to GR re-establishes cytoplasmic redistribution upon hormone treatment and subsequent withdrawal.

Graphical representation of the subcellular localization of wtGR and the N-terminally truncated mutants $GR_{407-795}$, $GR_{300-795}$, $GR_{200-795}$, and $GR_{100-795}$ upon one hour treatment with cortisol, followed by a 24 hour hormone withdrawal. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.

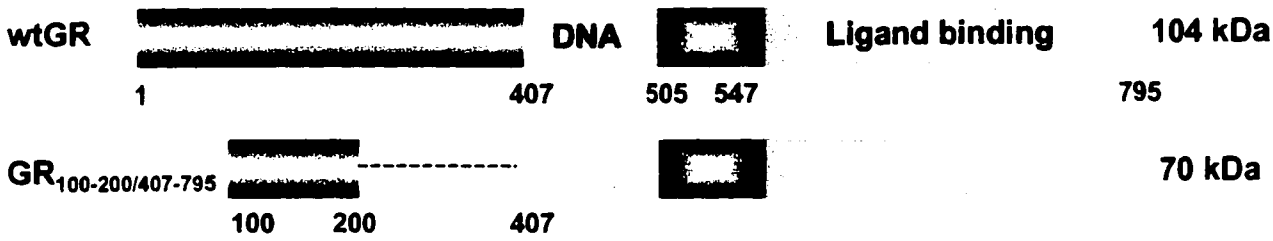

% Localization
110
100
90
80
70
60
50
40
30
20
10
0
N+N>C
N=C
C+C>N
wtGR
GR407-795
GR300-795
GR200-795
GR100-795

localized protein. To investigate whether a delimited sequence can function to invoke cytoplasmic localization on a GR construct found ubiquitously in the cell, I created a GR mutant, which places amino acids 100-200 directly upstream of the $GR_{407-795}$ construct, which is completely devoid of the N-terminus. This construct ($GR_{100-200/407-795}$) effectively deletes amino acids 201-406, and addresses the question of sufficiency since the original $GR_{407-795}$ construct appeared to lose its favored cytoplasmic compartmentalization in the cell. Upon visualizing cells transfected with this deletion construct, it was clear that the level of nuclear staining was appreciably higher (~46%) than cells transfected with wtGR (~7%) (Fig. 11). This result suggests that the sequence of amino acids ranging from 100-200 is not sufficient to impart cytoplasmic localization to a protein otherwise found ubiquitously in the cell.

Figure 11. Amino acids 100-200 of GR are not sufficient to impart cytoplasmic localization on a ubiquitously localized protein.

A. Schematic representation of full length rat wtGR (top), and the deletion construct $GR_{100\text{-}200/407\text{-}795}$ (bottom). **B.** Graphical representation of the subcellular localization of wtGR and deletion mutant $GR_{100\text{-}200/407\text{-}795}$ in the absence of steroid. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.

A


wtGR
DNA
Ligand binding
104 kDa
1
407
505
547
795
GR100-200/407-795
70 kDa
100
200
407

B

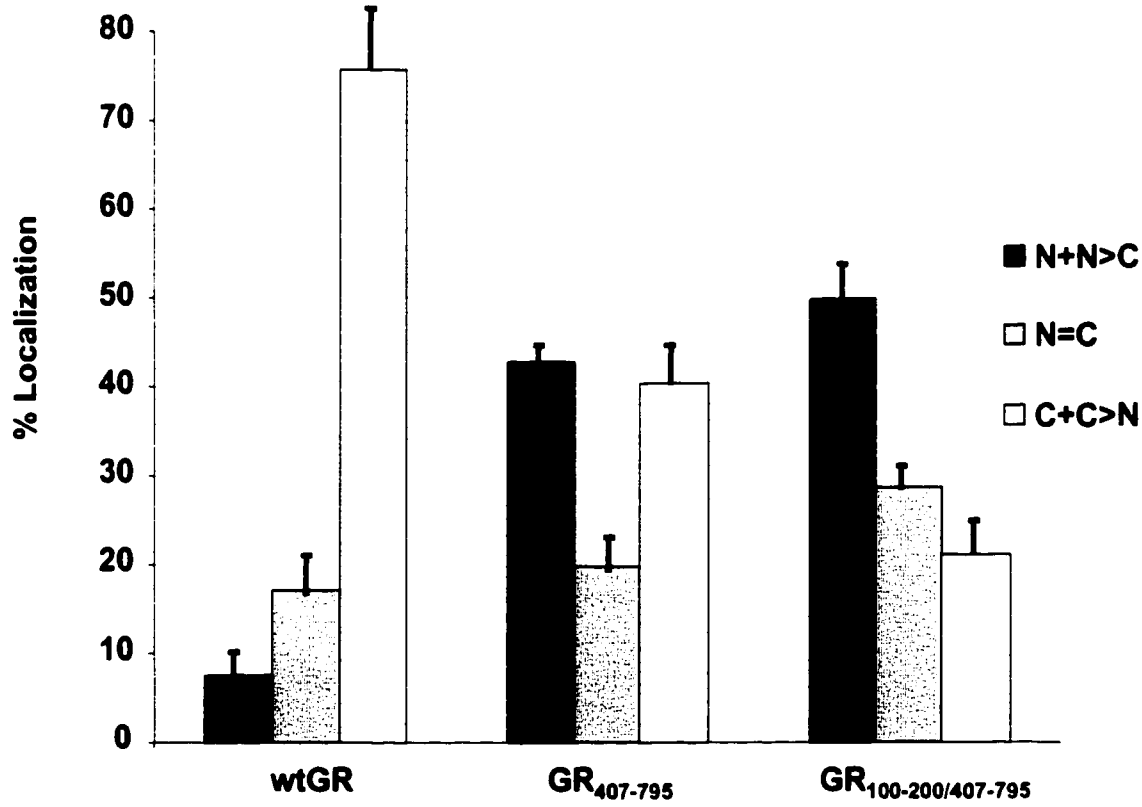

% Localization
80
70
60
50
40
30
20
10
0
N+N>C
N=C
C+C>N
wtGR
GR407-795
GR100-200/407-795

Table 1. Summary of results in the analysis of the subcellular distribution of various GR N-terminal truncation and deletion constructs in the absence of steroid.

COS7 cells were transiently transfected with plasmids encoding wtGR, $GR_{407\text{-}795}$, $GR_{300\text{-}795}$, $GR_{200\text{-}795}$, GFP-$GR_{200\text{-}795}$, $GR_{100\text{-}795}$, $GR_{.25\text{-}795}$, $GR_{175\text{-}795}$, and $GR_{100\text{-}200/407\text{-}795}$. Cells were cultured, synchronized to G_0, then fixed as described in Materials and Methods. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. Overall subcellular localization was determined by observing trends in compartmentalization representative of the majority of receptor population. Percentages listed for nuclear occupancy reflect those cells demonstrating exclusively nuclear and nuclear greater than cytoplasmic staining (N+N>C). Percentages listed for cytoplasmic occupancy reflect those cells demonstrating exclusively cytoplasmic and cytoplasmic greater than nuclear staining (C+C>N).

Table 1. Subcellular distribution of GR constructs in the absence of hormone

Constructs transfected	Size (kDa)	Subcellular Localization	
		% Nuclear	% Cytoplasmic
wtGR	104	7% ± 4	75% ± 7
$GR_{407-795}$	58	42% ± 4	40% ± 5
$GR_{300-795}$	70	48% ± 7	32% ± 7
$GR_{200-795}$	81	32% ± 8	39% ± 13
GFP-$GR_{200-795}$	109	36% ± 7	25% ± 11
$GR_{175-795}$	85	30% ± 6	35% ± 14
$GR_{125-795}$	89	20% ± 3	55% ± 10
$GR_{100-795}$	93	7% ± 5	72% ± 9
$GR_{100-200/407-795}$	70	48% ± 4	20% ± 4

Results Part II

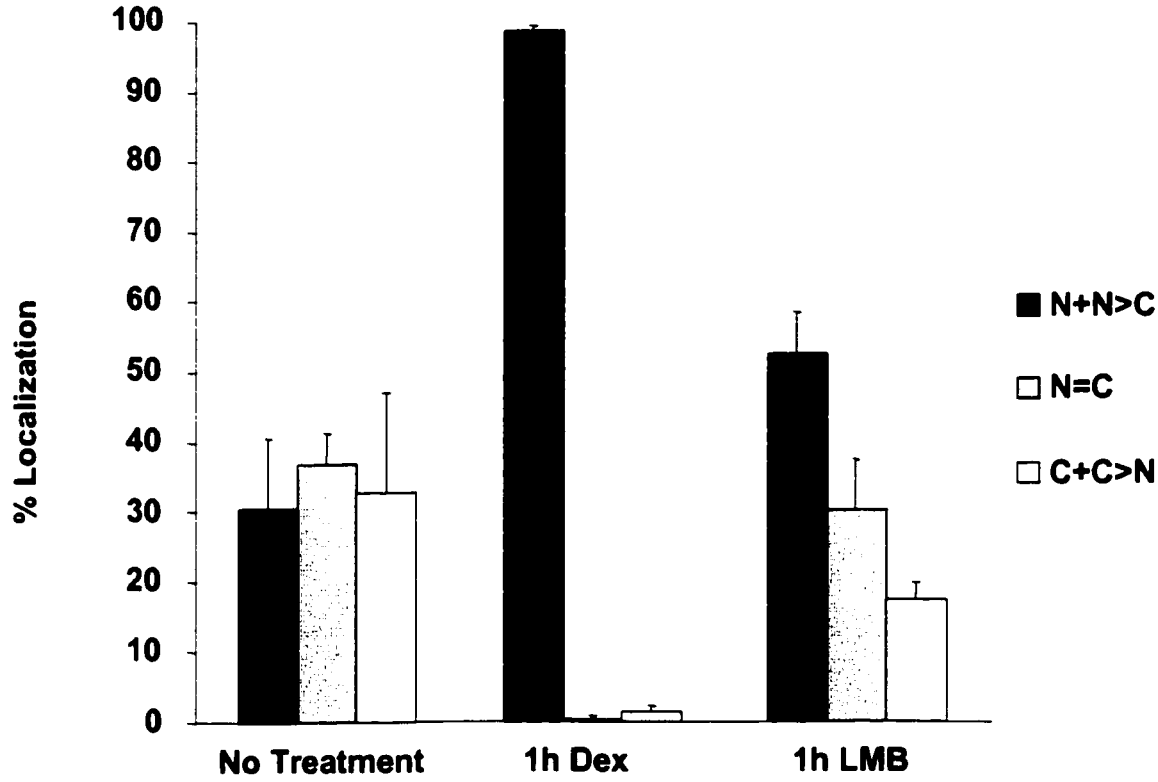
In addition to investigating determinants involved in the cytoplasmic localization of GR in the absence of steroid, I was interested in studying the effect of the newly reported export inhibitor leptomycin B on the redistribution of the wild type glucocorticoid receptor and an NL1 mutant GR. Furthermore, completing previous studies conducted in our laboratory, I was involved in a study regarding the NL2 mediated nuclear import of GR. More specifically, my focus was to assess the subcellular localization of an NL1 mutated GR construct (GR_{NL1-}), and characterize its distribution profile under varying hormone treatments, withdrawal, and subsequent restimulation. Moreover, I was interested in further investigating the effects of blocking export on the subcellular localization of this NL1 mutated GR construct because the results might help us determine constituents involved in the cytoplasmic redistribution of GR.

7. Naïve GR shuttles between the nucleus and cytoplasm in the absence of hormone in an NL1 dependent manner.

Previous work in our lab had shown that in the absence of hormone, GR resides predominantly in the cytoplasm but is capable of shuttling between the nucleus and cytoplasm (162, 165). I wanted to complete this work, but first had to reassess the subcellular localization profile of wtGR, because in these studies I evaluated the localization of a full-length rat wtGR with an SV40 origin of replication (4). Prior to hormone treatment, approximately 30% of cells expressing this wtGR demonstrated

Figure 12 The subcellular localization of wtGR under various treatments.

The 6RGR construct was transfected in COS7 cells, treated with cortisol or LMB for one hour and fixed as described in Materials and Methods. IIF was used to visualize and characterize subcellular localization. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.


100
90
80
70
60
50
40
30
20
10
0
% Localization
No Treatment
1h Dex
1h LMB
N+N>C
N=C
C+C>N

mostly nuclear staining (Fig. 12). This result is appreciably higher than previously published results, which ranged from 5-10% nuclear staining (163). However, the difference between the two previous studies was that in the first, as in the studies described in this part of my thesis, an SV40 origin of replication was used in the GR construct transfected into cells. Therefore the increase in mostly nuclear staining before exposure to hormone can be accounted for by the overexpression of GR which was replicating under the regulation of the SV40 origin of replication. Overexpression of GR has been shown to promote the partial transfer of unliganded, hsp associated receptor to the nucleus in an undefined NL1 dependent manner (162, 165). Upon treatment with the synthetic glucocorticoid dexamethasone (dex) for one hour, nuclear uptake of wtGR was almost complete with greater than 98% of cells displaying exclusively nuclear or mostly nuclear staining.

In these preliminary experiments, I also wanted to investigate the effect of the nuclear export inhibitor Leptomycin B (LMB) on wtGR since we had information that hsp bound GR prior to hormone treatment trafficked between the nucleus and cytoplasm (161). If GR was capable of shuttling between subcellular compartments, a block in export would trap the receptor in the nucleus, and a higher level of cells with nuclear GR would be visualized. To assess the optimal conditions for the experiment, I tried various concentrations of LMB, which included 2 nM, 20 nM, 200 nM, and 1 uM with times ranging from 30 min. to 7 hours (as described in (146, 181-183)). I found that concentrations of LMB less than 200 nM displayed only slight effects on the localization of GR to the nucleus. In addition, concentrations of LMB greater than 1uM, as well as

incubation times greater than 5 hours were shown to increase the potential for cytotoxicity in serum starved cells (results not shown).

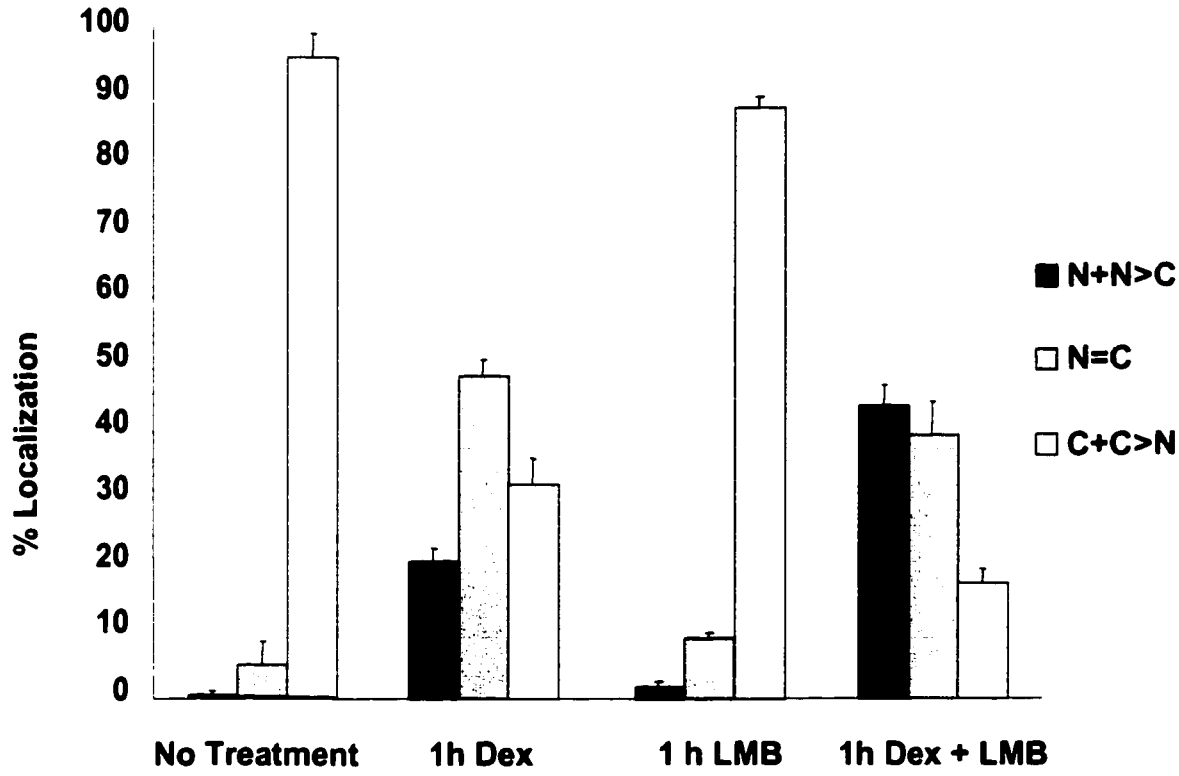
Complying with the treatment conditions of Engel et al. (183), I treated cells for one hour with 200 nM of LMB in the absence of steroid, and found that these cells displayed a higher nuclear occupancy of GR (approximately 52% nuclear staining) in comparison to untreated cells (Fig. 12). This result showed that GR, in the absence of ligand but in the presence of LMB, is capable of first entering, then secondly accumulating in the nucleus presumably due to a block in export. To confirm this finding we used an NL1 site-directed mutant (GR_{NL1-}) constructed in our lab whose nuclear import function is inactivated. Nuclear uptake of this mutant is essentially mediated by NL2, and was found to be agonist specific, act independent of binding to importin-α, and translocate more slowly than NL1 mediated nuclear import (162). Treatment of cells containing the NL1 mutant GR with LMB to a final concentration of 200nM in serum-free medium showed no appreciable rise in nuclear occupancy compared to wtGR, suggesting that nuclear import of unliganded hsp associated GR is NL1 dependent (Fig.13). Since LMB specifically inhibits the CRM1 regulated export pathway (144), this result also provides the first indirect evidence suggesting that the export of GR is mediated in a CRM1 dependent manner.

8. Inhibition of export promotes the nuclear localization of GR_{NL1-}

As stated above, studies with the export inhibitor LMB demonstrated that a block in export promoted an increase in the nuclear accumulation of wtGR prior to the exposure of cells with hormone, and that this rise in nuclear occupancy was dependent on NL1 (Figs. 12&13). To study the effects of blocking export on GR with an inactivated NL1, cells expressing GR_{NL1-} were subjected to similar treatments with LMB and dex (Fig. 13). Prior to hormone treatment, COS7 cells expressing GR_{NL1-} demonstrated predominantly cytoplasmic staining (~95%) as mentioned above. Upon treatment for one hour with 10^{-8} M dex, movement of the receptor towards the nucleus is evident (~20%), however levels of nuclear occupancy are substantially less than when compared to wtGR after the same treatment (~98%) (Fig. 12). Cells treated with the combination of dex (10^{-8} M) and LMB (200nM) however, displayed a two-fold increase in the percentage of nuclear stained cells (40%) when compared to cells treated with dex alone. This shift of GR_{NL1-} towards the nucleus was very similar to that observed with the WT unliganded receptor (Fig. 12). Treatment of GR_{NL1-} transfected cells with LMB alone showed no discernible difference from the non treated cells, further demonstrating how in the absence of ligand, GR_{NL1-} is incapable of entering the nucleus and subsequently accumulating when nuclear export is blocked. These results suggest that NL2 mediated nuclear translocation is ligand dependent, with levels of nuclear staining rising due to a block in nuclear export. Furthermore, this result indicates that the site-directed mutation that inactivated NL1 appeared to have little, if any effect on the nuclear export of GR through the CRM1 mediated pathway.

Figure 13 Leptomycin B promotes the nuclear localization of GR_{NLI}.

Graphical representation of the subcellular localization of GR_{NLI} following treatment for 1 h with dex or with dex and leptomycin B. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.


100
90
80
70
60
50
40
30
20
10
0
% Localization
N+N>C
N=C
C+C>N
No Treatment
1h Dex
1 h LMB
1h Dex + LMB

9. NL2 mediated nuclear uptake of GR occurs in a slow and incomplete manner.

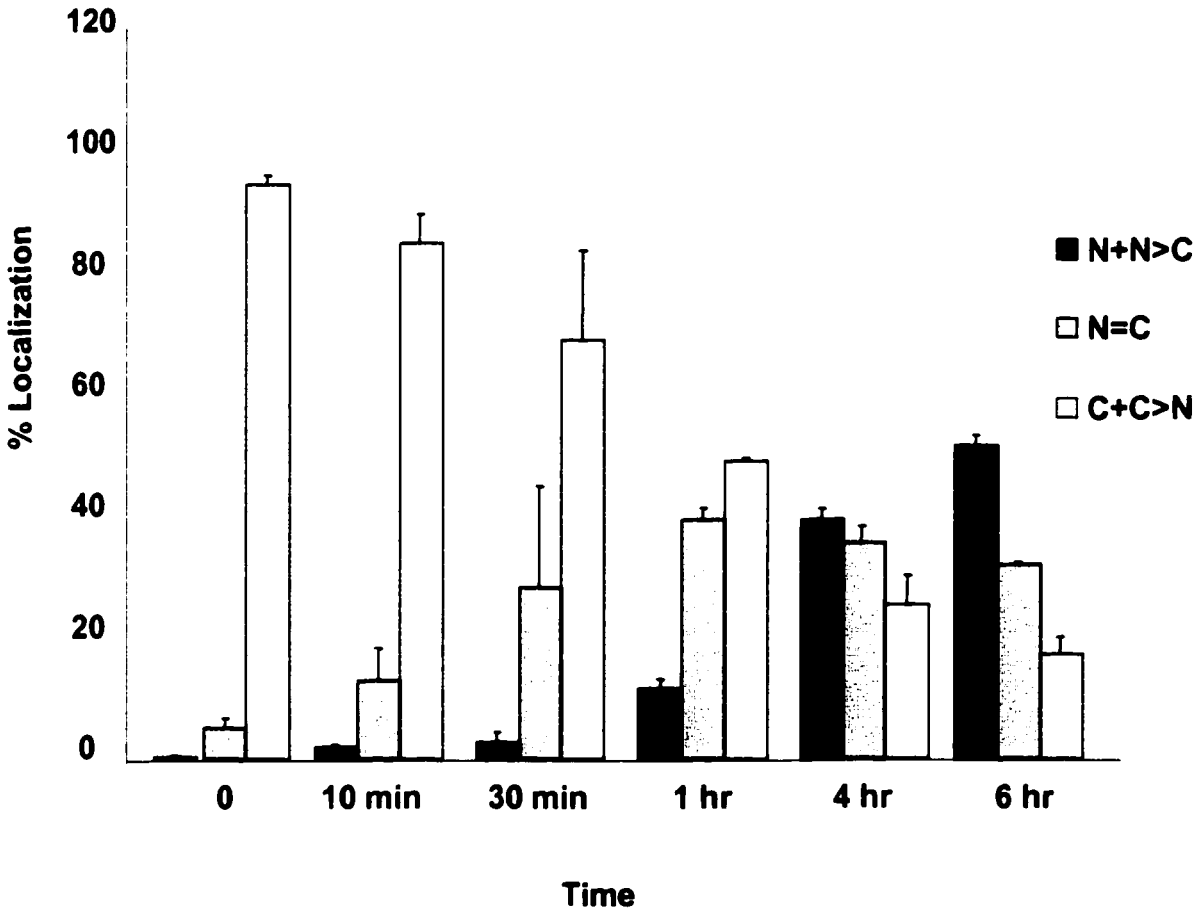
To further investigate the nuclear uptake of GR mediated by NL2, GR_{NL1-} was treated with cortisol to a final concentration of 10^{-8} M for times ranging from 10 minutes to 6 hours (Fig. 14). In the absence of steroid, GR_{NL1-} was shown to be localized almost entirely to the cytoplasm (~95%) having almost no traces of nuclear staining (0.2%). This is in contrast to wtGR which was shown have a lower cytoplasmic localization (~75%), and exhibit a mild nuclear occupancy prior to ligand treatment (~7%) (Figs. 5-8). Upon treatment with cortisol for one hour, cells expressing GR_{NL1} were observed to have minimal levels of nuclear staining (~12%), differing markedly from wtGR which was shown to localize almost completely to the nucleus (~90%) upon treatment with cortisol for the same period of time (Fig. 9). Furthermore, GR_{NL1-} never completely localized to the nucleus (~50%) even after prolonged (6 hour) treatment with hormone. This shows that nuclear transfer of GR_{NL1-}, mediated by NL2, occurs in a slow and inefficient manner.

10. GR_{NL1-} is rapidly redistributed to the cytoplasm upon hormone treatment and subsequent withdrawal.

Cytoplasmic redistribution upon hormone withdrawal is yet another interesting feature with regards to the nucleocytoplasmic trafficking of GR. As stated above, GR is found redistributed to the cytoplasm in an undefined, appreciably slow process ($t_{1/2}$ =12-

Figure 14 NL2 mediated nuclear uptake of GR occurs in a slow and incomplete manner.

Graphical representation of the subcellular localization of GR_{NL1} following treatment with cortisol for times ranging from 10 min to 6 hrs. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.


120
100
80
60
40
20
0
% Localization
N+N>C
N=C
C+C>N
0
10 min
30 min
1 hr
4 hr
6 hr
Time

24 hrs) (161, 163, 171). To study the effects on cytoplasmic redistribution caused by mutating the GR NL1, cells expressing GR_{NL1-} were treated for 6 hours with cortisol to a final concentration of 10^{-6} M, washed, then hormone withdrawn for times ranging from 15 minutes to 4 hours (Fig. 15). GR_{NL1-} was shown to rapidly redistribute to the cytoplasm, almost completely exiting the nucleus by 4 hours after withdrawal (~5%). By contrast, wtGR remains predominantly nuclear (~45%) even after a 24 hour withdrawal from hormone (Fig. 10). This result suggests that NL1 may mediate the prolonged nuclear retention of GR following hormone withdrawal.

11. Hormone restimulation promotes the return of GR_{NL1-} to the nucleus.

Retreatment of cortisol withdrawn cells expressing wtGR with dex is shown to promote the re-uptake of cytoplasmic receptors into the nucleus with kinetics and equilibrium distribution indistinguishable from a primary response (163). To assess the subcellular localization profile of an NL1 inactivated GR under hormone restimulation conditions, cells expressing GR_{NL1} were treated with cortisol (10^{-6} M) for 6 hours, hormone withdrawn for 4 hours, then retreated with dex (10^{-6} M) for times ranging from 10 minutes to 4 hours (Fig. 16). GR_{NL1-} was shown to re-enter the nucleus at a rate strikingly similar to that shown under primary cortisol stimulation (Fig. 14). Furthermore, GR_{NL1-} never completely relocalizes to the nucleus (~40%) even after prolonged (4 hour) secondary treatment with hormone. This result demonstrates that nuclear transfer mediated by NL2, upon hormone treatment, subsequent withdrawal, and secondary stimulation occurs in a slow and inefficient manner similar to what is observed upon primary response.

Figure 15 GR_{NLI} is rapidly redistributed to the cytoplasm upon hormone treatment and subsequent withdrawal.

Graphical representation of the subcellular localization of GR_{NLI} upon treatment with cortisol for 6 hrs, followed by hormone withdrawal for times ranging from 15 min. to 4 hrs. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate. Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.

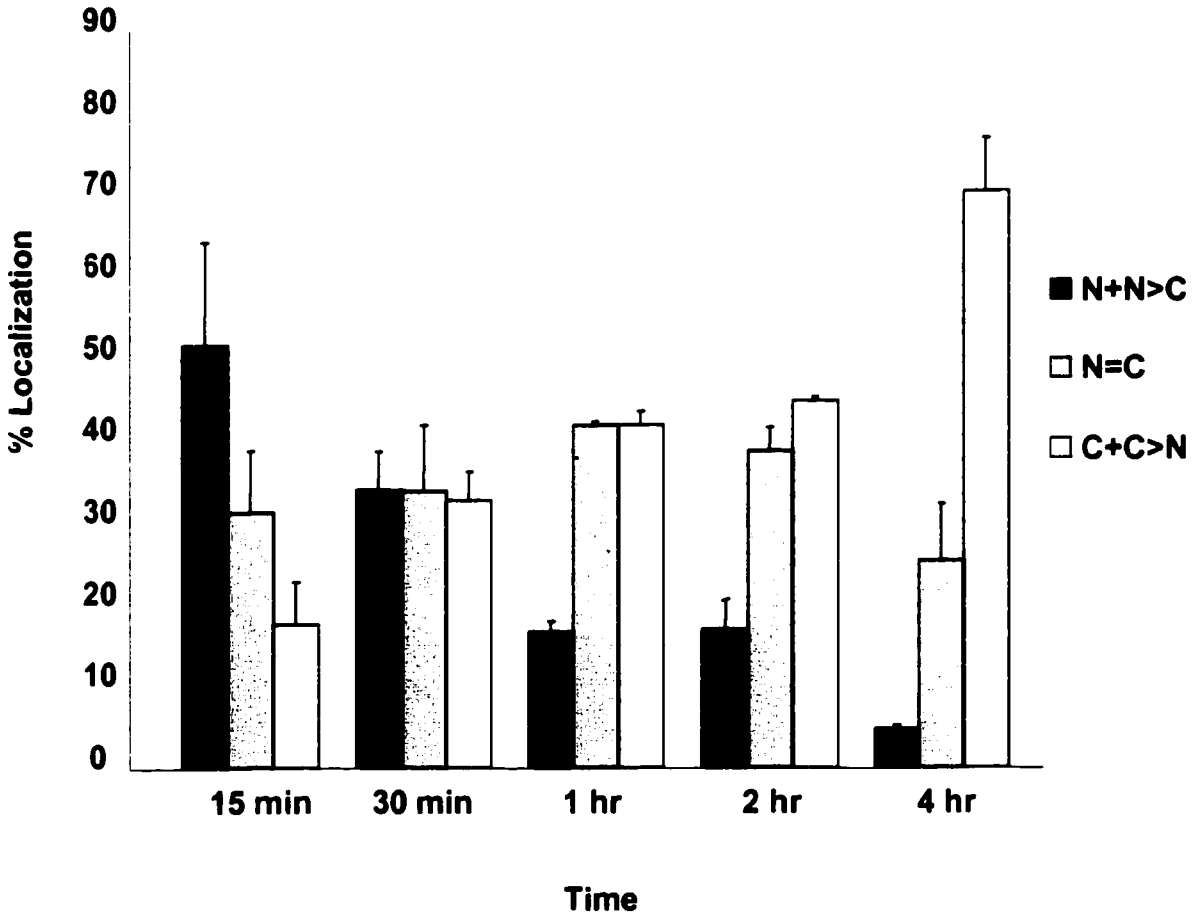
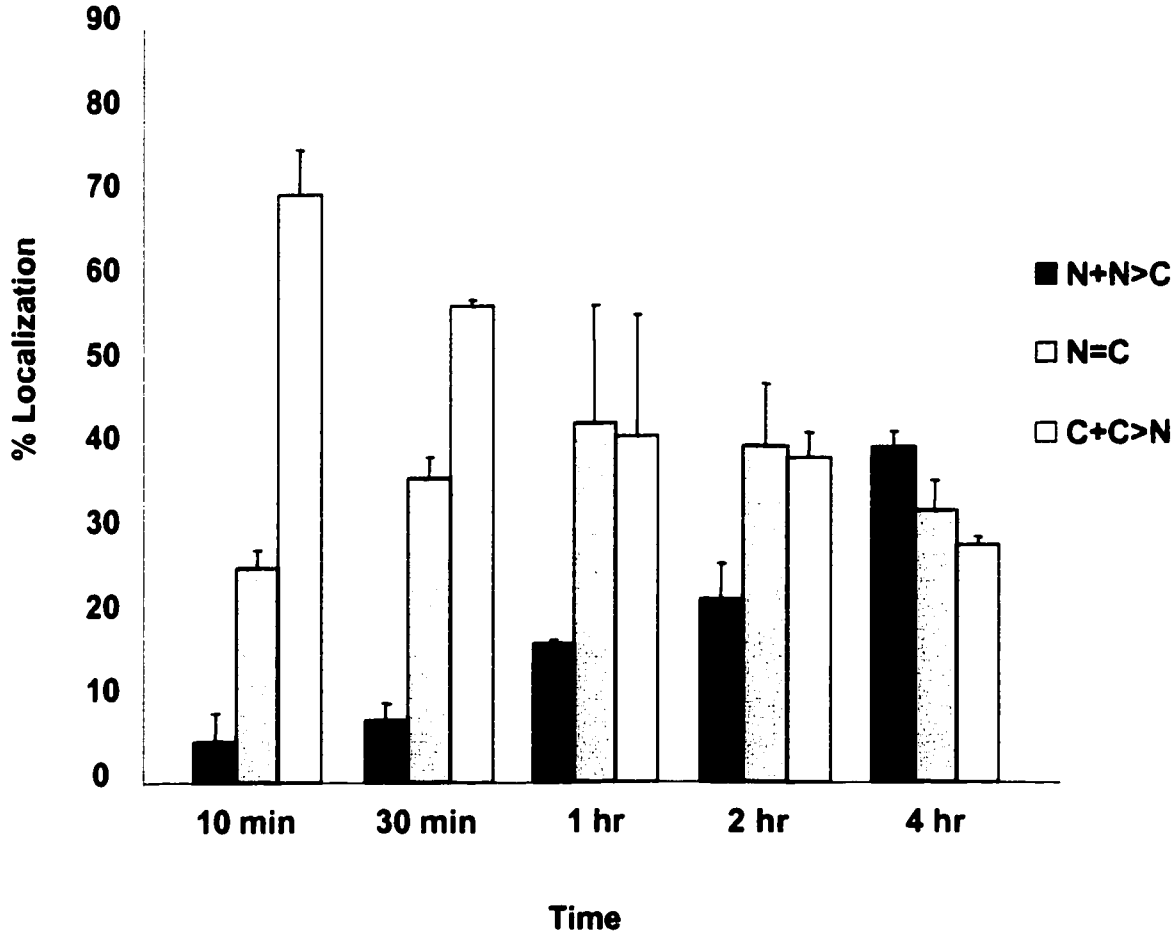

90
80
70
60
50
40
30
20
10
0
% Localization
15 min
30 min
1 hr
2 hr
4 hr
Time
N+N>C
N=C
C+C>N

Figure 16 Hormone restimulation promotes the return of GR_{NLI} to the nucleus.

Graphical representation of the subcellular localization of GR_{NLI} upon treatment with cortisol for 6 hrs, hormone withdrawal for 4 hrs, followed by treatment with dex for times ranging from 10 min. to 4 hrs. For each treatment type, a minimum of 400 cells was counted and experiments were performed in triplicate Quantification was carried out under blinded encryption and cells were classified into one of five categories of subcellular staining. To simplify further, categories of exclusively nuclear and mostly nuclear stained cells were combined (N+N>C), as well as the categories for exclusively cytoplasmic and mostly cytoplasmically stained cells (C+C>N). Categories for subcellular localization are represented in the form of bar graphs with error bars reflecting the standard deviations among three separate experiments.


90
80
70
60
50
40
30
20
10
0
% Localization
10 min
30 min
1 hr
2 hr
4 hr
Time
N+N>C
N=C
C+C>N

Discussion

Part 1

The three specific goals of my study were: 1) to delimit a sequence within GR responsible for its cytoplasmic localization prior to the addition of hormone, 2) to examine the effects of the export inhibitor leptomycin B (LMB) on the subcellular localization of GR, and 3) to assess the subcellular trafficking kinetics of an NL1 deficient GR mutant upon primary hormone stimulation, followed by steroid withdrawal and subsequent secondary hormone stimulation. The effects of LMB on this GR NL1 mutant were also assessed. The aim within all objectives was to further characterize the nucleocytoplasmic trafficking profile of GR using a semi-quantitative indirect immunofluorescence assay.

The first goal was achieved by constructing truncation mutants of GR and visualizing the subcellular localization patterns for each mutant as compared to a wtGR construct. Beginning with the removal of the N-terminus ($GR_{407-795}$), in the absence of hormone, we found that the subcellular localization of the mutant was distributed throughout both the cytoplasm and the nucleus. This result differed markedly from wtGR, which appeared to be almost totally localized to the cytoplasm. To ensure that the observed loss in cytoplasmic localization was not due to passive diffusion through the nuclear pore, 6 copies of a myc tag epitope were placed N-terminally to $GR_{407-795}$. Addition to the N-terminus of peptides which did not encode for an import or export signals have been shown to cause no alterations in characteristics regarding the

subcellular localization of GR (161, 162). This addition effectively increased the size of the construct to 58 kDa, a size well within or above the known 40-60 kDa limit permitting passive diffusion (See Table 1). Following the logical hypothesis that the removal of the N-terminus abolished the favored cytoplasmic localization viewed in the absence of hormone, further mutants were constructed which added successive hundred amino acid increments to the $GR_{407-795}$ backbone. To address differences amongst GR constructs, the myc tag, originally used to increase the size of $GR_{407-795}$, was fused N-terminally to wtGR, as well as each successive construct regardless of size. The subcellular localization profiles of the succeeding constructs ($GR_{300-795}$ and $GR_{200-795}$) were found to be similar to that of the N-terminal truncation mutant, in that they too were distributed evenly between the nucleus and cytoplasm. Furthermore, to determine whether an increase in size, as well as an addition to the N-terminus would affect the subcellular localization of GR, a green fluorescent protein was fused N-terminally to $GR_{200-795}$, adding another 28 kDa in size. Subcellular distribution profiles for $GR_{200-795}$ and the larger GFP-$GR_{200-795}$ were extremely similar, showing that additions to the N-terminus of GR, as well as size increases to the protein did not affect the subcellular localization of identical GR molecules.

In adding amino acids 100-199 to $GR_{200-795}$, we observed the return of cytoplasmic localization to levels nearly identical to wtGR. Endeavoring to further delimit a potential signal, additional constructs ($GR_{125-795}$ and $GR_{175-795}$) were created which essentially made cuts in the previously added hundred amino acids. Subcellular localization profiles between these two mutants were shown to differ markedly with regard to levels of nuclear and cytoplasmic occupancy. However both were observed to lose cytoplasmic

localization prior to hormone treatment. Since $GR_{100-795}$ portrayed a profile which most closely resembled that of wtGR, amino acid 100 was selected as the N-terminal boundary for a potential signal necessary for the cytoplasmic localization of GR in the absence of hormone. Upon treatment with hormone, we observed that all mutant constructs were able to enter the nucleus to levels comparable to wtGR, thus demonstrating that the truncations performed on the GR protein did not affect the ability to translocate to the nucleus. We also observed that upon adding steroid and subsequently withdrawing ligand for 24 hours, the $GR_{100-795}$ construct once again exhibited a subcellular localization profile, which most closely mimicked that of wtGR. This result implies that the addition of amino acids 100-200 was not only capable of imparting the function of cytoplasmic localization to GR in the absence of hormone, but was also crucial in directing the redistribution of GR to the cytoplasm upon hormone treatment and subsequent withdrawal.

Finally, to assess whether a specific signal exists to sufficiently impart cytoplasmic localization on an otherwise ubiquitously localized protein in the absence of steroid, a construct was created which fused amino acids 100-200 to the N-terminally truncated $GR_{407-795}$. Primarily we chose to select a large fragment to begin our analysis, anticipating to further delimit a discrete signal to within 25 or 50 amino acids. Unexpectedly, we observed that the subcellular distribution profile for this deletion mutant ($GR_{100-200/407-795}$) displayed a loss in cytoplasmic localization, showing that the sequence was necessary, but not sufficient to impart a cytoplasmic localization function to a ubiquitously dispersed molecule.

Several possible theories explain the outcomes observed in the above mentioned experiments. Acknowledging that the subcellular distribution of GR is itself a dynamic process possibly involving import, export, nuclear retention and/or cytoplasmic retention; a theory could be put forward in which the removal of amino acids 100-200 effectively eliminates a specific determinant, causing a change in the regular skewed, cytoplasmically localized equilibrium distribution observed in the absence of hormone. Experiments within this thesis, as well as recently published works have demonstrated that hsp-associated GR, although primarily localized to the cytoplasm, is capable of shuttling between the nuclear and cytoplasmic compartments in the absence of hormone. This dynamic process has been shown to depend on certain factors, including nuclear localization signals and nuclear import mechanisms which affect the intricately controlled transfer rates across the nuclear membrane (161, 162). It is therefore conceivable that the increased nuclear accumulation observed for the GR truncation mutants arise from the disruption of a certain trafficking equilibrium stemming from the removal of a sequence which itself may act to serve a role in nuclear export or active cytoplasmic anchoring. Characterization of nuclear export signals thus far have been primarily limited to the leucine rich variety which fall under the regulation of the CRM1 or exportin type nuclear export receptor (144, 147, 148). Consensus sequences within GR containing a leucine rich motif however, are not located within the N-terminus, strongly ruling out the possibility that the loss in favored compartmentalization is due to the elimination of a leucine rich export signal hindering export through the CRM1 pathway. This however does not completely abolish the idea that nuclear export of GR is affected by the removal of N-terminal portions. In recognizing that there are multiple receptors involved in different mechanisms of signal mediated nuclear protein import, it is a logical expectation

that additional export receptors exist and act via alternate export pathways, possibly by recognizing yet to be defined nuclear export signals. The same can be said for the argument that the removal of N-terminal portions of GR eliminated a discrete retention signal responsible for the cytoplasmic localization of GR observed in the absence of steroid. Protein sequence analyses of the region encompassing GR amino acids 100-200 did not provide a match to any known conventional cytoplasmic retention domains exemplified by the CRDs discovered in *Xenopus* factor xnf7 and *Xenopus* MyoD (156, 157). Although GR amino acids do not impart cytoplasmic localization such as was seen in deletion and transfer experiments carried out with the xnf7 and xMyoD, it does not rule out the possibility that there exists a non-conventional, undefined signal within this region, which acts in an entirely novel manner.

A body of evidence once strongly argued for the cytoplasmic sequestration of GR being attributed to NLS masking by hsp association. Since we have recently shown that naïve GR shuttles independent of hormone and hsp association (161), there must exist an alternative explanation for the initial cytoplasmic localization observed in the absence of steroid. Considering the findings of Smith et al.(16), whose work reports that hsp association to GR is not a static process, an inevitable question arises. Why, if GR continuously associates and disassociates from the multiprotein heterocomplex, is there not a higher level of GR in the nucleus considering the availability of the exposed NLS during instances where the receptor is dissociated from hsps? An interesting theory emerges which brings together a number of elements, stemming from the idea of intramolecular interactions, that is, interactions within the GR protein itself which ultimately influence its subcellular localization in the absence of steroid.

Evidence for the cytoplasmic retention through intramolecular interactions has already been shown to occur, particularly in the case of the p50 subunit of NF-κB (184). Here, intramolecular binding of the C-terminus of the p50 precursor (p110) to its own NLS, led to NLS masking, ultimately hindering the ability of p50 to translocate to the nucleus. Interestingly, removal of the C-terminus was shown to allow the nuclear import of p50, a result not unlike those seen for the removal of N-terminal portions of GR. In considering the information compiled in this thesis, a potential model emerges whereby there exists a determinant within amino acids 100-200 which allows a specific intramolecular interaction to occur within GR, which actively folds the molecule into a conformation that sequesters its ability to enter the nucleus. Upon removal of this sequence interface, intramolecular binding, and thus proper protein folding necessary to sequester the NLS sequence is abolished. Thus, at the moment the hsps disassociate from GR through their dynamic equilibrium process, GR is immediately shuttled into the nucleus due to the free exposure of the NLS to the nuclear import machinery.

Extending this model, information regarding conformational changes to steroid receptors upon agonist and antagonist exposure, lends evidence to the possibility that the intramolecular interaction occurs between the N-terminus and the C-terminal ligand binding domain. Exposure and thus binding to hormone induces a change in the highly structured ligand binding pocket of nuclear receptors (185, 186). In changing conformational structure, the possibility exists that a weaker intramolecular interaction between the N- terminus and C-terminus is broken, allowing full exposure of the NLS to

the nuclear import machinery upon ligand induced disassociation with the hsp heterocomplex.

After regulating transcription of target genes, GR is reassociated into its multiprotein heterocomplex and redistributed to the cytoplasm upon hormone withdrawal. It was interesting to note that GR N-terminal truncation mutants failed to redistribute to the cytoplasm to levels comparable to wtGR. Not until amino acids 100-200 were added to $GR_{100\text{-}795}$, did cytoplasmic redistribution profiles return to near wtGR levels. This suggests that amino acids 100-200 are not only necessary for cytoplasmic localization in the absence of steroid, but also for cytoplasmic redistribution upon hormone withdrawal. Interestingly, the reluctance of GR to return to the cytoplasm is also observed in another situation, specifically upon treatment of GR transfected cells with hormone antagonist RU486 (161-163). This observation coincides well with the proposed model of an existing intramolecular interaction between the N- terminus and the C-terminus of GR, since it has been shown that RU486 induces conformational changes to the LBD of steroid receptors, different from those changes observed upon agonist treatment (186, 187). The differences in LBD conformation displayed upon RU486 exposure may serve to negatively affect the intramolecular interaction within GR, inhibiting proper receptor folding. With regard to cytoplasmic redistribution, proper structural conformation may be a necessary requirement allowing the exposure of a possible nuclear export signal, or signaling the end to an active nuclear retention process. In the case of the N-terminal truncation mutants, steroid treatment propagated entry into the nucleus, however cytoplasmic redistribution was hindered, possibly due to the loss of essential portions of the N-terminus required for the refolding of GR into its proper

structural conformation. This idea of nuclear retention being due to the inhibition of an intramolecular interaction can also be used to explain the subcellular localization profiles of N-terminal mutants showing an increase in nuclear accumulation in the absence of steroid. In this case, entry into the nucleus is potentiated independent of ligand as explained above, and since truncation mutants lack the proposed amino acids necessary for proper conformational structure, nuclear accumulation is inevitable, due to the reduced ability of GR to return to the cytoplasm.

Furthermore, the observation that this N-terminal sequence was necessary, but not sufficient to impart cytoplasmic localization on a ubiquitously localized protein, tends to strengthen the argument for an intramolecular interaction directing the subcellular distribution profile of GR. With regard to receptor folding and intercommunication, the loss in cytoplasmic localization observed for the deletion construct ($GR_{100\text{-}200/407\text{-}795}$) may be explained by spacing requirements of the receptor itself. Amino acids 100-200 were fused directly N-terminally to the GR DNA and ligand binding domains. If, as postulated, an intramolecular interaction between the N-terminus and C-terminus is required for cytoplasmic compartmentalization in the absence of steroid, it could be argued that the number of amino acids between the two regions was not sufficient to allow the protein to correctly fold onto itself. Since the proper interaction and protein conformation was abrogated, so too was cytoplasmic localization.

Acknowledging that GR is a phosphoprotein whose phosphorylation state changes in response to the addition and withdrawal of hormone, it is tempting to integrate this theory of intramolecular interactions directing subcellular localization with a potentially

regulatable mechanism such as post-translational modification of GR by phosphorylation. Of particular interest is the observation that in response to RU486, GR is found to acquire a different hyperphosphorylation pattern from GR treated with glucocorticoid agonist (188, 189). Site-specific changes in GR phosphorylation patterns observed at different phases of the cell cycle coincide well with an observed change in nuclear occupancy and activation function (179). Phosphorylation is highly dependent upon cell cycle and mitogenic stimuli, with the peak in phosphorylation occurring during G_2 (190-192). Interestingly, GRs that translocate to the nuclei of G_2 synchronized cells in response to hormone, are not efficiently retained and thus redistribute to the cytoplasm (179). Ultimately, this supports the idea that cell cycle regulation of specific protein kinases and phosphatases could influence nuclear retention and receptor recycling. The prolonged nuclear occupancy shared between RU486 treated GR, and the N-terminal truncation mutants may infer an importance to the potential role phosphorylation plays in the subcellular localization of GR, since all phosphorylation sites lie within the N-terminal region of the receptor (193, 194). Although this provides a provocative model in terms of the subcellular localization of GR, it is important to acknowledge several results suggesting that the currently defined phosphorylation sites within GR are unlikely to play a role in localizing naïve GR to one compartment over another. Substitution of individual and multiple phosphorylation sites have been shown to have no effects on first, the localization of unliganded receptor in asynchronously growing cells (193), and second, the transcriptional activity of GR at the mouse mammary tumor virus promoter (194). In addition, experiments studying the subcellular localization of a receptor chimera fusing the GR N-terminus to the ER DNA and ligand binding domains resulted in a protein that was constitutively localized to the nucleus (195). Similar results were

also observed in the analyses of other GR-ER and GR-PR chimeras, suggesting that cytoplasmic localization of unliganded GR is determined, at least in part by its ligand binding domain (196). This last observation is of particular interest, since the findings of this thesis once again argue for the potential interaction between the N- and C-terminal domains of GR. It was also interesting to note, that the phosphorylation status of GR has profound effects on protein stability, playing a crucial role in regulating receptor levels and hence receptor functions (193).

These above listed observations, together with the results displayed in this thesis, provide a possible connection between the roles the N-terminus, C-terminus, and receptor phosphorylation play in the cytoplasmic localization of naïve GR. Future studies could be conducted to uncover a possible C-terminal determinant for cytoplasmic localization, at the same time further delimiting the N-terminal region roughly characterized thus far. In addition, experiments could be undertaken to assess potential interactions between the N- and C-termini of GR, at least in part to uncover if an intramolecular communication actually exists, as well as determining if there is a requirement for proper amino acid spacing to allow correct protein folding. The experimental strategy to create truncation mutants of GR and employ the indirect immunofluorescence assay to visualize their subcellular localizations was solid in theory and practice. Following this approach, employing another method such as the permeabilization of cells through digitonin, may serve to further complement our findings (80). In this method, regions of GR could be individually processed in terms of subcellular localization regardless of current function or association with other proteins. By creating finer truncation or deletion mutants, as well as using defined purified soluble factors, this assay would allow for the dissection of

the complete system, uncovering specific signals and additional players involved in the subcellular trafficking of GR. In any case, further researching this area will help in ultimately elucidating the biological significance for the energetically costly, yet seemingly constitutive cytoplasmic localization observed for GR in the absence of steroid.

Discussion

Part 2

The other project goals were achieved once again by the use of the semi-quantitative indirect immunofluorescence assay. These experiments however primarily dealt with the nucleocytoplasmic trafficking of a site-directed GR mutant lacking an active NL1. Experiments comparing the subcellular localization profiles of wtGR and the GR_{NL1-} mutant further strengthened the notion that GR is capable of shuttling between the nucleus and cytoplasm in its naive hsp-bound conformation. Observing that unliganded over-expressed wtGR is localized in the nucleus to a markedly higher level as compared to the NL1 mutant suggested that trafficking of unliganded hsp-associated GR is dependent on NL1. Trials with the export inhibitor leptomycin B served to further solidify these findings, as nuclear accumulation was observed with unliganded wtGR under LMB treatment alone, but not with the NL1 mutant. Nuclear accumulation occurred presumably by blocking export of the shuttling GR protein, giving strong evidence that the lack of nuclear entry and thus accumulation of unliganded GR_{NL1-} was due to the inactivation of NL1. Treatment with a combination of steroid and LMB also served to demonstrate that ligand induced nuclear import was not hindered since GR_{NL1-} was capable of first entering the nucleus, and secondly accumulating upon inhibiting export. Consistent with the findings of Kudo et al., who reported that LMB specifically inhibits signal mediated nuclear export by directly binding to CRM1, these findings provide the first indirect evidence suggesting that the nuclear export of GR is mediated through a CRM1 dependent manner.

Experiments dealing with the nuclear transfer and cytoplasmic redistribution of GR_{NL1-} were for the most part concordant with the findings of an earlier report published by Savory et al. In my experiments, the hormone dependent nuclear entry and accumulation of the GR_{NL1-} mutant was shown to increase at a rate much slower than that observed for wtGR. Furthermore, levels of nuclear occupancy never reached wtGR levels, which showed an almost complete transfer of receptor to the nucleus. Hormone withdrawal following treatment displayed a rapid redistribution to the cytoplasm ($t_{1/2}$ = 2-4 hrs), a result differing markedly from wtGR which is known to redistribute slowly to the cytoplasm ($t_{1/2}$ = 12-24 hrs) (163). The difference in kinetics, together with the observation that the GR_{NL1-} mutant is unable to interact with pendulin, the mouse homologue of importin-α (162), suggests that the NL2 mediated nuclear import of GR occurs through a pathway distinct from that involving the conventional importin-α type machinery. This theory also coincides well with the observation that the GR LBD lacks any type of basic motif required for importin-α recognition. Finally, in assessing the subcellular localization profile for GR_{NL1} following a primary steroid treatment, withdrawal, and subsequent re-stimulation, we observed that the return to the nucleus upon secondary stimulation closely mimics the trend, as well as the rates displayed for a primary response to hormone. This suggests that NL2 mediated import through a distinct pathway does not detrimentally affect receptor function, allowing GR to recycle and re-acquire hormone responsiveness.

With evidence pointing towards the theory of NL2 mediated nuclear entry occurring through a novel import pathway, future studies could be conducted to identify and isolate the NLS receptor responsible for NL2 mediated import. Since the subcellular

localization of GR is also dependent on a number of different factors, including cell cycle progression and receptor over-expression (162, 165, 179) identifying conditions under which NL2 is important for the localization of GR to the nucleus is also another area of potential interest. Ultimately, these findings will lead to progress towards further uncovering the physiological significance this novel manner of nuclear translocation plays in glucocorticoid hormone action.

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SUMMARY OF QUALIFICATIONS

- Fluently bilingual in French & English with excellent communication skills
- Approachable, enthusiastic worker proficient at problem solving
- Possesses leadership qualities & the ability to work within a team atmosphere
- Skilled in Marketing, Finance, Economics, Management Strategy & Organizational Behaviour
- Knowledgeable in Molecular Biology, Toxicology Research, Analytical Chemistry & Microbiology
- Certified in CPR and First Aid

EDUCATION

UNIVERSITY OF OTTAWA, Ottawa, Ontario (1999-present)
- Master's in Business Administration (Hi-tech/Health Admin)

UNIVERSITY OF OTTAWA, Ottawa, Ontario (1997-1999)
- Master of Science, Biochemistry

UNIVERSITY OF WATERLOO, Waterloo, Ontario (1992-1997)
- Honors Bachelor of Science, Biochemistry (Co-operative Program)

UNIVERSITY OF SUSSEX, Brighton, England, UK (January 1996 - May 1996)
- University exchange program (Department of Chemistry, Waterloo / School of Molecular Sciences, Sussex)
- Focus included Medicinal Chemistry, Cellular Regulation, & Elementary Formal Logic

AWARDS & PUBLICATIONS

- Ontario Graduate Student Science & Technology Scholarship
- University of Waterloo / Sussex International Exchange Scholarship

Discrimination between NL1- and NL2-Mediated Nuclear Localization of the Glucocorticoid Receptor
Joanne G. A. Savory, Brian Hsu, Ian R. Laquian, Ward Giffin, Terry Reich, Robert J. G. Hache, and Yvonne A. Lefebvre. MCB 19 (2), p. 1025-1037
Co-first authorship

REFERENCES AVAILABLE UPON REQUEST

WORK EXPERIENCE

RESEARCHER
Loeb Health Research Institute
Department of Hormones, Growth & Development

- Researched the subcellular distribution of glucocorticoid receptors for partial completion of a degree in the Master of Science in Biochemistry
- Thesis work includes molecular cloning, tissue culture studies, in-vitro translation, protein chemistry & immunocytochemistry

RESEARCH ASSISTANT
Spectral Diagnostics Inc.
Molecular Biology Division

- Involved in the molecular cloning of cardiac proteins to be used as markers for myocardial infarction
- Project work included expression vector design, molecular cloning & sequencing
- Procedures included PCR, restriction digests, ligations, DNA preparation, extraction, purification, transformation, and automated DNA sequencing

ASSISTANT CHEMICAL/MECHANICAL ENGINEER
Cominco Engineering Services Ltd.

- Designed and constructed sections of a large scale chemical agitation system for the extraction of precious metals from mining ores; duties included autocad design of a cascade process, impeller and motor construction, welding, physical labor & maintenance
- Supervised and tested small scale hydrological metal extraction processes; duties included troubleshooting, repair, maintenance, and analytical testing

TECHNICAL RESEARCH ASSISTANT
Kerry Ingredients
Research and Development Branch

- Aided in the creation & production of various products used in marketed snack food
- Project work included matching (color, taste, texture), blending, troubleshooting & formula revision
- Implemented a database system for raw material specifications & final product formulations allowing the transfer of finished products from Canada to the U.S. under federal legislation and guidelines
- Handled sample requisitions from purchasers & dealt in public relations with clients

RESEARCH ASSISTANT
National Department of Health Canada
Air Quality Health Effects Research

- Involved in research regarding the ambient effects of air particles on lung tissue (in vivo & in vitro)
- Project work included particle dosing of rats, micro dissection of rat lung tissues, necropsies & cell explant procedures, tissue preps, protein assays, SDS-PAGE & Western blotting (identifying HSP and CYP 1A1), as well as plasmid preparations and tissue culture maintenance

RESEARCH ASSISTANT
National Department of Health Canada
Environmental Contaminants & Occupational Toxicology

- Determined tissue absorption levels of CDE, HCB & PCB congeners by means of GC-ECD
- Assisted in necropsy procedures & performed analysis of Ethoxyresorufin Deethylase (EROD) enzyme activity by means of luminescent spectroscopy/fluorometry