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CHARACTERIZATION OF NUCLEAR IMPORT OF THE GLUCOCORTICOID RECEPTOR MEDIATED BY THE NUCLEAR LOCALIZATION SIGNAL IN THE LIGAND BINDING DOMAIN

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ABSTRACT

The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily of transcription factors. Before exposure to hormone, GR is situated in the cytoplasm in a heterocomplex consisting of heat shock proteins and immunophilins. Upon addition of hormone, GR dissociates from this complex and translocates to the nucleus. After hormone withdrawal, GR returns to the cytoplasm where it is found once again associated in a heterocomplex. GR contains two nuclear localization signals (NLS) in its primary sequence. Nuclear localization signal 1 (NL1), a hormone-independent NLS, is located in the hinge region from amino acid residue 486 to 524 in rat GR. Nuclear localization signal 2 (NL2), a hormone-dependent NLS, is found in the ligand binding domain but its sequence has not been delimited further. To determine the contribution by NL2 to the nucleocytoplasmic trafficking of the full length GR, a GR mutant (mutGR) with no NL1 activity was generated by mutation of the three lysine (K) residues at amino acid residues 513 to 515 to asparagine (N). Abolition of NL1 activity was confirmed with two constructs that expressed enhanced green fluorescent protein fused to the N-terminus of GRaa22-525. The construct that expressed the fusion protein containing the wildtype sequence of NL1 was constitutively nuclear as expected while the fusion protein possessing the NL1 mutation was localized to the cytoplasm. Characteristics of nuclear uptake and occupancy of the full length wildtype receptor (wtGR) and mutGR were determined by a semi-quantitative indirect immunofluorescence (IIF) assay. After

dexamethasone treatment, a delay in nuclear entry, a slowed rate of nuclear uptake and a decrease in nuclear retention by the mutated receptor were observed. After a cycle of agonist treatment and withdrawal, redistribution to the cytoplasm also occurred at a faster rate for mutGR. Treatment with the antagonist, RU486, resulted in a drastically reduced nuclear uptake and minimal accumulation of mutGR in the nucleus, compared to wtGR. Sucrose density gradient analysis of wtGR and mutGR indicated that multiprotein heterocomplex dissociation was not altered for mutGR. An electrophoretic mobility shift assay did not uncover a significant difference in DNA binding between wtGR and mutGR. Transcriptional activity of mutGR correlated with its observed maximum level of accumulation in the nucleus. These results suggested that the NL1 mutations, apart from abolishing the nuclear localization function, did not appear to affect other functions of GR. However, it was not conclusively proven that the NL1 mutations absolutely did not have any negative effects on transcriptional activation by GR. Further investigations by our laboratory revealed that NL2-mediated nuclear import may occur via an alternative import pathway to that of NL1. GR with a functional NL1, but not GR with a functionally impaired NL1 or GR with only NL2, bound to pendulin, a mouse homologue of importin α, the NLS receptor of the classical basic NLS import pathway. Future studies should elucidate the physiological role played by NL2 in glucocorticoid hormone action.

DEDICATION

I dedicate this thesis to my parents whose love, support and sacrifice has brought me to where I am today. I also want to thank my brothers and sisters for their support. I would like to make a special dedication to my father who passed away late last year. I regret that he is not going to be present to see this through to the end with me.

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A	alanine
aa	amino acid
Ah	arylhydrocarbon
AR	androgen receptor
Arnt	arylhydrocarbon nuclear translocator
ATP	adenosine triphosphate
ßgal	ß-galactosidase
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
C	cysteine or cytoplasm
C-Nup1	C-terminus of Nup1
CAS	cellular apoptosis susceptibility gene
CAT	chloramphenicol acetyltransferase
CBC	cap-binding protein complex
cDNA	complementary deoxyribonucleic acid
COUP-TF 1	chicken ovalbuin upstream promoter transcription factor 1
cpm	counts per minute

CRM1	chromosomal region maintenance 1
Cy-P	cyclosporin A-binding protein
D	aspartic acid
DBD	DNA binding domain
ddH_2O	deionized, distilled water
dex	dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DOPE	dioleoyl phosphatidylethanolamine
DOSPA	2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DTT	dithiothreitol
E	glutamic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetate
egfp or EGFP	enhanced green fluorescent protein
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor

F	phenylalanine
FBS	fetal bovine serum
FGF	fibroblast growth factor
FKBP	FK506 binding protein
G	glycine
GAP	GTPase activating protein
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GTP	guanine triphosphate
h	hour(s)
H	histidine
HEPES	N2-hydroxyethylpiperazine-N'2-ethanesulphonic acid
HIV	human immunodeficiency virus
HMG	high mobility group
hnRNP	heterogeneous ribonucleoprotein
hop	hsp90-hsp70 organizing protein
HRE	hormone response element

hsp	heat shock protein
I	isoleucine
IBB	importin ß binding
IIF	indirect immunofluorescence
IPTG	isopropyl β-D-thiogalactopyranoside
K	lysine
kDa	kilodalton
L	leucine
LBD	ligand binding domain
M	methionine
MDa	megadalton
MEM	Minimal Essential Medium
min	minute(s)
MMTV	mouse mammary tumour virus
MR	mineralocorticoid receptor
mRNA	messenger RNA
mutGR	mutant GR

N	asparagine or nuclear
NE	nuclear envelope
NES	nuclear export signal
NF-κB	nuclear factor-κB
ng	nanogram
NGFI-B	nerve growth factor inducible factor B
NGS	normal goat serum
NLS	nuclear localization signal
nm	nanometre
NPC	nuclear pore complex
NPI-1	nucleoprotein interactor 1
NTF	nuclear transport factor
p	phosphoprotein
P	proline
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKI	protein kinase inhibitor
PMSF	phenylmethylsulfonylfluoride
PP-1	protein phosphatase type 1
PP-2A	protein phosphatase type 2A

PP5	protein phosphatase 5
PR	progesterone receptor
py-f	pyrogen-free
Q	glutamine
R	arginine
Ran	Ras-related nuclear protein
RanBP1	Ran binding protein 1
RAR	retinoic acid receptor
RCC1	regulator of chromosomal condensation 1
Rch1	Rag cohort 1
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal RNA
RT	room temperature
RU486	RU38486
RXR	retinoic X receptor
S	serine
SDS	sodium dodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second(s)
snRNA	small nuclear ribonucleic acid
SRP1	suppressor of RNA polymerase I
SV	simian virus
T	threonine
TBE	Tris-boric acid-EDTA
TBS	Tris buffered saline
TEMED	N,N,N'-N'-tetramethylethylene-diamine
TPR	tetratricopeptide repeat
TR	thyroid receptor
tRNA	transfer RNA
µ	micron
V	valine
VDR	vitamin D_3 receptor
v/v	volume/volume

W ..... tryptophan

w/w ..... weight/ weight

WCE ..... whole cell extract

WGA ..... wheat germ agglutinin

wt ..... wildtype

wtGR ..... wildtype GR

Y ..... tyrosine

I. INTRODUCTION

Subcellular localization of the glucocorticoid receptor (GR) is tightly regulated. In the absence of hormone, the receptor resides primarily in the cytoplasm, in a complex with heat shock and other proteins. Upon hormone binding, the receptor transforms and enters the nucleus where it binds to specific DNA sequences to regulate expression of target genes. Entry into the nucleus is dependent upon the two nuclear localization signals located in the GR. The subject of this thesis is to determine the relative contribution by each nuclear localization signal in GR to its trafficking between the cytoplasm and the nucleus.

1. Steroid Hormone Receptors

In 1960, after the observation that ecdysone, an insect molting steroid hormone, induced puffing of the giant polytene chromosomes, it was proposed that steroid hormones might have a direct effect on gene expression [1]. The detection of an intracellular receptor using radiolabelled estradiol [2] and the movement of [^{14} C] cortisol from the cytoplasm to the nucleus (see [3]) led to a proposed model for steroid hormone action. In this model, the steroid hormone binds to its cytoplasmic receptor after entry into the cell and then this hormone-receptor complex translocates into the nucleus to influence gene expression [4]. The cloning of the steroid receptor genes during the mid-

1980's greatly facilitated studies on a molecular level but the general mechanism of action of steroid hormones remains generally unchanged.

a) Structure and Function of the Steroid Hormone Receptors

The gene for GR was the first of the steroid receptor genes to be cloned [5]. Subsequently, through the use of receptor-specific antibodies or by low stringency cDNA hybridization techniques, the genes for the other steroid receptors, estrogen (ER) [6], progesterone (PR) [7], androgen (AR) [8] and mineralocorticoid receptors (MR) [9], were cloned. The cDNA sequence for the thyroid (TR) [10, 11] and vitamin D_3 receptors (VDR) [12] also were obtained through the use of low stringency cDNA hybridization techniques. Sequence analysis of both receptors revealed that they were related to steroid receptors which led to the introduction of the concept of the nuclear receptor superfamily [13]. This superfamily now includes the steroid receptors, TR, VDR, retinoic acid and retinoid-X receptors (RAR & RXR) [14, 15], peroxisome proliferator-activated receptor (PPAR) [16] and many orphan receptors e.g. chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) [17] and nerve growth factor inducible factor B (NGFI-B) [18]. An orphan receptor is a protein which is structurally related to members of the nuclear receptor superfamily but does not have an identified, endogenous, physiological ligand.

Sequence analysis of members of the nuclear receptor superfamily revealed the presence of discrete structural domains and different degrees of homology among the

domains of the different receptors [13, 19] (Figure 1). The following general descriptions of structure and function are applicable to members of the nuclear receptor superfamily [13, 19] but this introduction will focus mainly on receptors from the steroid receptor family. The N-terminal domain or the A/B region is the most variable, in length and amino acid sequence, among the steroid receptors. It contains a number of phosphorylation sites as well as a ligand independent transactivation function (reviewed in [20, 21]).

The DNA binding domain (DBD) or the C region is highly conserved among the steroid receptor family members and contains two zinc fingers motifs generated by two zinc ions, each coordinating four cysteine residues. Sequence-specific DNA binding involves a subregion of the DNA binding domain, located at the base of the first zinc finger, called the P box [22-24]. A second subregion called the D box is located at the base of the second finger and is necessary for DNA-dependent dimer formation [24-26].

The hinge region (D), located between the DNA binding domain and the ligand binding domain, is variable in length and sequence. This region contains an identified, hormone-independent nuclear localization signal (NLS) [27-29]. For ER, PR and GR, the NLS is composed of three clusters of basic amino acids [28, 30]. This NLS in GR, by itself, has been shown to be capable of directing a cytoplasmic protein, ß-galactosidase, into the nucleus [27].

Finally, the C-terminal ligand binding domain (LBD) (region E/F) displays some conservation between receptors and contains a number of functions in addition to ligand

Figure 1 Schematic representation of GR structure

This figure is adapted from S. Simons, Jr. [31]

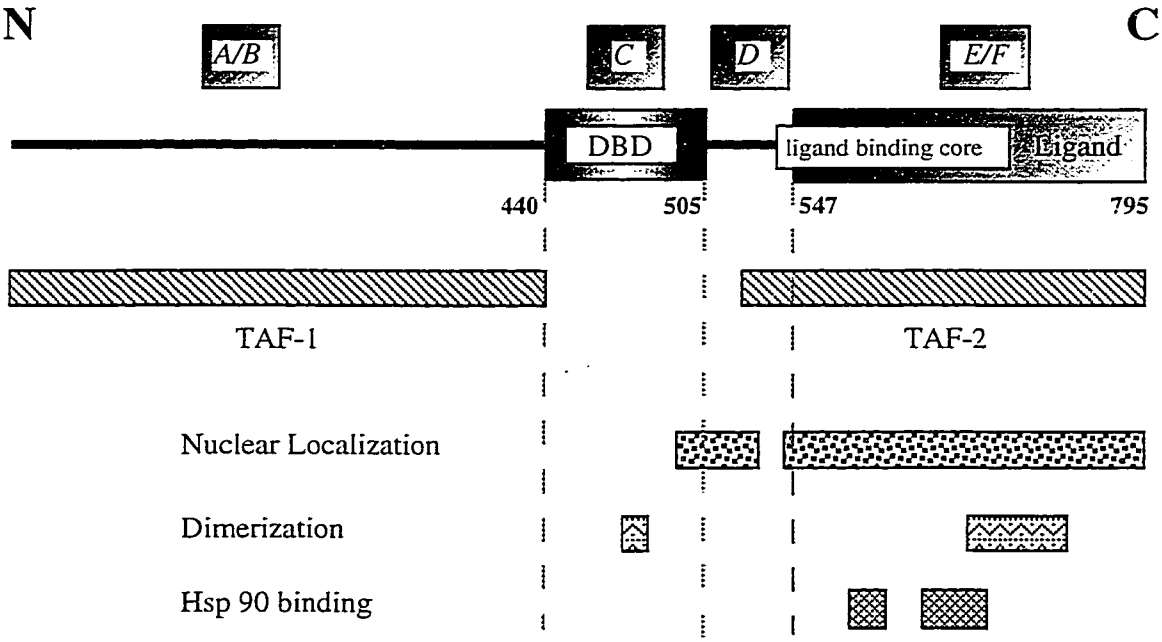
GR is represented as a black line with the DNA binding and ligand binding domains highlighted. Regions responsible for the various attributes of GR i.e. nuclear localization, are outlined. All numbered amino acid residues correspond to those of rat GR. The following provides details on correlation of function to regions of GR.

Two areas are responsible for transactivation by GR. Transcriptional activation function 1 (TAF-1) is located at the N-terminus [32, 33] and TAF-2 is positioned at the C-terminus [32, 33].

Sequences responsible for nuclear localization are found in two regions. NL1 is in the hinge region, delimited to aa 486-524 [27, 30]. NL2 is localized to the ligand binding domain (LBD) (aa 540-795) [27].

Dimerization, another property of GR, occurs in two regions. DNA-dependent dimerization is located in a subregion of the DNA-binding domain called the D box [25, 26]. This is delimited to aa 477-481. A second dimerization domain at aa 730-764 is proposed based on homology to a dimerization sequence implicated for estrogen receptor [34].

Along with nuclear localization and dimerization, a number of other properties are associated with the LBD. Obviously, one property is ligand binding. A core sequence (aa 537-673) was discovered that maintained considerable affinity and all the specificity to ligand [35, 36]. Another attribute is binding to hsp90. From peptide competition studies, two sites (aa 599-618 and aa 644-671) have been identified [37].


N
C
A/B
C
D
E/F
DBD
ligand binding core
Ligand
440
505
547
795
TAF-1
TAF-2
Nuclear Localization
Dimerization
Hsp 90 binding

binding. For GR, this domain is also involved in binding heat shock proteins, dimerization, transactivation and possesses a second NLS whose activity is hormone-dependent [31]. The exact amino acid sequence in the LBD required for nuclear localization has not been determined. However, a region in the LBD contains sequence similarity to the developmentally regulated NLS of the adenovirus E1a protein ((unpublished observations), [38]).

b) Steroid Hormone Action

Members of the steroid receptor family are transcription factors that ultimately act in the nucleus to regulate expression of specific genes. They all bind as a homodimer to specific DNA sequences called hormone response elements (HRE), located in the enhancer or enhancer-promoter regions of target genes (reviewed in [39, 40]). These sequences consist of inverted hexameric palindromes with a three base-pair space that always separates the two six base-pair half-site sequences. Once bound to the specific DNA sequence, the steroid receptors can enhance transcription of target genes. Recent results suggest that transcriptional enhancement by steroid receptors occurs through proteins called co-activators (reviewed in [41]). Co-activators are adapter proteins that bridge the steroid receptor with the basal transcriptional machinery. They possess intrinsic histone acetyltransferase activity but they also associate with histone acetyltransferases [42, 43] and appear to enhance transcription by increasing accessibility to DNA [44].

c) Proteins of the Steroid Receptor Heterocomplex

Steroid receptors exist in two forms as demonstrated by their sedimentation at different positions on a sucrose or glycerol gradient. This was first shown with ER [45]. A gradient fraction containing ER was found to sediment at 8-10S and was called the untransformed receptor because it could bind hormone but not DNA. After hormone treatment, the ER-containing fraction sedimented at 4S. It was called the transformed receptor as it was able to bind DNA (reviewed in [46, 47]). It is now known that the 4S form is a receptor monomer capable of binding DNA and that the larger 8-10S form consists of the steroid receptor in association with a dimer of a 90 kDa heat shock protein, hsp90 [48, 49], hsp70 [50], along with an immunophilin (FKBP51, FKBP52/hsp56 or CyP-40) [51-53] or protein phosphatase 5 (PP5) [54], and a 23 kDa acidic protein, p23 [55]. There also may be other additional factors still to be discovered.

Hsp90 is an ubiquitous, abundant, highly conserved protein that binds to all steroid receptors as well as other proteins [56]. Hsp90 binds to GR through the ligand binding domain [57, 58] and is necessary to maintain the receptor in a high affinity, ligand binding state [59]. Recent experiments uncovered a minimal chaperone heterocomplex associated with GR, which consisted of hsp90, hsp70, p60 and p23, that enables GR to bind steroid [60-62]. Association of hsp90 with GR represses the other functions such as DNA binding, transactivation, dimerization and nuclear localization [63, 64]. Hsp90 performs the same functions for the other steroid receptors [65-67] except that repression of nuclear localization seems to be specific only for GR [68-70]

and MR [68, 71-73] but not PR [28, 68, 74, 75] and ER [68, 76]. When either ER or PR is bound to hsp90, they still localize to the nucleus in the absence of ligand as opposed to being in the cytoplasm. In contrast, the non-steroidal receptors e.g. TR [77] and RAR [78], in the absence or presence of ligand, do not bind hsp90 and are localized to the nucleus, bound to DNA.

Another component of the GR minimal chaperone heterocomplex, hsp70, is also a heat shock protein [62]. Hsp56/FKBP52/FKBP59 (FK506 binding protein) is a heat shock protein as well as an immunophilin [52, 79, 80]. Immunophilins are proteins that bind to immunosuppressent drugs such as FK506, cyclosporin A and rapamycin with high affinity and specificity. Members of the immunophilin family possess a peptidylprolyl isomerase activity *in vitro* [81]. The immunophilins, FKBP51, FKBP52 or CyP-40 or PP5 are all found in separate heterocomplexes, bound to a common site in hsp90 called the tetratricopeptide repeat (TPR) domain [54]. The p60 protein or Hop for hsp90-hsp70 organizing protein, is a necessary but transient component of the heterocomplex assembly pathway of GR [60, 62]. The acidic protein, p23, is an ubiquitous, conserved protein that contains a number of C-terminal aspartic residues [55]. It binds to hsp90 in an ATP-dependent manner [82] and it functions to stabilize the heterocomplex bound to GR [61].

2. Nuclear Import and Export of Proteins

a) Nuclear Pore Structure

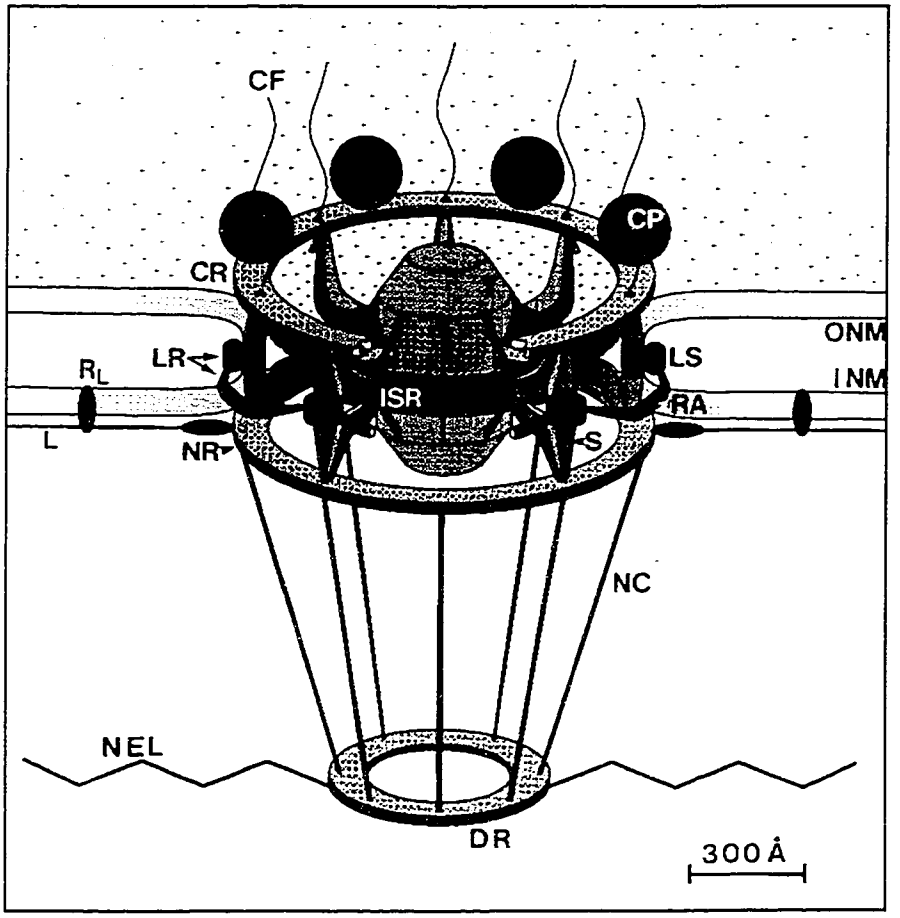
In all eukaryotic cells, the nucleoplasm is separated from the cytoplasm by the nuclear envelope (NE) (reviewed in [83]). The NE represents a barrier where the traffic of material into and out of the nucleus can be regulated. The NE is made up of 3 components: i) a double lipid bilayer; ii) lamins and iii) nucleoporins. Lamins are a meshwork of proteins that underlie the double lipid bilayer to provide structural integrity to the nucleus as well as points of attachment for chromosomal DNA [83]. Nucleoporins are proteins inserted in the double lipid bilayer to form a structure known as the nuclear pore complex (NPC) where the passage of cellular material occurs e.g. RNA, proteins (reviewed in [84]).

The nuclear pore complex is a large (~125 MDa) proteinaceous complex [84] (Figure 2). From electron microscopic studies, the physical structure is revealed to be basically cylindrical, with an 8-2-2 symmetry and consists of a three part structure where a central framework that spans the nuclear envelope lies between a cytoplasmic ring and a nuclear basket-like structure. The cytoplasmic ring has eight 30-50 nm long filaments that extend into the cytoplasm. The nuclear basket-like structure consists of a nuclear ring with eight 50-100 nm filaments that extend into the nucleoplasm and end at a nucleoplasmic ring of 30-50 nm in diameter. The central framework is an eight-spoked structure connected to a central plug or transporter. The nuclear pore has been shown to

Figure 2 A three-dimensional ribbon representation of the nuclear pore complex

This figure is reproduced from Akey and Radermacher [85].

Abbreviations: **CR**-cytoplasmic ring; **ISR**-inner spoke ring; **NR**-nucleoplasmic ring; **LS**-lumenal spoke domain; **RA**-radial arm dimer; **CP**-cytoplasmic particle; **S**-spoke; **T**-tripartite transporter; **CF**-cytoplasmic filament; **ONM**-outer nuclear membrane; **INM**-inner nuclear membrane; $\mathbf{R_L}$-membrane receptor; **NC**-nucleoplasmic cage; **NEL**-nuclear envelope lattice; **DR**-distal ring; **L**-nuclear lamina; **C**-cytoplasm; **N**-nucleus


CF
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CR
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300 Å

be highly "elastic". Rigid gold particles, up to 25 nm in diameter and covered with karyophilic (nuclear seeking) proteins, were capable of being transported into the nucleus [86]. This elasticity suggests that the pore is able to support the import of large proteins without unfolding them unlike transport of proteins across membranes of organelles such as mitochondria [87, 88].

b) Entry Into the Nucleus: Passive Diffusion vs. Signal-Mediated Import

Entry into the nucleus can occur in one of two ways, either by passive diffusion or signal-mediated import (reviewed in [89]). Passive diffusion is non-saturable and energy-independent. Channels (~10 nm in diameter) in the NPC allow ions and small molecules to exchange freely between the cytoplasm and nucleus. Some recent studies have shown that nuclear entry for some ions e.g. calcium, occurs through specific routes [90].

Signal-mediated import is required for karyophilic proteins larger than 40-60 kDa since they cannot enter the nucleus by passive diffusion. However, this process is saturable and requires energy. Signal-directed entry is dependent upon: a) a nuclear localization signal (NLS) within the imported protein; b) proteins e.g. importin α and β which are components of the general import machinery and c) GTP (energy).

i) Nuclear Localization Signals (NLSs)

Signal-mediated import requires an NLS which is a signal that enables a protein to enter the nucleus. The NLS is part of the amino acid sequence of the protein. Unlike signals for transport across membranes into other organelles, the NLS is not cleaved from the protein as it enters the nucleus [91, 92]. Usually, only proteins greater than 40-60 kDa require such a signal but proteins of smaller size sometimes possess such a signal e.g. histone H1 [93]. Two approaches are used to determine if a sequence is truly an NLS. The first approach is deletion of the sequence. This assesses the necessity of the sequence for protein nuclear import. The second approach is the addition of the putative NLS sequence to a normally cytoplasmic protein e.g. ß-galactosidase. This determines if the sequence is sufficient for nuclear import. The two methods are complementary since neither approach alone is adequate. If the deletional approach is used exclusively, additional amino acids of the putative NLS may be missed. In only using the additional approach, potential additional NLSs within the protein may be missed or the putative NLS may not function in nuclear import for the original protein since it may be masked by the structure of the protein. It has been shown that the protein context of the NLS affects its nuclear localization ability [94].

There are different types of NLSs but the NLSs for a majority of nuclear proteins fall mainly into one of two categories: the basic motif NLS or the bipartite type NLS [95]. The most extensively studied NLSs are the basic motif NLSs which consist of short stretches (~6) of basic amino acids (usually lysine or arginine residues) [95, 96]. An

example of a basic motif NLS comes from the SV40 large T antigen, PKKKRKV, which was the first sequence to be identified as an NLS [97, 98]. Fusion of this sequence to a non-nuclear protein such as ß-galactosidase or pyruvate kinase, directs the protein into the nucleus. However, threonine substitution of the second lysine residue from the N-terminus abolishes the nuclear localization ability of this sequence. Sequences similar to the SV40 NLS have been found in other proteins and shown to be NLSs [95, 96]. The other major category of NLS is the bipartite type NLS and it is made up of two sets of basic residues of either lysine or arginine that are separated by an ~10 amino acid spacer sequence. The first example of a bipartite NLS was discovered in the *Xenopus* nucleoplasmin protein whose NLS sequence is KRPAATKKAGQAKKKK [99]. The severity of disruption of the nuclear localization activity of this sequence depends upon how many amino acids and which ones were changed. Changes to the spacer amino acids had no effect. One amino acid change at either the N or C-terminal lysine or arginine residues somewhat impaired nuclear localization activity. Total disruption occurred when at least two of the lysine residues at the C-terminus end (excluding the third lysine residue) were mutated. Another type of NLS is one found in the yeast mating-type switch factor, Matα2 [100, 101]. It consists of a short stretch of hydrophobic amino acids interspersed with one or more basic amino acids. Matα2 contains two such NLSs, one from amino acid residues 1 to 13, NKIPIKD [100], and another one from residues 141 to 159, VRILESWFAKNI [101]. Other examples of NLSs are the hydrophobic NLS from the adenovirus E1a protein or the M9 domain from the protein, heterogeneous

ribonucleoprotein A1 (hnRNP A1). The hydrophobic NLS, $FV(X)_{7-20}MXSLXYM(X)_4MF$, is unique since it does not contain any basic residues. This protein is targetted to the nucleus in *Xenopus* oocytes in a developmentally regulated manner [38, 102]. The M9 domain, NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY, discovered in the hnRNP A1 protein, is conserved across species and a homologous sequence is found in other hnRNP proteins like hnRNP A2 [103]. The domain is notably different than the basic motif or bipartite type NLS since it does not contain basic amino acid clusters, is rich in glycine residues and can also function as a nuclear export signal (NES) [104].

Interestingly, not all proteins that get into the nucleus contain an NLS. Their transport into the nucleus can occur by binding to or "piggybacking" with a protein that does contain an NLS. Two such examples are the import of the arylhydrocarbon (Ah) or dioxin receptor [105] and the import of an artificially created cytoplasmic PR mutant by wildtype PR [106]. The Ah receptor is a basic helix-loop-helix (bHLH) transcription factor with similar characteristics as the steroid receptors but which shares no homology in the DBD. It does not possess an NLS but binds to another bHLH protein called Arnt, the arylhydrocarbon nuclear translocator, which does have an NLS for entry into the nucleus. Normally, wildtype PR is localized in the nucleus in the absence of hormone. However, the PR mutant remains in the cytoplasm in the absence or presence of hormone. Cotransfection of wildtype PR results in nuclear localization of the cytoplasmic PR

mutant in the presence of agonist or antagonist, presumably by "piggybacking" onto the wildtype receptor.

ii) Factors Required For Nuclear Import

The identification of factors that are required for nuclear import has been greatly facilitated by the development of an *in vitro* digitonin-permeabilized cell nuclear import assay [107]. Mammalian cells plated onto coverslips are selectively permeabilized with digitonin such that the plasma membrane, but not the nuclear envelope, is disrupted. The cytosol is lost so that only exogenously added soluble factors required for import will reconstitute the import process. Recently, there has been an explosion of new information regarding factors that play a role in the process of protein nuclear import. Previously, a 54/56 kDa protein was isolated from bovine erythrocytes that was a possible candidate for an NLS receptor [108]. However, a cDNA clone was not obtained. At the end of 1994, the cDNA clone of a *Xenopus* NLS receptor was isolated and named importin [109]. It is now known that there are at least four factors required for import to occur as demonstrated by the digitonin-permeabilized cell assay. These are importin α/karyopherin α/Kap60p, importin ß/karyopherin ß/Kap95p, Ran/TC4 and nuclear transport factor (NTF) 2/p10/B2 (reviewed in [110]).

Importin α, the NLS receptor, has been isolated and cloned from a number of species. Its molecular weight varies between species and ranges between 54 to 60 kDa [108, 109]. The N-terminus contains an importin ß binding domain (IBB) [111, 112] and

the C-terminus is responsible for binding to an NLS-containing substrate [113]. The structure of the rest of the protein consists of eight internal repeats made up of ~42 hydrophobic amino acids. These repeats are known as "arm" motifs and are shared by proteins of diverse functions [114]. Importin α is localized in the cytoplasm, on the NE and in the nucleus [84]. It binds as a complex with the NLS substrate to importin ß to mediate entry of the substrate into the nucleus [109, 115]. Evidence collected so far suggests that importin α only binds to NLSs that are of the basic motif or bipartite type NLS [109, 116]. Variants of importin α have been identified suggesting the existence of a family of these proteins. They can be classified into two main groups based on primary sequence conservation [117, 118]. One group consists of the yeast protein SRP1 (suppressor of RNA polymerase I), mouse SRP1, karyopherin α_1, human SRP1 and human NPI-1 (nucleoprotein interactor 1). Members of the second group are *Xenopus* importin α, mouse pendulin, karyopherin α_2, human SRP1α and human Rch1 (Rag cohort 1). Different expression levels between the two importin α groups have been found in various tissues [118, 119]. In addition, recent evidence indicates that members of the two groups bind substrates with different basic NLSs with distinct affinities [117-120].

Importin ß, a ~97 kDa protein, was also originally purified from bovine erythrocytes [115, 121, 122]. It binds to importin α, Ran and specific nucleoporins. Several studies conclude that the N-terminus is involved in Ran-GTP binding, the C-terminus is responsible for importin α binding and an internal region in the first half of

the protein for binding to specific nucleoporins [123, 124]. It is found in the cytoplasm and on both sides of the NE [121, 125]. It mediates the docking of the importin α-NLS substrate complex to the nuclear pore [115]. Recently, proteins that are distantly related to importin ß have been described that perform both the NLS binding function of importin α and the pore docking function of importin ß. They are transporters involved in alternative nuclear import pathways. Transportin/karyopherin ß2/Kap104p is responsible for the nuclear import of mRNA-binding proteins that carry the M9 domain NLS [116, 126]. Kap123p/Pse1p/karyopherin ß3 is a third NLS receptor that has been cloned [127] and is involved in the nuclear targetting of some ribosomal proteins. These recent discoveries suggest that different NLS receptors, specific for NLSs associated with discrete classes of proteins (mRNA versus ribosomal proteins), mediate multiple, noncompetitive nuclear import pathways.

Ran/TC4 is a Ras-related nuclear protein GTPase. It is an abundant, nuclear protein which has been linked to a number of cellular functions other than nuclear import (reviewed in [128]). Although it is primarily a nuclear protein, it can be found in the cytoplasm. Ran is required to stimulate nuclear import and export of proteins and RNA. Ran acts as a "molecular switch" and it cycles between two states, an active GTP-bound state and an inactive GDP-bound state [128]. Since the intrinsic GTPase activity of Ran is low, there are accessory proteins that control and regulate the GTPase activity. One such protein is RanGAP1 (GTPase activating protein) which enhances the GTPase activity. It is located on the cytoplasmic side of the nuclear pore. Another protein is

RCC1 (for regulator of chromosomal condensation). It acts as a guanine nucleotide exchange factor (GEP) for Ran and is located in the nucleus. A third factor is RanBP1 (Ran binding protein 1) and it binds to Ran-GTP and increase the activity of RanGAP1. In the absence of RanGAP1, it can inhibit the nucleotide exchange of Ran-GTP. Ran not only interacts with its regulatory factors, it also binds to importin ß, nucleoporins and NTF2.

The cytosolic factor, NTF2, is initially isolated as a protein that interacted with a nuclear pore glycoprotein, p62 [129] and the gene encoding for this protein has been cloned. In addition to p62, it also can bind to importin β and Ran-GDP at distinct sites [130, 131]. It is required for nuclear import of proteins [132] and acts in concert with Ran to stimulate nuclear import [133, 134].

iii) Process of Signal-Mediated Protein Nuclear Import

The process of protein nuclear import is now believed to occur in the following manner (reviewed in [110]) (Figure 3). First, a NLS-containing protein binds to a heterodimer of importin α/ß which then docks at the nuclear pore. Energy (GTP) is not required for the NLS substrate to bind to the importin heterodimer or to dock at the pore. Next, the heteromeric complex translocates across the nuclear envelope via the pore in a process that requires Ran/TC4 and NTF2. This step requires Ran-GDP and GTP since this translocation can be blocked by nonhydrolyzable analogs of GTP [135]. Once inside the nucleus, this protein complex is dissociated by Ran-GTP binding to

Figure 3 Process of protein nuclear import

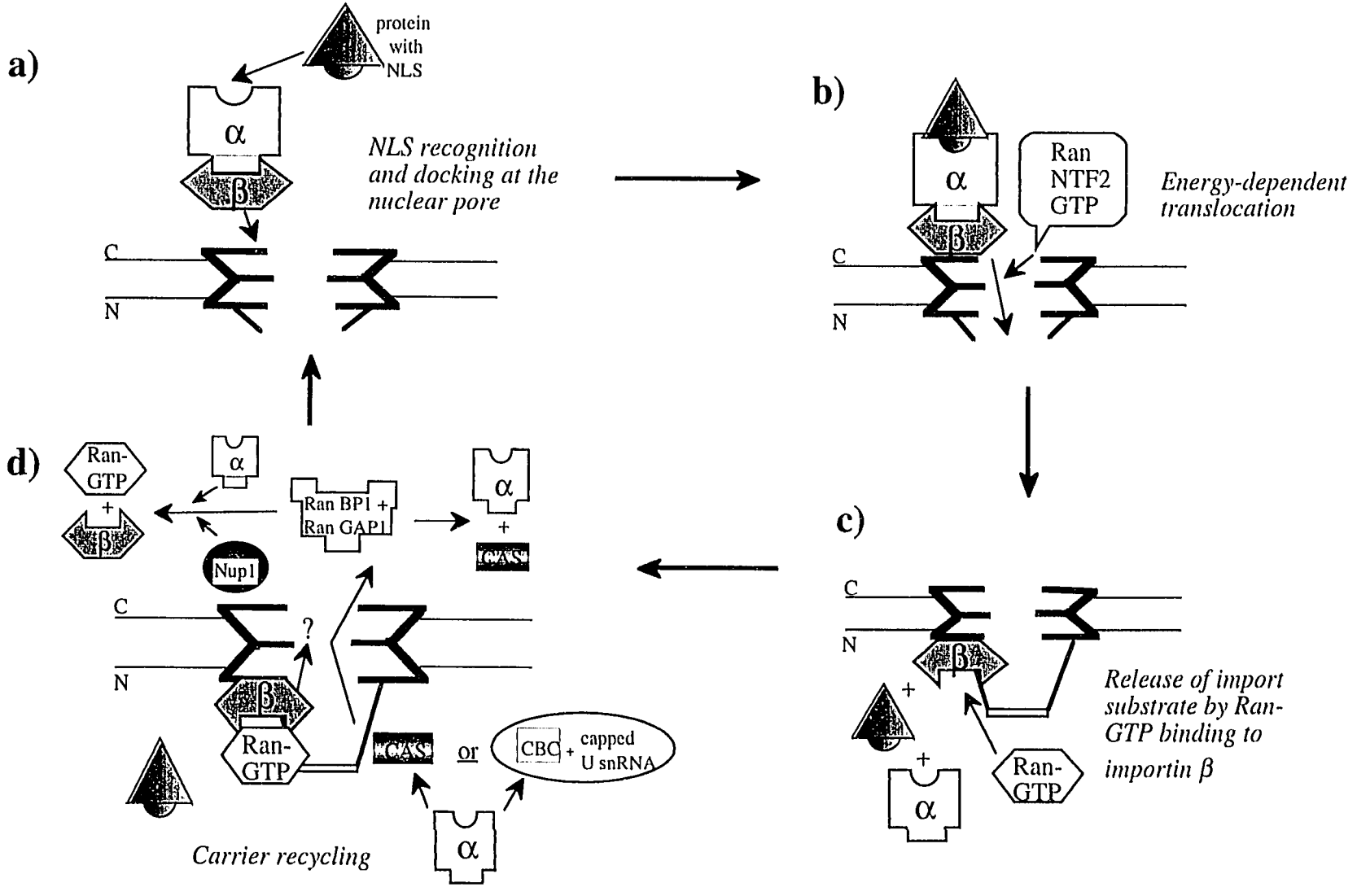
Protein import occurs in several steps [136]. Refer to the text for a more detailed explanation.

a) NLS binding by importin α/β heterodimer and subsequent docking of the heteromeric complex at the nuclear pore.

b) Involvement of Ran, NTF2 and GTP in the energy-dependent translocation of the complex across the nuclear envelope

c) Release of import substrate by Ran-GTP binding to importin β which dissociates the import complex.

d) Recycling of importin α and β to the cytoplasm. Importin α recycling occurs by two different mechanisms. The use of CAS protein is one method and the other pathway involves the formation of a complex consisting of importin α, cap binding protein complex (CBC) and U snRNA. The mechanism of importin β recycling is currently unknown. However, it is speculated that importin β is recycled back to the cytoplasm in a complex with Ran-GTP. Details on dissociation of the Ran-GTP/importin β complex in the cytoplasm are in the text.


a)
protein with NLS
α
β
NLS recognition and docking at the nuclear pore
C
N
b)
α
β
Ran NTF2 GTP
Energy-dependent translocation
C
N
c)
C
N
β
+
α
+
Ran-GTP
Release of import substrate by Ran-GTP binding to importin β
d)
Ran-GTP
+
β
α
Ran BP1 + Ran GAP1
α
+
CAS
Nup1
C
N
?
β
Ran-GTP
CAS
or
CBC + capped U snRNA
α
Carrier recycling

importin ß [133, 137]. However, importin ß remains docked on the nuclear side of the pore [115]. Importin α and ß are then recycled separately back to the cytoplasm.

Importin α is exported back to the cytoplasm by one of two mechanisms. The first mechanism requires the action of the protein, CAS (Cellular Apoptosis Susceptibility gene), recently identified to be the export factor for importin α [138]. It begins with CAS binding to importin α in the presence of Ran-GTP, forming a trimeric complex. CAS preferentially binds to importin α that is free of the NLS substrate. The complex is exported and then dissociated through the combined action of RanBP1 and RanGAP1. The second mechanism commences with the formation of a complex consisting of importin α, the nuclear cap-binding protein complex (CBC) and capped U snRNAs [139]. Next, this complex is exported from the nucleus where it is dissociated by importin ß binding. It is not yet clear whether or not CAS or another export factor is also involved in this export mechanism. Importin α is then free to start another round of import.

The mechanism for importin ß recycling is unknown. However, Ran-GTP, after displacing the complex of importin α and the NLS substrate, may still be bound to importin ß as it is recycled back to the cytoplasm. Details on the dissociation of the complex containing importin ß and Ran-GTP have been reported [140]. The factors involved are importin α, the C-terminus of Nup1 (C-Nup1), a nuclear pore protein, RanGAP and RanBP1. First, importin ß is released from Ran-GTP by importin α. Ran-GTP is then converted to Ran-GDP by the actions of RanGAP and RanBP1. RanBP1 also prevents reformation of the importin ß-RanGTP complex, in the presence of importin

α and C-Nup1. The released importin ß forms a ternary complex with importin α and C-Nup1 which makes rebinding of importin ß to Ran-GTP less favourable. This will enable importin ß, in association with importin α, to begin another round of nuclear import.

c) Nuclear Export of RNA and Proteins

The NPC is not only involved in protein nuclear import but also the export of RNA and proteins. It is previously thought that protein nuclear export occurs by default [141]. However, with the discovery of NESs and NES receptors (Crm1/exportin1), it seems that nuclear export can be an active and/or default process [142-144]. Nuclear export signals have been isolated from proteins such as HIV-1 Rev and the protein kinase inhibitor (PKI). The NES sequences of PKI (LALKLAGLDI) [145] and HIV-1 Rev (LQLPPLERLTL) [146] are rich in leucine residues and bear no resemblance to NLSs. A receptor that binds to this sequence has been isolated and proposed to be a nuclear export receptor. The NES receptor, exportin/CRM1/Crm1p (chromosomal region maintenance 1), is a distant member of the importin ß family [147, 148]. Another export factor, CAS, has also been discovered and is involved in the export of importin α [138]. Nuclear export of the substrate (importin α or NES-containing protein) is thought to occur beginning with the formation of a complex consisting of exportin (CAS), the NES substrate (importin α or PKI) and RanGTP [138, 148]. The complex then translocates across the pore to the cytoplasm. With the CAS protein, importin α is released in the cytoplasm through the concerted action of RanGAP1 and RanBP1 [138].

The export of RNA has been observed to be energy dependent and it has been proposed that RNA is exported in a complex with proteins [110]. Competition experiments studying export of different types of RNA (tRNA, U snRNA and mRNA) suggest that they each utilize a different pathway [149]. However, there is growing evidence that export of certain proteins and RNA shares a common pathway although specific factors for RNA export have not yet been discovered. Competition studies done with the NES of Rev protein uncovered inhibition of export of 5S rRNA and U snRNA [146]. Additionally, experiments using saturating amounts of the M9 NLS/NES domain of hnRNP A1 inhibited export of mRNA but not other RNA classes [150].

d) Influences on Protein Nuclear Import

There are certain conditions and factors that affect protein nuclear import. They can affect nuclear transfer by: i) modification of the NLS; ii) disrupting the formation of the importin α/β heterodimer and binding of importin α to the NLS-containing protein; iii) disruption of docking at the nuclear pore; iv) alteration of the nuclear pore; and v) disruption of translocation across the nuclear pore.

Modification of the NLS can occur in at least two ways. First, the sequence of NLS can be mutated so it no longer functions in nuclear import [97, 99, 118]. Second, phosphorylation of serine or threonine residues that flank an NLS can affect the kinetics of nuclear import [151]. It has been demonstrated that an increase in the rate of import

can be due to enhanced binding by the importin heterodimer to the phosphorylated sequence [152].

Inhibition of importin α binding to the NLS also can prevent nuclear import. The NLS of a protein may be hidden or "masked" either by an inhibitor protein or through an intramolecular interaction. These two possibilities are exemplified by the protein, nuclear factor-κB (NF-κB). NF-κB can be retained in the cytoplasm through the interaction with an inhibitor protein, inhibitor of κB (I-κB) [153]. Release from I-κB results in the exposure of the NLS and transport of NF-κB to the nucleus. Retention in the cytoplasm can also occur through an intramolecular interaction. Intramolecular binding of the C-terminus of the precursor (p110) of the p50 subunit of NF-κB to its own NLS effectively masks the NLS, leading to retention in the cytoplasm. Removal of the C-terminus allows nuclear import of p50 [154]. Disruption of the formation of the importin α/β heterodimer also has been shown to prevent nuclear import utilizing artificially created mutants which contain only the domain for binding importin β [124]. These mutants disrupt import by competing with wildtype importin α for binding to importin β. However, import by transportin, the mediator of an alternative import pathway, is not affected [116].

Docking of the complex containing the importin heterodimer and the NLS substrate to the nuclear pore can be disrupted to prevent nuclear import. This can be done using a lectin, wheat germ agglutinin (WGA) [155-157]. The suggested mechanism of inhibition by WGA is its binding to O-linked glycoproteins present in the NPC. Antibodies to the components of the NPC can be used to inhibit nuclear import [158].

Presumably, this is by blocking access of the importin heterodimer to the antibody-bound nucleoporins. Docking at the nuclear pore also can be disrupted by importin ß mutants that bind to the NPC but not Ran [124]. These mutants can inhibit not only NLS-mediated and M9 signal-mediated nuclear import but also export of mRNA, U snRNA and the NES-containing Rev protein from the nucleus. Thus, importin ß binds to sites at the NPC that may be shared by mediators of the other import and export pathways.

Nuclear import can be affected by alterations to the NPC. An increase has been reported in the rate and size of gold particles imported into the nucleus in proliferating cells as compared to quiescent cells [159]. Similar differences are also observed for virally transformed cells versus untransformed cells [160].

Finally, translocation across the pore can be inhibited to prevent nuclear transport. This can be done by a decrease in temperature to 0°C, the use of energy inhibitors or a Ran mutant that does not stably bind GTP. They interfere with the energy-dependent translocation step of import [161-164].

3. Nucleocytoplasmic Trafficking of GR

The glucocorticoid receptor is a nuclear transcription factor whose subcellular localization is regulated. In addition to regulation imposed on all proteins targetted to the nucleus, GR also has to overcome the inhibitory effect on nuclear localization by the ligand binding domain of GR [69, 70]. Unliganded GR resides in the cytoplasm, bound in a multiprotein heterocomplex [165]. Binding of hormone initiates a cycle whereby GR

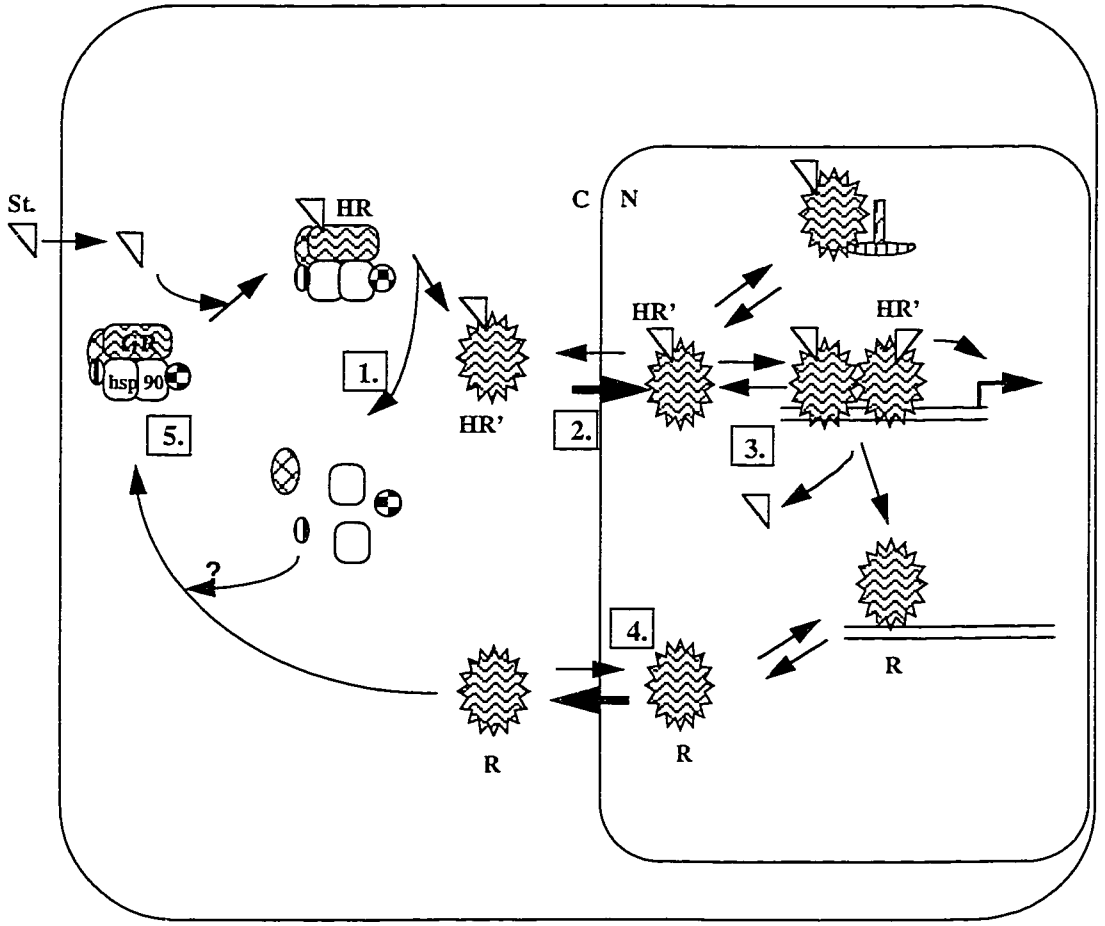
dissociates from the heterocomplex and enters the nucleus to modulate expression of certain genes. Upon loss of ligand, the cycle is completed with GR relocating back to the cytoplasm, repackaged in the multiprotein complex.

This cycle of GR action can be divided into a number of different steps (Figure 4). The first step is the process of transformation which occurs once GR has bound ligand. This involves a conformational change of GR, dissociation of GR from the heterocomplex which contains mostly heat shock proteins and exposure of the NLSs of GR (reviewed in [21]). The second step is nuclear import where GR is thought to interact with the general import machinery and translocates across the nuclear pore. The third step is nuclear retention of GR. GR can bind to DNA [165], the nuclear matrix [166], nuclear envelope [167, 168] and other nuclear proteins [169-171], thus providing a means of nuclear retention. However, our laboratory has shown that DNA binding is a significant determinant of GR nuclear retention. After hormone treatment, a DNA-binding mutant of GR translocates to the nucleus but attains a lower nuclear equilibrium level [172]. Additionally, after a cycle of hormone treatment and withdrawal, this GR mutant redistributes more rapidly to the cytoplasm than its wildtype counterpart [172]. The fourth step is nuclear export. It is not yet known whether GR is exported by a default process or is actively transported out of the nucleus. The final step is recycling of GR where GR is redistributed and retained in the cytoplasm, bound to the multiprotein complex as it awaits the next hormonal stimulus.

Figure 4 Cycle of GR transport

See text for detailed explanation of the cycle of GR transport [21]. Briefly, step **1** is the transformation of GR upon ligand binding. Step **2** is the nuclear import of GR. Step **3** is nuclear retention of GR through DNA binding, interaction with other nuclear proteins and the nuclear matrix. Step **4** is nuclear export of GR. Step **5** is cytoplasmic retention of GR and the ability to respond to the next hormonal stimulus.

Abbreviations: **St.**-steroid; **HR**- receptor bound to hormone;
HR'- transformed receptor bound to ligand; **R**- receptor


St.
HR
C
N
HR'
HR'
hsp
90
1.
HR'
2.
3.
5.
0
?
4.
R
R
R

In addition, localization of liganded GR is not a static state. Instead, it is a dynamic equilibrium of the receptor that shuttles quickly and continuously between the cytoplasmic and nuclear compartments. This was observed for GR in a heterokaryon assay. In this assay, cells from two different cell lines are fused so that the cytoplasm is shared but the nuclei remain distinct. GR present in one nucleus can be seen to accumulate in the other nucleus even when protein synthesis is arrested [173]. The most reasonable explanation is that GR rapidly exits one nucleus and enters the other one. This phenomenon of nucleocytoplasmic shuttling is not restricted to GR. PR [174] as well as other functionally unrelated proteins such as some members of the heterogeneous nuclear ribonucleoprotein family also possess this ability [175].

Various conditions and factors can regulate GR transport. First, the stability of the GR heterocomplex can be altered to affect nuclear translocation of GR. *In vivo* studies reveal that the liposomal delivery of a known *in vitro* GR heterocomplex stabilizer, sodium molybdate, can negatively affect nuclear import of GR by dexamethasone (dex), a synthetic glucocorticoid agonist [176]. As well, heat shock has been shown to promote nuclear uptake of unliganded GR [177, 178]. Presumably, this is due to dissociation of heat shock proteins and exposure of the NLSs. It is well established that ligand (such as cortisol) binding causes a conformational change in GR whereby GR dissociates from the heat shock protein complex and enters the nucleus [21]. In contrast, ursodeoxycholic acid, in a ligand-independent fashion, also can promote GR accumulation in the nucleus [179]. The pH indicator, phenol red, and fetal calf serum

have been able to induce nuclear import [27]. The effect of phenol red may be due to its resemblance to ligand. The effect of fetal calf serum may derive from low levels of endogenous steroid or from some unidentified factors present in the serum. It appears that a variety of substances can alter the interaction of GR with the heterocomplex and influence GR transport into the nucleus.

Second, nuclear retention of GR can be affected. The phase of the cell cycle is implicated in this effect. In G_2 synchronized mouse L fibroblasts, GR, which has translocated to the nucleus after dex treatment, is not efficiently retained in the nucleus and redistributes to the cytoplasm [180]. Although changes in GR phosphorylation have not been correlated with changes in gene expression [181], they are linked to changes in GR nucleocytoplasmic shuttling capability [182]. In *v-mos* transformed cells, GR redistributes to the cytoplasm after translocating to the nucleus in the continuous presence of hormone [183]. The redistributed GR is not able to re-enter the nucleus upon re-exposure to hormone. Inefficient nuclear retention is also observed for GR in cells treated with agonist and okadaic acid, an inhibitor of serine/threonine protein phosphatase types 1 (PP-1) and 2A (PP-2A) [182]. To produce this effect, okadaic acid is required at the export step of GR cycling. Inefficient nuclear retention of the okadaic acid-agonist treated receptor is correlated to hyperphosphorylation of sites which are dephosphorylated in vitro by PP-1 and PP-2A. Since a similar effect is seen between GR in *v-mos* transformed cells and okadaic acid-agonist treated receptor, it has been proposed

that the *v-mos* oncoprotein has an effect on the phosphatases or a specific group of their targets [182].

Finally, cytoplasmic retention of GR can be altered. GR treated with RU486, an antiglucocorticoid and antiprogestin, is able to shuttle (demonstrated by a heterokaryon assay) [173] but it is unable to redistribute to the cytoplasm unlike the agonist-treated receptor [172, 180, 184]. The mechanism responsible for this phenomenon is unknown.

4. NLS in Steroid Receptors

An NLS is one requirement for nuclear import. All the steroid receptors (ER, PR, AR and MR) contain an NLS in the hinge region. For ER and PR, the NLS possesses a tripartite structure. It extends over a large stretch (>40) of amino acids and consists of three clusters of basic amino acids that cooperate to direct a protein into the nucleus since each cluster, on its own, is unable to do so [28]. For AR, deletion of a bipartite NLS in the hinge region results in its cytoplasmic localization [29]. However, it appears that this sequence is insufficient to target pyruvate kinase (a cytoplasmic protein) into the nucleus. Thus, for AR, residues outside the proposed NLS sequence appear to be required for nuclear targetting.

In the original study done by Picard and Yamamoto, they showed that GR contained two NLSs [27]. One signal (NL1) is located in the hinge region and part of it overlaps the C-terminal end of the DNA binding domain. The amino acid sequence has been identified and it bears a close resemblance to a bipartite type NLS. The sequence

identified in the rat GR is YRK(X_{10})RKTKKKIKG and starts from residues 497 to 524. A more recent report proposed that additional amino acid (aa) residues upstream of aa 497 (starting from aa 486) are also part of NL1 and are involved in its activity [30]. NL1 is a true NLS since its deletion prevents nuclear entry of GR in the absence of the second NLS and fusion to a cytoplasmic protein, ß-galactosidase, promotes its nuclear import. The second NLS (NL2) is localized to the LBD (residue 540 to 795 in rat GR) but it has not been delimited further. Interestingly, its nuclear localization ability is hormone dependent, unlike NL1, which is constitutive when taken out of context of the full length receptor. Presently, NL2 appears to be unique to GR since strong evidence has not emerged supporting the presence of a hormone-inducible NLS in the ligand binding domain of ER and PR. Sequence comparison of the LBD to other proteins has uncovered a region with sequence similarity to the developmentally regulated NLS of the adenovirus E1a protein [38]. This may be the putative sequence of NL2. However. it is also speculated that NL2 is not a continuous amino acid stretch but instead, it is "assembled" from conformational changes associated with hormone binding [27].

5. Statement of the Problem

Many proteins contain more than one NLS. Examples include SRY, SOX9, fibroblast growth factor 3 (FGF3), hnRNP K, adenovirus E1a, *Agrobacterium* VirE2 and Abl. Some studies have sought to understand the necessity for multiple NLSs and a

number of different reasons have been uncovered. First, it has been demonstrated multiple NLSs can increase the rate of nuclear import [185]. For a protein with multiple weak NLSs, studies had shown that the weak NLSs could cooperate with one another to increase the rate of nuclear import of the protein so that it could localize completely to the nucleus [28, 94, 186]. Individually, the weak NLSs were not able to transfer the protein exclusively to the nucleus. This is the case for the high mobility group (HMG) domain transcription factors, SRY and SOX9 [186]. On the other hand, multiple NLSs may be needed to overcome another targetting signal present within the protein. For example, in FGF3, two NLSs within the protein are competing with a secretory signal so that ultimately, some of the protein can be imported into the nucleus as well as being secreted [187-189]. A third reason for multiple NLSs is to gain access to more than one import pathway. This is exemplified by hnRNP K, a pre-mRNA-binding protein, which has two different types of NLSs [190]. One is the bipartite NLS which can access the importin pathway whereas the other novel NLS can utilize a different pathway which is RNA polymerase II dependent. A fourth reason is that NLSs may play different roles at different times during development; e.g. for the adenovirus E1a protein, one of the two NLSs is developmentally regulated [38, 102]. This also is true for the two NLSs in the VirE2 protein [191]. Finally, the role of the NLSs may be cell specific; e.g. two of the three NLSs of the protein Abl function in one cell type but not in another [192, 193].

Picard and Yamamoto concluded that NL2 of GR appears to be the major contributor to the rate of import of GR [27]. This conclusion was based on experiments

where a ß-galactosidase (ßgal) fusion protein with the GR ligand binding domain alone was shown to have the same rate of import as a fusion consisting of both NL1 and NL2. However, neither fusion proteins were full length receptors which might be important for proper protein conformation and/or possible intramolecular interactions between the N- and C-terminus. Several early time points, after initiation of dex treatment, were done to observe nuclear uptake of the LBD-ß-galactosidase fusion protein. This only provided limited information on the rate of import and not on the equilibrium distribution of the receptor. Additionally, quantification was not provided on the percentage of cells with exclusively nuclear or mostly nuclear staining of GR at each of the time points. This data would have been useful to highlight any differences occurring at the early time points. Also, parallel results were not shown for the full length wildtype receptor for comparison.

Subsequent studies conducted by Cadepond *et al.* [194] and Jewell *et al.* [195] reached a different conclusion. Both groups used a construct expressing full length human GR where the C-terminal basic cluster of the tripartite NLS had been deleted. Although a time course was not done, they observed that after over an hour of agonist treatment, there was still some cytoplasmic staining of the mutated receptor in comparison with the wildtype receptor, which was totally nuclear. This contradicted the earlier finding by Picard and Yamamoto. In conclusion, the contribution of each NLS of GR to the subcellular localization of GR remains controversial.

6. Project Goals

To resolve the controversy about the relative contribution of NL2 to nuclear import and subcellular trafficking of GR and to gain insight into the functional significance of multiple NLSs in GR, the following approach was taken. Kinetics of nuclear uptake after ligand treatment and of exit from the nucleus after withdrawal of ligand of wildtype GR (wtGR) and of full-length GR in which the NL1 activity had been abolished were determined. Studies were carried out in COS-7 cells which had been transfected with either wtGR or mutant GR and had been synchronized in G_0. Since it is known that the cell cycle affects trafficking, it is preferable to use cells synchronized to one phase of the cell cycle. G_0 was chosen as the phase of the cell cycle for two reasons: first, as protein synthesis and degradation were reduced in G_0, a stable pool of receptor would be present for the duration of the experiment and second, most differentiated glucocorticoid target tissues in the adult would be in the G_0-G_1 state.

Specific project goals were the following:

a) Generation of a mutant GR (mutGR) with abolished NL1 activity in the context of the full-length receptor by mutation of the three consecutive lysine residues in the NL1 from residues 513 to 515. Control constructs, egfp-wtGRN525 and egfp-mutGRN524, were obtained to confirm abolition of NL1 function.

b) Nuclear uptake and nuclear exit kinetics of GR after treatment with agonist or antagonist will be determined by semi-quantitative indirect immunofluorescence of cells in G_0 which had been transfected with either wildtype or mutant GR.

II. MATERIALS AND METHODS

1. Materials

The Sculptor™ *in vitro* mutagenesis kit was purchased from Amersham (Oakville, ON). PCR primers, oligonucleotides containing the sequence of the consensus glucocorticoid response element (GRE) and the oligonucleotide used for *in vitro* mutagenesis of NL1 were synthesized using a Beckman Oligo 1000 DNA synthesizer (Mississauga, ON). Reagents for making the oligonucleotides were also purchased from Beckman. Restriction and DNA modifying enzymes were obtained from New England Biolabs (NEB) (Mississauga, ON), GIBCO Bethesda Research Laboratories (GIBCO BRL) (Burlington, ON), or Pharmacia (Baie d'Urfé, Que). Electrophoresis grade agarose, Tris-base, boric acid and ethidium bromide were from BDH (Toronto, ON). Ethylenediamine tetra-acetate (EDTA) was purchased from Fisher Scientific (Nepean, ON). All tissue culture materials (trypsin, fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM)) were obtained from GIBCO BRL. Dexamethasone (dex) and cortisol were from Steroloids (Wilton, NH). RU38486 (RU486), an antiglucocorticoid and antiprogestin, was kindly provided by Roussel Uclef. The BuGR-2 antibody was from Affinity Bioreagents (Golden, CO). Fluorescein-conjugated anti-mouse sheep antibody was purchased from Boehringer Mannheim (Laval, Que). The sheep anti-mouse and donkey anti-rabbit horseradish peroxidase-conjugated antibodies

and the Enhanced Chemiluminescence (ECL) Western blot detection kits were bought from Amersham. Protein A-Sepharose, buffers, dithiothreitol (DTT) and bovine serum albumin (BSA) were from Sigma (Oakville, ON). Electrophoresis grade acrylamide, N',N'-methylene bisacrylamide, sodium dodecyl sulphate (SDS) and glycine were purchased from BDH. N,N,N'-N'-tetramethylethylene-diamine (TEMED) was from GIBCO BRL. The radioisotopes, [α^{35}S] dATP (10 mCi/mL) and [γ^{32}P]ATP (10 mCi/mL), were bought from Amersham. The [^{14}C] chloramphenicol (50 μCi/mL) came from Mandel-Dupont (Guelph. ON).

2. Construction of GR NL1 Mutant

The rat GR NL1 mutant was generated by site-directed mutagenesis of the three consecutive lysine (K) residues, at aa 513 to 515, to asparagine (N). A BspEI/PstI fragment, corresponding to aa 391 to 524 of wtGR, was excised from the plasmid 6RGR [196] and subcloned into the corresponding sites in the phagemid vector, pBlueScript II KS- (Stratagene , La Jolla, CA). Cells of *E. coli* NM522, a bacterial strain capable of being infected by phage, was transformed with the phagemid vector containing the GR insert. The helper phage, M13KO7, produced single-stranded, anti-sense DNA containing the wtGR sequence (corresponding to aa 391 to 524) from the modified phagemid vector. The single-stranded DNA was packaged into phage particles and released into the culture broth. The released phage particles were then collected to isolate

the single-stranded DNA. To proceed with the site-directed mutagenesis reaction, single-stranded, anti-sense DNA with the wtGR sequence and an oligonucleotide with the desired mutations were required. The sequence of the 44 base pair (bp) oligonucleotide with the NL1 mutations is 5'-CCTTGAAGCTCGAAAAACAAACAACAACATCAAAGGGATTCAGC-3' with the altered bases underlined. The mutagenesis reaction was done according to manufacturer's instructions using the Sculptor™ *in vitro* mutagenesis kit from Amersham. Plasmid DNA was isolated from bacteria transformed with the mutagenized DNA and screened by dideoxy sequencing for potential NL1 mutants. A clone with the NL1 mutation was isolated but additional, unanticipated base changes had also occurred. Those additional changes were reversed to the wildtype sequence using PCR mutagenesis. The resulting PCR fragment was digested with BspEI and PstI restriction enzymes and ligated into 6RGR which had been digested with the same restriction enzymes. Plasmid DNA was isolated from bacteria transformed with products from the ligation reaction. The NL1 mutant, without any other additional mutations, was identified by sequencing and the isolated clone was called p6RGRmutNL1.

3. Construction of Control Constructs That Express GR-β-galactosidase Fusion Proteins or Enhanced Green Fluorescent Protein-GR Fusion Proteins

In addition to the construct of full length GR with the mutated NL1 (p6RGRmutNL1), two control constructs were also generated. The first set of control constructs that were created had expressed β-galactosidase fused to the C-terminus of GRN524. To generate the wtGRN524.z construct, a PstI fragment, corresponding to GR aa 1-524, was excised from the GRN525 plasmid [196] and inserted into the PstI site in the GR 497-524.z plasmid [27]. The mutGRN524.z construct was created by removing a PstI/NheI fragment, which contained the cDNA for β-galactosidase, from the GR 497-524.z plasmid [27] and ligated into the PstI/NheI sites in p6RGRmutNL1.

The second set of control constructs that were generated had expressed the enhanced green fluorescent protein (egfp or EGFP) fused to the amino-terminus of GR aa 22-525 or aa 22-524. To generate the egfp-wtGRaa22-525 construct, which contained GR with the wildtype NL1 sequence, a MscI/BamHI fragment, corresponding to aa 22-525 of GR, was removed from the GRN525 plasmid [196] and ligated into the SmaI/BamHI sites of pEGFP-C1 (Clontech, Palo Alto, CA). Construction of the egfp-mutGRaa22-524 expression vector (which contained GR with the mutated NL1 sequence) involved insertion of a linker, that contained a stop codon, into the PstI site of the p6RGRmutNL1 vector. Expression of this construct (mutGRN524) would result in the production of a carboxy-terminally truncated GR with the mutated hinge NLS. A MscI/BamHI fragment, corresponding to aa 22-524 of mutGR, was excised from the

mutGRN524 plasmid and inserted into SmaI/BamHI sites of pEGFP-C1 to obtain the egfp-mutGRaa22-524 construct. Clones were screened by restriction digest to ensure the presence and correct orientation of the insert.

4. Insertion of SV40 Origin of Replication Into Constructs Expressing Full Length WtGR or MutGR or β-galactosidase Fusion Proteins Containing WtGRN524 or MutGRN524

To ensure high expression levels of the various GR constructs (for detection in an indirect immunofluorescence (IIF) assay), a SV40 origin of replication was inserted into the 6RGR and p6RGRmutNL1 plasmids, which express full length wildtype and mutated NL1 GR, respectively. The fragment containing the sequence of the SV40 origin of replication was isolated after pRShGRα [197] was digested with NdeI. The ends of the NdeI fragment were filled in with Klenow polymerase (NEB) and ligated into the NaeI site in 6RGR and p6RGRmutNL1. Plasmid DNA isolated from transformants were screened by restriction digest for the presence of the insert. The isolated, positive clones were called 6RGRori (wtGRo) and p6RGRmutNL1ori (mutGRo).

A SV40 origin of replication was also inserted into the vectors, wtGRN524.z and mutGRNA524.z, to express high levels of the β-galactosidase fusion proteins. After restriction digestion of the wtGRN524.z and mutGRN524.z plasmids with NheI, the overhanging ends were filled in with Klenow polymerase (NEB). The blunt-ended NdeI

fragment (containing the sequence of the SV40 origin of replication) was then ligated into the blunted NheI site. Plasmid DNA isolated from transformed bacterial cells were screened by restriction digest for the presence of the insert. The isolated, positive clones were called wtGRN524ori.z and mutGRN524ori.z

5. Construction of Vectors To Express GR Fragment (aa 394-552) That Contain Either the Wildtype or the Mutated NL1 Sequence

A cDNA segment of GR that corresponded to aa 394-552 (with either the wildtype or mutated NL1 sequence) was cloned into the pET (30a) vector (Invitrogen, Carlsbad, CA). This is a bacterial expression vector that contains the coding sequence for a histidine tag. The GR DNA fragment used for cloning was obtained by PCR amplification. The 5' and 3' primers annealed to the coding sequence that corresponded to amino acid 394 and 552 and contained the restriction sequence for BamHI and XhoI, respectively. Once the PCR fragment was isolated and digested with BamHI and XhoI, it was inserted into the corresponding sites in the pET expression vector. Positive clones were verified by restriction digestion and sequencing. The isolated, positive clones were called pETwt and pETmutNL1.

6. DNA Preparation and DNA Electrophoresis

Plasmid DNA was isolated using a standard alkaline lysis maxi-preparation protocol [198, 199] after transformation of competent *E. coli* DH5α bacterial cells [200]. The DNA was purified twice by cesium chloride centrifugation in a Beckman TL-100 ultracentrifuge [201], dialyzed against Tris-EDTA buffer (10 mM Tris pH 8, 1 mM EDTA), aliquoted and ethanol precipitated. Plasmid DNA, purified by cesium chloride, consisted of 80-95% supercoiled DNA, as estimated by agarose gel electrophoresis and ethidium bromide staining. For use in transient transfections, ethanol-precipitated DNA was centrifuged, allowed to dry under sterile conditions and resuspended in sterile, pyrogen-free (py-f), deionized and distilled water (ddH_2O).

Preparation of agarose gels and agarose gel electrophoresis were done according to a standard protocol [202, 203]. Records of gels were stored initially on Polaroid 55 black and white film (Polaroid, Cambridge, MA) but later, the gels were visualized and stored on a Bio-Rad Gel Doc 1000 system.

7. Preparation of Charcoal-Stripped Fetal Bovine Serum

To remove any endogenous steroids present in FBS, the FBS was incubated with activated charcoal (Sigma) and dextran T500 (Pharmacia) at 5% and 0.5% (w/v) respectively and was shaken continuously for 16 h at room temperature (RT). The mixture was then centrifuged in the Beckman J2-21 centrifuge at 8500 rpm for 30 min

and the incubation step with activated charcoal and dextran was repeated. The following day, the mixture was centrifuged at 8500 rpm for 30 min, then 30 000 rpm for 35 min in a Beckman L8-70M centrifuge. The supernatant was then sequentially filtered through 8 μ, 5 μ and finally, 0.22 μ filters, to sterilize the serum.

8. Cell Culture

SV40 transformed African Green Monkey kidney cells, COS-7 (ATCC# CRL 1651), were grown in DMEM containing heat-inactivated fetal bovine serum (FBS) to 10% (v/v). The cells were maintained in 75 cm^2 tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) at 37°C in a humidified atmosphere of 5% CO_2. Before the cells reached confluency, they were passaged by treatment with 0.25% (v/v) trypsin-EDTA (GIBCO).

9. Semi-Quantitative Indirect Immunofluorescent (IIF) Assay

a) Transient Transfection of COS-7 cells

Transient transfection of COS-7 cells was done by the lipofection method in 60 mm tissue culture dishes (Becton Dickinson). Lipofectamine™ (GIBCO), a 3:1 (w/w) liposome formulation of the polycationic lipid, 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate

(DOSPA) and the neutral lipid, dioleoyl phosphatidylethanolamine (DOPE), in membrane filtered water, was used. The transfection mixture consisted of 3 mL of Opti-MEM® medium (GIBCO), an antibiotic-free, modified Eagle's Minimal Essential Medium (MEM), 15 μg of Lipofectamine™ and 0.75 μg and 0.5 μg of constructs that expressed wtGR and mutGR, respectively. After incubation of the DNA/lipid mixture with the cells at 37°C for 8-10 h, the transfection was stopped by the addition of an equal volume (3 mL) of DMEM with 20% FBS. Beginning at this step and with all subsequent steps, DMEM containing charcoal stripped serum but no phenol red was used. The next morning, the medium was replaced with DMEM supplemented with 10% FBS. Approximately 6 h later, the cells were removed from the 60 mm dishes and replated onto poly L-lysine-coated coverslips in 35 mm tissue culture dishes and incubated overnight in DMEM with 10% FBS. Cells were synchronized to the G_0 state by washing twice with 1x phosphate-buffered saline (PBS) and incubating in serum-free DMEM at 37°C for a further 21 h before starting experimental treatments. For induction experiments, dex or RU486 was added to 10^{-6} M. To determine export after hormone withdrawal, cells were pretreated with cortisol or RU486 to 10^{-6} M for greater than 6 h. Hormone withdrawal was initiated by multiple washes (3x with PBS, 1x with serum-free DMEM supplemented with BSA to 5%) followed by incubation in serum-free DMEM with 5% BSA.

b) Preparation of Poly L-Lysine Coated Coverslips

Poly L-lysine-coated coverslips were prepared the same day the cells were transfected. The coverslips were sterilized by quickly rinsing in ethanol followed by heating in the flame of an alcohol lamp. After application of 500 µl of a 15 µg/mL poly L-lysine solution onto the coverslips, the solution was allowed to evaporate overnight. The coverslips were washed the next day by dipping in py-f water and air dried.

c) Preparation of Cells For Immunofluorescence

After experimental treatments, cells on coverslips were fixed by incubation with a freshly made solution of 3% paraformaldehyde/1X PBS for 30 min at RT. Intrinsic fluorescence due to fixation was quenched for 5-7 min in 0.1 M glycine/PBS. Cells were permeabilized by incubation with 0.5% Triton X-100/PBS for 30 min and then blocked for 1-1.5 h with 5% normal goat serum (NGS)/PBS (GIBCO BRL). After aspiration of NGS, 500 µl of a 5 µg/ml solution of the primary antibody, BuGR-2 (a monoclonal anti-GR antibody), were added to the coverslips and incubated overnight at 4°C. After washing 5x with PBS, 500 µl of a 6 µg/ml solution of fluorescein-conjugated anti-mouse sheep antibody were added and incubated with the cells at RT for 40 min in the dark with gentle shaking. After washing 4x with PBS, glycerol/PBS (1:1 (v/v)) was added to the coverslips before mounting onto slides. The coverslip edges were then sealed with nail polish.

d) Visualization of Cells By Immunofluorescence Microscopy

The cells were viewed at 200x magnification by epifluorescence with a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). They were classified into one of five categories of subcellular localization, from exclusively nuclear to exclusively cytoplasmic as previously described [172]. A minimum of 250 cells were counted for each time point in each experiment and each experiment was repeated 2-5 times. Fields for representative micrographs were chosen at random and photographed with Kodak T-Max film (ASA 3200) (Kodak, Rochester, NY).

10. Protein Determination and Protein Gel Electrophoresis

Protein amount was measured by the Bradford method [204], using BSA as a standard. The samples were incubated for at least 5 min at RT for colour development after the addition of the colormetric reagent. Absorbance of the samples was read at 595 nm.

Solutions for making and running SDS polyacrylamide gels were prepared according to a standard protocol [205]. The minigel casting equipment and accessories were purchased from Bio-Rad (Mississauga, ON). Unless otherwise specified, SDS-PAGE was performed on 8% gels using the discontinuous buffer system by Laemmli [205]. SDS protein sample buffer (63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS

(w/v), 0.05% ß-mercaptoethanol (v/v) and 0.0013% bromophenol blue (w/v)) was added to samples before heat denaturation for 5 min at 100°C. Gels were run at 170 V for 1.5 h.

11. Western Blot Analysis Using Enhanced Chemiluminescence Detection System

After SDS-PAGE, proteins were electrophoretically transferred onto Immobilon-P™ membrane at 100V for 1h [206]. The transblot buffer consisted of 25 mM Tris-HCl pH 7.7 at RT, 192 mM glycine, 20% methanol and 0.1% SDS. The Immobilon-P™ membrane was hydrated with methanol before use. After the transfer, the membrane was rinsed with buffer consisting of Tris buffered saline (TBS) and tween (20 mM Tris pH 7.5, 500 mM NaCl, 0.1% tween) and blocked in TBS-tween buffer with skim milk added to 10% for 1h at RT with gentle mixing. The membrane was then washed for 15 min at RT before incubation with the primary antibody, BuGR-2 (0.25 µg/ml), for 16 h at 4°C. After extensive washing (3x for 20 min each) with TBS-tween buffer, the membrane was incubated with the secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse antibody diluted 1:50 000 in TBS-tween buffer, for 1 h at RT. Again, the blot was washed extensively (3x for 30 min each) in TBS-tween buffer and then developed using the Enhanced Chemiluminescence (ECL) system (Amersham). Solutions of luminol and hydrogen peroxide were mixed at a 1:1 ratio and applied to the blot. The membrane was incubated with this mixture for 1 min before removing the excess solution and placed between two acetate sheets. The acetate sheets were transferred to an autoradiography

cassette and exposed to autoradiography film (NEN™, Guelph, ON) for a set period of time.

12. Preparation of Whole Cell Extracts Containing Either Wildtype or Mutant GR For Sucrose Density Gradient Analysis

COS-7 cells, transiently transfected with constructs that express either wtGR or mutGR with an N-terminal myc tag [207], were treated in the same manner as the cells prepared for the IIF assay. After serum withdrawal, cells either remained untreated or treated with dex for 15 min for those cells expressing myc-tagged wtGR or 15 min and 2 h for the cells expressing myc-tagged mutGR. After agonist treatment, the cells were harvested in cold 1x PBS and centrifuged at 8000 rpm for 1 min at 4°C. All solutions used during and after harvesting the cells contained 20 mM sodium molybdate. The cells were resuspended in 100 µl of whole cell extract (WCE) buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol) and sonicated with a small probe three times for 10 sec each at 35% duty cycle at 4°C and centrifuged at 11 000 rpm for 1 min. The protein content of the supernatant was determined by Bradford assay [204] and 200 µg of supernatant was used for sucrose density gradient analysis.

13. Preparation and Centrifugation of Sucrose Gradients

Four solutions of sucrose (a 10%, 19%, 28% and 35% sucrose (w/v)) were prepared in homogenization buffer (20 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 20 mM sodium molybdate. Sucrose density gradients were prepared by underlaying (with a spinal needle) 1 ml of each of the four different sucrose solutions in a 5 ml centrifuge tube (Beckman) starting with the least concentrated sucrose solution [208, 209]. Gradients were equilibriated by incubation at 4°C for 16-24 h. WCE samples (200 μg) were layered on top of the gradient prior to centrifugation at 55 000 rpm in a SW55Ti rotor (Beckman) at 4°C for a minimum of 16 h. A tube containing proteins with known sedimentation values, BSA (4.3S) and aldolase (7.6S), was run in parallel. After centrifugation, four drop fractions were collected from the centrifuge tubes using a peristaltic pump. The fractions were diluted to 800 μl with homogenization buffer and GR was immunoprecipitated for 16 h at 4°C with gentle mixing after addition of 2 μl of 0.5 μg/μl stock of BuGR-2 antibody. Protein A-Sepharose beads were added to each fraction the next day and mixed on the multipurpose rotator (Bohemia, NY) for 1 h at RT. The beads were then washed 3x with 500 μl of wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) and 1x with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). SDS protein sample buffer was then added and the samples were heated for 5 min at 100°C to elute and denature the proteins. The tubes were centrifuged briefly and the supernatant was run on SDS-PAGE and transferred to Immobilon-P™ (Millipore, Mississauga, ON) membrane for Western blot analysis.

14. Isolation of Bacterially Expressed, His-tagged GR Fragments To Use In Electrophoretic Mobility Shift Assay

The pETwt and pETmutNL1 vectors, which express GR fragments aa 394-552 that contained either the wt or mutNL1 sequence, respectively, were individually transformed into bacterial cells of the *E. coli* strain BL21 (DES) pLys. This bacterial strain contains a stable integrant of the T7 gene 1 (RNA polymerase) under the control of the inducible *lac* UV5 promoter. A fresh transformant was selected and grown to OD (600nm) of 1. Next, the T7 RNA polymerase was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) to express the GR fragments. After inducing overnight at RT, cells were collected by centrifugation at 5000 rpm for 5 min at 4°C and resuspended in nickel column binding buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 2 mM imidazole, 0.2 mM PMSF). The suspension was then sonicated 3x with 10 sec pulses with a small probe at 35% duty cycle. The extract was cleared of insoluble material by centrifugation at 19 600 rpm for 20 min in a Beckman Ti-60 rotor and further purification was done using the his-tagged protein isolation kit from Invitrogen, according to manufacturer's instructions. The isolated protein was eluted with nickel column elution buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 1 M imidazole) and the purified protein was stored at 4°C in the elution buffer but with the addition of glycerol to 12% and sodium azide (NaN_3) to 0.02%.

15. Electrophoretic Mobility Shift Assay

Two, 40 bp single-stranded oligonucleotides that contain the consensus GRE sequence were synthesized for use in the electrophoretic mobility shift assay (EMSA). The sequence of the upper strand is 5'-ACAGGTCGACATAGAACAAACTGTTCTTAAAAGGTACCCA-3' and of the lower strand, it is 5'-TGGGTACCTTTTAAGAACAGTTTGTTCTATGTCGACCTGT-3' [179, 210]. The bases that correspond to the consensus GRE sequence are underlined. T4 polynucleotide kinase (NEB) was used to end-label 500 ng of the upper strand with [γ^{32}P]dATP. After incubation at 37°C for 1 h, the kinase was heat inactivated and the unincorporated, radiolabelled nucleotides were removed by a Sephadex G-50 spin column. Double-stranded, radiolabelled probe was prepared by annealing the radiolabelled upper strand in the presence of 2x excess of the cold, lower strand. The probe was then gel purified on a 4% non-denaturing polyacrylamide gel by electrophoresis in 1x TBE buffer and then the gel slice containing the isolated probe was excised. The probe was eluted from the gel by crushing the gel slice and then incubated in water with gentle mixing. The probe was concentrated by ethanol precipitation.

The mobility shift assay done with the two, different GR fragments was performed essentially as described [211] using 1 µl of purified protein with 5000 cpm of the ^{32}P-labelled probe in a total volume of 20 µl of DNA binding buffer (12 mM HEPES pH 7.9, 60 mM KCl, 0.6 mM EDTA, 12% glycerol, 0.6 mM DTT and 0.12 mM PMSF). BSA and poly dI•dC were also added at 1 µg to the binding reaction before incubation for

20 min at RT. Unlabelled, double-stranded GRE or IAP [212] oligonucleotides were added at 2x the amount of the labelled probe in experiments to assess the specificity of binding of the GR fragments. To detect the formation of higher order protein-DNA complexes, 1 µl of BuGR-2 (0.5 µg/µl) was added. A 4% non-denaturing, polyacrylamide gel with a 37.5:1 ratio of acrylamide:bisacrylamide was pre-run before samples were loaded and run at 150 V for 1.5 h in 0.5x TBE buffer. After electrophoresis, the gel was dried at 80°C under vacuum and protein-DNA complex formation was visualized by autoradiography.

16. Chloramphenicol Acetyltransferase Assay

Transient co-transfection of COS-7 cells was performed with 12 µg of Lipofectamine™(GIBCO). The vectors used in the transfection were: 6RGR (30 ng), a rat wtGR expression vector with a RSV-LTR promoter or p6RGRmutNL1 (10 ng), the mutant GR NL1 expression construct; pMMTVCAT (200 ng), the reporter construct with the mouse mammary tumour virus (MMTV) promoter (MMTV-631/+105) fused upstream to the chloramphenicol acetyltransferase (CAT) gene; and RSV-βgal (200 ng), a β-galactosidase expression vector, to monitor transfection efficiency. To prevent transcriptional inhibition of the CAT gene by production of high levels of GR due to a SV40 origin of replication contained in the full length GR expression constructs, low plasmid DNA levels (nanograms) were transfected. Twenty-four h after stopping transfection, cells were switched from serum-containing medium to incubation in

medium without serum. Following an additional 16 h of incubation, cells either remained untreated or treated with 10^{-6} M dex for 24 h. Afterwards, cells were harvested and processed for CAT assays essentially as described [213].

III. RESULTS

1. Subcellular Localization of Control Constructs

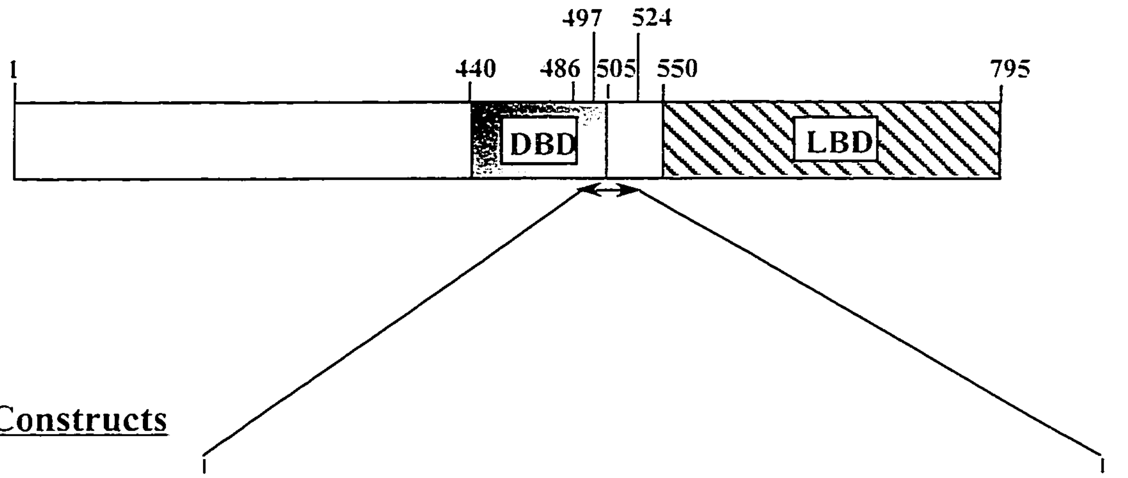
Experiments described in this thesis that monitored nucleocytoplasmic trafficking of wtGRs and mutated GRs were conducted in transiently transfected COS-7 cells, which did not contain endogenous GR, and were synchronized to G_0 of the cell cycle by serum starvation. G_0 was chosen for a number of reasons. First, it has been demonstrated that the cell cycle influences the nucleocytoplasmic trafficking of GR [180]. In G_2 synchronized cells, GR, which had translocated to the nucleus after dex treatment, was not efficiently retained in the nucleus. In contrast, in either G_0 or in the S phase of the cell cycle, GR remained in the nucleus after transferring from the cytoplasm upon treatment of cells with dex. By restricting our experiments to the G_0 phase of the cell cycle, we were assured of observing an uniform response. Second, in the adult, most target tissues of the glucocorticoid hormone are in the G_0/G_1-like state of the cell cycle i.e. the cells are not actively proliferating [214]. Thus, the results obtained for GR trafficking from experiments done with cells in G_0 more accurately reflect transport of GR under physiological conditions. Finally, in G_0, synthesis and degradation of proteins, including GR, are inhibited. Therefore, a stable pool of GR is available for the study of its trafficking [214]. This system previously has been utilized successfully to study the effect of DNA binding on nucleocytoplasmic trafficking of GR [172].

Transient transfection of COS-7 cells using lipofectamine resulted in a strong immunofluorescent signal from greater than 10% of cells (results not shown). Serum was withdrawn from cells for over 16 h to synchronize them to G_0. Cells were incubated with hormone for defined periods before fixation with paraformaldehyde and processed for the IIF assay. Classification of receptor localization in cells was done as described previously [28, 172]. The cells were classified into one of five categories: cells with exclusively nuclear staining of GR (N); cells with greater nuclear than cytoplasmic staining (N>C); cells with equal nuclear and cytoplasmic staining (N=C); cells with greater cytoplasmic than nuclear staining (C>N); cells with totally cytoplasmic staining of GR (C). Results are presented as the percentage of cells that had either totally nuclear (N) or mostly nuclear (N+N>C) staining of GR. Only these two categories are displayed because we are interested in the amount of GR that has transferred to and is retained in the nucleus. Although considerable variation was seen between experiments but the general trend was always the same. Therefore, a representative result from an experiment was shown for almost all the figures.

To study nucleocytoplasmic trafficking of GR mediated by NL2 alone in the context of the full length receptor, a rat GR NL1 mutant was generated by mutating the group of lysine residues, ^{513}KKK^{515}, to asparagine, ^{513}NNN^{515} (Figure 5). One of the conclusions reached in a study by Tang *et al.* was that NL1 had a tripartite structure which consisted of three proto-NLS; pNLS1 from aa 510-517; pNLS2 from aa 496-499; pNLS3 from 486-490 [30]. Replacement of aa 507-515 (i.e. consisting most of pNLS1)

Figure 5 Schematic Representation of GR Constructs Transiently Transfected Into COS-7 Cells

GR is represented by a bar with the gray, shaded segment indicating the DNA binding domain (DBD) and the diagonally striped segment portraying the ligand binding domain (LBD). The numbers correspond to the amino acid residues in the primary sequence of rat GR. The wildtype NL1 sequence is shown with amino acid residues at 513 to 515 highlighted to indicate that these residues are mutated from lysine (K) to asparagine (N). Segments of the NL1 sequence are numbered and underlined to indicate the proto-NLSs that are contained in NL1 (as described by Tang *et al.*) [30].



Test Constructs

wtNL1 (wtGR)	486	KIRRKNCPACRYRKCLQAGMNLEARKTKKKIKGIQQATA (524); underlined motifs: KIRRK (3), RYRK (2), RKTKKKIK (1)
mutated NL1 (mutGR)	486	KIRRKNCPACRYRKCLQAGMNLEARKTNNNIKGIQQATA (524); underlined motifs: KIRRK (3), RYRK (2), RKTNNNIK (1)

in rat GR with proline, aspartic acid and leucine residues in the context of the carboxy-teminal deletion mutant, N525, effectively eliminated NL1 activity. Since the region between aa 510-517 needed to be intact for nuclear import to occur, mutation of the three consecutive lysine residues to asparagine should abolish NL1 activity. Additionally, these residues were chosen because it had been demonstrated that for the protein, nucleoplasmin, substitution of a similar group of lysine residues to asparagine abolished all nuclear localization activity [99].

To confirm that the NL1 function had been destroyed by the mutation of ^{513}KKK^{515} to ^{513}NNN^{515}, constructs were originally made in which β-galactosidase was fused to the C-terminus of GRN524, which contained either the wildtype NL1 sequence (wtGRN524.z) or the mutated sequence (mutGRN524.z) (Figure 6A). A C-terminal truncated GR (containing only the first 525 amino acids) had been demonstrated previously to be constitutively nuclear [27, 30]. However, the β-galactosidase-wtGRN524 chimeric protein did not localize exclusively in the nucleus as expected. Instead, only 24% of cells expressing the wtGRN524 chimera displayed mostly nuclear staining (Figure 6B). The mutGRN524 fusion protein was located exclusively in the cytoplasm. However, since the wtGRN524 fusion protein did not localize to the nucleus as expected, new constructs were generated in which both the protein to be fused to GR and the terminus of GR to which it was fused were altered. Enhanced green fluorescent protein was fused to GR aa22-525 and the orientation was changed such that EGFP was

Figure 6 Subcellular Distribution of the GRN524-β-galactosidase Fusion Proteins In COS-7 Cells

a) Schematic depiction of constructs that express GRN524-β-galactosidase fusion proteins to confirm elimination of NL1 activity by mutation of ^{513}KKK^{515} to ^{513}NNN^{515}

The numbers correspond to amino acid positions in rat GR (**A**). The wtGRN524.z construct expresses the wtGRN524-β-gal fusion protein which is expected to be localized exclusively to the nucleus. The mutGRN524.z construct expresses the mutGRN524 chimeric protein that contains the mutated NL1 sequence. The vertically striped box at the C-terminal end of mutGRN524 indicates the mutated amino acid residues in NL1.

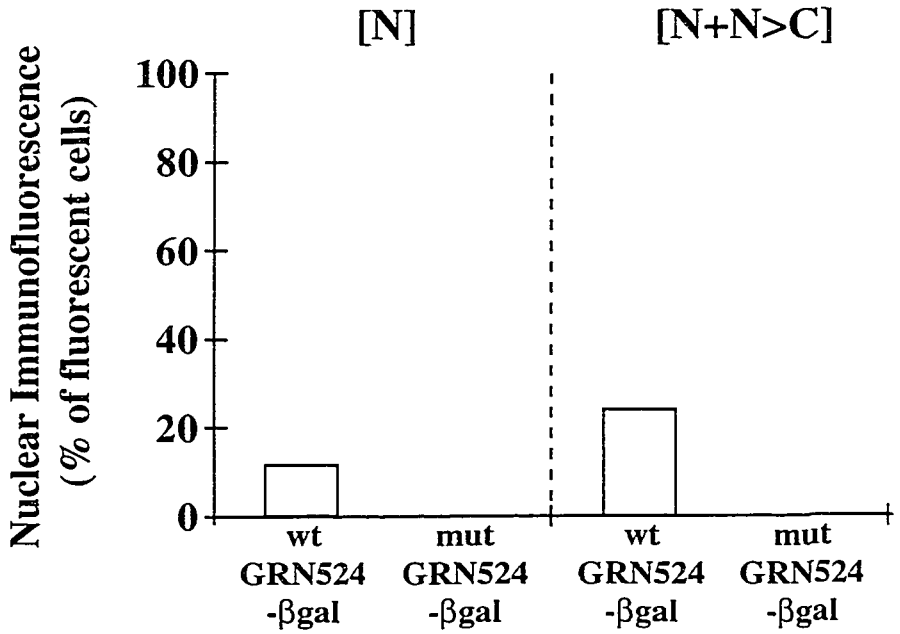
b) WtGRN524-β-galactosidase fusion protein did not completely localize to the nucleus as expected

After transfection, cells were incubated with medium containing serum for 24 h before initiating serum withdrawal by incubating in serum-free medium for an additional 21 h. Cells were fixed with a 3% paraformaldehyde/PBS solution before processing for the IIF assay. BuGR-2, an anti-GR monoclonal antibody, was used to detect the GR fusion proteins. Quantification was done by double-blind encryption. Cells were classified into one of five categories: N- exclusively nuclear immunofluorescence; N>C- cells with greater nuclear staining than cytoplasmic staining; N=C- cells with even nuclear and cytoplasmic staining; C>N- greater cytoplasmic than nuclear immunofluorescence; C- cells with staining entirely in the cytoplasm [172]. The change in the percentage of cells with mostly nuclear staining (N+N>C) and exclusively nuclear staining (N) of the β-gal-GR fusion proteins (**B**) are displayed. A minimum of 250 cells were counted for each experiment and the experiment was performed three times. Representative values from an experiment are shown.

A


wtGRN524.z (wtGRN524-βgal)
1
524
βgal
mutGRN524.z (mutGRN524-βgal)
1
524
βgal

B


[N]
[N+N>C]
Nuclear Immunofluorescence
(% of fluorescent cells)
100
80
60
40
20
0
wt GRN524 -βgal
mut GRN524 -βgal
wt GRN524 -βgal
mut GRN524 -βgal

fused to the N-terminus of GR aa22-525 instead of the C-terminus. The final constructs expressed EGFP fused amino-terminus to aa22-525 or aa22-524 amino acids of GR containing either the wildtype (egfp-wtGRaa22-525) or mutated NL1 sequence (egfp-mutGRaa22-524), respectively (Figure 7A). The enhanced green fluorescent protein was chosen because of its enhanced fluorescent signal [215] and protein production [216] and most important, to increase the total molecular weight of the GRaa22-525 protein. The fusion protein would then exceed the 60 kDa limit for passive diffusion of proteins into the nucleus. Therefore, import into the nucleus of the EGFP fusion proteins would have to be signal-mediated. Thus, if mutant NL1 fusion proteins were to localize to the nucleus, the only explanation would be that the mutated NL1 sequence still retained some nuclear localization activity and not that the small size of the fusion protein (<60 kDA) allowed nuclear import due to passive diffusion [89].

The fusion constructs, expressing EGFP-wtGRaa22-525 or EGFP-mutGRaa22-524, were transiently transfected into COS-7 cells. The predicted molecular weight of both GR chimeric proteins were approximately 85 kDa. Western analysis confirmed that both fusion proteins were present in extracts of transfected COS-7 cells. The EGFP-mutGRaa22-524 chimera was expressed at levels six times higher than that of the wtGR aa22-525 fusion protein as determined by quantitation with a densitometer (Figure 7B). Subcellular localization of the expressed fusions was then examined using the IIF assay introduced above. Representative micrographs display the subcellular localization of the

Figure 7 Expression and Subcellular Localization of Transiently Transfected EGFP-GRaa22-525 Control Constructs

a) Schematic diagram of constructs that express the EGFP-GRaa22-525 fusion proteins to confirm abolition of NL1 activity of the mutated NL1 sequence

The egfp-wtGRaa22-525 construct expresses the wtGR carboxy-terminal truncated fusion protein which is expected to be nuclear [30] and the egfp-mutGRaa22-524 construct expresses the EGFP-mutGRaa22-524 chimera that contains the mutated NL1 sequence (**A**). The vertically striped box at the carboxy-terminal end of EGFP-mutGRaa22-524 indicates the mutated residues. For these constructs, EGFP is fused to the N-terminus of GR.

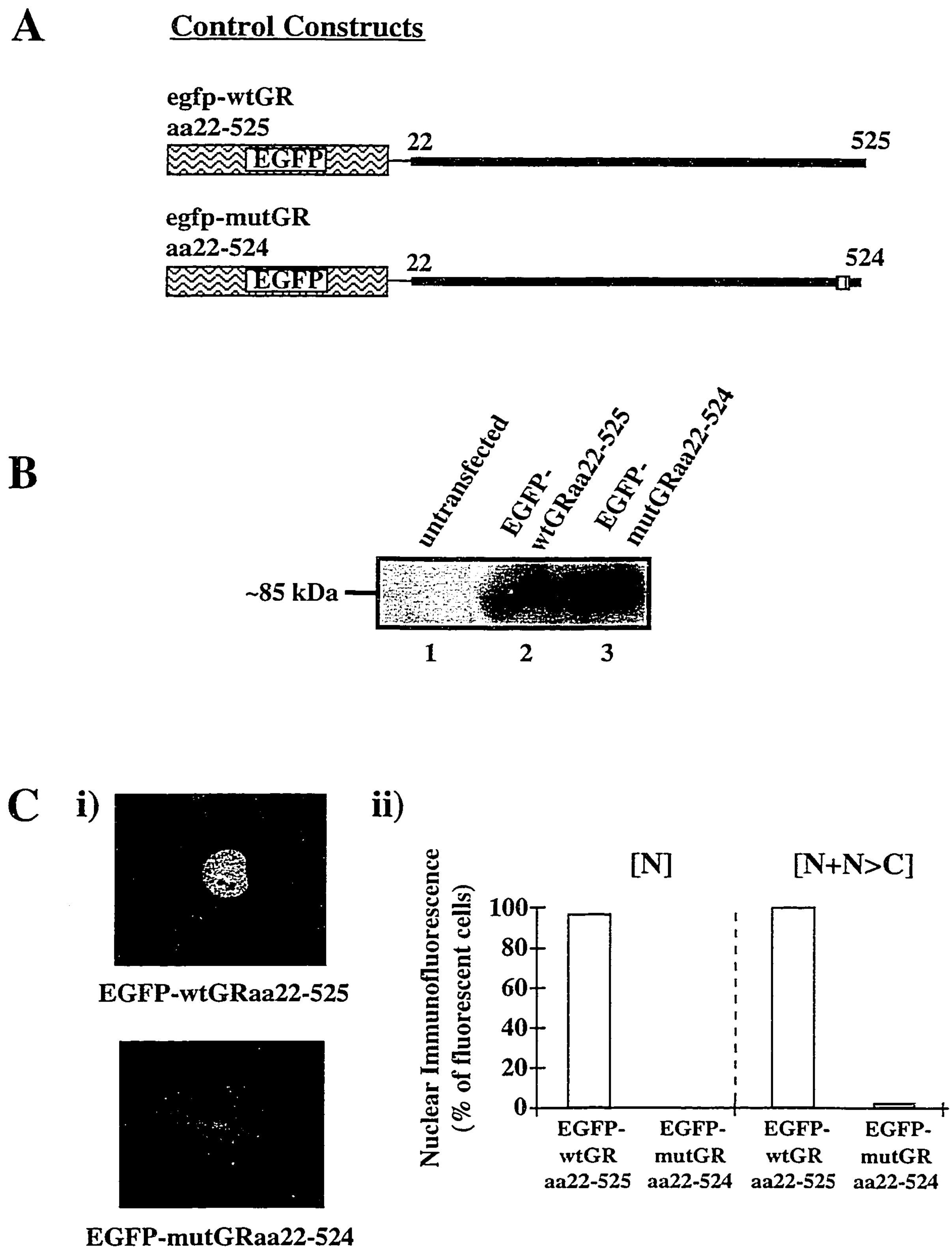
The two control constructs expressing the EGFP-wtGRaa22-525 and EGFP-mutGRaa22-524 fusion proteins were transiently transfected into COS-7 cells. Cells were incubated in serum-containing medium for 24 h after stopping transfection. After cells were synchronized in G_0 by incubation in serum-free medium for 21 h, they were either used for Western analysis (**B**) or IIF studies (**C**).

b) Level of Expression of EGFP-GR fusion proteins

Whole cell extracts, prepared from COS-7 cells transiently transfected with either egfp-GRaa22-525 or egfp-GRaa22-524 expression constructs, were subjected to SDS-PAGE and electroblotted for Western analysis (**B**). Lane **1**- extracts from untransfected cells; Lane **2**- extracts from cells transfected with the egfp-wtGRaa22-525 construct; lane **3**- extracts from cells transfected with the egfp-mutGRaa22-524 construct. The GR monoclonal antibody, BuGR-2, was used to detect GR.

c) Subcellular localization of expressed EGFP-GR carboxy-terminal truncated chimeras.

Cells were fixed with paraformaldehyde and processed for the IIF assay. Quantification was performed by double-blind encryption. Cells were classified into one of five categories as described in **Figure 6**. GR fusion proteins were visualized by fluorescence intrinsic to the EGFP protein. Representative micrographs (**C(i)**) and quantification of subcellular distribution (**C(ii)**) of the fusion proteins are shown. Panel C(**ii**) shows the percentage of cells with mostly nuclear staining (N+N>C) and exclusively nuclear staining (N) of the EGFP-GR fusion proteins. A minimum of 250 cells were counted for each experiment and the experiment was performed two times. The averaged values of the two experiments are shown.


A
Control Constructs
egfp-wtGR
aa22-525
EGFP
22
525
egfp-mutGR
aa22-524
EGFP
22
524
B
untransfected
EGFP-wtGRaa22-525
EGFP-mutGRaa22-524
~85 kDa
1
2
3
C
i)
EGFP-wtGRaa22-525
EGFP-mutGRaa22-524
ii)
Nuclear Immunofluorescence (% of fluorescent cells)
[N]
[N+N>C]
100
80
60
40
20
0
EGFP-wtGR aa22-525
EGFP-mutGR aa22-524
EGFP-wtGR aa22-525
EGFP-mutGR aa22-524

expressed fusion proteins (Figure 7C(i)). The percentage of cells identified in each category of subcellular localization, after transfection of either the wtGRaa22-525 and themutGRaa22-524 cDNAs, are shown in Table 1. Values were calculated for all categories but only the percentage of cells with mostly nuclear staining (N+N>C) and exclusively nuclear staining (N) of GR were displayed for this experiment and for the following IIF assays (Figure 7C(ii)). The EGFP-wtGRaa22-525 protein localized almost exclusively to the nucleus with 99.8% of transfected cells displaying mostly nuclear staining (N+N>C) and 96.4% of cells with exclusively nuclear staining. By contrast, the EGFP-mutant GR chimera was found almost entirely in the cytoplasm with less than 2% of cells demonstrating mostly nuclear staining and 0.2% of cells with exclusively nuclear staining. This experiment proved that the nuclear localization activity of NL1 had been abolished by the amino acid substitutions, KKK to NNN, in the primary sequence of NL1.

2. Characteristics of GR Nuclear Uptake and Equilibrium Distribution Were Dramatically Altered By Destruction of NL1 Activity

Once ablation of the NLS function of the mutated NL1 had been confirmed, nucleocytoplasmic trafficking of wtGR and mutGR were monitored by the IIF assay. To evaluate the function of NL2 in the context of the full length receptor, kinetics of nuclear uptake and subcellular distribution of wtGR and mutGR after treatment with dex, a synthetic glucocorticoid agonist, were compared. Expression of both full length wtGR

Table 1 Classification of cells expressing EGFP fused to carboxy-terminal truncated GR into one of five categories of subcellular localization

	N	N>C	N=C	C>N	C
EGFP-wtGRaa22-525	**96.4**	**3.4**	**0.2**	**0.0**	**0.0**
EGFP-mutGRaa22-524	**0.2**	**1.5**	**24.5**	**73.2**	**0.6**

COS-7 cells are transiently transfected with constructs encoding the EGFP-GRaa22-525 fusion proteins. After stopping transfection, cells were incubated with serum-containing medium for 24 h before incubation in serum-free medium for a further 21 h. Cells were fixed with paraformaldehyde before processing for the IIF assay. Quantification was performed by double-blind encryption. Values are calculated as a percentage of total number of cell counted. A minimum of 250 cells were counted for each sample in each experiment. The averaged values from two experiments are indicated.

and mutGR was confirmed by Western blot analysis prior to performing the IIF assay (Figure 8A). The level of expression of mutGR was 2.7 times higher than that of wtGRfrom cells transfected with the same amount of both constructs. Since an abundant amount of the NLS transporter proteins, importin α and ß, are present in cells [109, 217], nuclear import of both GRs should not be affected by limiting amounts of the NLS receptor proteins.

For the IIF assay, COS-7 cells transiently transfected with either full length wtGR or mutGR constructs were incubated in medium containing serum for 24 h followed by incubation in serum-free medium for over 21 h before hormone treatment was initiated. Following this treatment, the cells were fixed at certain times after hormone treatment to determine nuclear uptake of wtGR and mutGR. In a previous study, before dex treatment, less than 5% of cells containing wtGR displayed mostly nuclear staining (N+N>C) and less than 2% of cells showed exclusively nuclear localization of GR [172]. In my experiments, however, prior to hormone treatment, greater than 30% of cells expressing wtGR displayed mostly nuclear staining (Figure 8B (i)) but less than 1% of cells showed exclusively nuclear localization of GR (Figure 8B (ii)). For mutGR, less than 1% of cells displayed mostly nuclear staining (N+N>C) and no cells showed exclusively nuclear GR (N). After dex treatment, a marked difference was observed between the nucleocytoplasmic trafficking of wtGR and mutGR. Nuclear uptake of wtGR proceeded rapidly with a $t_{1/2}$ of <5 min. This was similar to the reported value of 4-5 min from an earlier study [172]. After 10 min of dex treatment, the amount of wtGR

Figure 8 Level of Expression and Kinetics of Nuclear Import of Full Length Wildtype and Mutated NL1 GR After Treatment with 10^{-6} M Dexamethasone

COS-7 cells were transiently transfected with constructs expressing full length wtGR or mutGR. After transfection was stopped, cells were incubated in serum-containing media for another 24 h before serum withdrawal for a further 21 h.

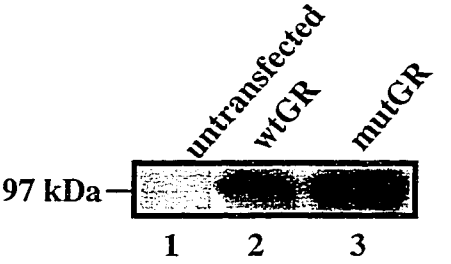
a) Detection of expression of full length wtGR and mutGR

After serum withdrawal, transiently transfected COS-7 cells were harvested to prepare whole cell extracts which were subjected to SDS-PAGE and further processed for Western analysis (**A**). Lane **1**- extracts from untransfected cells; Lane **2**- extracts of cells transfected with wtGR; lane **3**- extracts of cells transfected with mutGR.

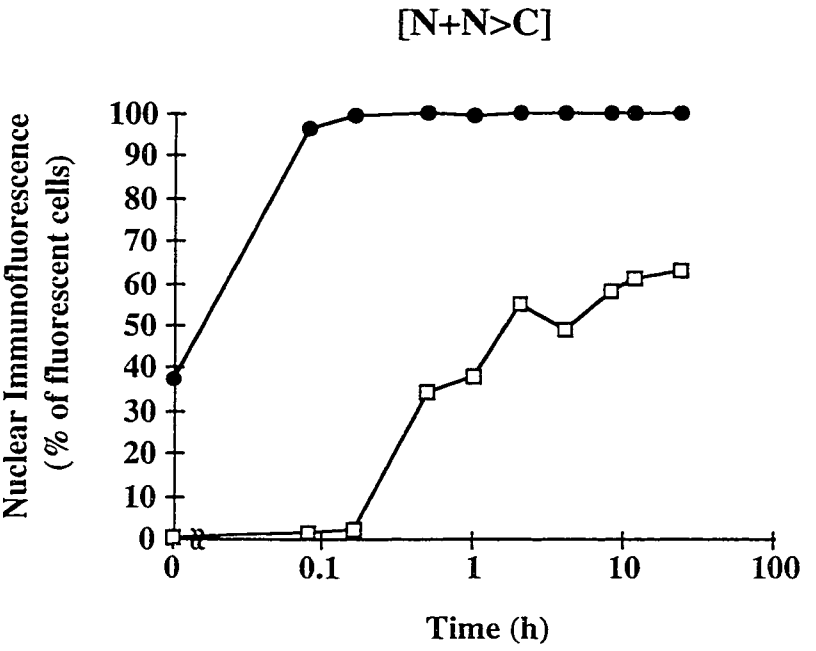
b) Effect of dex treatment on subcellular distribution of wtGR and the GR NL1 mutant

After serum withdrawal for 21 h, cells were incubated with serum-free medium containing 10^{-6} M dex. The cells were fixed at specified times after addition of hormone and processed for the IIF assay as described in Materials and Methods. GR was detected using the BuGR-2 antibody. WtGR (●) and mutGR (□). Classification of subcellular distribution was performed as described in Materials and Methods. The changes in the percentage of cells with mostly nuclear GR staining (N+N>C) are shown in panel **B(i)** and the changes in percentage of cells with exclusively nuclear staining (N) are displayed in panel **B(ii)**. A logarithmic time scale was used to clarify the early events in GR nuclear import. A minimum of 250 cells were counted for each time point within an experiment. Values for each time point was collected from three to five independent experiments repeated over a period of several months. A representative time course is shown.

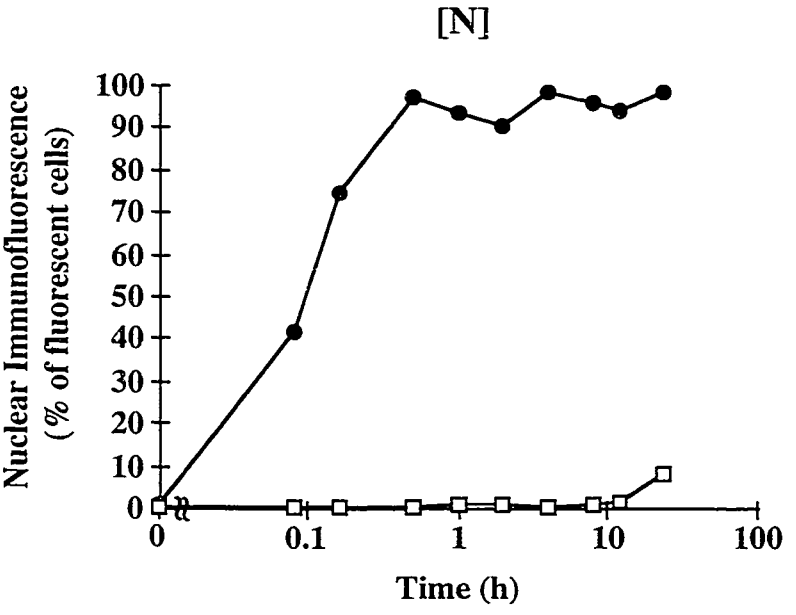
A


untransfected
wtGR
mutGR
97 kDa
1
2
3

B i)


[N+N>C]
Nuclear Immunofluorescence
(% of fluorescent cells)
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
10
100
Time (h)

ii)


[N]
Nuclear Immunofluorescence
(% of fluorescent cells)
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
10
100
Time (h)

in the nucleus reached a steady-state concentration at which greater than 95% of cells displayed mostly nuclear staining and >90% of the cells showed exclusively nuclear staining (Figure 8B(i, ii)). In contrast, mutGR displayed a noticeable lag time in nuclear uptake which persisted for 30 min before any nuclear accumulation occurred. Nuclear uptake of mutGR had a $t_{1/2}$ of >30 min (Figure 8B(i)). However, at this time, only a very small percentage of the cells (1%) had receptors wholly in the nucleus (Figure 8B(ii)). Additionally, at equilibrium, after dex treatment of over 4 h, only 60% of cells exhibited mostly nuclear staining and approximately 5% of cells contained solely nuclear staining of mutGR. Thus, import into the nucleus occurred at a slower rate and was maintained at a lower nuclear equilibrium level when NL1 activity was abolished.

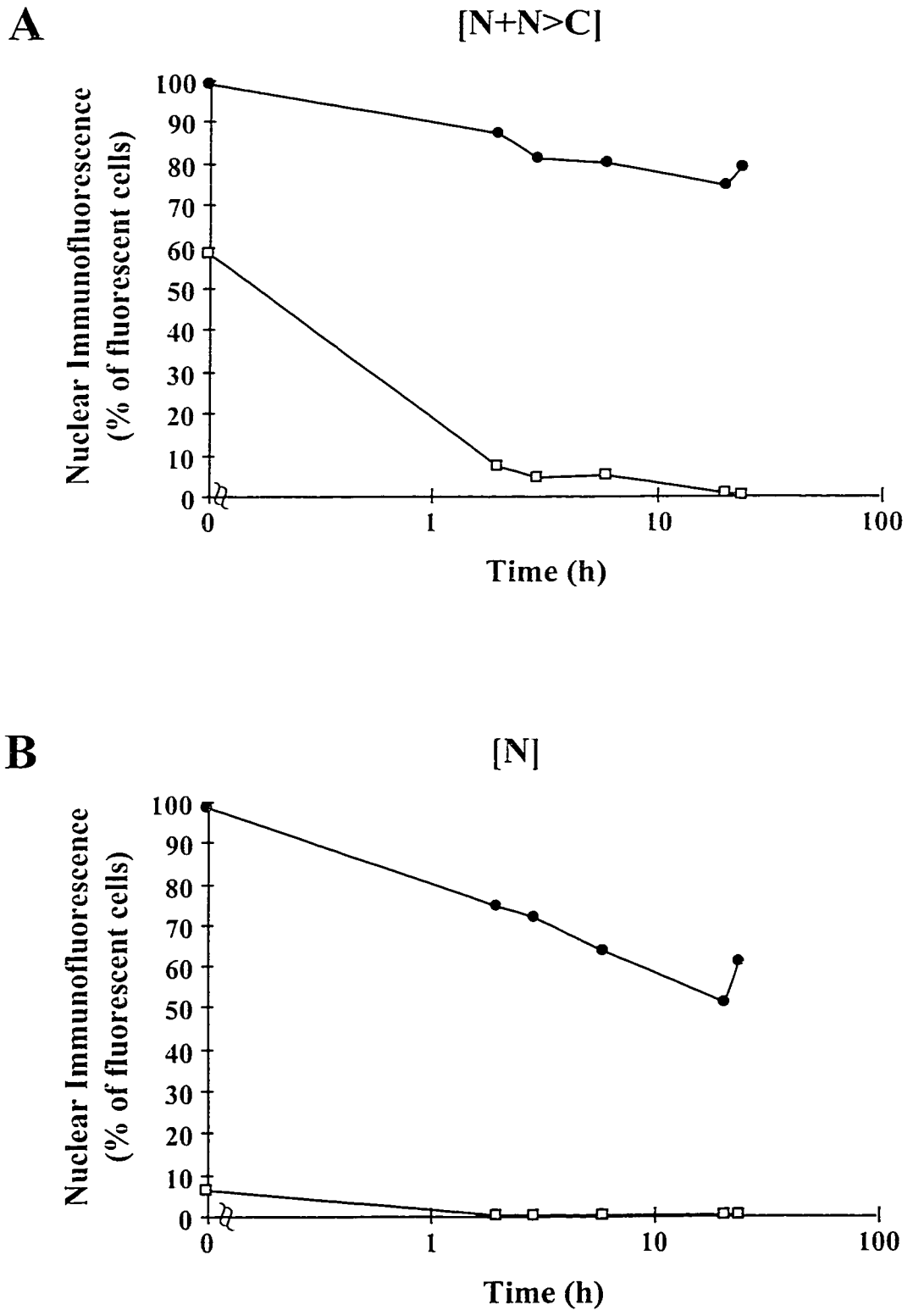
3. Redistribution To the Cytoplasm After Hormone Withdrawal Occurs More Rapidly For MutGR Than WtGR

It has been shown that after hormone-bound GR enters the nucleus and binds to DNA, the hormone then dissociates from GR. GR then returns to the cytoplasm where it can be found in the multiprotein complex until the next hormonal signal [21]. Although the steroid hormone dissociates from the receptor in less than 10 min [218], the wildtype receptor returns slowly to the cytoplasm after withdrawal of hormone ($t_{1/2}$ of 24 h for the mouse GR) [172]. To determine whether the rate of cytoplasmic return of GR was also affected by the inactivation of NL1, trafficking of the mutant NL1 receptor was monitored after a cycle of agonist treatment and withdrawal. COS-7 cells transiently

transfected with either wtGR or mutGR expression constructs were initially administered cortisol (10^{-6} M) for 6 h or more before withdrawal from hormone. Cortisol, a natural glucocorticoid, is used because it is rapidly metabolized by the cell [219]. This should prevent residual hormone from influencing the loss of GR from the nucleus. The lengthy period of cortisol treatment was to ensure that the maximum amount of mutGR had accumulated in the nucleus before hormone withdrawal had begun. Hormone withdrawal was initiated by removal of hormone-containing serum-free medium followed by several washes with PBS and serum-free medium before final incubation of cells in serum-free medium supplemented with 5% BSA (to bind nonspecifically to any trace amounts of steroid). Cells were fixed after withdrawal from hormone for certain periods of time. Previous results indicated that after cortisol withdrawal, redistribution of GR to the cytoplasm from cells displaying mostly nuclear wtGR and exclusively nuclear wtGR had a half-time of 24 h and 12 h for the mouse receptor and 19 h and 8 h for the human receptor [172]. In the study reported here, the rate of return for the rat wtGR was even slower. Loss of the rat wildtype receptor from the nucleus had a half-time of greater than 24 h after agonist withdrawal. Even at 24 h, wtGR remained largely nuclear with greater than 70% of cells displaying mostly nuclear staining and greater than 60% of cells showing exclusively nuclear GR (Figure 9A & B). The slight increase in the percentage of cells displaying mostly or exclusively staining of wtGR at 24 h was not reproducible. Again, as with nuclear uptake and accumulation, the rate of cytoplasmic return for GR NL1 mutant was different than for wtGR. Before cortisol withdrawal, less than 60% of

Figure 9 Redistribution of WtGR and MutGR after Treatment and Withdrawal of 10^{-6} M Cortisol

Cells expressing wtGR or mutGR were treated with cortisol for over 6 h before initiating agonist withdrawal by multiple washes with PBS followed by washes with serum- and hormone-free medium before final incubation in serum-free medium supplemented with 5% BSA. Cells were fixed at specified times after withdrawal was initiated and prepared for IIF assay. WtGR (●) and mutGR (□). To compare the rate of loss of GR from the nucleus, values for nuclear occupancy are expressed as the percentage of cells displaying mostly nuclear staining at equilibrium after cortisol treatment for over 6 h. Changes to the percentage of cells exhibiting mostly nuclear staining (N+N>C) are displayed in panel **A** and changes in the percentage of cells with wholly nuclear staining (N) are shown in panel **B**. A logarithmic time scale was used to clarify the early events in cytoplasmic redistribution. Values for each time point was collected from two to four independent experiments. A representative time course is shown.


A
[N+N>C]
Nuclear Immunofluorescence
(% of fluorescent cells)
100
90
80
70
60
50
40
30
20
10
0
0
1
10
100
Time (h)
B
[N]
Nuclear Immunofluorescence
(% of fluorescent cells)
100
90
80
70
60
50
40
30
20
10
0
0
1
10
100
Time (h)

cells displayed mostly nuclear staining of mutGR (Figure 9A & B). MutGR was rapidly depleted from the nucleus with a $t_{1/2}$ of less than 1 h. After 2 h, most of the NL1 mutant receptors had redistributed to thecytoplasm with less than 10% of cells displayed mostly nuclear staining and less than 2% of cells showed exclusively nuclear staining of GR. Thus, following cortisol treatment and withdrawal, inactivation of NL1 function resulted in a much more rapid redistribution of GR to the cytoplasm.

4. Effect of RU486 on Nucleocytoplasmic Trafficking of MutGR Is Strikingly Different Than From Dex Treatment

RU486, a synthetic compound with antiglucocorticoid and antiprogestin abilities, is bound by GR with high affinity and specificity. However, RU486-bound GR activates transcription poorly [220]. Our laboratory had shown that nuclear import of wtGR, in G_0, was slowed after treatment with RU486 with a half-time of 6-7 min compared to 4-5 min after dex treatment but the nuclear equilibrium level that was reached was virtually the same after both treatments [172]. Interestingly, in cycling cells, RU486-treated GR, after translocation to the nucleus, was observed to remain associated with the nucleus, unable to redistribute to the cytoplasm after antagonist withdrawal [184]. This was also observed with nuclear-transferred RU486-bound GR in cells in G_0 [172]. To determine if destruction of NL1 activity had an effect on the subcellular distribution of mutGR after RU486 treatment, transiently transfected cells that expressed either wtGR or mutGR were incubated with 10^{-6} M RU486 prior to fixing with paraformaldehyde and processing for

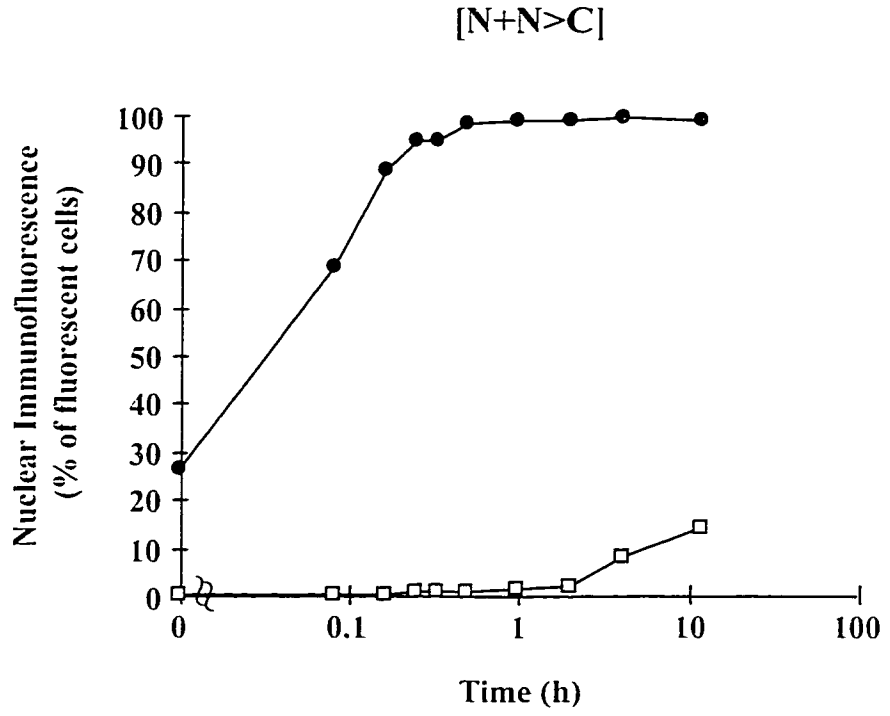
the IIF assay. In my experiments, differences in half-times for nuclear import of wtGR between dex and RU486 treatments were not observed. A half-time of less than 5 min was obtained after RU486 treatment, similar to the value received after dex treatment. As previously reported, at equilibrium after RU486 treatment, >95% of cells displayed mostly nuclear staining of wtGR and >90% of cells showed exclusively nuclear staining of GR (Figure 10A & B). Treatment with RU486 produced an even more dramatic difference between wtGR and mutGR in nuclear uptake and equilibrium distribution than dex treatment. In fact, mutGR transferred so poorly to the nucleus after RU486 treatment that the $t_{1/2}$ was >4 h in comparison to >30 min for dex treatment (Figure 10A). Even after 24 h incubation with the antagonist, the maximum nuclear accumulation of mutGR was low with less than 20% of cells displaying mostly nuclear distribution of mutGR and less than 1% of cells showing wholly nuclear mutGR (Figure 10A & B). Remarkably, it appeared that nuclear uptake and accumulation of mutGR was even more impaired after RU486 treatment than after dex treatment.

An earlier report stated that after antagonist withdrawal, the mouse wildtype receptor remained in the nucleus at levels similar to the equilibrium levels attained after agonist or antagonist treatment (>95%, N+N>C; >90%, N) [172]. Nuclear retention of RU486-withdrawn GR persisted even after 24 h of RU486 withdrawal. My study revealed a similar finding. The percentage of cells with mostly nuclear staining of rat wtGR never dropped below 90% or below 83% for cells with exclusively nuclear staining even after 24 h of RU486 withdrawal (Figure 11A & B). The slight increase in the

Figure 10 Subcellular Distribution of WtGR and MutGR After Treatment With 10^{-6} M RU486

GR-transfected COS-7 cells underwent 21 h of serum withdrawal before antagonist treatment. Cells were fixed at specific time points after RU486 withdrawal and processed for the IIF assay. WtGR (●) and mutGR (□). The changes in percentage of cells with mostly nuclear GR staining (N+N>C) are shown in panel **A** and the changes in totally nuclear GR distribution (N) are presented in panel **B**. A logarithmic time scale was used to clarify the early events in nuclear transfer. Values for each time point was collected from three to five independent experiments. A representative time course is shown.

A


[N+N>C]
Nuclear Immunofluorescence
(% of fluorescent cells)
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
10
100
Time (h)

B

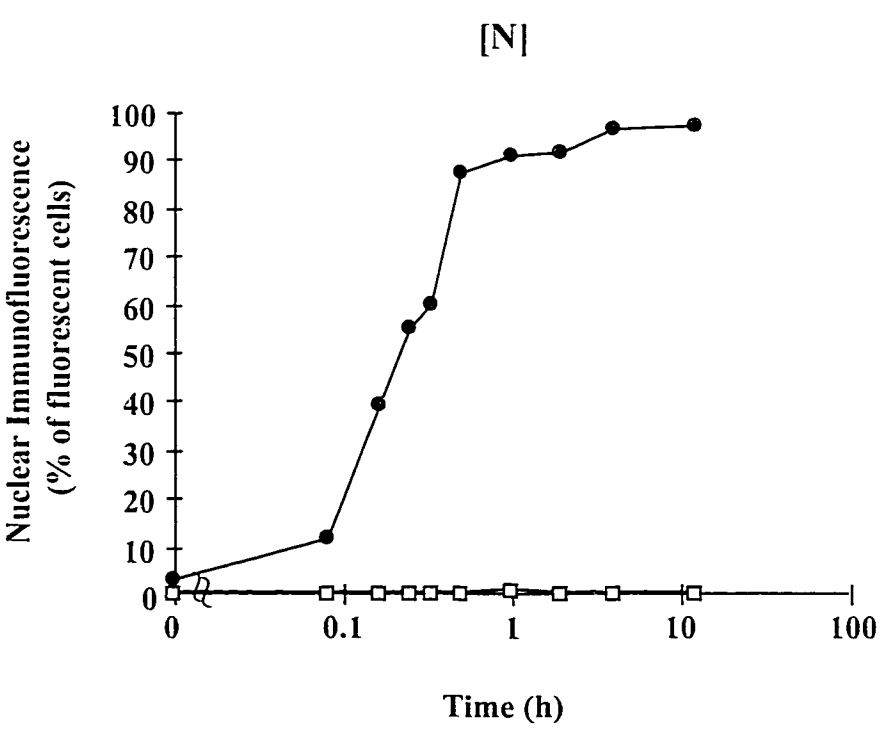
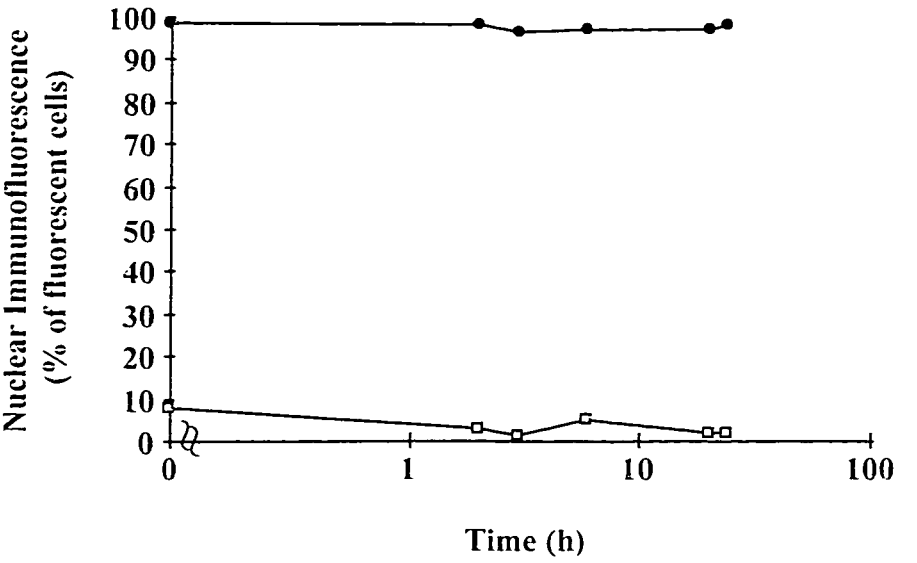

[N]
Nuclear Immunofluorescence
(% of fluorescent cells)
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
10
100
Time (h)

Figure 11 Subcellular Distribution of Wildtype and Mutant GR After A Cycle of Treatment With, and Withdrawal From, 10^{-6} M RU486

COS-7 cells transfected with wildtype or mutant GR constructs were treated with RU486 for over 6 h before antagonist withdrawal was started by multiple washes with PBS and serum- and hormone-free medium followed by incubation in serum-free medium supplemented with 5% BSA. Cells were fixed at set time points and prepared for IIF. WtGR (●) and mutGR (□). Changes to the percentage of cells exhibiting mostly nuclear staining (N+N>C) are displayed in panel **A** and changes in the percentage of cells with exclusively nuclear distribution (N) are shown in panel **B**. A logarithmic time scale was used to clarify the early events in cytoplasmic redistribution. Values for each time point was collected from two to four independent experiments. A representative time course is shown.

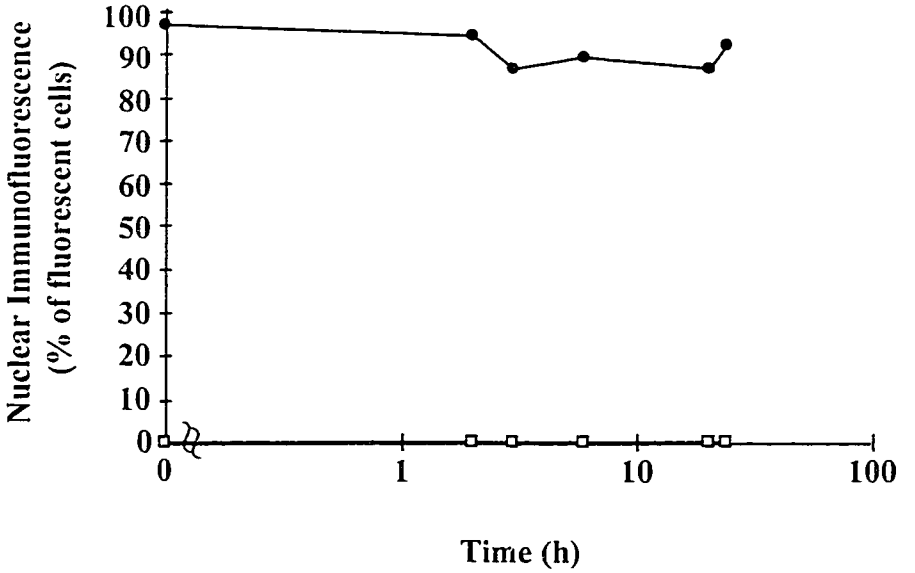
A

[N+N>C]


Nuclear Immunofluorescence
(% of fluorescent cells)
100
90
80
70
60
50
40
30
20
10
0
0
1
10
100
Time (h)

B

[N]


Nuclear Immunofluorescence
(% of fluorescent cells)
100
90
80
70
60
50
40
30
20
10
0
0
1
10
100
Time (h)

percentage of cells showing exclusively nuclear staining of wtGR at 24 h was not reproducible. Since mutGR did not accumulate to a significant amount in the nucleus upon RU486 treatment (<10% of cells with mostly nuclear GR staining) (Figure 11A), it was not possible to determine if mutGR was also unable to redistribute to the cytoplasm after antagonist withdrawal.

5. Rate of Transformation of MutGR Is Not Affected By NL1 Mutations

Two series of events must occur after the hormone binds GR and before GR is imported into the nucleus. First, GR must disassociate from hsp90 and other proteins of the heterocomplex. A previous study had demonstrated that hsp90 binding to the LBD was involved in the repression of nuclear localization activity (either NL1 or NL2) of GR fusion proteins and this repression was relieved upon ligand binding by hsp90 dissociating from the LBD [70]. Second, GR must interact with components of the transport machinery. My findings revealed that after initiation of dex treatment, an apparent sustained cytoplasmic presence before a slowed rate of nuclear uptake was observed for the GR NL1 mutant. Two possible explanations for the lag and slowed nuclear uptake are: i) transformation of GR (which includes first, a conformational change and then dissociation from proteins of the heterocomplex e.g. dissociation of hsp90) is not proceeding as efficiently for mutGR as it is for wtGR after agonist binding;

ii) alteration in the as40sociation of GR with members of the import machinery which resulted in the slowed nuclear uptake of mutGR.

To evaluate whether the rate of transformation of mutGR was slowed relative to wtGR, extracts from transiently transfected COS-7 cells expressing myc-tagged GR were subjected to sucrose density gradient analysis. This analysis relies on the fact that the untransformed, heterocomplex-bound GR and transformed free GR sediment at approximately 8S and 4S, respectively [221]. Therefore, once hormone treatment has begun, the oligomeric status of the GR-heterocomplex entity for both wtGR and GR NL1 mutant can be determined. Constructs expressing myc-tagged GR (wildtype or mutant) were used because these constructs were expressed at higher levels than other GR constructs, as determined by Western analysis. COS-7 cells transiently transfected with myc-tagged GR expression constructs were withdrawn from serum for over 21 h before experimental treatment with dex for 15 min for wtGR or 15 min and 2 h for mutGR. The 15 min duration was chosen as one of the treatment periods because at this time point, wtGR was mostly nuclear in the majority of cells but mutGR was mainly in the cytoplasm. Whole cell extracts were prepared from harvested cells and layered on 10%-35% linear sucrose gradients for centrifugation. Protein markers with known sedimentation values (BSA-4.6S, aldolase-7.3S) were prepared simultaneously to be centrifuged in parallel with the GR samples to determine the sedimentation position of GR. Sodium molybdate (20 mM) was present at all times to prevent any spontaneous dissociation of GR from the heterocomplex. It has been shown previously that certain

metal oxyanions such as molybdate and tungstate stabilize the interaction between GR and hsp90 [222]. With the GR-hsp90 complex stabilized, any GR found associated with the 4S fraction would be the result of heterocomplex dissociation due to ligand binding and not from spontaneous dissociation of the multiprotein complex due to manipulation of the samples. Following centrifugation, gradient fractions were collected and immunoprecipitated with BuGR-2. The immunoprecipitated samples were resolved by SDS-PAGE and prepared for Western analysis to determine the fraction in which GR had sedimented. Before dex treatment, both myc-tagged wtGR and mutGR sedimented with the 8S fraction (Figure 12A and B). Strikingly, both wtGR and mutGR sedimented at 4S after 15 min of dex treatment. The GR NL1 mutant also sedimented at 4S after 2 h of dex treatment (Figure 12B). The decreased amount of mutGR recovered with the 4S fraction after 15 min of dex treatment might be due to inefficient immunoprecipitation by BuGR-2 antibody for this particular sample. It appeared that mutGR transformed just as efficiently as wtGR since no mutGR was detected in the 8S fraction after dex treatment for 15 min or 2 h. This excluded the possibility that after ligand binding, inefficient dissociation of mutGR from the heterocomplex was responsible for the long lag time or slower rate of import seen for mutGR.

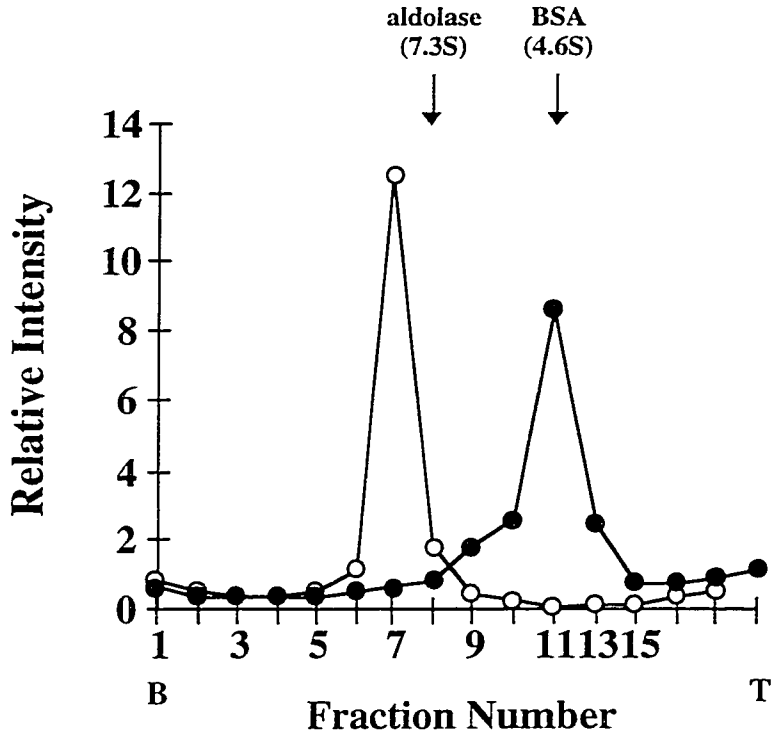
6. Comparison of DNA Binding Affinity of WtGR Versus MutGR

Results previously published from our laboratory had demonstrated that a mutation that impaired DNA binding affected the steady-state distribution of the GR

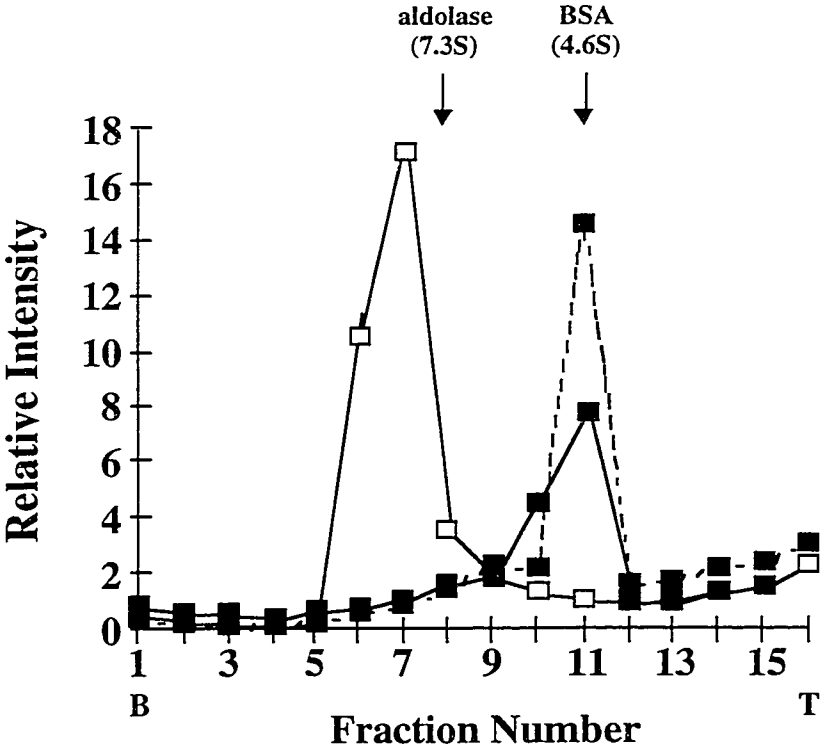
Figure 12 Sucrose Density Gradient Analysis of WtGR and MutGR After Treatment With 10^{-6} M Dexamethasone

Whole cell extracts of COS-7 cells transiently transfected with myc-tagged wtGR or mutGR expression constructs were prepared for sucrose density gradient analysis before (wtGR-○; mutGR-□) and after cells were treated with dex for 15 min (●) for myc-tagged wtGR and 15 min (■—■) and 2 h (■---■) for the myc-tagged mutGR. Whole cell extract samples were layered on a linear 10-35% sucrose gradient. After overnight centrifugation, GR was immunoprecipitated from collected fractions and processed for Western analysis. Sedimentation positions of wtGR and mutGR are shown in panel **A** and panel **B**, respectively. Sedimentation of external markers, aldolase (7.6S) and BSA (4.3S), are indicated at the top of each graph. The letters, B and T, located on the x-axis, indicate the top and bottom positions of the centrifuge tubes.

A


aldolase
(7.3S)
BSA
(4.6S)
Relative Intensity
0
2
4
6
8
10
12
14
1
3
5
7
9
11
13
15
B
T
Fraction Number

B


aldolase
(7.3S)
BSA
(4.6S)
Relative Intensity
0
2
4
6
8
10
12
14
16
18
1
3
5
7
9
11
13
15
B
T
Fraction Number

DNA-binding mutant in that a lower nuclear distribution of GR was observed at equilibrium [172]. This mutation also increased the rate of redistribution of the GR DNA-binding mutant to the cytoplasm. Therefore, DNA binding is a important determinant in the nuclear retention of GR. Since the mutations in the NL1 sequence overlap with the C-terminal end of the DNA binding domain, this raised the distinct possibility that the decrease in the nuclear equilibrium level and apparent increased rate of return of mutGR might be due to altered DNA binding. To evaluate if DNA binding had been compromised in the GR NL1 mutant, an electrophoretic mobility shift assay (EMSA) was performed. Constructs that expressed his-tagged GR fragments, from aa 394-552, that either did or did not include the mutated NL1 sequence, were transformed into bacteria. These constructs were used because it had been demonstrated that a GR fragment, comprised mainly of the DNA binding domain (aa 407-556), had successfully bound to a sequence-specific site on a radiolabelled DNA fragment [211, 223]. The bacterial expression vector, pET-30a, was used because it carried the DNA sequence for a histidine tag which would be expressed with the recombinant protein to enable easy purification from transformed bacterial extracts. The expressed GR fragments were purified from bacterial extracts by passage over a nickel column. The molecular weight of the purified GR fragments was approximately 24 kDa. Equal volumes of extracts containing either fragments of wtGR or mutGR were loaded onto a polyacrylamide gel and subjected to SDS-PAGE. Proteins on the gel were visualized using the protein dye,

Coomassie blue. Majority of the proteins eluted from the nickel column contained the GR recombinant protein but some bacterial proteins also were present (Figure 13A). Comparing equal volumes of extract, wtGR fragments were present at levelsapproximately three times higher than that of the mutGR fragments, as determined by densitometry. Therefore, the amount of the partially purified proteins used in the electrophoretic mobility shift assay was adjusted to reflect the difference. Equivalent amounts of the purified GR fragment containing either the wildtype NL1 sequence or the mutated sequence were incubated with a ^{32}P-labelled, 40 base-pair (bp) probe that contained a consensus glucocorticoid response element (GRE) sequence to assess DNA binding affinity. Protein-DNA complexes were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography.

The results did not uncover any conspicuous differences in the DNA binding affinity between wtGR (Figure 13, lane 3) and mutGR (Figure 13, lane 4). Furthermore, DNA binding by the his-tagged GR fragments to the radiolabelled probe was specific since competition with an unlabelled GRE oligonucleotide (Figure 13, lanes 5 and 6), but not a non-specific oligonucleotide, could disrupt the protein-DNA complex (Figure 13, lanes 7 and 8). Additional proof of specificity was illustrated with the formation of a higher order protein-DNA complex caused by the addition of BuGR-2, a GR monoclonal antibody (Figure 13, lane 9 and 10). This ruled out DNA binding as a possible cause of decreased nuclear occupancy since similar DNA binding affinities were observed

Figure 13 Comparison of DNA Binding Between WtGR and MutGR By Electrophoretic Mobility Shift Assay

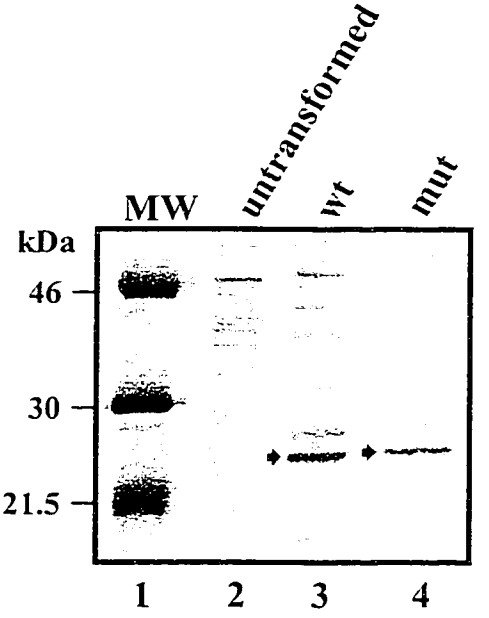
a) Expression And Isolation of His-tagged GR Fragment aa394-552 Containing Either the Wildtype or Mutated NL1 Sequence

Bacterially expressed, his-tagged wildtype or mutant GR fragment aa394-552 was isolated from extracts of BL21 (DES) pLys cells transformed with their respective pET expression vectors. Isolation of the GR protein was performed using a his-tagged protein isolation kit from Invitrogen, according to manufacturer's instructions. Lane **1**-protein molecular weight marker; lane **2**-extract from untransformed bacterial cells; lane **3**-extract from bacterial cells transformed with wtGR aa394-552 prokaryotic expression vector; lane **4**-extract from bacterial cells transformed with mutGR aa394-552 prokaryotic expression vector

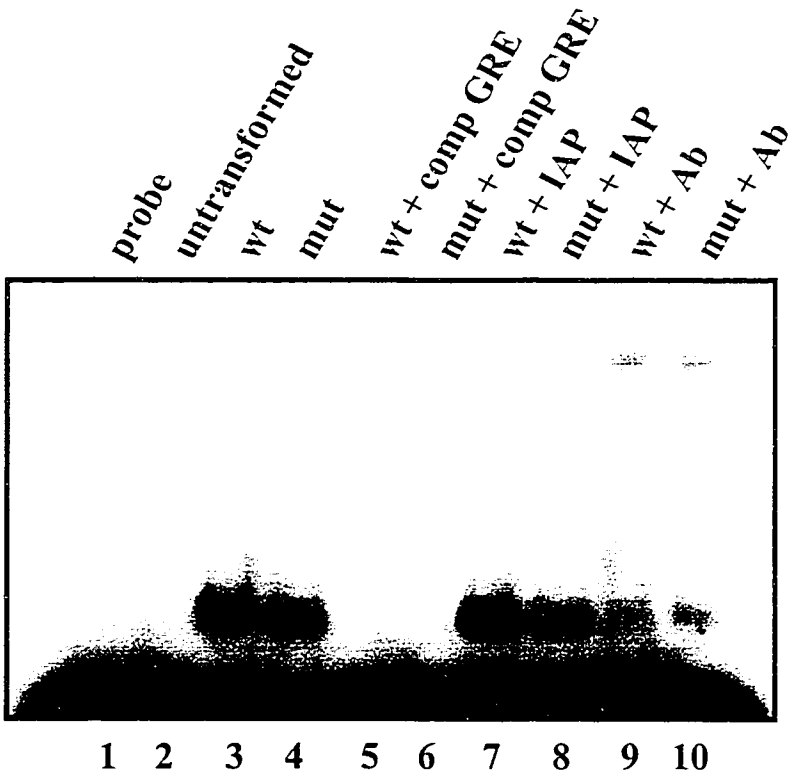
b) Assessment of DNA Binding by WtGR or MutGR Fragments

Determination of DNA binding affinity was performed by incubation of 1 μl of bacterially expressed, his-tagged purified GR fragments (aa 394-552) with or without the mutated NL1 sequence with 5000 cpm of ^{32}P-labelled, 40 bp GRE probe for 20 min at 25°C. Resulting protein-DNA complexes were resolved on a non-denaturing 4% polyacrylamide gel and visualized by autoradiography. Incubation of probe with DNA binding buffer or extract from untransformed bacterial cells was displayed in Lanes **1** and **2**, respectively. DNA binding by wtGR or mutGR fragments was seen in lanes **3** and **4**, respectively. Competitive binding by excess cold GRE oligonucleotides to the GR fragments was displayed in lanes **5** and **6**. Competition with excess of a cold, non-specific oligonucleotide (IAP) [212] for binding to the GR fragments were shown in lanes **7** and **8**. Protein-DNA complexes that migrated slower after the addition of 1 μl of BuGR-2, an anti-GR monoclonal antibody (0.5 μg/μl), to the binding reaction containing wtGR aa394-552 or mutGR aa394-552 were observed in lanes **9** and **10**, respectively.

A


MW
untransformed
wt
mut
kDa
46
30
21.5
1
2
3
4

B


probe
untransformed
wt
mut
wt + comp GRE
mut + comp GRE
wt + IAP
mut + IAP
wt + Ab
mut + Ab
1
2
3
4
5
6
7
8
9
10

between GR bacterially-expressed fragments that had either the wildtype or mutated NL1 sequence.

7. Transcriptional Activity of the Full Length Crippled NL1 GR Mutant

To ensure that the NL1 mutation had not disrupted any other functions of GR, the transcriptional activiation of a reporter gene by mutGR was compared with that of wtGR. COS-7 cells were co-transfected with a GR-expressing construct and the pMMTVCAT plasmid which contained a chloramphenicol acetyltransferase (CAT) gene fused downstream to a hormone-responsive promoter, the mouse mammary tumour virus (MMTV) promoter. β-galactosidase activity derived from the co-transfection and expression of the plasmid, RSV-β-gal, was used to correct for transfection efficiency. After serum withdrawal for 16 h, cells were either untreated or treated with dex at 10^{-6} M for 24 h. This initiates a cascade of events leading to the initiation of transcription of the CAT gene by GR. Assay of CAT activity was performed essentially as described previously [213] and analyzed by phosphorimager. The amount of CAT activity derived from the expressed enzyme is directly correlated to the amount of transcriptional activity by GR. However, before the CAT assay experiments were conducted, the amount of GR cDNAs used for transfection for the assay was titrated to: i) obtain equivalent levels of GR protein expression so that changes in the amount of transcriptional activity between the two receptors would be a reflection of the transactivation function of the receptors and

not of the amount of receptor present; and ii) prevent transcriptional squelching from overproduction of GR due to the presence of the SV40 origin of replication on plasmids that expressed either wtGR and mutGR when transiently transfected into COS-7 cells.

As determined by Western analysis, equivalent amounts of receptor were produced when DNA expressing wtGR and mutGR were transfected at a ratio of 3:1 (Figure 14A). In addition, the amount of supercoiled DNA to be transfected was determined to be 30 ng and 10 ng for the constructs expressing wtGR and mutGR, respectively. Activity by β-galactosidase reflects the level of its expression which may be used as an indication of the general transcriptional activation by the cell. Comparison of the level of β-galactosidase activity derived from extracts of cells that did not contain GR with samples that did have GR revealed no significant differences. This suggested that transfection with this amount of DNA did not lead to transcriptional squelching due to overproduction of GR. Interestingly, wtGR and mutGR both displayed a small amount of CAT activity in the untreated condition (Figure 14B(i), lanes 1 & 3). However, upon dex treatment, mutGR had approximately 50% of the CAT activity (18.8) of the wildtype receptor (32.1) (Figure 14B(i), lane 2 & 4). Comparison of transcriptional activity by fold activation revealed a 2.5-fold decrease in CAT activity for mutGR (Figure 14B(ii)). This correlated roughly to the immunofluorescence data where the nuclear equilibrium level of mutGR was approximately 1.7 fold less than of wtGR. These results suggest that the level of transcriptional activity by the GR NL1 mutant was equivalent to the amount of receptor present in the nucleus and therefore, transcriptional activation was not

Figure 14 Transcriptional Activation by Wildtype or Mutant GR

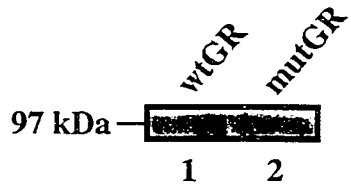
a) Comparison of expression of wtGR and mutGR from constructs that contain a SV40 origin of replication

COS-7 cells expressing wtGR and mutGR were transiently transfected with their respective expression constructs at a ratio of 3 to 1, respectively. Whole cell extracts were prepared, fractionated on SDS-PAGE and processed for Western analysis (**A**). Lane **1**-extracts from cells transiently transfected with 3 μg of wtGR expression construct; Lane **2**-extracts from cells transiently transfected with 1 μg of mutGR expression construct.

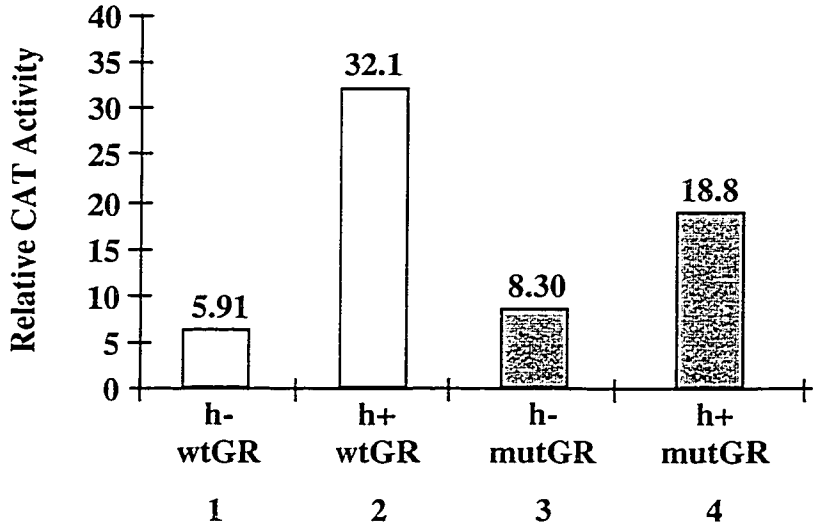
b) CAT activity of wtGR and mutGR

COS-7 cells, transiently co-transfected with either wtGR (30 ng) or mutGR (10 ng) expression constructs containing a SV40 origin of replication and the pMMTVCAT reporter plasmid (200 ng), were incubated in serum-containing media for 24 h before serum withdrawal for the next 16 h. After treatment with 10^{-6} M dex for 0 and 24 h, the cells were harvested and processed for CAT assays [213]. βgalactosidase activity derived from co-transfection and expression of RSV-βgal was used to correct for transfection efficiency. **i)** Relative CAT activity were determined for wtGR (lanes 1 & 2) or GR NL1 mutant (lanes 3 & 4). Cells were incubated in serum-free medium (lanes 1 & 3) or incubated in serum-free medium containing dex for 24 h (lanes 2 & 4). Averaged values from two experiments are shown. **ii)** To compare the increased accumulation of CAT activity after dex challenge, the values in lanes 2 & 4 were divided by the values in lanes 1 & 3 to obtain fold CAT activity by wtGR or mutGR, respectively.

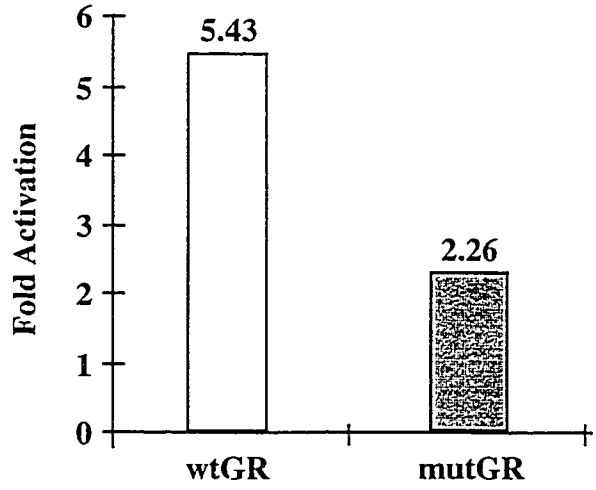
A


wtGR
mutGR
97 kDa
1
2

B **i)**


Relative CAT Activity
40
35
30
25
20
15
10
5
0
5.91
32.1
8.30
18.8
h-
wtGR
h+
wtGR
h-
mutGR
h+
mutGR
1
2
3
4

ii)


Fold Activation
6
5
4
3
2
1
0
5.43
2.26
wtGR
mutGR

affected by the mutations in the NL1 sequence. However, we have not, as yet, conclusively demonstrated that full transcriptional activity of GR requires 100% nuclear occupancy. Thus, although suggested, it remains to be proven that there is absolutely no negative effects of the NL1 mutations on transcriptional activation by GR.

IV. DISCUSSION

The two specific goals of my study were: i) to generate a GR mutant without NL1 activity by mutation of the three lysine residues at the carboxy-terminal end of the NL1 sequence and ii) to examine the pattern of nucleocytoplasmic trafficking of the mutated receptor in cells synchronized to G_0 using the semi-quantitative indirect immunofluorescence assay. The first goal was achieved with the generation of the GR NL1 mutant. Originally, confirmation of abolition of NL1 activity was attempted with constructs that expressed GRN524-β-galactosidase fusion proteins. However, the wtGRN524 chimeric protein did not localize completely to the nucleus as expected. One possible explanation for this result is that the chimeric protein may be too large to pass efficiently through the pores due to the ability of β-galactosidase to form tetramers [27]. Since β-galactosidase has a molecular weight of 116 kDa, the tetramer will be four times this molecular weight plus the molecular weight contributed by the GRN524 portion of the fusion protein. Thus, the predicted molecular weight of the complex of tetrameric fusion proteins is approximately 700 kDa. However, the first study investigating nuclear import of GR utilized different sized fragments of GR fused to β-galactosidase [27] and problems in observing full length GR fusion proteins localizing to the nucleus, after dex treatment, were not reported. Therefore, a more likely possibility for the decrease in nuclear localization of the wtGRN524 chimera is that fusion of β-galactosidase to the

carboxy-terminus of GRN524 is partially blocking access of NL1 to the import machinery by steric hindrance. Although the above mentioned initial study fused β-galactosidase to the C-terminus of different sized fragments of GR, the NL1 sequence was not immediately N-terminal (as in our GRN524 fusion proteins) to β-galactosidase. Therefore, interaction of NL1 with the import machinery was not hindered by the presence of β-galactosidase. Two changes were made to address these concerns. One change was to fuse a different protein, EGFP, to GRN525. The molecular weight of EGFP of ~26 kDa is fairly low but high enough to increase the molecular weight of the GRN525 protein to above 60 kDa so that import of the chimeric protein will have to be signal-mediated. The second change was the fusion of EGFP to the amino-terminus of GRN525 instead of the carboxy-terminus. This should allow NL1 to interact effectively with the general import machinery. Constructs that were generated expressed GR with the first 21 amino acids deleted because the first convenient restriction site available for cloning corresponded to amino acid residue 21 of GR. The deleted amino acids only had a minor impact on the molecular weight of the fusion proteins such that they were of approximately 85 kDa instead of 87 kDa. Constructs that expressed the EGFP-wtGRaa22-525 or EGFP-mutGRaa22-524 chimeric proteins were utilized to verify that NL1 function had been eliminated. The fusion proteins localized to the expected subcellular compartments with the EGFP-wtGRaa22-525 fusion protein located in the nucleus and the EGFP-mutGRaa22-524 chimera remaining in the cytoplasm. This

confirmed that the NL1 function had been abolished by the mutation of ^{513}KKK^{515} to ^{513}NNN^{515}.

The second goal was also achieved in experiments that revealed a striking difference in the nucleocytoplasmic trafficking of GR possessing an intact NL1 and NL2 versus GR possessing only an active NL2. However, prior to hormone treatment, greater than 30% of cells displayed mostly nuclear staining of wtGR in my study while less than 5% of cells showed mostly nuclear staining of wtGR in a previously published report. This may be explained by different transfection conditions used in the two studies. It had been shown previously that overexpression of wtGR could lead to partial nuclear transfer of the unliganded receptor [168]. Transient transfection of COS-7 cells with plasmids containing SV40 origins of replication results in the production of multiple copies of the plasmid and a corresponding high level of expression of the cloned gene. The previous study demonstrated that lipofectin-mediated transfection of a vector expressing wtGR that contained a SV40 origin of replication resulted in overexpression of wtGR that localized mostly to the cytoplasm in the absence of ligand. However, in my study, lipofectin was replaced with lipofectamine which further increased the transfection efficiency and expressed even higher levels of wtGR which resulted in the partial nuclear transfer of the receptor in the absence of hormone. Upon dex treatment, mutGR, compared to wtGR, exhibited an apparent sustained cytoplasmic presence before transferring to the nucleus at a slow rate and attaining a decreased nuclear equilibrium level. Additionally, upon RU486 treatment, the time for nuclear uptake of mutGR was similarly slow with even

lower levels of nuclear retention. Finally, after cortisol treatment and withdrawal, mutGR redistributed more rapidly to the cytoplasm than wtGR.

Several experiments were conducted to determine the cause of these effects. A decrease in the rate of dissociation of mutGR from the multiprotein complex after dex treatment was one possible reason for the slow rate of nuclear import of mutGR. This hypothesis was investigated by sucrose gradient analysis. If mutGR was inefficiently dissociated from the heterocomplex after agonist treatment, some of the receptors would sediment with the 8S fraction from samples collected shortly after dex administration. However, no mutGR was detected with the 8S fraction after treatment with dex for 15 min or 2 h. All of the mutGR had sedimented with the 4S fraction. This ruled out the possibility that stable association of mutGR with the heterocomplex prevented its import into the nucleus.

Previously, mutations that affect DNA binding of GR had been demonstrated to decrease the level of nuclear GR at equilibrium and also increase the rate of cytoplasmic redistribution by GR [172]. The amino acid substitutions in NL1 might have affected the DNA binding affinity of mutGR which caused the decreased nuclear equilibrium level and accelerated rate of redistribution to the cytoplasm. Therefore, differences in affinity for sequence-specific DNA binding of wtGR and mutGR were explored. However, no appreciable differences were seen in the DNA binding affinity of the two receptors.

Transcriptional activation similar to wtGR would indicate that mutGR was fully competent to carry out all of its functions except for nuclear localization by NL1. CAT

assays were done to determine the transactivation ability of mutGR. Although the total amount of transcriptional activation was lower for the mutated NL1 receptor, the fold activation closely correlated with the maximum accumulation of the GR NL1 mutant in the nucleus. This corroborated the result that DNA binding was not affected and also suggested, but not conclusively proven, that the transactivation function of mutGR was not significantly impaired. This conclusion is supported by previous studies utilizing a human GR NL1 deletion mutant [194, 195, 224]. In that study, the region removed in the deletion mutant was aa 491-515 which corresponded to aa 509-533 in rat GR. Part of the deleted region contained the lysine residues that were mutated to asparagine in the NL1 of rat mutGR used in our studies. It was demonstrated that after dex treatment, the human GR NL1 deletion mutant had 50% of the transcriptional activity of wildtype receptor [224] but similar to the results of my study, accumulation in the nucleus of the human GR NL1 deletion mutant was similarly negatively affected [194, 195]. Therefore, this is consistent with the decrease in the level of transcriptional activation by the GR NL1 deletion mutant being the result of impaired nuclear import. However, the possibility that the NL1 mutations affected DNA binding which altered the level of transcriptional activation could not be absolutely ruled out.

In the first study on nuclear localization of GR conducted by Picard and Yamamoto, in cycling cells, the β-galactosidase-LBD chimera (i.e. thus containing only NL2) was able to localize completely to the nucleus within 15-30 min after dex treatment [27]. Additionally, nuclear import of this fusion protein was stated to be similar to the

import of a β-galactosidase-GR fusion protein which did not have the N-terminus of GR but did possess the hinge NLS (NL1) in addition to NL2. Supported by this evidence, they concluded that NL2 was the major NLS in GR nuclear import. However, subsequent studies by two different groups did not support this claim [194, 195]. Instead, it was postulated that both NL1 and NL2 contributed equally to the nuclear import of the liganded GR [194]. Both groups used a human GR NL1 deletion mutant in which the C-terminal region of NL1, that included the three consecutive lysine residues mutated in my study, were removed. Their proposal was based on the observation that in cycling cells, exclusively nuclear staining seen for wtGR after 1 h of agonist treatment did not occur for the NL1 deletion mutant. Instead, cells expressing the NL1 deletion mutant displayed moderately nuclear staining in conjunction with some staining in the cytoplasm [194, 195].

My study also demonstrated that NL2 was not the major NLS in the nuclear transfer of GR. In addition, my data suggested that instead of NL2, NL1 was the signal favoured in GR transport to the nucleus. Destruction of NL1 activity resulted in a marked decrease in the rate of nuclear uptake as well as a lower nuclear distribution, at equilibrium, of GR. Thus, NL1 appeared to be responsible not only for the quick appearance of GR in the nucleus ($t_{1/2}$=<5 min) but also for a mostly nuclear steady-state distribution of liganded GR. Studies by Cadepond *et al.* [194] and Jewell *et al.* [195], using the full length GR NL1 deletion mutant, indicated that nuclear transfer by NL2 alone was also slower than wtGR (containing an active NL1 and NL2). Therefore, in the

context of the full length receptor, nuclear uptake by NL2 was quite different than for the LBD fusion protein used by Picard and Yamamoto [27].

Two possible reasons for the difference in the rate of nuclear transfer of GR in my study compared to that of Picard and Yamamoto [27] were: i) the phase of the cell cycle used in the studies; and ii) the GR constructs used in the studies. I used cells synchronized in the G_0 phase of the cell cycle while Picard and Yamamoto [27] used asynchronous cycling cells for a majority of their experiments in their study. However, two other groups, Cadepond *et al.* [194] and Jewell *et al.* [195], also utilized cycling cells for their studies but they obtained results which indicated that NL2-mediated nuclear import of the full length GR NL1 deletion mutant was not equivalent to wtGR. However, quantification of the subcellular localization of GR in transfected cells at additional time points was not performed so it was not known whether a result similar to our study was obtained by them. Therefore, the phase of the cell cycle as a possible reason for the difference in nuclear uptake observed between the full length GR NL1 mutant and the LBD fusion protein remains controversial. From the four studies, a more likely explanation to account for the difference in the rate of NL2-mediated nuclear import of GR, was that we (as reported here), Cadepond *et al.* [194] and Jewell *et al.* [195] used constructs that express full length receptors while Picard *et al.* [27] used constructs that express LBD as a fusion with β-galactosidase. The presence of the amino-terminus of GR might account for the difference in the rate of NL2-mediated nuclear transfer observed between the full length GR NL1 mutant and the LBD fusion protein. If the N-

terminus possessed an activity that partially inhibited nuclear localization, its removal by deletion would allow more rapid nuclear transfer of the β-galactosidase-LBD chimera to occur after dex treatment, compared to full length GR NL1 mutants. The partial inhibition on nuclear localization may be particular to the N-terminal sequence of GR since the LBD chimeric protein, which contains β-galactosidase fused to the N-terminus of the LBD, is still able to translocate to the nucleus [27].

Redistribution of GR after cortisol withdrawal is a slow process. Loss of wtGR from the nucleus of cells displaying mostly nuclear staining of GR has half times of 24 h and 19 h for the mouse and human receptors, respectively [172]. This process had been shown by our laboratory to be affected by DNA binding, utilizing a mouse GR DNA binding mutant that was able to redistribute more quickly to the cytoplasm with a $t_{1/2}$ of 9-11 h [172]. From my study, it is clear that elimination of NL1 function can also increase the rate of cytoplasmic return. In this instance, it is not lack of nuclear retention through DNA binding but lack of NL1 function that increases the apparent rate of return of mutGR to the cytoplasm. The observation that GR shuttles between the nucleus and cytoplasm during hormone treatment is presumably dependent upon the NLSs present within the protein. Upon removal of hormone, if both NLSs are masked by repackaging GR into the inactive, multiprotein complex that is required for hormone binding, re-entry into the nucleus will not occur. However, reassociation of GR with the heterocomplex occurs more quickly than cytoplasmic redistribution from the nucleus [172, 225]. This suggests that the reassociation of GR with proteins of the heterocomplex is not stable and

the hormone-independent NLS, NL1, may still be exposed to interact with the import machinery. NL2 is probably masked by hsp90 bound to LBD [70] and requires ligand to activate the NL2 activity again. If NL1 is masked or its function inactivated, as in mutGR, the receptor does not have an active NLS to return GR to the nucleus once it has exited, thereby increasing the apparent rate of cytoplasmic return. This hypothesis is consistent with the finding that steroid-treated GR localizes more readily to the cytoplasm after incubation of transfected cells with molybdate, a substance that stabilizes the interaction with hsp90, a member of the GR heterocomplex [176]. The stable interaction of GR with hsp90 presumably masks both NLSs thereby inhibiting the receptor from returning to the nucleus through NL1-mediated nuclear import.

RU486 is a synthetic steroid that possesses both antiglucocorticoid and antiprogestin activities. It binds to GR and PR with high affinity and specificity (dissociation constant of 10^{-9} M) but has low affinity for AR and does not bind to MR or ER [226]. Studies of RU486-bound GRs *in vitro* showed a decreased rate of dissociation of GR from the multiprotein heterocomplex [227-229]. Following transformation, however, GRs bound to RU486 translocate to the nucleus where they bind to glucocorticoid response elements but do not activate transcription well [220]. This type of activity is typical of a type I antagonist (a group of which RU486 is a member) in which the antagonist-bound receptor is capable of sequence-specific DNA binding but is transcriptionally unproductive [226, 230]. This is in contrast to type II antagonists e.g. RU43044 in which receptors bound to these compounds do not bind their hormone

response elements and therefore, are transcriptionally inactive [226]. Differences in conformation have been observed between receptors bound to agonists or to antagonists such as RU486. For PR, differences in conformation after agonist (progestin) or antagonist (RU486) binding were detected by either differential antibody binding [231] or dissimilar susceptibility to digestion with proteolytic enzymes [232, 233]. For GR, differences in conformation after agonist or antagonist binding is controversial [234]. Proteolytic digestion of GR after binding several different antagonists did not reveal a consistent digestion pattern. Additionally, no consistent difference in the digestion pattern was observed for GR bound to antagonist or agonist. However, a consistent difference was observed in the pattern of proteolytic fragments obtained for GR after dex or RU486 binding. This indicated that conformational differences in GR were elicited by dex and RU486 binding to GR.

In this study, agonist treatment of the mutated NL1 receptor uncovered alterations that negatively affected the kinetics of nuclear uptake and cytoplasmic redistribution as well as altering equilibrium levels of nuclear GR after dex treatment. However, treatment with RU486 demonstrated an even more profound effect on the nucleocytoplasmic trafficking of mutGR. Following RU486 treatment, less than 20% of cells displayed mostly nuclear staining of mutGR and less than 1% of cells showed mutGR localized exclusively in the nucleus. This was in contrast to the earlier finding by Picard *et al.* [27] where it was demonstrated that the β-galactosidase-LBD fusion protein was capable of entering the nucleus after treatment with RU486. However, Picard and Yamamoto were

using N-terminal truncated GRs in their experiments while we used full length receptors. The difference in the rate of nuclear import between the N-terminal truncated GR and full length GR can be explained by: i) possible inhibition on nuclear localization by the N-terminus of GR; and ii) conformational changes that may have occurred with the GR LBD after full length or N-terminal truncated GR binds to RU486. As mentioned above, the N-terminus might possess a partial inhibitory activity on nuclear localization. Also mentioned previously, conformational differences were also seen after dex or RU486 was bound to GR [234]. Thus, inhibition on nuclear localization by the N-terminus and a RU486-induced LBD conformation might act in concert to alter the interaction between NL2 and members of the import machinery resulting in the poor nuclear uptake and accumulation of mutGR.

Different proteins contain multiple NLSs for a variety of reasons. One reason is that the number of NLSs can influence the rate of nuclear import [94, 185, 186, 235]. A study was done to determine if the number of NLSs affected transport of proteins into the nucleus [185]. Synthetic peptides that possessed the SV40 NLS sequence were chemically cross-linked to bovine serum albumin (BSA) such that different numbers of synthetic peptides were linked per BSA molecule. The SV40 NLS-conjugated BSA was then used to coat gold particles which were injected into cells from Xenopus oocytes and subsequently fixed for electron microscopy. The study concluded that as the numbers of NLSs increased, both the relative rate of nuclear uptake as well as the functional size of the transport channels increased. Other examples explored the effect of multiple weak

NLSs on increasing nuclear uptake of proteins. A study was conducted to investigate the effect of inserting multiple mutant SV40 NLS sequences, that contain partial NLS function, into the primary sequence of pyruvate kinase, a normally cytoplasmic protein [94]. The altered protein displayed increasing nuclear accumulation as more partially active SV40 NLSs were inserted. Within a physiological context, the concerted action of two weak NLSs within the HMG domain transcription factor, SRY, promoted exclusively nuclear localization of the protein [186]. Upon deletion of one of the two NLSs, a subcellular shift occurred in which the protein was no longer located solely in the nucleus but instead, some of the protein appeared in the cytoplasm. This also occurred similarly for the SOX9 protein [186]. For the p53 protein, removal of one of the three NLSs within the protein resulted in its appearance in the cytoplasm and reduced accumulation in the nucleus [235].

Another reason for multiple NLSs is that they are needed to overcome another signal within the protein that targets a different pathway e.g. the secretory pathway. An example is the fibroblast growth factor, FGF3, which contains two weak NLSs and a secretory signal [189]. Each NLS, by itself, was unable to redirect the protein from the secretory pathway [187, 189]. However, through the combined action of both NLSs, the protein was similarly targetted to both the secretory and nuclear import pathways [189].

A third reason for multiple NLSs is that a protein, with different types of NLSs, may be able to gain access to additional, alternative nuclear import pathways in addition to the "classical" pathway utilized by basic motif or bipartite type NLSs. This non-

classical NLS may be developmentally regulated so that nuclear import of proteins mediated by this NLS may occur up to a certain stage in development but not after this stage has passed. The adenovirus protein, E1a, is an example of a protein that utilizes such a pathway [38, 102]. One of the two NLSs was shown, using Xenopus oocytes as the nuclear import system, to be developmentally regulated. The developmentally regulated NLS conferred nuclear import up to the early neurula stage after which it ceased to function. A different example is a NLS that functions in a cell-type specific manner. The Abl protein contains three NLSs in which two of its three NLSs function in one cell type but not in another cell type [192, 193]. Presumably, this is due to the expression of a cell-type specific factor that mediates nuclear import by this particular NLS. A third example is a NLS found in the mRNA binding protein, hnRNP K. It contains two NLSs, one which is the classical basic NLS and a second NLS that is dependent on transcription by RNA polymerase II for nuclear import by this NLS to occur [190].

With the possibility that different types of NLSs employ alternative nuclear import pathways, transport mediators of the non-competitive, different nuclear import pathways had been recently reported. They involved the import of M9 domain-containing mRNA binding proteins and certain ribosomal proteins [116, 126]. Even for the classical basic NLS pathway, variants of the NLS receptor, importin α, had been identified and it had been demonstrated that they bound with distinct affinities to specific basic NLSs [117-119] even to the extent where one variant was excluded from binding to

a specific basic NLS. This was illustrated with the Stat1 protein which contained a basic motif NLS that was bound by the importin α variant of one group, NPI-1, but not by an importin α variant from another group, Rch1 [120].

For GR, access to an alternative import pathway may be one reason why it has different two NLSs. Indeed, further research from our laboratory seeking the reason for the difference in the kinetics of nuclear uptake and accumulation between NL1 and NL2 has led to the conclusion that nuclear import by these two NLSs may occur through different pathways [Hsu, B. *et al.*, manuscript in preparation]. Nuclear import mediated by a basic NLS begins by binding of the NLS to the NLS receptor, importin α, which is responsible for import through this pathway. Interaction between pendulin, a mouse homologue of importin α [119], with either GR, with intact NL1 and NL2 (wtGR), or GR with the mutated NL1 sequence (mutGR), was tested in an *in vitro* GST binding assay. Equivalent amounts of ^{35}S-labelled full length GR were incubated with GST-pendulin fusion proteins. Specifically bound proteins were fractionated on SDS-PAGE and analyzed by phosphorimager. The results obtained indicated that pendulin bound only to GR containing a functional NL1 but not to GR containing a mutated NL1 or to GR possessing only NL2. This result was confirmed using another method, the *in vivo* yeast two-hybrid assay. Constructs expressing the Gal4DNA binding domain-GR fusion proteins that contained either the wildtype or mutated NL1 sequence [Hsu, B. *et al.*, manuscript in preparation] were co-transformed with constructs that express pendulin fused to the Gal4 activation domain into cells of the yeast strain, Y190. β-galactosidase

activity was observed when constructs expressing GR with a functional NL1 and pendulin were co-transfected and only in the presence of hormone. No interaction was detected when a defective NL1 or NL2 alone was present. The result from the yeast two-hybrid assay corroborated the finding from the GST-pull down assay that only GR containing a functional NL1 interacts with importin α but not GR with a functionally impaired NL1 or GR with only NL2.

As discussed above, the possibility that a slow process of nuclear translocation by mutGR due to impaired association or dissociation from the multiprotein heterocomplex was refuted since mutGR transformed at a similar rate as wtGR upon dex treatment. Since NL2-mediated nuclear import may occur through an alternative pathway, this provides another explanation for slowed rate of nuclear uptake of mutGR. Import mediated by NL1 (a basic NLS) occurs through the importin α pathway which appears to be used only by proteins that contain a basic NLS. Import through this pathway has been demonstrated to occur rapidly [107]. However, it is possible that the kinetics of nuclear transfer and occupancy of the pathway used by NL2 (a non-basic NLS) is slower compared to the classical basic NLS pathway. This can potentially explain the general decrease in the rate of nuclear transfer seen with mutGR.

With evidence indicating that nuclear import by NL2 may occur through a novel pathway, future studies can be conducted to isolate the NLS receptor that mediates import into the nucleus by NL2. Additionally, competition analyses can be done to uncover any potential interactions between the different pathways as well as experiments to compare

the kinetics of nuclear uptake and accumulation between the different import pathways. Finally, further research may elucidate the physiological role this pathway plays in glucocorticoid hormone action.

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ABSTRACTS:

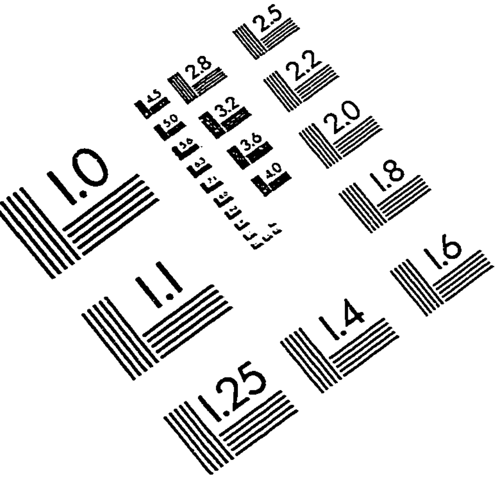
1. Kwast-Welfeld, J., Hsu, B., Tse, R., Hache, R.J.G. and Lefebvre, Y.A. "Function of the Ligand Binding Domain in Intracellular Trafficking of the Glucocorticoid Receptor" The Endocrine Society Annual Meeting, Washington, D.C., June 14-17, 1995.

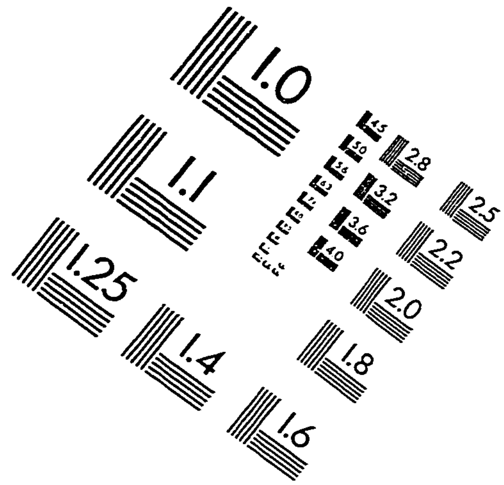
2. Hsu, B., Hache, R.J.G. and Lefebvre, Y.A. "Properties of Ligand Binding Domain Mediated Nuclear Localization of the Glucocorticoid Receptor (GR)" The Endocrine Society Annual Meeting, Minneapolis, MN, June 10-15, 1997.

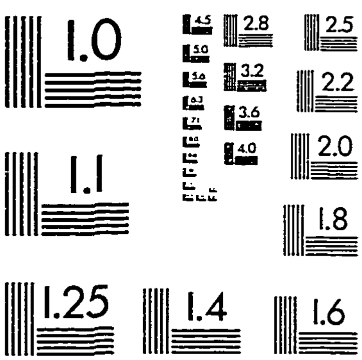
3. Hsu, B., Hache, R.J.G. and Lefebvre, Y.A. "Characterization of Nuclear Import of the Glucocorticoid Receptor (GR) Mediated by the Nuclear Localization Signal in the Ligand Binding Domain" [Manuscript in preparation]

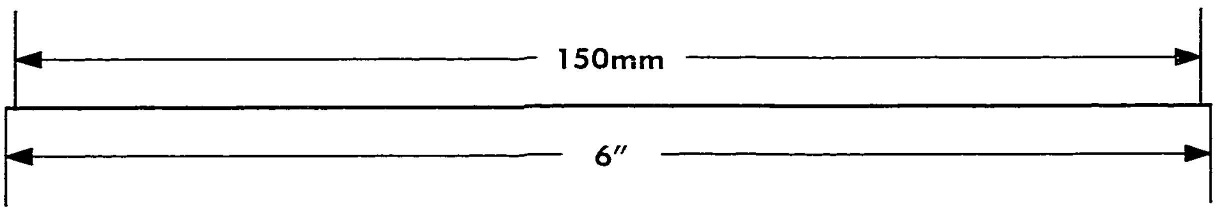
4. Hsu, B., Hache, R.J.G. and Lefebvre, Y.A. "Characterization of Nuclear Import of the Glucocorticoid Receptor (GR) Mediated by the Nuclear Localization Signal in the Ligand Binding Domain" Xth International Congress on Hormonal Steroids, Quebec City, Quebec, June 17-21, 1998.

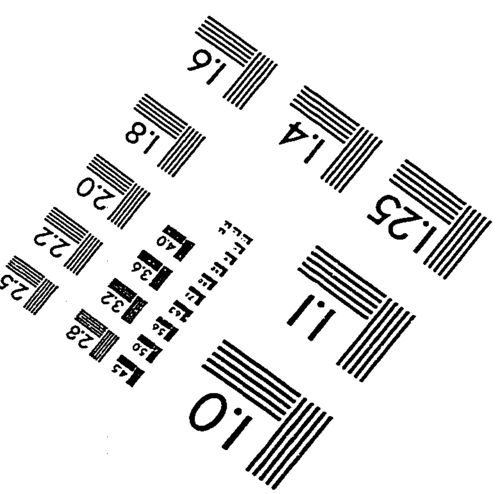
IMAGE EVALUATION TEST TARGET (QA-3)











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