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Mechanisms of Nucleocytoplasmic Trafficking of the Glucocorticoid Receptor

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Mechanisms of Nucleocytoplasmic Trafficking of the Glucocorticoid Receptor

Rhian Walther

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Abstract

Nuclear hormone receptors (NHRs) are ligand-inducible transcription factors that regulate the activity of a wide variety of target genes. The glucocorticoid receptor (GR) is a NHR that is found predominantly in the cytoplasm prior to ligand binding. Upon addition of ligand, GR rapidly translocates to the nucleus and is then slowly exported from the nucleus following ligand withdrawal. It has been proposed that nuclear export of GR is mediated through calreticulin, a protein localised primarily to the endoplasmic reticulum. Based on the results of heterokaryon fusion experiments, it is widely believed that even though liganded GR and other NHRs are predominantly nuclear, they are rapidly and continuously exported from the nucleus and then re-imported. Nucleo-cytoplasmic shuttling has been proposed to play an important role in the regulation of NHR function by allowing for continuous communication between the nuclear receptor and cytosolic signalling pathways. For GR, the relatively rapid rate of nuclear export of the liganded receptor that is believed to underlie continuous shuttling contrasts with the slow rate of redistribution to the cytoplasm observed following ligand withdrawal.

To study shuttling in a setting that resembles the cellular environment I developed a fluorescence recovery after photobleaching (FRAP) technique using randomly occurring multinucleated Cos7 cells. We found little if any shuttling of the liganded receptor 4 h after photobleaching, which was consistent with the slow rate of redistribution following ligand withdrawal but not consistent with the previously described rapid export that is believed to underlie nucleocytoplasmic shuttling of GR. This persistent nuclear localisation of the liganded receptor was confirmed using two independent assays. However, experiments performed in cells fused by treatment with

polyethylene glycol (4000) confirmed earlier reports that liganded GR shuttles rapidly between nucleus and cytoplasm in a manner that is dependent upon direct binding of GR to calreticulin. In contrast, disruption of calreticulin binding did not abrogate the slow export of GR observed by FRAP. Further, redistribution of the receptor to the cytoplasm following ligand withdrawal was not dependent on calreticulin binding. Our results suggest that cell fusion activates a specialised mechanism of GR export that is dependent on calreticulin, and that under normal cellular conditions NHRs may not be able to respond rapidly to cytoplasmic signalling pathways. Additionally, our findings demonstrate that nucleocytoplasmic shuttling as suggested by cell fusion-based assays may not always reflect trafficking behaviour in a normal cell.

Within the course of these studies, we noted that the slow rate of GR nuclear export is accelerated upon mutation of the GR nuclear localisation sequence 1 (NL1). This suggested that in addition to mediating nuclear import of GR by binding with importin α, the NL1 sequence might function to retain GR in the nucleus. We determined that the hinge region of GR, which includes the NL1 sequence, was sufficient to efficiently repress export mediated by the CRM1-dependent HIV Rev nuclear export sequence (NES). This property appears to be specific to the GR hinge region as the basic NLS from SV40 had no significant effect on CRM1-mediated export in the same assay system. Our results indicate that a sequence motif within the GR hinge region that overlaps with NL1 acts as a nuclear retention signal in a manner that exerts a transdominant negative effect on export mediated through an active NES. We hypothesise that modulation of GR nuclear retention may provide a mechanism to regulate glucocorticoid signaling.

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Abbreviations

aldo	aldosterone
AR	androgen receptor
BDV	Borna disease virus
β-gal	beta galactosidase
CAS	cellular apoptosis susceptibility gene
CAT	chloramphenicol acetyl-transferase
CLX	calnexin
Cort	cortisol
CPM	counts per minute
CRM1	chromosomal region maintenance protein 1
CRT	calreticulin
DBD	DNA binding domain
Dex	dexamethasone
DMEM	Dubelco's modified Eagle medium
EDTA	ethylenediamine tetra-acetic acid
ER	estrogen receptor
ERR	estrogen-related receptor
FBS	fetal bovine serum
FLIP	fluorescence loss in photobleaching
FRAP	fluorescence recovery after photobleaching
GFP	green fluorescent protein
GR	glucocorticoid receptor

GRE	glucocorticoid response element
GST	glutathione S-transferase
HDAC	histone deacetylase
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRE	hormone response element
Hsp	heat shock protein
IBB	importin β binding domain
IIF	indirect immunofluorescence
JNK	c-Jun activated kinase
LBD	ligand binding domain
LMB	leptomycin B
LRP	low density lipoprotein receptor-related protein
MMTV	mouse mammary tumour virus
MTA1	metastatic tumour antigen 1
MR	mineralocorticoid receptor
NES	nuclear export sequence
NHR	nuclear hormone receptor
NL1	nuclear localisation factor 1 of the glucocorticoid receptor
NL2	nuclear localisation factor 2 of the glucocorticoid receptor
NLS	nuclear localisation sequence
NES	nuclear export sequence
NPC	nuclear pore complex
Nups	nucleoporins

ONPG	o-nitrophenyl-β-D-galactose
PBS	phosphate buffered saline
PEG	polyethylene glycol
PK	pyruvate kinase
PKC	protein kinase C
PKI	protein kinase A inhibitor
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
RanBP1	Ran binding protein 1
RanGAP	Ran guanine activating protein
RanGEF	Ran guanine exchange factor
RAP46	receptor-associating protein 46
RAR	retinoic acid receptor
RXR	retinoid X receptor
RRE	Rev recognition element
SFBS	stripped fetal bovine serum
SHR	steroid hormone receptor
SLO	streptolysin O
SRC	steroid receptor coactivator
SV40	simian virus 40
TR	thyroid hormone receptor
VDR	vitamin D receptor

Table of Contents

List of Figures and Tables

Introduction

Overview of Glucocorticoid Signalling

Endocrine signalling through cholesterol derivatives has long been recognised as an important physiological mediator of various signalling pathways in a number of target tissues. Glucocorticoids, which are produced in the adrenal cortex, are the primary physiological mediators of stress response. In humans glucocorticoid signalling is mediated primarily by cortisol, which was first isolated from adrenal extracts in the 1930's (1). Production of cortisol is regulated by signalling through the hypothalamic – pituitary – adrenal axis in response to both physiological stressors and diurnal rhythms. The physiological effects of cortisol are wide ranging and include mobilisation of metabolic resources through increased gluconeogenesis (2), protein degradation (3) and lipolysis (4), increased catecholamine synthesis (5), adipogenesis (6), and alteration of memory consolidation (7,8). Chronic increases in cortisol levels due to physiological defects in cortisol signalling, as is observed in patients with Cushing's syndrome, prolonged stress or pharmacological use of glucocorticoid receptor agonists can have additional physiological consequences including immunosuppression and anti-inflammatory effects (9,10), induction of apoptosis (11,12), changes in fat storage (13), osteoporosis (14,15) and depression (16,17).

The physiological effects of cortisol are mediated predominantly by the action of the glucocorticoid receptor (GR). Early studies performed using preparations of steroid-sensitive tissues hinted at a role for nuclear receptors, including GR, in modulating gene activity through transcriptional activation [reviewed in (18)]. It is now well established

that nuclear hormone receptors (NHRs) including GR are ligand-inducible transcription factors that regulate transcriptional activity through direct binding to hormone response elements (HREs) within the promoter region of target genes [for review see (19,20)]. The preparation of highly purified GR was facilitated by the development of dexamethasone 21-mesylate, a covalent affinity label (21), and production of a GR monoclonal antibody (22). These advances and subsequent partial peptide sequencing led to the isolation of the GR cDNA sequence. The cloning of the rat GR cDNA by Yamamoto and colleagues was the first identification of a NHR cDNA (23), followed closely by the cloning of the human GR (24), the estrogen receptor (25,26) and the progesterone receptor (27). Soon after these initial advances, the cDNAs of many other NHRs were identified using molecular approaches. Subsequent genome sequencing efforts have allowed for further identification of NHR genes [reviewed in (28)]. Comparison of the NHR cDNA sequences reveals that nuclear receptors share a very high degree of homology within their DNA binding domains (DBDs), and to a lesser extent within their ligand binding domains (LBDs).

Analysis of the emerging cDNA sequences in the mid 1980's allowed for the formal recognition of evolutionary relationships between the nuclear receptors. The NHR superfamily includes receptor molecules for a wide range of hormone ligands, including steroids, retinoic acid derivatives, thyroid hormones, vitamin D derivatives, bile acids and fatty acids, among others (28,29). Additionally, there is a substantial group of NHRs known as orphan receptors for which the specific ligand has yet to be determined. GR belongs to the steroid receptor subfamily of the NHR superfamily. Other members of this family include the mineralocorticoid receptor (MR), progesterone receptor (PR),

androgen receptor (AR), and the less closely related estrogen receptor (ER) and the estrogen related receptors (ERR) (30). These steroid hormone receptors (SHRs) display significant sequence similarity and share several similarities in their mode of action. The distinguishing feature of SHRs is that prior to ligand binding they are found as inactive complexes with heat shock proteins (hsps) and immunophilins. Upon addition of hormone ligand this inactive complex is dissociated and the receptor becomes biologically active. The unliganded and liganded SHRs are often referred to as the 8S and 4S receptors respectively, reflecting the sedimentation values on a sucrose gradient of these receptors [reviewed in (31,32)].

The subcellular localisation of these unliganded complexes varies among receptors. Prior to ligand binding, ER (33-36) and PR (35,37-41) are found predominantly in the nucleus. Conversely, GR is found predominantly in the cytoplasm prior to ligand binding (42-47) although overexpression of the receptor has been shown to result in partial nuclear localisation (48,49). The subcellular localisation of unliganded MR and AR appears to differ depending on the cellular context in which localisation is studied and potentially the assay used to assess receptor localisation. Naïve AR has been reported to range from exclusively nuclear (50,51) to exclusively cytoplasmic (52-57) or evenly distributed between nucleus and cytoplasm (58). Similarly, naïve MR has been observed predominantly in the cytoplasm (59-62) or equally distributed between nucleus and cytoplasm (63-70). These reported differences might be due to the differential expression of factors that either mediate cytoplasmic sequestration (71) or nuclear targeting of unliganded receptors (56) or they may be a consequence of overexpression (48,49). Although ERR1 and ERR2 were the first orphan receptors to be identified (72)

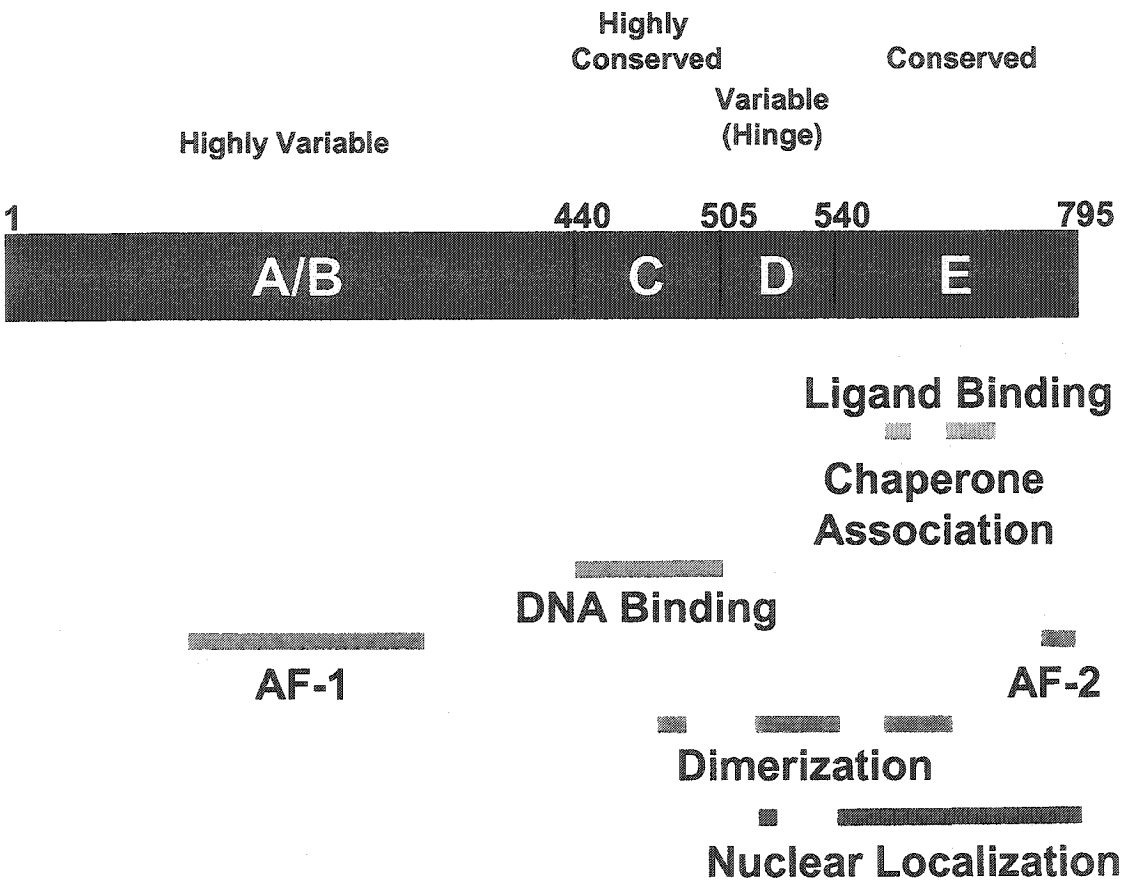
they and ERR3 (73) remain largely uncharacterised. As such, the precise localisation of the ERRs remains unknown.

The cloning of the GR cDNA sequence allowed for the molecular dissection of the receptor. It has been shown that GR and other SHRs are characterised by the presence of modular domains which retain their function outside of the context of the full-length protein (74-79). The modular organisation of GR is outlined in Figure 1. The various molecular functions of the receptor can be mapped to discrete regions of the protein.

The LBD is found in the E domain at the C-terminus of the receptor, and shares extensive homology with the LBDs of other steroid hormone receptors. Prior to ligand binding, the GR LBD is held in an inactive state through association with hsps and immunophilins. This inactive GR complex contains a dimer of hsp90, and an immunophilin molecule that can be either the FK506 binding protein (FKBP) 52, FKBP51, Cyp40 or PP5. Additionally, chaperones including hsp70, hsp40, HOP, and p23 are required for the proper maturation of the naïve receptor complex [reviewed in (31)]. The maintenance of this heteromeric complex, in particular binding to hsp90, is required in order to enable GR to bind ligand (80-82). Binding of ligand results in the dissociation of this complex and subsequent regulation of target genes. X-ray crystallography has shown that the GR LBD is comprised of eleven α helices that are arranged in a three-layer sandwich when bound to hormone ligand. These α helices are arranged in a manner which forms a binding pocket that is specific for glucocorticoid ligands (83).

Figure 1: Outline of the modular structure of the glucocorticoid receptor

The degree of conservation of individual domains across the nuclear receptor superfamily is summarised at the top. The localisation of individual functional motifs is summarised below. The sequence of the N-terminal domain is highly variable amongst receptors. It contains the AF-1 activation domain and sites for phosphorylation of the receptor. The C region is highly conserved and contains the DNA binding region and a DNA-dependent dimerisation domain. The variable D, or hinge region contains sequences that mediate the formation of GR dimers in solution and the basic NL1 nuclear localisation signal. The E region contains the receptor ligand binding domain. It contains the AF-2 transcriptional activation function, chaperone interaction domains, mediates solution dimerisation of ER and PR and contains the NL2 nuclear localisation function of GR.


Highly
Conserved
Conserved
Variable
(Hinge)
Highly Variable
1
440
505
540
795
A/B
C
D
E
Ligand Binding
Chaperone
Association
DNA Binding
AF-1
AF-2
Dimerization
Nuclear Localization

The highly conserved DBD is localised to the centrally positioned C domain. Structural analysis has shown that the GR DBD contains two zinc finger structures in which two zinc atoms are each co-ordinated by four highly conserved cysteine residues (84-86). Following ligand binding, this region is exposed and then mediates the recognition of glucocorticoid response elements (GREs) within the promoter regions of target genes. The GRE is an inverted imperfect palindrome separated by three base pairs with the consensus sequence GGTACAnnnTGTTCT (87,88). In addition to serving as the DNA recognition site for GR, this sequence is recognised by the related MR (89), PR (90) and AR (91). This sequence is similar to the related estrogen receptor response element (92) and specificity of binding is defined by amino acid residues within the first zinc finger of the DBD known as the P-box (93-96). When bound to DNA two GR DBDs form a dimer through a DNA-dependent dimerisation interface present within the descending α helix of the C-terminal zinc finger termed the D-box (96-98). Each of the two DBDs binds to one half of the palindrome in a head to head orientation. The N-terminal zinc finger lies in the major groove of the DNA helix making base specific contacts while non-specific contacts with the phosphate backbone are made through the second zinc finger (98). Intriguingly, while the GR is essential for survival (99), DNA binding of GR may be dispensable for survival, as mice carrying a mutant receptor deficient in DNA binding are viable (100).

Two separate regions of the receptor mediate transcriptional activity. The activation function 1, or AF-1 lies within the N-terminal A/B region of the receptor. AF-1 was first mapped to a 200 amino acid region by Hollenberg and Evans (101) and has been delineated to a core region comprising amino acid residues 187-244 of human GR

(102). Though the full length receptor is not functionally active in the absence of hormone, AF-1 has been shown to activate transcription when fused to a DNA binding domain irrespective of its relative position within the fusion protein, indicating that this GR activity is ligand independent (101-103). At the C-terminus of the receptor is a second activation function known as AF-2. AF-2 is ligand-dependent and has been mapped to the extreme C-terminal helix of the GR ligand binding domain (83,101,104,105). Upon ligand binding, a conformational change in the LBD results in the exposure of the AF-2 helix (83,106-110). Once exposed, AF-2 interacts with members of the p160 family of coactivator proteins (111-120) and a number of other coactivators including, but not limited to, p300/CBP (121,122) and P/CAF (123,124) and GRIP170 (125). The interaction of AF-2 with coactivator proteins leads to the eventual recruitment of a large complex of regulatory proteins possessing histone acetyl transferase and methylase activities. Through acetylation of lysine residues within the histone octamer and modification of protein components of the regulatory complex, recruitment of coactivator molecules through AF-2 results in the ligand-dependent activation of target genes [for review see (20,126,127)].

The liganded, active form of GR exists as a homodimer. Two sequences have been shown to mediate dimer formation. The DNA dependent dimerisation domain is found between the two zinc fingers of the GR DBD, and is required for maintaining GR as a dimer when bound to DNA (96-98). A second dimerisation domain found within the flexible hinge region of the receptor mediates the formation of GR homodimers in solution (128). Analysis of the GR LBD crystal structure reveals another potential solution dimerisation domain within the LBD that is novel to GR. An intermolecular β

sheet forms this second solution dimerisation domain, with core hydrophobic contacts made between amino acid residues P625 and I628 of human GR (P643 and I646 of the rat GR). As this interaction is thought to occur with low affinity, it is possible that in the context of the full-length protein both solution dimerisation domains are required to mediate the stable formation of GR homodimers (83).

Of particular interest to the work described in this thesis are the two nuclear localisation functions that mediate nuclear import of GR following ligand binding (42). Nuclear localisation sequence 1 (NL1) is found in the hinge region of the receptor and is comprised of a series of basic amino acid residues spanning positions 510-517 of rat GR. This region has been shown to bind with importin α, and import through NL1 is proposed to be mediated by the classical nuclear import pathway (129,130). Deletion or mutation of the NL1 core sequence results in decreased nuclear accumulation of GR in response to steroid (130,131). This decrease in nuclear accumulation is not complete due to the presence of an additional import signal, NL2. NL2 resides within the C terminal LBD of the receptor. Analysis of the GR LBD amino acid sequence does not reveal a typical NLS-like motif rich in basic residues, and the NL2 activity has not been mapped to a discrete amino acid sequence (42). This indicates that the GR NL2 motif may be an atypical NLS sequence.

General Mechanisms of Protein Nucleocytoplasmic Trafficking

The partitioning of the cell into membrane-bound organelles that are organised to perform specific cellular functions is a defining feature of all eukaryotes. This compartmentalisation allows the cell to function more efficiently by gathering

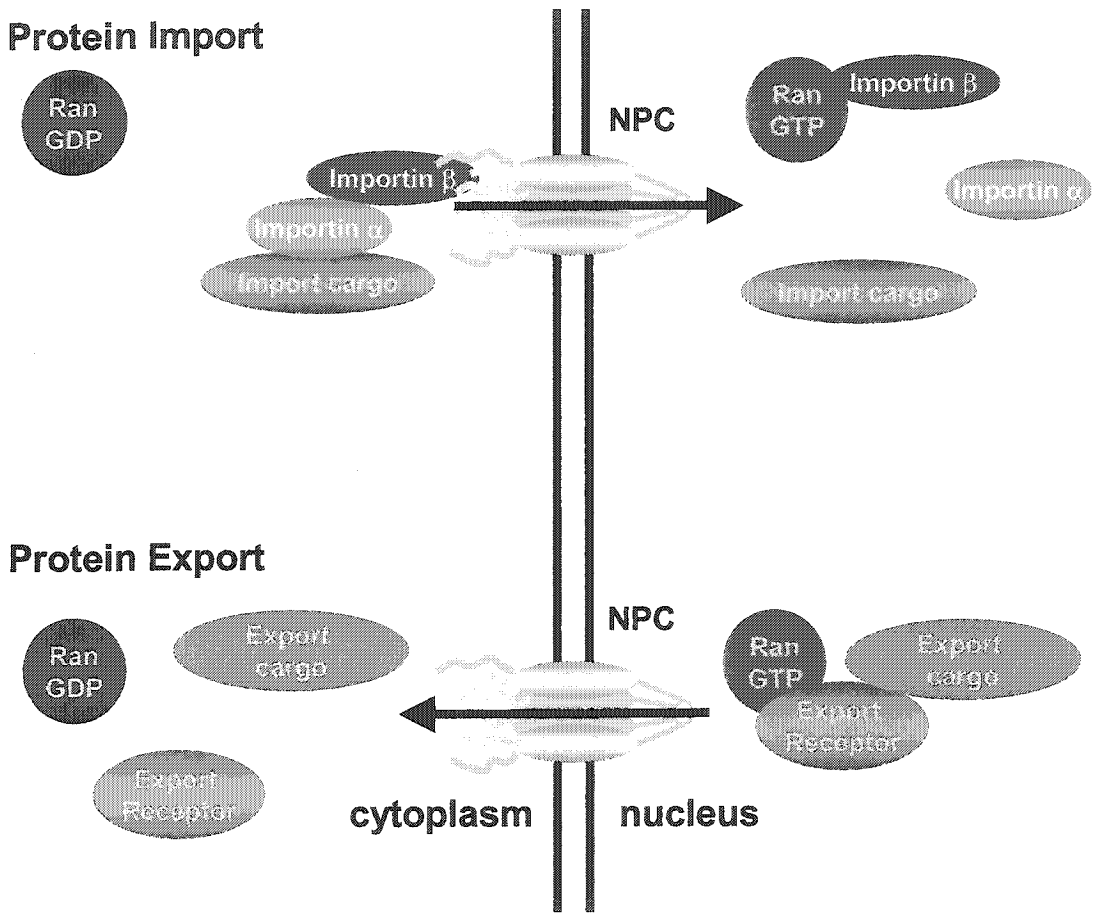
macromolecules that are involved in specific functions into spatially contained regions. However, with partitioning of the cell comes the need for regulated communication and transport of macromolecules across membrane barriers. The principle processes involved in nucleocytoplasmic transport are outlined in Figure 2 [for reviews see (132-138)].

Briefly, nucleocytoplasmic trafficking involves the interplay of two separate transport pathways – nuclear import and nuclear export. A signal resident within the cargo molecule itself initiates the formation of a complex with either import or export receptors. A key regulator of this process is the small guanine nucleotide binding protein Ran. In the cytoplasm Ran is primarily bound to GDP, while in the nucleus the GTP-bound form of Ran is predominant. These two forms of Ran have the ability to differentially influence the formation and maintenance of cargo-receptor complexes, resulting in regulated association and dissolution of transport complexes. This ensures that the directionality of import and export is established and maintained.

The nucleus represents the largest subcellular organelle in the eukaryotic cell and is surrounded by a nuclear envelope that is comprised of two membrane layers [reviewed in (139)]. The outer nuclear membrane is continuous with the endoplasmic reticulum. Consequently, changes within the lumen of the endoplasmic reticulum directly impact structures within the nuclear envelope. The inner nuclear membrane lies within the nucleoplasm. Underneath the inner membrane lies the nuclear lamina. The nuclear lamina is comprised principally of oligomers of A and B-type lamin proteins that are located beneath the nuclear envelope and also extend into the nucleoplasm (139). This network of dense intermediate filament-like fibres plays an important role in maintaining structural strength and directing the proper assembly of the nuclear envelope (140). In

Figure 2: Overview of protein nuclear import and export.

In the cytoplasm, in the presence of the GDP-bound form of Ran, a complex forms between the import substrate, importin α, and importin β. Importin β docks this complex at the NPC where it translocates to the nucleus. In the nucleus, the presence of RanGTP induces dissociation of the import complex, releasing the import cargo. Protein export is mediated by the formation of a trimeric complex between the export cargo, RanGTP and an export receptor. This complex traverses the NPC. In the cytoplasm, RanGTP is hydrolysed to RanGDP, leading to the dissociation of the trimeric complex. As a result, the export cargo is deposited in the cytoplasm.


Protein Import
Ran GDP
NPC
Ran GTP
Importin β
Importin β
Importin α
Import cargo
Importin α
Import cargo
Protein Export
Ran GDP
Export cargo
NPC
Ran GTP
Export cargo
Export Receptor
Export Receptor
cytoplasm
nucleus

addition to providing structural support, the nuclear lamina is known to influence chromatin organisation and has recently been shown to have an influence on gene expression (141,142).

Transport between nucleus and cytoplasm occurs through perforations in the nuclear envelope known as nuclear pore complexes (NPCs). The NPC is a selectively permeable barrier that spans the nuclear envelope and allows for the regulated transport of proteins and other macromolecules from nucleus to cytoplasm. This structure allows the free diffusion of small molecules, but restricts the movement of larger molecules such as nucleic acids and proteins greater than 20 kDa in size [for reviews see (132,143)]. The NPC is a rather large structure with an estimated molecular mass of approximately 50 mDa in yeast (144) and 125 mDa in vertebrates (145). The yeast NPC is comprised of 29 proteins termed nucleoporins or nups, (146) while the larger vertebrate NPC is comprised of approximately 45 nups (147) which are all present in multiple copies. Many of these nups, particularly those in the NPC core, contain repeats of phenylalanine and glycine residues, which are thought to be play a key role in the translocation of imported or exported substrates through the NPC (148). It has long been recognised that the NPC is arranged symmetrically along an eight-fold axis perpendicular to the nuclear envelope around a central aqueous passage of approximately 9nm in diameter [reviewed in (132,143)] that can expand to a maximum diameter of approximately 40 nm (149). Structural analysis has shown that fibrils extend from both the cytoplasmic and nuclear face of NPC, and on the nuclear face these fibrils join together to form a basket-like structure [for review see (132,143)]. While many nups are localised symmetrically around the central core, there are particular nups that localise to either the nuclear or

cytoplasmic face of the nuclear envelope that may play a role in maintaining directionality of transport within the NPC (146,150-154). Additionally, while nups that localise to the core region of the NPC are relatively immobile (155), some nups dynamically associate with the NPC complex (156,157) (Rabut and Ellenberg unpublished) and may serve to help target transport cargos to the pore (158).

Passage of a molecule through the nuclear pore is triggered by specific signals resident within the cargo itself. In the case of nuclear import, these signals are referred to as nuclear localisation signals (NLSs). The most extensively characterised class of NLSs contains clusters of basic amino acid residues. These signals may consist of a single cluster of lysine or arginine residues, as in the case of the simian virus 40 (SV40) large T antigen (159,160), or the NLS may be bipartite, as is observed for the nuclear protein nucleoplasmin (161). Bipartite NLSs consist of two interdependent clusters of basic amino acids separated by 10-12 amino acids. The N-terminal cluster contains two arginine or lysine residues, while the C-terminal cluster typically resembles a monopartite NLS (161). These NLS mediate nuclear import through direct binding to the importin α subunit (162,163), which in turn binds to the importin β of the import receptor (164,165) followed by docking of this trimeric complex to the nuclear pore (163,166,167).

NLS-mediated transport through the NPC is directed by a number of proteins belonging to the karyopherin transporter family. Both nuclear import and nuclear export are mediated in large part by karyopherins. In general, the karyopherins have an elongated, curved shape with characteristic HEAT or ARM repeats that span the entire length of the protein. HEAT repeats are comprised of two alpha helices that are joined by a sharp turn to form a hairpin-like structure. The N-terminal A helix localises to the

convex outer surface of the protein while the B helix is oriented to the inner concave surface (168,169). ARM repeats are closely related structures found within some karyopherins, including importin α family members, and are comprised of three helices. The two N-terminal helices are separated from the third helix by a sharp turn that orients the third helix along the inner concave surface while helices 1 and 2 lie along the outer convex surface (170). Binding to the GTP- bound form of Ran is a property shared by all karyopherins, and is mediated through the N-terminal region of the protein. The C-terminal portion of the protein is generally involved in binding to the cargo protein that is to be transported through the NPC.

Nuclear import is mediated by a subset of karyopherins known as importins. Members of the importin α subset of karyopherins were first identified as cytosolic proteins of approximately 60 kDa that mediate the nuclear import of NLS-bearing substrates (171-173). In yeast there is only one importin α homologue, while in vertebrates there are at least seven importin α homologues (133,134,138). In vertebrates these various importin α homologues display different affinities towards a variety of import substrates (174-178). Importin α functions as an adapter protein that binds to the basic NLS of import substrates and then forms a trimeric complex with another importin protein, importin β (163). Importin α contains two binding sites for basic sequences, with a major binding site that is optimised to recognise five lysine residues and a minor binding site that allows for recognition of bipartite NLSs (170,179,180). Binding of importin α to importin β is mediated through an N-terminal importin β binding domain (IBB) (164,165). The IBB can act as a transferable nuclear import signal as a non-imported cargo protein becomes predominantly nuclear when fused to this motif (164).

The IBB resembles a basic NLS, and upon loss of cargo binding in the nucleus this region binds to the NLS-binding pocket in a cis fashion (181). Following nuclear import of a cargo protein, importin α is exported back into the cytoplasm through a nuclear export signal found at the extreme C-terminus that is recognised by the nuclear export receptor CAS (182,183).

Importin β karyopherins are cytosolic proteins that act as binding partners for importin α and mediate docking of the importin α/cargo complex to the pore (184). Several importin β homologues have been identified in yeast and many more exist in vertebrates. Importin β adopts a superhelical structure with a large, flexible acidic loop joining the N and C-terminal regions of the protein (168). Binding to RanGTP and the IBB domain of importin α occurs on the concave side of the protein through the N-terminal (185) and C-terminal HEAT repeats (168), respectively. Docking to the nuclear pore is mediated by the convex face of the N-terminal region of importin β (148). Following translocation through the pore, the importin α/β-cargo complex is dissociated by the GTP-bound form of the small guanine binding protein Ran (164,186-188). When bound to GTP, Ran has a high affinity for the N-terminal region of importin β. RanGTP then makes contacts with the acidic loop that spans the N and C-terminal regions. This destabilises binding with importin α resulting in the deposition of the cargo into the nucleoplasm (168).

In addition to serving as an adapter for importin α-mediated nuclear import, importin β has also been shown to act directly as a transport receptor that binds to and mediates nuclear transport of cargo molecules in the absence of importin α. A wide variety of proteins are imported into the nucleus through direct interaction with importin

β, including transcription factors (189-191) ribosomal proteins (192), viral factors (193,194) and other proteins including cyclin B1 (195) and the parathyroid hormone-related protein (196). Direct binding of cargo to importin β is mediated through a second cargo binding domain in the N-terminal portion of the protein that partially overlaps with the C-terminal IBB domain binding site (197). Additionally, importin β family members may have a function that is independent of their role in trafficking, as they have been proposed to act as chaperone proteins, preventing the formation of aggregates of proteins containing exposed basic sequences (198).

A subset of karyopherins bearing homology to the importin β karyopherins has been shown to participate in nuclear export. The most extensively characterised nuclear export receptor is CRM1. CRM1, which stands for chromosomal region maintenance protein 1, mediates export of proteins from the nucleus by binding to leucine-rich nuclear export sequences (NESs) present on export cargos (199-202). These leucine-rich sequences were first identified in the protein kinase A inhibitor (PKI) and the HIV Rev protein (203,204). CRM1 has since been shown to mediate nuclear export of a wide variety of proteins, ranging from transcription factors such as STATs (205,206) and Smad1(207), to coactivators (208), to cyclins (209-213), to proteins that inhibit the activity of other signalling proteins including IκB (214) and the adenamatous polpyposis coli (APC) protein (215,216). Nuclear export through the CRM1 pathway is mediated by the formation of a trimeric complex with the GTP-bound form of Ran and the export cargo (199) that is sensitive to the drug leptomycin B (199,217). This complex translocates through the NPC and is dissociated in the cytoplasm upon hydrolysis of RanGTP to RanGDP (182,199). In addition to mediating the nuclear export of protein

cargos, CRM1 has been shown to mediate nuclear export of U snRNA (199,203,218) and may also mediate 5S rRNA export (203), although this function may be specific to differentiated oocytes (219).

In addition to the CRM1 pathway, other proteins belonging to the importin β family have been shown to mediate nuclear export of protein cargos. The cellular apoptosis susceptibility gene (CAS) and its yeast homologue Cse1p recycle importin α by re-exporting it to the cytoplasm (182,220-222). Exportin-4 mediates nuclear export of the eukaryotic translation initiation factor eIF5A following modification of a critical lysine residue (223). The nuclear export of double-stranded RNA-binding proteins is mediated by exportin-5 (224). In yeast the Msn5 export receptor has been implicated in phosphorylation-dependent nuclear export of a number of signalling factors including transcription factors Pho4 (225), Crz1p (226) and Far1p (227), a protein involved in pheromone signalling.

Non-importin β family members have also been implicated in protein nuclear export. As will be discussed in more detail in a later section, calreticulin, a chaperone protein that also binds and stores Ca^{2+} in the endoplasmic reticulum, has been proposed to mediate nuclear export of the protein kinase A inhibitor (PKI), GR and other nuclear receptors (228,229). Members of the 14-3-3 family of proteins have been proposed to enhance cytoplasmic localisation of a number of proteins including Cdc25 (230,231), class II histone deacetylases (HDACs) (232,233), and GR (234). The mechanism through which 14-3-3 proteins promote cytoplasmic localisation is controversial. Given that 14-3-3 does contain a leucine-rich sequence that resembles a CRM1 dependent NES, it has been proposed that 14-3-3 acts as an adapter that piggybacks other proteins into the

cytoplasm (235) in a manner that resembles the CRM1-mediated nuclear export of unspliced HIV RNA molecules through the Rev adapter protein (203). However, other studies have suggested that mutations within this domain affect direct binding of 14-3-3 to target proteins (230,236,237). In the case of Cdc25, nuclear export is mediated through a constitutively active intrinsic NES that functions independently of 14-3-3 binding. 14-3-3 binding masks the Cdc25 NLS, resulting in increased cytoplasmic localisation (230,231). Export of HDAC4/5 and the pro-apoptotic forkhead transcription factor FKHRL1 are both dependent upon intrinsic NES sequences that are activated by phosphorylation (238,239). In both of these cases, phosphorylation-dependent binding of 14-3-3 promotes cytoplasmic localisation through two mechanisms. Similar to Cdc25, binding of 14-3-3 masks the NLS, preventing association with importin α and thus blocking nuclear import. However, unlike Cdc25, the NESs of HDAC 4/5 and FKHRL1 are not constitutively active. Binding to 14-3-3 appears to be required to activate the intrinsic NES activities within these proteins (232,233,237). The mechanism through which the 14-3-3σ isoform affects GR localisation is yet unclear, although 14-3-3 appears to potentiate GR nuclear export following ligand withdrawal (234).

While most nuclear import and export sequences mediate uni-directional transport, there is a subset of trafficking signals that mediate bi-directional trafficking. One notable example is the M9 signal that mediates both import and export of the hnRNP A1 protein (240). Nuclear import of hnRNP A1 thorough M9 is mediated by transportin-1, which is related to importin β (241). While it has been suggested that transportin-1 also mediates hnRNP A1 export (241), and the closely related transportin-2 protein has been implicated in mRNA export (242), analysis of the crystal structure indicates that the

presence of RanGTP favours dissociation of a transportin-1/cargo complex (243). Another importin β family member, importin-13, has been shown to participate in both import and export of different substrates. While importin-13 appears to function primarily as an import receptor for the SUMO-1/sentrin-conjugating enzyme hUBC9 and the RNA binding motif protein 8, it can also mediate nuclear export of the translation initiation factor eIF1A (244).

Directionality of nucleocytoplasmic transport is maintained by the small guanine nucleotide binding protein Ran. Ran can bind to both GDP and GTP and adopts distinct conformations depending on its binding partner (243,245,246). In the cytoplasm Ran is present primarily in the GDP-bound form, while in the nucleus RanGTP is predominant [reviewed in (247,248)]. This gradient is essential for nucleocytoplasmic transport (187) and is maintained by the preferential localisation of several proteins. In the nucleus high levels of RanGTP are maintained by the Ran guanine exchange factor (RanGEF; also known as RCC1), which catalyses the exchange of GDP for GTP (249,250). The Ran GTPase activating protein (RanGAP) is found on the cytoplasmic face of the nuclear envelope (251) and catalyses the hydrolysis of RanGTP to RanGDP (252,253). The activity of RanGAP is stimulated by interaction with Ran binding protein 1 (RanBP1) (254-257). Re-import of RanGDP into the nucleus is mediated by NTF2 (258,259).

Translocation through the NPC is a key event in nucleocytoplasmic trafficking. The capacity of the NPC is quite high, with approximately 1000 translocation events occurring per second (260). Three models have been proposed to describe the mechanism of transport through the NPC. The Brownian affinity gate model was originally proposed by Rout and colleagues (146). This model proposes that the docking

of protein complexes to filamentous nucleoporins on either face of the NPC increases their residence time at the NPC opening, thus facilitating their diffusion through the NPC. According to this model, following the docking of a receptor/cargo complex to the pore, translocation through the pore occurs by simple diffusion (146). The graded affinity model is based on the observation that a subset of nups localise to either the cytoplasmic or nuclear face of the NPC. This model proposes that as a receptor/cargo complex travels through the NPC, directionality of transport is determined by a series of interactions of increasing affinity (152). The selective phase model proposes that the phenylalanine-rich FG repeats of the many nups that comprise the NPC form a highly hydrophobic permeability barrier within the central channel. Passage through the NPC is proposed to be dependent on hydrophobic interactions between these FG repeats and transporter molecules (260,261). While the actual mechanism of transport through the NPC most likely incorporates features of all models, the selective phase model appears to best fit the available data. The graded affinity model is not able to explain the observation that passage through the nuclear pore appears to be reversible (187) and that the reversal of the RanGTP/GDP gradient results in the reversal of the direction of trafficking (262). One potential shortcoming of the Brownian affinity gate model is that the addition of multiple transport signals to a synthetic cargo protein has a synergistic effect on the rate of transport through the pore. If the rate-limiting step in translocation were the initial docking of the cargo to the pore, then multiple signals would be expected to only have an additive effect on the rate of translocation (261).

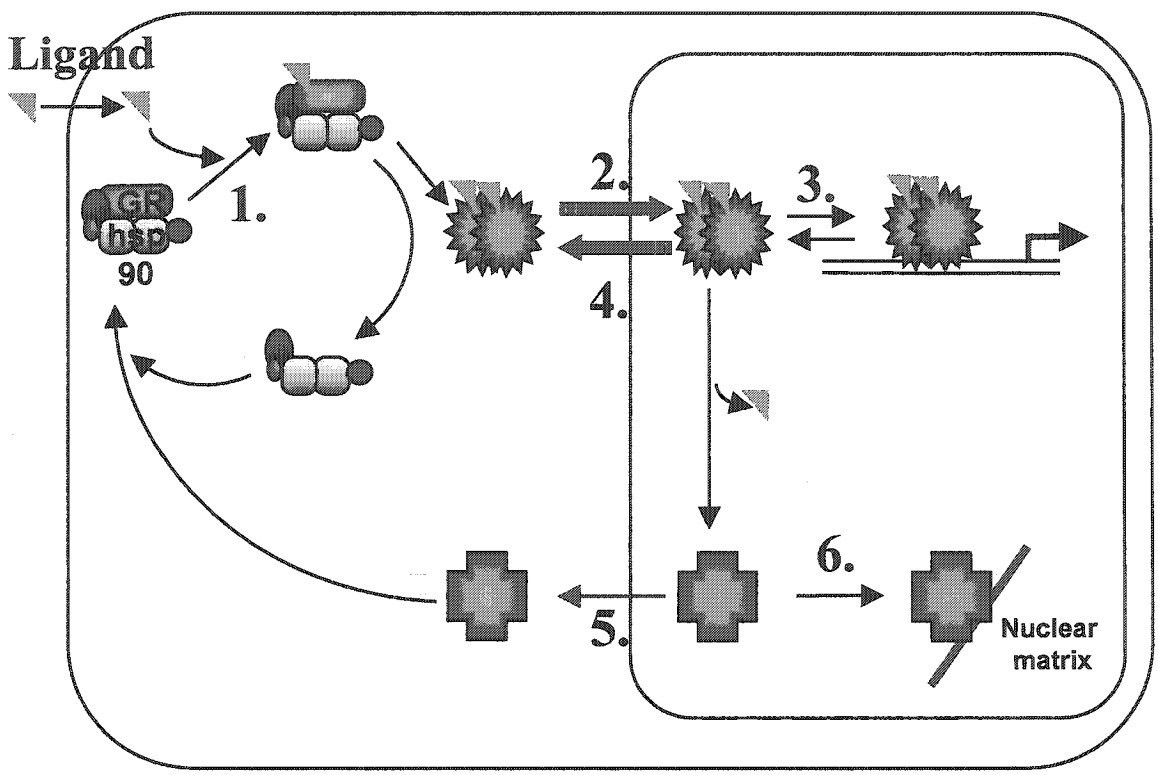
Regulation of GR Nucleocytoplasmic Trafficking

Regulation of nucleocytoplasmic trafficking provides an important means of influencing protein activity. The effects of modulating protein localisation on cell function are wide-ranging. For example, progression through the cell cycle is tightly regulated through phosphorylation-dependent control of cyclin subcellular localisation [reviewed in (263,264)]. In the developing myocyte, key pathways are activated by nuclear export of a transcriptional repressor in response to external signals (265). In the case of GR, regulation of nucleocytoplasmic trafficking ensures tight control over receptor activity, as access to its sites of action is severely limited until the receptor is specifically activated by the addition of hormone ligand.

GR localisation is regulated at several different points. These various points of control are illustrated in Figure 3 [for reviews see (266-269)]. Prior to ligand binding, the inactive 8S complex resides in the cytoplasm. Following the ligand-induced dissociation of this complex, GR forms a homodimer and rapidly translocates into the nucleus. Once in the nucleus, this active 4S form of GR regulates gene activity through transient association with GREs present in the promoter regions of target genes. A continuous nucleocytoplasmic shuttling mechanism is thought to underlie the complete nuclear occupancy of liganded GR. This proposed shuttling would potentially allow for the interplay of the predominantly nuclear receptor with cytosolic signalling factors. Upon removal of ligand, GR undergoes a second conformational change and is slowly redistributed back to the cytoplasm. Association of GR with the nuclear matrix may influence this slow nuclear export. Following redistribution of GR to the cytoplasm, a second round of hormone exposure results in the re-import of GR into the nucleus.

Figure 3: The subcellular localisation of GR is influenced by several mechanisms

Prior to ligand binding the receptor is localised to the cytoplasm where it resides as an inactive complex with heat shock proteins and immunophillins (1). Following exposure to steroid, cytoplasmic GR dissociates from the hsp-containing chaperone complex, dimerises and rapidly transfers to the nucleus (2) where it binds transiently to DNA as a homodimer (3). Nuclear liganded GR rapidly exchanges with DNA and has been proposed to shuttle continuously between the nucleus and cytoplasm (4). Upon loss of steroid, GR returns to the cytoplasm slowly (5). This slow return to the cytoplasm may be in part due to nuclear retention through interaction with the nuclear matrix (6).


Ligand
GR
hsp
90
1.
2.
3.
4.
5.
6.
Nuclear
matrix

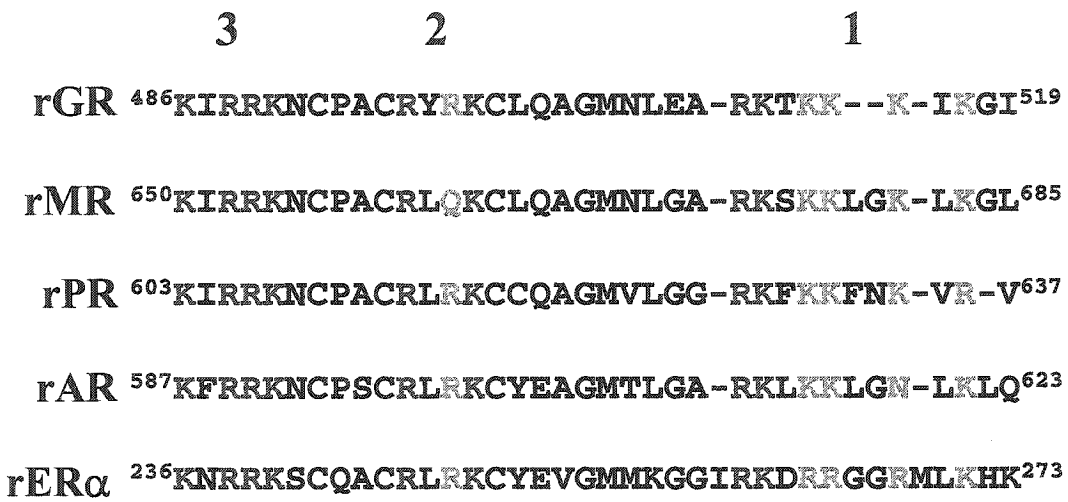
Early studies demonstrated that following exposure of target tissues to radio-labelled ligand, GR translocates from the cytoplasm into the nucleus resulting in the formation of high affinity binding sites that are largely localised within the nucleus, providing early evidence that liganded SHRs localise predominantly to the nucleus (270-272). Addition of hormone ligand results in the dissociation of the GR-hsp complex followed by the translocation of the liganded receptor from the cytoplasm to the nucleus (273). This nuclear translocation in response to steroid occurs rapidly with a $t_{1/2}$ of approximately 4.5 min (42,130,273,274), with the dissociation of the hsp complex being the rate-limiting step. Following dissociation of the hsp complex, GR is rapidly imported into the nucleus within approximately 30 sec (273,275), where it is then targeted to discrete subnuclear foci that are thought to correlate with regions of active transcription (49,276).

As previously mentioned, GR nuclear import is mediated by two separate nuclear localisation signals (42). Characterisation of the ligand-dependent NL2 sequence within the GR LBD remains a challenge as conventional mapping approaches, which rely primarily on deletion analysis, render the LBD non-functional. It is quite likely that the critical amino acid residues of the NL2 sequence are brought together through the three-dimensional conformation of the LBD. Among the steroid hormone receptors, the NL2 activity appears to be poorly conserved as a ligand-dependent nuclear localisation activity similar to NL2 has been observed for ER, but not for PR (35).

In contrast to the elusive nature of the GR NL2 sequence, the NL1 sequence has been well characterised. The GR NLS is located within the flexible hinge region and overlaps with the C-terminal portion of the DBD (42,277). As illustrated in Figure 4, the

Figure 4: NL1 is comprised of three clusters of basic amino acids.

NL1 amino acid residues that are identical among the steroid receptors are indicated in red, while residues that are similar are indicated in green. The NL1 core region, indicated by yellow shading, is required for transport, while regions 2 and 3 enhance the NLS activity.


3
2
1
rGR 486KIRRKNCPACRYRKCLQAGMNLEA-RKTKK--K-IKGI519
rMR 650KIRRKNCPACRLQKCLQAGMNLGA-RKSKKLGK-LKGL685
rPR 603KIRRKNCPACRLRKCCQAGMVLGG-RKFKKFNK-VR-V637
rAR 587KFRRKNCPSCRLRKCYEAGMTLGA-RKLKKLGN-LKLQ623
rERα 236KNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHK273

GR NL1 sequence is comprised of three clusters of basic amino acids, referred to as protoNLS-1, pNLS-2 and pNLS-3 (35,131). These basic sequences are highly conserved between GR and other SHRs and are sufficient to mediate nuclear accumulation when fused to a cytosolic protein (42,278,279). While the NL1 sequence does not directly require ligand for its activity, dissociation of the hsp-immunophilin complex is required to allow the NL1 to function in the context of full length GR (42,280). The core region of the NL1 sequence, pNLS-1, is absolutely required to mediate nuclear import and maps to amino acid residues 510-517 of the hinge region of rat GR (131). This sequence mediates nuclear import through direct binding to importin α (129,130). Mutation of key lysine residues ^{513}KKK^{515} results in the abrogation of importin α binding and the reduced accumulation of GR in the nucleus in response to steroid (130).

Basic residues within the DBD of GR and other SHRs are also thought to play a role in establishing nuclear localisation of the liganded receptor (35,131). Mutation of GR amino acid K490, found within pNLS-3, results in the partial abrogation of NL1-mediated nuclear import (131). This effect may be due in part to a loss of binding to DNA rather than a direct effect on nuclear localisation as the crystal structure of importin α reveals only two potential binding sites for basic clusters of amino acids (170). Although the pNLS-2 cluster is properly positioned to allow for interaction with the second binding pocket of importin α, mutations within pNLS-2 do not appear to have a direct effect on GR nuclear import as mutation at amino acids R496, R498 and K499 do not result in a decrease in NL1-mediated nuclear occupancy (47,131). However, mutation at residue R496 has been shown to affect GR subnuclear targeting. While

mutations at position R496 do not have any notable effect on GR import in the context of the full length receptor, when combined with deletion of the LBD, this mutation results in the formation of large nuclear foci (131).

Nuclear trafficking of GR may also be influenced by hsp90. While the hsp-containing complex is thought to mask NL1 function prior to ligand binding (42,280), it may also play a more direct role in actively retaining GR in the cytoplasm as has been demonstrated for the dioxin receptor (281). Stabilisation of receptor/hsp complexes inhibits ligand-dependent nuclear import of GR (274,280). This inhibitory effect on GR nuclear import may be a consequence of hsp-mediated targeting of GR to cytoplasmic microtubule networks (282), as inhibition of nuclear import is relieved by agents that disrupt the microtubule network (274). Interestingly, phosphorylation may also affect nuclear import. Inhibition of phosphatase activity during ligand withdrawal inhibits the re-import of GR upon a second hormonal stimulus (283). This inhibition of nuclear import by phosphatase inhibitors is alleviated by the disruption of microtubules, indicating that hyper-phosphorylation of GR may result in increased targeting of the receptor to cytoplasmic microtubule networks (284).

Removal of ligand results in the gradual return of GR to the cytoplasm. In contrast to the fairly well understood process of GR nuclear import, nuclear export of GR remains poorly characterised. Several studies have revealed that, in contrast to the rapid nuclear import of GR, nuclear export following ligand withdrawal occurs very slowly, with a $t_{1/2}$ of between 12 and 24 h (47,130,285,286). This slow rate of GR nuclear export may be influenced by DNA binding as a mutation at residue R496 has been observed to

increase the rate of redistribution of GR to the cytoplasm following ligand withdrawal (47).

Nuclear retention of GR through targeting to the nuclear matrix may influence the rate of GR redistribution to the cytoplasm (286,287). The association of the receptor with hsps may affect this process. Stabilisation of the interaction between GR and hsp90 in the presence of ligand by treatment with sodium molybdate results in a slow redistribution of the liganded receptor from the nucleus to the cytoplasm. The rate at which this redistribution occurs is similar to that observed when GR is withdrawn from ligand (280). Stabilisation of the GR-hsp90 complex also enhances the rate of nuclear export of ligand-withdrawn GR observed in permeabilised cells (286). Hsp70 and the receptor-associating protein 46 (RAP46) may potentially mediate GR nuclear retention. RAP46 is a co-chaperone that binds to GR through association with hsp70, resulting in the downregultion of GR DNA binding and transcriptional activity (288). Overexpression of GR results in a shift in the subcellular localisation of hsp70 from predominantly cytoplasmic to evenly distributed between the nucleus and cytoplasm. Binding of GR to hsp70 and RAP46 occurs through the GR NL1 sequence. Interestingly this shift in the subcellular localization of hsp70 is not observed in the presence of a mutant form of GR lacking the NL1 sequence (289). Additionally, hsp70 has been shown to colocalise with misfolded GR in nuclear foci. These foci are formed by the overexpression of a truncated form of GR lacking the LBD and containing a DNA binding mutation at amino acid residue R496 (131). These findings agree with previous observations that liganded GR binds to hsp70 (290,291) and suggest a possible nuclear

retention mechanism through subnuclear targeting of liganded GR by association with chaperone proteins.

The pathway through which GR is exported from the nucleus is as yet unclear. It has been suggested that GR nuclear export is dependent on the CRM1 export receptor (130), however these results are controversial (285). Nuclear export may be influenced by the phosphorylation state of the receptor. Inhibition of tyrosine phosphatase activity has been shown to enhance redistribution of ligand-withdrawn GR from the nucleus in a manner similar to the stabilisation of the interaction between GR and hsp90 (286). Interestingly, the pattern of GR phosphorylation is known to change upon ligand treatment (292-296) and through the cell cycle (297-301), raising the possibility that the cell cycle may influence GR nuclear export (298,302). Phosphorylation of GR by the c-Jun N-terminal kinase (JNK) has also been shown to influence GR nuclear export (303). However, the effect of JNK-mediated phosphorylation on GR export appears to be rather subtle (M. Adachi, personal communication) and may represent a specialised mechanism for the removal of active GR from the nucleus upon UV exposure rather than a general mechanism for GR export under normal physiological conditions.

GR nuclear export may be influenced by p53. p53 is a key tumor suppressor involved in many cellular functions, including cell cycle arrest, apoptosis and DNA repair [for reviews see (304-307)]. The subcellular localisation of p53 is tightly controlled through regulated nuclear import and export [reviewed in (308,309)]. It has recently been shown that inactive p53 is sequestered in the cytoplasm in a complex with Parc, a Parkin-like ubiquitin ligase (310). Overexpression of p53 results in the co-localisation of GR and p53 in the cytoplasm (311). The formation of this complex may target GR for

degradation (312). It is unclear whether the p53-dependent cytoplasmic localisation of GR is due to enhanced nuclear export or inhibition of nuclear import or both.

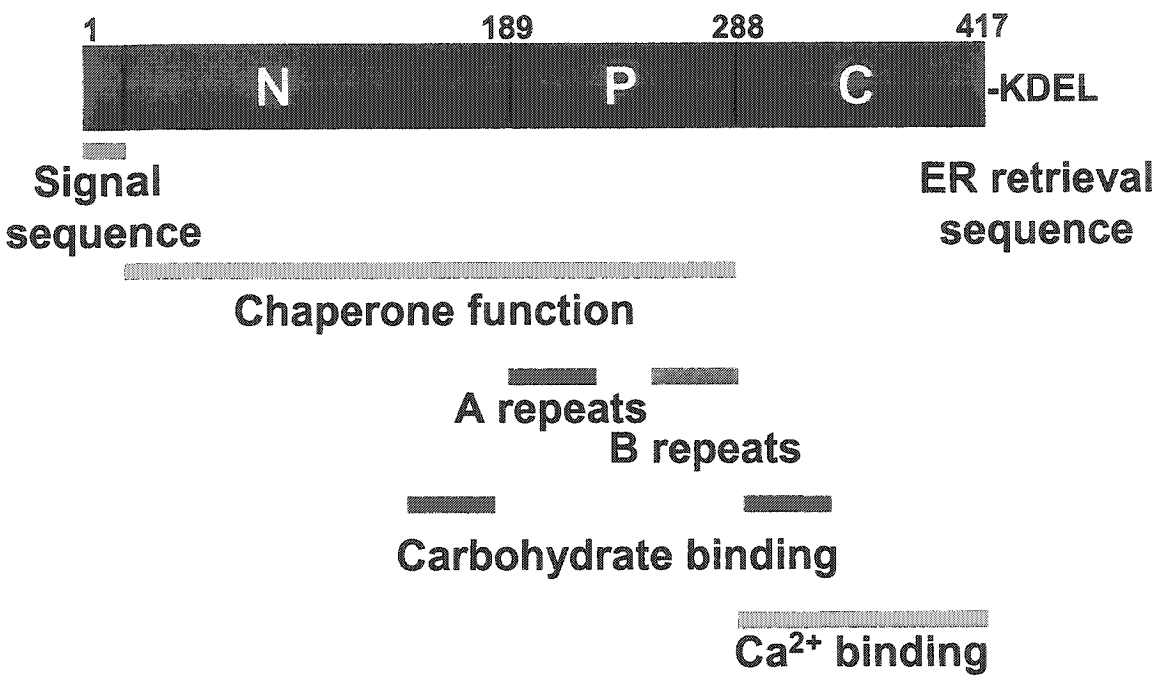
Calreticulin Function and Influence on GR Nuclear Export

Calreticulin is a protein localised to endoplasmic reticulum (313) that plays an important role in maintaining Ca^{2+} levels (314,315) and acts as a chaperone protein to ensure proper folding of glycosylated proteins, in particular immunoglobulins (316). Figure 5 provides a representation of the functional domains of calreticulin (CRT) [reviewed in (317)]. At the extreme N-terminus is a signal peptide sequence that mediates the translocation of CRT into the lumen of the endoplasmic reticulum and a retrieval sequence at the extreme C-terminus that maintains CRT in the lumen of the ER (313). The chaperone function of CRT is mediated primarily by the N domain and the P domain (315). The central P domain, which shares homology with the related endoplasmic reticulum chaperone protein calnexin, is particularly important for chaperone function. The P domain is comprised of two sets of short proline-rich repeats referred to as the A and B repeats. Three adjacent copies of each repeat are arranged in an AAABBB fashion. These repeats adopt a flexible hairpin-like structure that holds and shields maturing proteins (318). Carbohydrate binding is thought to be mediated by regions flanking the P domain (318). The C domain contains a high affinity, low capacity Ca^{2+} binding domain that allows CRT to function as a key regulator of Ca^{2+} homeostasis (315).

The generation of calreticulin knockout mice provided an indication that CRT may have some effect on nucleocytoplasmic trafficking. Homozygous deletion of CRT is

Figure 5: Representation of the modular structure of calreticulin

The modular organisation of calreticulin is indicated with the localisation of individual functional motifs summarised below. At the extreme N-terminus is a short signal sequence that mediates translocation of calreticulin into the endoplasmic reticulum. The N domain is unique to calreticulin and mediates binding to glycosylated proteins. The N domain is also required for chaperone function. The centrally located P domain plays an important role in mediating the folding of target proteins. The A and B maintain the P domain in the proper conformation required to function effectively as a chaperone. The C domain binds Ca^{2+} with high capacity and plays a role in Ca^{2+} storage within the endoplasmic reticulum. At the extreme C terminus is an ER retrieval sequence that mediates the retention of calreticulin within the lumen of the endoplasmic reticulum.


1
189
288
417
N
P
C
-KDEL
Signal sequence
ER retrieval sequence
Chaperone function
A repeats
B repeats
Carbohydrate binding
Ca2+ binding

embryonic lethal due to defects in cardiac development (319,320). This phenotype is attributable to a block in nuclear import of a key regulator MEF2C, and can be reversed by increasing the concentration of cytosolic Ca^{2+} (321). Nuclear import of the NF-AT3 transcription factor is also impaired in the absence of calreticulin (319). The observation that depletion of CRT affects nuclear import may be associated with the alterations in the Ca^{2+} storage capacity of the endoplasmic reticulum that occurs in $CRT^{-/-}$ cells (315) and is consistent with a previous finding that depletion of Ca^{2+} levels within the endoplasmic reticulum inhibits both passive diffusion and signal-mediated nuclear import (322). Since the outer membrane of the endoplasmic reticulum is continuous with the outer membrane of the nuclear envelope, alterations of the lumenal environment may directly affect properties of the NPC (322).

A possible connection between CRT and GR was first revealed through *in vitro* binding studies that suggested that there is a direct binding interaction between GR and CRT through a conserved KXFFKR calreticulin binding site (323,324). Overexpression of calreticulin was found to decrease the DNA binding and transactivation functions of both GR and AR (323), but not PPAR/RXR heterodimers (325), both of which bind CRT in vitro. GR-dependent transcription of the tyrosine amino transferase gene is decreased upon overexpression of CRT (326), however this effect may be indirect as overexpression of a form of CRT that is localised to the cytoplasm has a significantly lesser effect on GR-dependent transcription in comparison to the overexpression of CRT that is localised to the endoplasmic reticulum (327).

Recent reports have implicated calreticulin in GR nuclear export (228,229). CRT was identified as a cytosolic activity that mediates the export of PKI and GR (228,328).

It has been proposed to mediate the export of a number of nuclear hormone receptors including GR (229) in a manner that is sensitive to the depletion of intracellular Ca^{2+} (329). One challenge in defining the role of calreticulin in GR export has been to elucidate how a protein that is localised almost exclusively to the lumen of the endoplasmic reticulum (313) can directly act to influence the trafficking behaviour of a protein that is not targeted to the endoplasmic reticulum. It has been argued that although CRT is almost exclusively localised within the endoplasmic reticulum, there are sufficient levels of CRT present in the cytoplasm to allow for it to function in trafficking (228). However, this assertion is controversial as the methodology used to establish that CRT is significantly cytoplasmic subject the cell to relatively harsh centrifugal forces that could damage the endoplasmic reticulum resulting in leakage of lumenal proteins that are not membrane-associated (M. Michalak, personal communication).

Subcellular Trafficking is a Dynamic Process

The study of nucleocytoplasmic trafficking behaviour using fixed cells can lead to the impression that subcellular localisation is a static process, in which proteins are restricted to well-defined locations within the cell. Recent advances in imaging technology have challenged this view of protein trafficking, allowing the direct visualisation of protein function in real time. The development of fluorescent tags such as the green fluorescent protein (GFP) has allowed for the direct study of protein dynamics in a live cell. This has led to the direct demonstration that proteins are mobile to varying degrees within a subcellular organelle such as the nucleus, and may be subject to constant shuttling between organelles [for reviews see (330-335)].

A number of nuclear proteins have been shown to be mobile within the nucleus, ranging from heterochromatin binding proteins (336,337) to a subset of components of the basal transcription machinery such as TFIIB (338) and TFIIH (339) and the histone H1 subunit (340,341). Nuclear receptors share this property. Liganded GR and transcription factors in general, are thought to regulate gene activity through a series of transient interactions with DNA (342-344). This process appears to involve two distinct modes of association – a relatively short non-specific association and a longer, yet still transient, specific association with GREs (345) NHR intranuclear dynamics are affected by a number of different factors. For the estrogen receptor, addition of hormone agonist results in a decrease in intranuclear mobility, and addition of antagonist results in an even less-mobile receptor (346-348). In contrast, addition of ligand does not appear to affect the intranuclear movement of the thyroid hormone receptor (TR) or of the retinoic acid receptor (RAR). This is consistent with the fact that these receptors bind DNA in the absence of hormone (348). For GR, it has been suggested that the relative nuclear mobility of the receptor is affected more by the relative affinity of a particular ligand than by whether it acts as an agonist or antagonist (349). Additionally, it has been suggested that blocking GR turnover through inhibition of the 26S proteasome decreases the intranuclear mobility of GR, presumably through increased association of the receptor with the nuclear matrix (350).

A continuous shuttling mechanism between nucleus and cytoplasm is thought to underlie the apparently static nature of GR localisation. Guiochon-Mantel and colleagues first demonstrated continuous nucleocytoplasmic shuttling of a predominantly nuclear receptor for the progesterone receptor in 1989 (39). This finding was based on the use of

the heterokaryon cell fusion assay, which involves the fusion of two distinct cell populations – one that expresses the protein of interest and one that does not. Following cell fusion, nuclear export is inferred by the detection of the protein of interest in the nucleus that did not originally express that protein. The initial findings of Guiochon-Mantel *et al.* were soon followed by reports indicating that a number of receptors that are predominantly localised to the nucleus appear to shuttle between nucleus and cytoplasm (40,351-355) including GR bound to both hormone agonist (353) and antagonist (354). The rate of this shuttling process in heterokaryon fusions appears to be quite rapid, as equilibration of GR between heterokaryon nuclei generally occurs within 1 h (353,354). These findings suggest that nuclear receptor trafficking is a dynamic process where the net localisation of the receptor is determined by the relative rates of import and export.

Continuous shuttling of a nuclear protein would allow for rapid and direct communication with cytosolic signalling pathways (356). For proteins such as STAT1, prolonged activity is maintained only if the protein is able to continuously interact with signalling pathways resident in the cytoplasm through shuttling. This provides a means of ensuring that active STAT molecules in the nucleus can be rapidly shut down following the removal as extracellular signal (206). The function of GR shuttling remains elusive. It has been proposed that GR is preferentially degraded in the cytoplasm (285). A complicating issue in understanding the apparently rapid shuttling of nuclear GR is the slow redistribution to the cytoplasm that is observed following ligand withdrawal. Following ligand withdrawal, re-association of GR with heat shock proteins occurs within 30 min (354,357). Since hsp association blocks nuclear import (42,280), removal of ligand would be expected to result in rapid GR export. This suggests that GR nuclear

export may be subject to additional regulatory mechanisms, which could include active nuclear retention. While many different proteins have been implicated in the control of GR export, nuclear retention and shuttling, the precise mechanisms controlling these processes remain unclear. Clarification of the interplay between these processes will provide valuable insight into the control of GR signalling.

Project Goal

The regulation of GR trafficking has a direct impact on GR activity. While the process of GR nuclear import is fairly well understood GR, nuclear export remains largely uncharacterised. Thus, the principle goal of my Ph.D. project was **to study the mechanism of GR nuclear export and to identify signals that modify the rate of GR nucleocytoplasmic shuttling.** To accomplish this aim, I have relied primarily on a novel methodology that I have developed to study protein trafficking in live cells without the need for cell fusion. This offers the benefit of studying protein trafficking in real time under cellular conditions that more closely resemble the normal physiological environment.

Materials and Methods

Plasmids

The pNES-GFP-PK-NLS plasmid was a gift of Dr. Stephen Lee and has been described elsewhere (201,358). The pGST-GFP-NLS plasmid expresses a fusion protein comprised of glutathione s transferase (GST) and green fluorescent protein (GFP), with the sequence of the SV40 NLS at the C-terminus. This expression vector was derived from the FVHL-GFP vector described by Lee *et al.* (358). The sequence encoding VHL was removed by restriction digest and replaced with an oligonucleotide linker. Oligonucleotide sequences for all primers used in plasmid subcloning are listed in Appendix A. The GST cDNA was amplified by PCR using the proofreading polymerase Vent (New England Biolabs) and then cloned N-terminal to the GFP coding sequence to produce pGST-GFP-NLS. pNES-GST-GFP-NLS was directly derived from pGST-GFP-NLS by inserting an oligonucleotide encoding the HIV Rev NES sequence N-terminal to the GST coding sequence by linker tailing (359). For both plasmids, positive clones were identified by screening with restriction enzymes followed by sequencing to ensure that the reading frame of each construct was correct. Western blotting was performed using the GFP antibody JL8 (Clontech) to verify the expected size of each expression product.

The pGFP-GR and pGFP-GR_{NL1-} plasmids used in this study were derivatives of the GFP-labelled GR constructs described by Savory *et al.* (130). These previously derived GFP-GR plasmids, denoted herein as pGFP-$GR_{\Delta 1\text{-}20}$ and pGFP-$GR_{NL1\text{-}/\Delta 1\text{-}20}$, lack the first twenty amino acids of the GR coding sequence. To create plasmids encoding full length GR, pGFP-$GR_{\Delta 1\text{-}20}$ and pGFP-$GR_{NL1\text{-}/\Delta 1\text{-}20}$ were digested with XhoI and SalI to

remove the N-terminal portion of GR spanning amino acids 20-153. Oligonucleotide primers were used to PCR amplify the complete GR N-terminus using Vent Polymerase and p6RGR as a template (23). The 5' primer used incorporated a XhoI restriction site and the 3' primer used amplified GR from downstream of the SalI site present at amino acid residue S153. This PCR product was digested with XhoI and SalI and then ligated to the pGFP-$GR_{\Delta 1\text{-}20}$ and pGFP-$GR_{NL1\text{-}/\Delta 1\text{-}20}$ XhoI / SalI digests to create full length pGFP-GR and pGFP-$GR_{NL1\text{-}}$. Positive clones were identified by restriction digestion and Western blotting was performed to ensure that the protein product was of the expected size.

pGFP-$GR_{F463,464A,}$ pGFP-GR_{R496H} and pGFP-GR_{C500Y} were derived from full-length pGFP-GR. Complementary oligonucleotides encoding the desired mutations were synthesised (Gibco BRL) and purified on a non-denaturing polyacrylamide gel followed by gel extraction. The purified oligonucleotides were then used to introduce the desired mutations using the Stratagene QuikChange™ mutagenesis kit and the Pfu proofreading polymerase (Stratagene). Similarly, pGFP-$GR_{NL1\text{-}/F463,464A}$ was cloned using the QuikChange™ mutagenesis kit using the full-length pGFP-$GR_{NL1\text{-}}$ construct as a template. Mutations were confirmed by restriction digest and sequencing.

The pDM128, pRev-Rex and pRev-IκB$\alpha_{1\text{-}72}$, plasmids used in the HIV-Rev complementation assay were the kind gift of Dr. Thomas Hope (214). The pRSV β-gal plasmid used has been previously described (360). pRev-GR was derived from pRevΔNES (214). The full length GR coding sequence was PCR amplified from p6RGR with Vent polymerase using primers encoding BglII and XbaI restriction sites and inserted into pRevΔNES to produce pRev-GR. Sequencing of the N-terminal junction

was performed to verify that the GR cDNA was cloned in the correct reading frame. Western blotting using the GR Ma10 antibody (Affinity Bioreagents) was performed to verify that the expression product was of the correct size. Transcriptional activity of the pRev-GR expression construct in 293T cells was verified by co-transfecting 1 μg of pRev-GR with 200 ng of pRSV β-gal and 200 ng of the pHC17 MMTV plasmid, which contains the CAT reporter gene, by calcium phosphate. Twenty-four hours following the start of transfection, cells were treated with 1 μM dexamethasone for a further 24 h, harvested and assayed for CAT activity using the protocol of Seed and Sheen described below (361).

The pNES-$GR_{407\text{-}556}$-GFP-NLS and pNES-$GR_{407\text{-}556NL1\text{-}}$-GFP-NLS plasmids were both constructed from the pNES-GST-GFP-NLS plasmid. The pNES-GST-GFP-NLS plasmid was digested with EcoRV and XhoI to remove the GST moiety. The $GR_{407\text{-}556}$ and $GR_{407\text{-}556NL1\text{-}}$ cDNAs were PCR amplified using Vent polymerase from the p6RGR template and primers encoding the appropriate restriction enzyme sequences. The $GR_{407\text{-}556}$ and $GR_{407\text{-}556NL1\text{-}}$ sequences were then cloned N-terminal to the GFP coding sequence to produce pNES-$GR_{407\text{-}556}$-GFP-NLS and pNES-$GR_{407\text{-}556NL1\text{-}}$-GFP-NLS respectively. Positive clones were identified by restriction digestion and western blotting was performed to ensure that the expression products were of the expected size. Similarly, pNES-GR_{WT}-GFP-NLS and pNES-$GR_{NL1\text{-}}$-GFP-NLS full length GR constructs were also derived from the pNES-GST-GFP-NLS vector. The GR_{WT} and $GR_{NL1\text{-}}$ cDNA sequences were PCR amplified from p6RGR and cloned in place of GSTN-terminal to the GFP coding sequence.

The pNES-GST-$GR_{500\text{-}525}$-GFP-NLS, pNES-GST-$GR_{500\text{-}525NL1}$-GFP-NLS and pNES-GST-$GR_{525\text{-}500}$-GFP-NLS plasmids were all based upon the pNES-GST-GFP-NLS plasmid. pNES-GST-GFP-NLS was digested with XhoI and the $GR_{500\text{-}525}$, $GR_{500\text{-}525NL1}$-, and $GR_{525\text{-}500}$ peptides were all generated by PCR amplification from p6RGR using primers encoding XhoI recognition sequences. The PCR products were digested with XhoI and then cloned into the pNES-GST-GFP-NLS backbone C-terminal to the GST sequence. Positive clones were selected by restriction digestion, and Western blotting confirmed that the expression products were of the expected size.

Large Scale Plasmid DNA Preparation

Large scale preparation of DNA for transfection was carried out using a standard alkaline lysis protocol followed by centrifugation on a CsCl gradient. Bacterial cultures of *E. coli* DH5α containing the plasmid of interest were grown at 37°C in selective LB media. Following overnight incubation, bacteria were harvested by centrifugation at 4500 × g. The bacterial pellet was resuspended in GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0) and incubated in lysis solution (0.2 M NaOH, 1% SDS) for 10 min on ice. Following 10 min incubation on ice in potassium acetate solution (3 M K^+, 5 M OAc), the solution was centrifuged at 15 000 × g. Plasmid DNA was precipitated from the supernatant with isopropanol and then centrifuged at 4000 × g. The pellet was resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and precipitated with an equal volume of 5 M LiCl to remove excess RNA. Following centrifugation at 4000 × g, three volumes of 100% ethanol were added to the supernatant. The precipitated plasmid

DNA was collected by centrifugation at 4000 × g and resuspended with CsCl (1.1 g/mL) in TE containing ethidium bromide (20 μg/mL). The refractive index of this solution was between 1.397 and 1.399. This solution was transferred to a polyallomer tube, sealed and centrifuged at room temperature overnight at 95000 rpm in a Beckman TLN 100 fixed angle rotor. The DNA band was removed and then centrifuged at 95000 rpm on a second CsCl gradient for 6 h. The DNA band was removed and extracted with salt/water saturated isopropanol to remove ethidium bromide. Following dialysis with TE buffer the plasmid DNA was precipitated with NaOAc and 100% ethanol and stored at -20°C until use.

Small Scale Plasmid DNA Preparation

Small scale plasmid preparation for the purpose of screening potential clones was carried out using a modified version of the large scale plasmid preparation described above. Briefly, 1.5 mL *E. coli* DH5α cultures containing potential clones were grown in selective LB media. Following overnight incubation the bacteria were collected by centrifugation at 13000 × g and resuspended in GTE. Lysis buffer was added followed by potassium acetate solution. The precipitate was removed by centrifugation at 13000 × g and the supernatant was extracted with an equal volume of 25:24:1 phenol:chloroform: isoamyl alcohol (v/v/v). Plasmid DNA was precipitated with 100% ethanol, resuspended in TE containing RNAseA (20 μg/mL) and stored at -20°C.

Cell Culture and Transient Transfection

Cos7 (ATCC #CRL-1651) and HeLa (ATCC #CCL-2) cells were maintained in high glucose Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% FBS and non-essential amino acids. 293T cells (obtained from Dr G. Nolan, Stanford University) were maintained in DMEM supplemented with 10% FBS. Lipofectamine™ (Invitrogen) was used, using 10 µL of Lipofectamine™ per 60 mm dish to transiently transfect Cos7 cells. For each plate, between 0.5 and 1.0 µg of DNA was transfected, as indicated in the Figure legends. Prior to transfection, cells were rinsed twice in PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2PO_4 \bullet 7H_2O$, 1.5 mM KH_2PO_4) and once in antibiotic-free Opti-MEM™ reduced serum media (Invitrogen). All Lipofectamine transfection mixtures were prepared in Opti-MEM™. Following 45 min incubation at room temperature to allow for complex formation, the transfection mixture was added to the cell media. The cells were incubated with the transfection mixture at 37°C for 16 h. The transfection was then stopped by adding an equal volume of phenol red-free DMEM supplemented with 20% stripped FBS, such that the final concentration of FBS was 10%.

To transiently transfect HeLa cells, ExGen500™ (MBI) was used at a ratio of 8 µL ExGen500™ reagent per µg DNA. Prior to transfection the cells were rinsed twice in PBS and once in OptiMEM™. The ExGen transfection mixtures were prepared in 150mM NaCl and incubated at room temperature for 10 min to allow complex formation. The cells were incubated with the transfection mixture at 37°C for 4 to 6 h. To stop the transfection, an equal volume of phenol red-free DMEM supplemented with 20% stripped FBS was added.

The calcium phosphate method was used to transiently transfect 293T cells. Prior to transfection, the cell media was changed to complete media containing 25 μM chloroquine to improve transfection efficiency by inhibiting the lysosomal pathway. DNA solutions were diluted in $CaCl_2$ at a final concentration of 250 mM $CaCl_2$ in 500 μL. An equal volume of 2X HBS (50 mM HEPES, 50 mM KCl, 280 mM NaCl, 1.5 mM Na_2HPO_4, 0.22% dextrose) was added and the tube was immediately mixed by inversion. The transfection mixture was added to the cells in a dropwise fashion and mixed. Following incubation at 37°C for 16 h the transfection media was removed and replaced with complete media.

SDS-PAGE and Western Blotting

To prepare cell extracts for Western analysis, transfected cells were harvested 48 h post-transfection. Each 60 mm dish was rinsed once in PBS and the cells were scraped into 1 mL PBS using a rubber policeman. Cells were collected by centrifugation at 2000 × g for 2 min at 4°C and then resuspended in TEDG buffer (10 mM Tris pH 7.4, 1 mM EDTA, 10% glycerol, 1mM DTT, 1X Complete™ protease inhibitor cocktail (Roche)). The cell pellet was incubated on ice for 10 min followed by three freeze-thaw cycles. The extract was cleared by centrifugation at 13000 rpm at 4°C and then assayed for protein concentration using the Bio-Rad Protein Assay reagent according to the manufacturer's instructions. Protein samples were loaded onto a SDS-PAGE gel prepared according to the method of Laemmli (362). Typically 50-100 μg of protein was used per lane.

The SDS-PAGE gel was electophoresed in electrode buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and then transferred in transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS) to a PVDF membrane (BioRad) prepared according to the manufacturer's instructions. The blot was then blocked for 1 h at 20°C in PBST (140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2PO_4 \bullet 7H_2O$, 1.5 mM KH_2PO_4, 0.05% Tween, pH 7.4) containing 5% w/v skim milk powder. Primary antibody was diluted according to the manufacturer's recommendation in PBST and incubated for either 1 h at 20°C or overnight at 4°C. Excess primary antibody was removed by washing three times in PBST and then the blot was incubated for 1 h at 20°C with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in PBST. The blot was washed three times in PBST and then developed using the Western Lightning™ chemiluminescence reagent (Perkin Elmer) according to the supplier's protocol. In situations where more stringent washing was required due to high background signal, TBST (20 mM Tris, 500 mM NaCl, 0.5% Tween, pH 7.4) was used in place of PBST.

Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss of Imaging in Photobleaching (FLIP) Assays.

Cos7 or HeLa cells were seeded onto 40 mm round coverslips (Bioptechs) and transfected with 1 μg of the indicated GFP-tagged expression construct using 10 μL Lipofectamine™ (Invitrogen). Following overnight incubation in OptiMEM™ reduced serum media (Invitrogen) the transfection was stopped by addition of charcoal stripped serum. Cells were cultured in complete serum for 8 h and then in serum-free medium for

16 h prior to treatment. Cycloheximide (20 μg/μL) was added 1 h prior to FRAP. Treatment with ligand at 1 μM or leptomycin B at 10 nM was initiated 1 h prior to analysis. To initiate steroid withdrawal, cells were rinsed four times in PBS and then twice in serum-free media. This rinsing cycle was repeated after a 5 min incubation at 37°C. After a second rinsing cycle followed by a 5 min incubation at 37°C, the cells were rinsed a final time in serum-free media and incubated at 37°C for the indicated withdrawal period.

To initiate the FRAP nuclear export assay, coverslips were placed in a Bioptechs FCS2 environmental chamber maintained at 37°C and visualised on a BioRAD MRC 1024 confocal microscope. Bi-nucleated cells were identified by visual inspection of the cell population. Once identified, an image of the unbleached cell was obtained at 3% laser power. Using the zoom function of the LaserSharp image acquisition software program, one nucleus was selected for photobleaching. The fluorescent signal was completely ablated by exposing the selected nucleus to five to ten laser pulses at 100% laser power (approximate power output = 20 mW). Each experiment included a minimum of three independent trials performed over several months with each involving 5-50 individual repetitions of FRAP.

To quantify the amount of recovery observed following photobleaching, the J-Image software package (National Institutes of Health) was used. Using the image analysis tools provided within the software package, the regions of the image representing both the donor and acceptor nuclei were defined manually. The mean pixel intensity within each nucleus was measured and corrected for background levels by subtracting the mean pixel intensity obtained for a randomly selected area within a blank

portion of the same image. The percentage of recovery within the acceptor nucleus was determined using the following formula:

$$\% \text{ recovery} = [F_{\text{acceptor nucleus}} / (F_{\text{donor nucleus}} + F_{\text{acceptor nucleus}})] \times 100$$

where F represents the mean pixel intensity within the specified nucleus. Using this formula, at equilibrium recovery is expected to plateau at 50%.

For FLIP experiments, Cos7 cells were transfected with the indicated GFP-labelled constructs and treated as described for FRAP analysis. Discrete portions of selected nuclei were subjected to a single laser pulse at 100% power and then immediately imaged at 3% laser power. This cycle was repeated at 30 sec intervals and images were recorded over a period of 5 min.

Digitonin Permeabilisation Export Assay

Cos7 cells were transiently transfected with the indicated expression constructs using Lipofectamine™ as described previously. Complete media was added to the cells 16 h after beginning transfection. After a further 24 h incubation in complete media the cells were treated for 1 h with 1 μM cortisol. The cells were then rinsed three times in ice cold transport buffer (20 mM HEPES pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, 0.5 μg/mL leupeptin, 1.0 μg/mL aprotinin). Digitonin was added to a final concentration of 50 μg/mL and the cells were incubated for 5 min at 4°C. Cells were then rinsed three times in transport buffer, incubating 5 min at 4°C between each wash. To initiate nuclear export, HeLa cell lysate

containing 20 U/mL creatine phosphokinase, 5 mM creatine phosphate, 2 mM ATP and 2 mM GTP was then added to each plate. Cells were placed at 20°C and export was monitored on a Zeiss Axiovert S100TV microscope equipped with an Empix digital charge-coupled device (CCD) camera using Northern Eclipse software.

HIV Rev Complementation Assay.

Analysis of HIV Rev co-transport of CAT encoding RNA in 293T cells was performed following the protocol outlined by Hope and colleagues (214,363,364). Cells were seeded into 35 mm dishes and co-transfected by calcium phosphate with 1 μg of the indicated RevΔNES fusion constructs (IκB NES, HIV Rex NES and GR) 200 ng of pRSV β-gal and 200 ng of the pDM128 CAT reporter. Sixteen hours after starting the transfection cells were treated with 1 μM dex as indicated for a further 24 h period. Cells were harvested and resuspended in 100 μL FT buffer (25 mM sucrose, 10 mM Tris pH 7.4, 10 mM EDTA). Following three freeze-thaw cycles the samples were centrifuged at 13000 × g to remove cellular debris and the supernatants were collected.

CAT activity was measured by liquid scintillation using the method of Seed and Sheen (361). Briefly, 10-50 μL cell extract was incubated at 65°C for 10 min to eliminate endogenous deacetylase activity. 2 μL of ^{14}C chloramphenicol (0.05 mCi/mL) and 5 μL n-Butyrl CoA (5 mg/mL in 0.25 M Tris-HCl pH 7.8) were then added and 0.25 M Tris-HCl pH 7.8 was added to a final volume of 125 μL. Following incubation at 37°C for 2 h 300 μL of mixed xylenes was added. The samples were vortexed for 30 sec and then centrifuged for 3 min at maximum speed to separate the phases. The upper

organic phase (250 μL) was transferred to a new tube and back extracted by adding 100 μL of 0.25 M Tris-HCl. The samples were again vortexed for 30 sec and centrifuged for 3 min at maximum speed. The upper phase (200 μL) was subjected to liquid scintillation counting. CPM values specific to the n-butyrylated chloramphenicol were obtained by subtracting the CPM values obtained for a blank sample containing no cell extract and then normalised to β-galactosidase (β-gal) activity to account for potential variations in transfection efficiency. To determine β-gal activity 40 μL cell extract was incubated with 80 μL ONPG (o-nitrophenyl-β-D-galactose, 40 mg/mL in 100 mM phosphate buffer pH 7.0) and Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 50 mM β-mercaptoethanol, pH 7.0) to a final volume of 480 μL. Samples were incubated at 30°C until the samples had turned light yellow. The reaction was stopped with 1 M Na_2CO_3 (200 μL) and the absorbance of each sample was read at 420 nm. Error bars represent the standard error of the mean of a minimum of three independent experiments performed in duplicate.

Homokaryon Cell Fusion

Cos7 cells were separately transfected with the dsRed-C1 expression construct or the indicated GFP constructs. Following transfection cells were cultured in complete serum overnight and harvested by trypsinization. Cells expressing dsRed-C1 were mixed 1:1 with cells expressing the indicated GFP construct and seeded onto 40 mm round coverslips. Cells were allowed to attach for 8 h and then withdrawn from serum for 16 h. Plates were coded using a double blind encryption technique to avoid introducing a bias

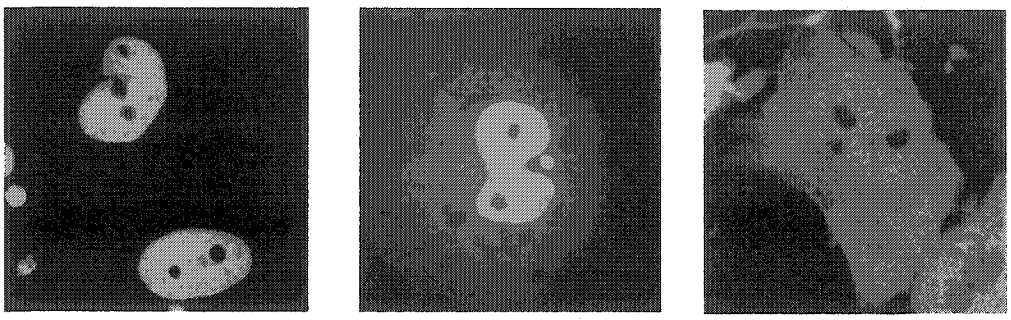
when collecting images. One hour prior to initiation of cell fusion, all cells were treated with 1 μM cortisol. Cell fusion was initiated by incubation with 50% w/v PEG-4000 in Ca^{2+}/Mg^{2+} free Hanks balanced salt solution (HBSS) for 2 min at 37°C. Cells were then washed five times in Ca^{2+}/Mg^{2+} free HBSS and incubated at 37°C with serum-free DMEM for 1 h. The cells were then placed in a Bioptechs FCS2 environmental chamber maintained at 37°C and visualised on a Nikon TE300 microscope. Live cell images were obtained using an Orca ER camera (Hamamatsu) and C-imaging software (Compix).

Quantification of Subcellular Distribution and Indirect Immunofluorescence

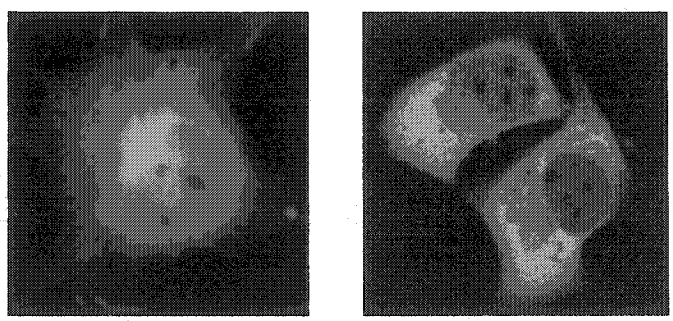
For direct analysis of subcellular distribution, Cos7 cells were transfected using Lipofectamine™ as previously described. Following transfection, cells were cultured in complete serum overnight and then seeded onto 22 mm square coverslips. Cells were allowed to attach for 8 h and then withdrawn from serum for 16 h. Cells were treated with ligand at 1 μM and then withdrawn from ligand as described for FRAP for the indicated time period. Following ligand withdrawal, cells were fixed with 3% paraformaldehyde for 30 min at 4°C followed by incubation with PBS containing 0.2 M glycine for 10 min at 20°C. Coverslips were mounted onto microscope slides, overlaid with 50% glycerol in PBS and sealed with nail polish. Cells were scored into five categories from exclusively nuclear (N) to exclusively cytoplasmic (C) as previously described (130). Representative images corresponding to each of these five categories are shown in Figure 6. Quantification was performed using double-blind encryption with individual data points derived from a minimum of 1000 cells quantified over four independent experiments performed in duplicate.

Figure 6: Representative images of subcellular localisation categories

To quantify subcellular localisation, a semi-quantitative approach based on the categorisation of nucleocytoplasmic distribution within individual cells was employed. Images shown are representative of the five categories utilised, ranging from exclusively nuclear (N) to exclusively cytoplasmic (C).



N N > C N = C



C > N C

For indirect immunofluorescence, cells were transfected and processed as described for direct analysis of subcellular distribution. Following fixation in 3% paraformaldehyde and incubation with 0.2 mM glycine in PBS, cells were permeabilised by incubation with Triton X-100 (0.5% in PBS) for 30 min at 20°C. Cells were blocked with 5% normal goat serum in PBS for 1 h at 20°C and then incubated at 4°C in primary antibody diluted according to the manufacturer's recommendation in PBS. Following overnight incubation, the coverslips were washed three times in PBS and then incubated at 20°C for 45 min with rhodamine red-X conjugated secondary antibody (Jackson Immunoresearch Laboratories) diluted in PBS [1/150 (v/v)]. Cells were mounted onto glass coverslips as described for direct analysis of subcellular distribution. Following double-blind encryption subcellular distribution was quantified as described previously (47,130).

Analysis of Calreticulin and Calnexin Exposure

Selective permeabilisation experiments were performed using a modification of the protocol of Du et al (365). Cells were plated onto 22 mm square coverslips and treated as described were fixed with 3% paraformaldehyde in PBS for 30 min at 4°C, followed by incubation with PBS containing 0.1 M glycine for 10 min at 20°C. Permeabilisation with Triton X-100 (0.5% in PBS) was for 30 min at 20°C. For streptolysin O (SLO) permeabilisation, cells were incubated with SLO (250U/mL) in BBII buffer (25 mM HEPES pH 7.5, 75 mM KAc) for 15 min on ice. Cells were washed once in cold BBII to remove unbound SLO and then incubated at 37°C for 15 min. Following permeabilisation, cells were pre-blocked with 5% IgG-free BSA in PBS for 1

h at 20°C. Cells were then incubated overnight at 4°C with primary antibodies to calreticulin (C-17, Santa Cruz) and calnexin (H-70, Santa Cruz) diluted in PBS [1/150 (v/v)] containing 5% IgG-free BSA. Following three washes with PBS, cells were incubated at 20°C for 45 min with secondary antibody conjugated to rhodamine red X (Jackson Immunoresearch Laboratories) diluted in PBS [1/150 (v/v)] containing 5% IgG-free BSA. Cells were washed three times in PBS and then mounted on microscope slides. Images were recorded with an Orca ER camera (Hamamatsu) on a Nikon TE300 microscope using C-imaging software. For each primary antibody the optimal exposure time required to record an image following Triton X-100 permeabilisation was determined. This exposure time was used to gather all subsequent images within that repetition.

Results

Establishment of an in vivo Assay to Study Nucleocytoplasmic Shuttling

Previous results based on the heterokaryon cell fusion assay had suggested that liganded nuclear hormone receptors, including GR, shuttle rapidly between nucleus and cytoplasm despite being predominantly localised to the nucleus (39,351-355). While cell fusion-based systems have been used extensively to study the trafficking behaviour of nuclear proteins, the fact that these assays rely on membrane fusion represents a limitation to this technique. Membrane fusion is induced by treatment with relatively harsh detergents over a period of several minutes, which could potentially alter the cellular environment in a manner that is not representative of the physiological state. Further, in order to detect nuclear export using fusion-based techniques, a recovery period is required. This recovery period restricts the ability to detect rapid export events using a fusion-based system. As my goal was to study rapid and dynamic movement of GR between the nucleus and cytoplasm, I sought to develop an assay system that would allow me to measure nucleocytoplasmic trafficking in real time based on a fluorescence recovery after photobleaching (FRAP) based assay.

The development of variants of the *Aequorea victoria* green fluorescent protein (GFP) suitable for imaging in live cells has led to the development of several experimental strategies designed to study intracellular protein movement [for reviews see (334,366)]. Prolonged excitation of GFP results in the complete and irreversible loss of fluorescence signal. This process is referred to as photobleaching. By monitoring changes in the intensity of the level of GFP within a defined area of a cell following

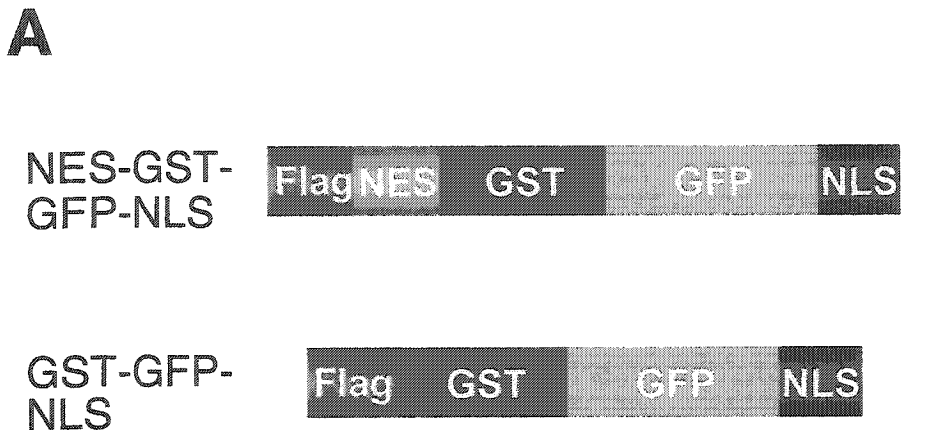
photobleaching, movement of a protein within a target subcellular region can be monitored live and in real time. Photobleaching techiniques were first used by McNally and colleagues to demonstrate that liganded GR is mobile within the nucleus (344). By adapting the methodology used by McNally *et al.*, I have developed a FRAP-based nuclear export assay that allows for the detection of rapid and dynamic nuclear export in live cells.

The FRAP-based nuclear export assay that I have developed takes advantage of a sub-population of cells present in most established cell lines that are maintained in a multinucleated state. Multinucleated cells are thought to result from the de-coupling of DNA replication and cell division that can occur in immortalized cell lines. The FRAP-based export assay has the advantage of allowing real-time visualisation of protein trafficking in live cells under conditions that more closely resemble a physiological environment. Following transient transfection with a GFP-labelled construct, cells are placed in a live cell chamber and visualised by confocal microscopy to identify bi-nucleated cells. The confocal laser is then focused exclusively on one nucleus. The GFP signal within that nucleus can then be completely ablated by scanning the nucleus at maximum laser power. Active nuclear export is indicated by the re-appearance of GFP signal in the bleached nucleus following ablation of GFP fluorescence by photobleaching. The addition of cycloheximide to prevent *de novo* protein synthesis ensures that recovery of fluorescence in the bleached nucleus reflects the transfer of GFP-labelled protein from the unbleached donor nucleus to the photobleached acceptor and not synthesis of new GFP-labelled receptor.

To establish the reliability of this assay, I first tested the trafficking behaviour of two control constructs (Fig. 7a). As a positive control, a pNES-GST-GFP-NLS fusion construct was prepared. This construct contains the leucine rich CRM1-dependent NES from the HIV Rev protein fused N-terminal to the full length GST protein. The GFP protein is located C-terminal to the GST moiety, and at the extreme C-terminus is the nuclear localisation sequence from the SV40 large T antigen that mediates the initial nuclear import of this fusion protein. The pNES-GST-GFP-NLS fusion construct produces a 55kDa protein product that was expected to be larger than the uppermost estimated limit for passive diffusion through the NPC of approximately 40 kDa (132). Western blotting confirmed the production of a 55kDa protein, thus it is unlikely that any observed transfer between nuclei is the result of passive diffusion. When subjected to FRAP analysis, this positive control construct was found to equilibrate completely between nuclei within 10 min after photobleaching (Fig. 7b, upper panel), suggesting that the NES-GST-GFP-NLS fusion protein trafficks rapidly between nucleus and cytoplasm through rapid nuclear export. To show that this observed behaviour was the result of CRM1-mediated nuclear export and not a consequence of possible cell damage resulting from exposure to the confocal laser, the effect of adding LMB was tested. LMB is a specific inhibitor of CRM1-mediated nuclear export. In the presence of LMB, I observed a minimal appearance of GFP signal in the bleached nucleus up to 30 min following photobleaching (Fig. 7b, middle panel). This confirmed that the observed equilibration of GFP signal between nuclei is a consequence of active nuclear export of the GFP fusion protein and not due to photodamage or diffusion. To further demonstrate that photobleaching does not damage the cell in a way that promotes specific protein export

Figure 7: FRAP based analysis of nucleocytoplasmic trafficking of synthetic GFP constructs in multinucleated Cos7 cells

(A) Representation of the functional features of the control proteins employed to establish the FRAP based nucleocytoplasmic trafficking assay. (B) Cos7 cells transiently transfected to express the indicated constructs were treated as indicated in a Bioptechs FCS2 environmental chamber maintained at 37°C on a BioRAD confocal microscope (Before). The GFP signal in one nuclei of a multinucleated cell was irreversibly bleached by focusing a laser on this nucleus and adjusting the power to the maximum output (T_0). Fluorescence recovery at the times indicated is shown in the third panel of each series. All experiments were performed in the presence of 20 μg/mL cycloheximide to prevent de novo protein synthesis.


A
NES-GST-GFP-NLS
Flag
NES
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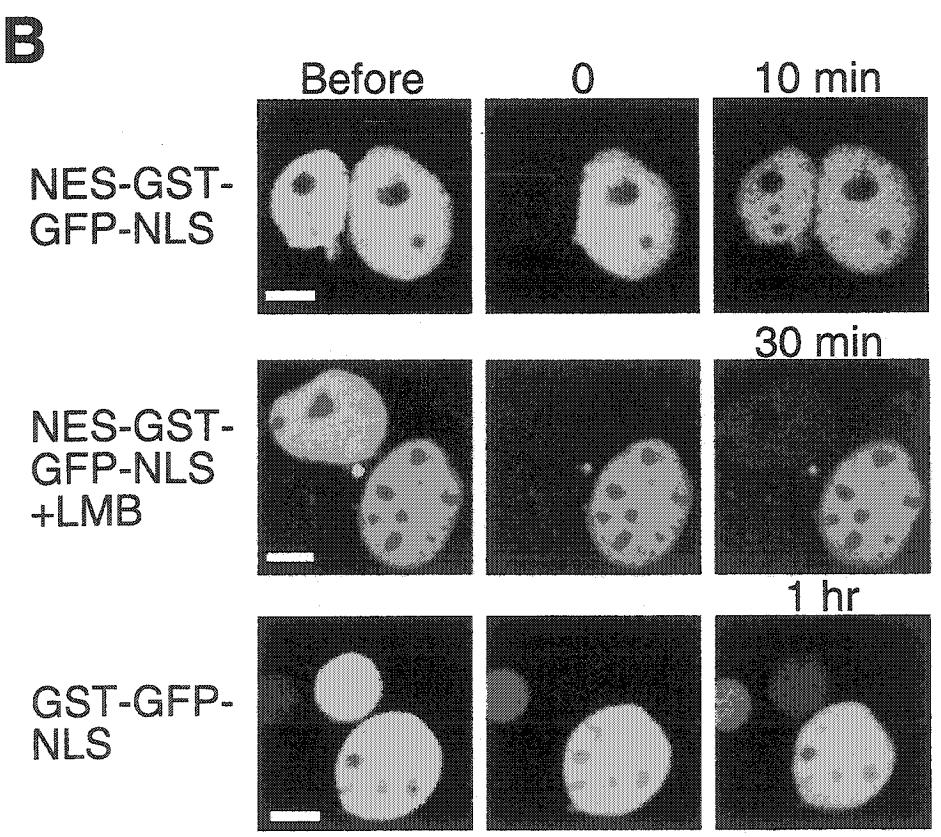
GFP
NLS
GST-GFP-NLS
Flag
GST
GFP
NLS


B
Before
0
10 min
NES-GST-GFP-NLS
30 min
NES-GST-GFP-NLS +LMB
1 hr
GST-GFP-NLS

from the nucleus, a negative control construct lacking the Rev NES was tested. pGST-GFP-NLS is identical to pNES-GST-GFP-NLS except that it lacks an NES motif (Fig. 7a). At up to 1 h following photobleaching minimal GFP signal was detected in the bleached nucleus (Fig. 7b, bottom panel). This result indicates that in this assay nuclear export is dependent on the presence of a NES. Together these three experiments establish that the FRAP-based nuclear export assay is able to accurately recapitulate the expected trafficking behaviour of control constructs.

GR Nuclear Export is a Slow Process

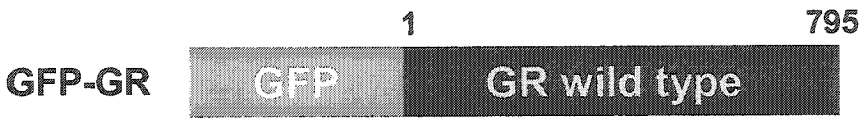
Having established a system to study nuclear export in live cells, I then examined the trafficking behaviour of GR in this assay. Cos7 cells were transiently transfected with the GFP-GR_{wt} construct (Fig. 8a) and treated with 1 μM cortisol to induce complete nuclear localisation of the receptor (Fig. 8b, upper panel). Cycloheximide was included at 20 μg/mL to ensure that any recovery of GFP signal observed was the result of nuclear transfer and not *de novo* synthesis of receptor. Based on previous observations obtained using the heterokaryon cell fusion assay, I had expected to detect equal distribution of GFP-GR signal between nuclei within 1 h after photobleachng, indicative of rapid nuclear export. Unexpectedly, no significant recovery of GFP signal was observed in the bleached nucleus 1 h after photobleaching. Further, at 4 h following photobleaching only a modest transfer of GFP fluorescence of 19% ± 6% was observed. This observation suggests that the rate of constitutive nuclear shuttling for the liganded receptor might be slower than originally suggested.

To confirm that this finding was not limited to the Cos7 cell line, the FRAP

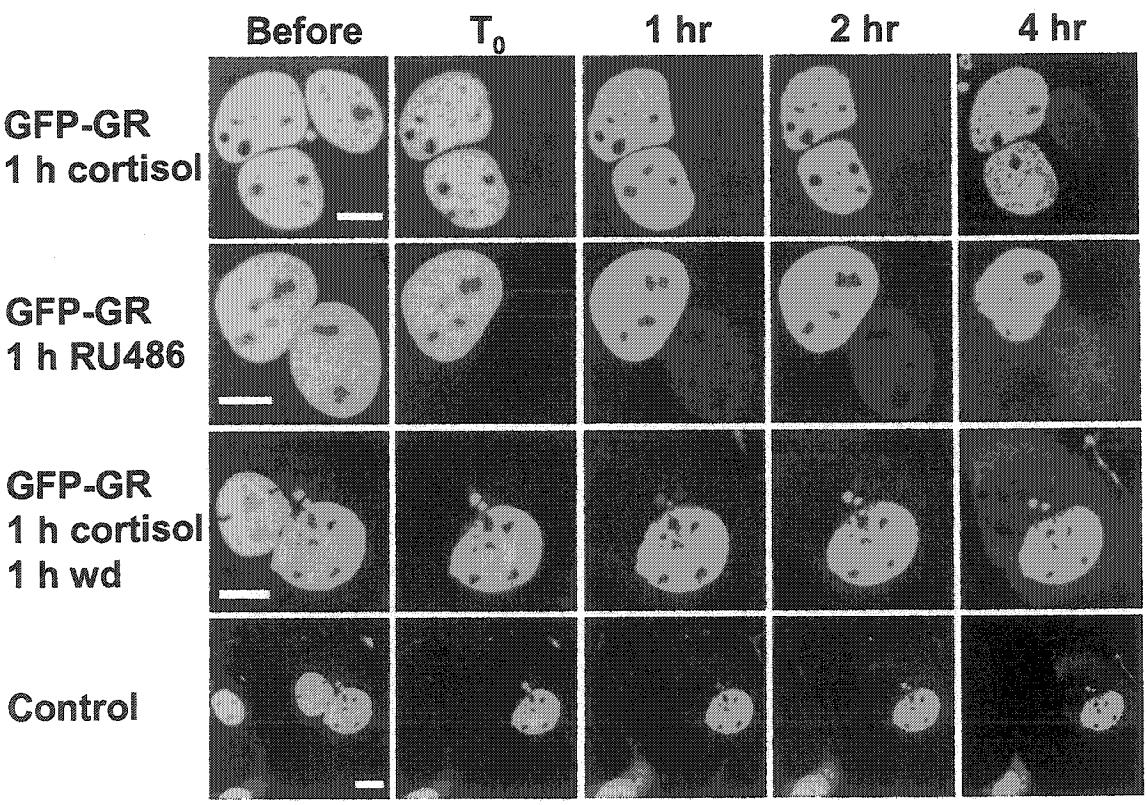
Figure 8: GR transfers only very slowly between nuclei in FRAP experiments

(A) Representation of the GFP-GR construct utilised in this study. The GFP moiety has been fused to the N-terminus of the receptor to avoid disrupting AF-2 function. (B) Cos7 cells transiently transfected to express GFP-GR were treated as indicated in a Bioptechs FCS2 environmental chamber maintained at 37°C on a BioRAD confocal microscope (Before). The GFP signal in one nuclei of a multinucleated cell was irreversibly bleached by focusing a laser on the nucleus and adjusting the power to the maximum output (T_0). Fluorescence recovery at the times indicated is shown in the third panel of each series. All experiments were performed in the presence of cycloheximide (20 μg/mL) to prevent *de novo* protein synthesis. Minimal recovery of signal in the bleached nucleus was observed at 4 h post-bleach. Scale bar = 10 μm.

A


1
795
GFP-GR
GFP
GR wild type

B


Before
T0
1 hr
2 hr
4 hr
GFP-GR
1 h cortisol
GFP-GR
1 h RU486
GFP-GR
1 h cortisol
1 h wd
Control

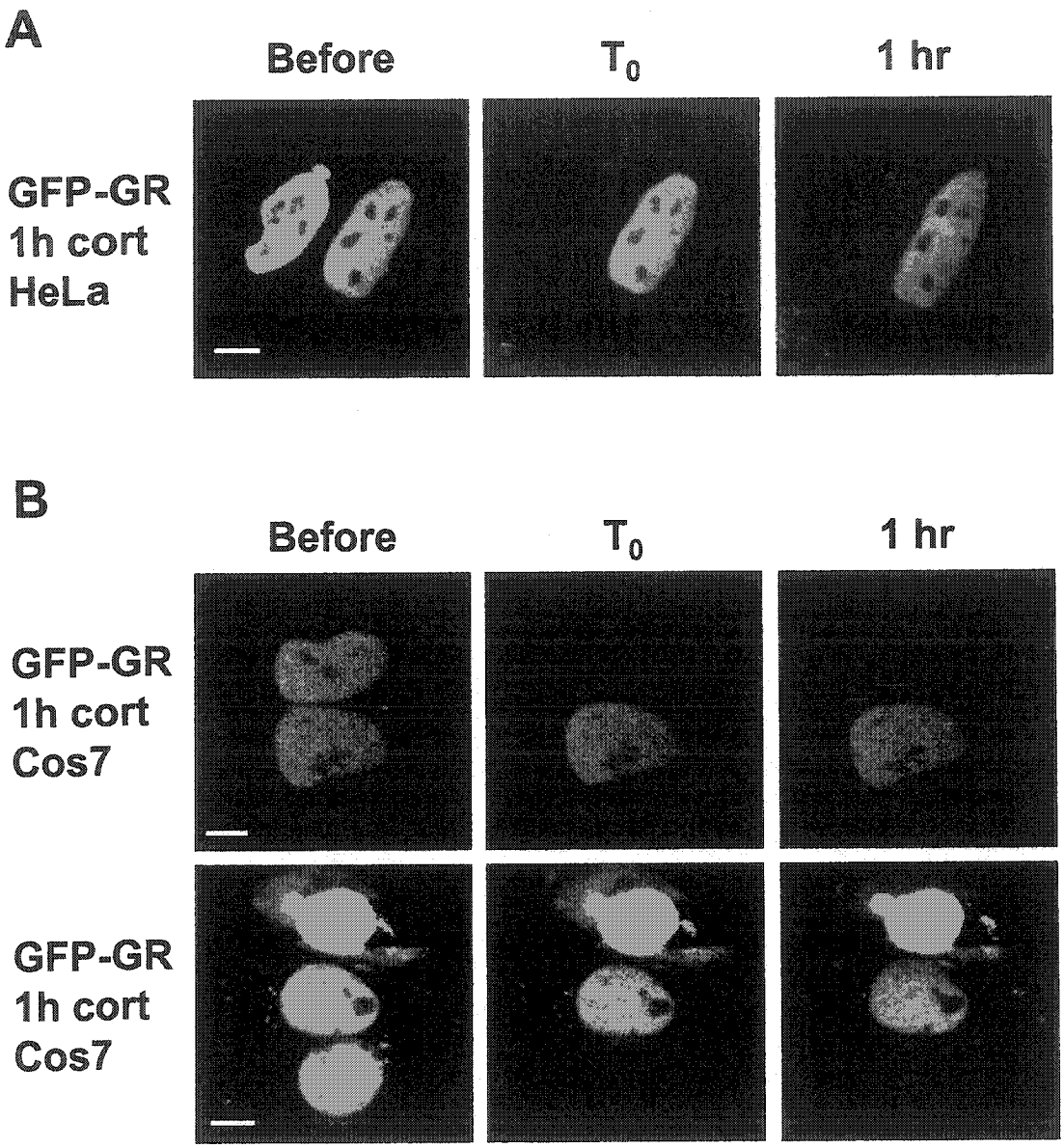
experiment was repeated using HeLa cells. As shown in Figure 9a, no significant recovery of liganded GFP-GR_{wt} was observed in the bleached nucleus 1 h after photobleaching. This indicates that the slow rate of nuclear export observed in this assay is not specific to the Cos7 cell line. The observed slow rate of nuclear export is not a consequence of GR over-expression as this finding was reproduced using Cos7 cells expressing very low levels of receptor as determined by visual inspection (Fig. 9b). These results confirmed that the trafficking behaviour of agonist bound GR is characterised by slow nuclear export in this assay.

To investigate whether this slow rate of nuclear export is specific to the agonist-bound receptor, the FRAP experiment was performed following treatment with the GR antagonist RU486 (Fig. 8b, second panel). Similar to the observations made with the agonist-bound receptor, minimal recovery of GFP-GR_{wt} was detected in the bleached nucleus 1 h following photobleaching. Only a modest transfer of GFP-labelled receptor of 19% ± 2% was observed 4 h following photobleaching, indicating that the antagonist-bound receptor is also slowly exported from the nucleus.

Following ligand withdrawal GR is exported, albeit very slowly, to the cytoplasm. This suggested that perhaps a conformational change of the receptor associated with ligand withdrawal might be required to fully stimulate nuclear export of GR. To address this possibility, FRAP analysis was performed 1 h following withdrawal of ligand (Fig. 8b, third panel). Only a 12% ± 3% increase in cytoplasmic GFP-GR_{wt} was observed 4 h after photobleaching, indicating nuclear export of the ligand-withdrawn GR occurs slowly. The rate of cytoplasmic transfer observed by FRAP is consistent with the slow rate of return to the cytoplasm observed following cortisol withdrawal (130,285).

Figure 9: GR transfers slowly between nuclei in HeLa cells and at low levels of protein expression in Cos7 cells

(A) HeLa cells transiently transfected to express GFP-GR were treated with 1μM cortisol and 20μg/mL cycloheximide 1 h prior to photobleaching and then placed in a Bioptechs FCS2 environmental chamber maintained at 37°C on a BioRAD confocal microscope (Before). The GFP signal in one nuclei of a multinucleated cell was irreversibly bleached by focusing a laser on this nucleus and then adjusting the power to the maximum output (T_0). Fluorescence recovery at the times indicated is shown in the third panel of each series. Minimal recovery of signal in the bleached nucleus was observed at 1 h post-bleach. (B) Cos7 cells were transiently transfected to express GFP-GR and then treated with 1 μM cortisol and 20 μg/mL cycloheximide 1 h prior to photobleaching. Cells were placed in a Bioptechs FCS2 environmental chamber maintained at 37°C on a BioRAD confocal microscope (Before), and then bleached as described for panel A. In order to visualise GFP-GR_{wt} at low levels of expression, images were acquired using the maximum gain and iris settings, resulting in a degradation of image quality. Minimal recovery of signal was observed 1 h post-bleach. In the second panel an overexposed cell is included to demonstrate that the level of expression within the bi-nucleated cell is below average. Scale bar = 10 μm.


A
Before
T_0
1 hr
GFP-GR
1h cort
HeLa
B
Before
T_0
1 hr
GFP-GR
1h cort
Cos7
GFP-GR
1h cort
Cos7

Due to the inclusion of cycloheximide in the cell media and the relatively long maturation time of GFP (367), it is unlikely that the low level of fluorescence recovery observed in the acceptor nucleus is the result of *de novo* synthesis of new GFP-labelled receptor. However, if the GFP protein was not irreversibly bleached it seemed possible that recovery of signal might be observed within 4 h. To determine whether the modest recovery of signal observed following photobleaching was the result of slow nuclear export or recovery from photobleaching, an additional control was routinely included in which a mono-nucleated cell was bleached and tracked over the course of 4 h (Fig. 8b, bottom panel). No recovery of signal was observed in these nuclei 4 h after photobleaching, confirming that the bleaching procedure results in the irreversible ablation of GFP signal and that synthesis of new GFP-labelled receptor is not significant. Further, this control indicates that any recovery of GFP signal observed in either the bleached nucleus or the cytoplasm does indeed reflect the slow trafficking behaviour of GR.

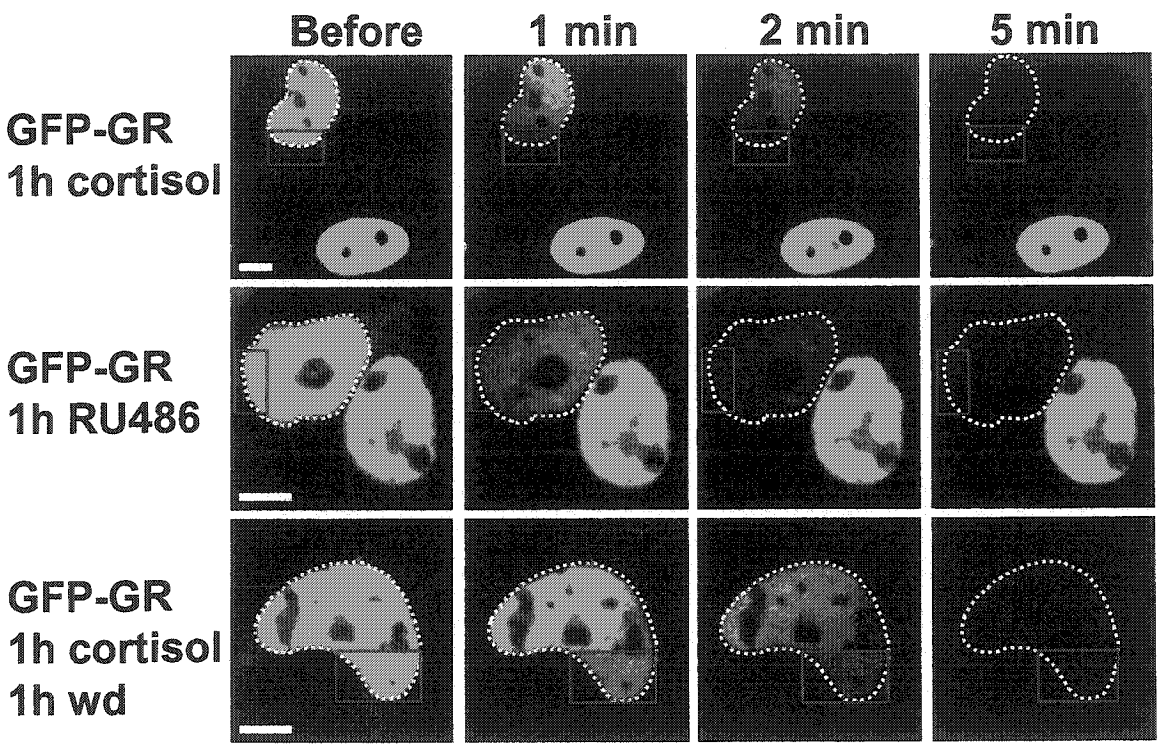
While FRAP analysis clearly showed that GR was slowly exported from the nucleus, it is possible that this observation reflects retention of the receptor at discrete locations within the nucleus. If GR were restricted to discrete locations within the nucleus, then it could have decreased access to the nuclear export machinery resulting in a decreased rate of nuclear export. To investigate this possibility, a qualitative fluorescence loss in photobleaching (FLIP) assay was performed. Briefly, at 30 sec intervals a discrete portion of the nucleus was bleached and then an image of the entire nucleus was obtained within 5 sec of bleaching. If the GFP-labelled protein were mobile, photobleaching of a discrete area of the nucleus would be expected to result in the

complete loss of signal from the entire nucleus. As shown in Figure 10, when FLIP is performed using the GFP-GR_{wt} construct 1 h after addition of hormone agonist, photobleaching an area of the nucleus equivalent to approximately 5% - 10% of the total nuclear volume results in the complete loss of GFP signal from the entire nucleus within 5 min (Fig. 10, upper panel). This indicates that agonist bound GFP-GR_{wt} is relatively mobile within the nucleus, in agreement with previously published observations (344,349). Similarly, FLIP analysis performed in the presence of RU486 reveals that antagonist-bound GR is also relatively mobile within the nucleus (Fig. 10, middle panel). This particular example also confirms that while GR is very mobile within the nucleus of a bi-nucleated cell, there is no measurable rapid transfer between nuclei. Further, FLIP analysis revealed that GR is also relatively mobile within the nucleus following hormone withdrawal (Fig. 10, bottom panel). Together these results indicate that the slow rate of GR nuclear export is not a consequence of sequestration of GR within a fixed compartment of the nucleus.

To further validate my finding that GR is exported slowly from the nucleus, two additional independent assay systems were implemented to monitor GR trafficking behaviour in the absence of cell fusion. The first confirmation of my initial finding was obtained using the digitonin permeabilisation export assay. Digitonin selectively permeabilises the plasma membrane while leaving the nuclear membrane intact. A protein that is exported from the nucleus will be cleared from the nucleus and diluted into the surrounding media. Thus, in this assay loss of visible GFP signal from the nucleus indicates active nuclear export. As a control to ensure that the digitonin system was functioning effectively, export of a positive control construct consisting of the export-

Figure 10: Liganded and hormone withdrawn GR moves rapidly within nuclei as determined by rapid fluorescence loss of imaging after photobleaching (FLIP)

Cos7 cells transiently transfected to express GFP-GR were treated as indicated in a Bioptechs FCS2 environmental chamber maintained at 37°C on a BioRAD confocal microscope (Before). A discrete region of the nucleus, outlined in red, was irreversibly bleached by focusing a laser on this region and adjusting the power to maximum. Following one complete scan of the targeted region the laser power was readjusted to 3% of maximum and an image of the entire nucleus was acquired. This cycle was repeated at 30 sec intervals over a period of 5 min. Images recorded at the indicated time points show depletion of GFP signal from the entire nucleus. Scale bar = 10 μm.

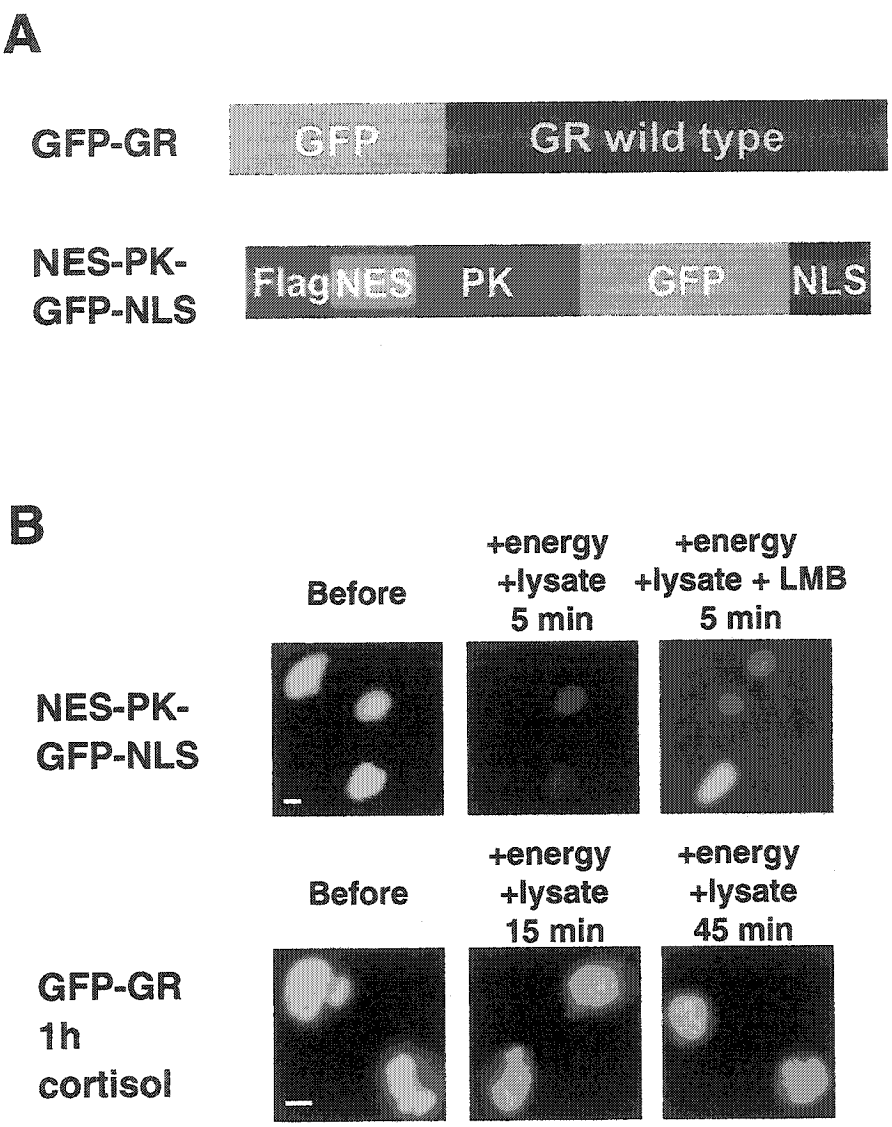

Before
1 min
2 min
5 min
GFP-GR
1h cortisol
GFP-GR
1h RU486
GFP-GR
1h cortisol
1h wd

deficient pyruvate kinase (PK) fused to GFP and the Rev NES was examined. As shown in Figure 11, following permeabilisation of the plasma membrane with digitonin and addition of HeLa cell lysate, which provides cytosolic factors required for nuclear export, plus a source of energy, export of the NES-PK-GFP-NLS control construct is observed within 5 min of the start of the assay (Fig. 11b, upper panel). This specifically reflects CRM1-mediated nuclear export as the addition of LMB inhibits the loss of GFP signal from the nucleus up to 15 minutes after the addition of cell lysate. When this assay was repeated using GFP-GR 1 h after addition of cortisol, GR was found to remain in the nucleus up to 45 min (Fig. 11b, bottom panel) in agreement with previous results (286). This result provides independent confirmation that GR is not rapidly exported from the nucleus.

The second confirmation that GR export occurs at a slower rate than previously anticipated was obtained using the previously described HIV Rev complementation assay (214,363,364). HIV replication requires the translation of proteins from incompletely spliced viral RNA transcripts [for reviews see (219,368)]. As unspliced RNA transcripts are not exported by the normal cellular RNA export pathways, complex retroviruses such as HIV have developed a mechanism that allows for the export of incompletely processed RNA transcripts. The HIV Rev protein binds to viral RNA sequences bearing a Rev recognition element (RRE) and then mediates nuclear export of these viral RNAs by binding to CRM1. By redirecting the viral RNA transcripts from the host cell's RNA export machinery to the cellular pathway for protein export, Rev circumvents the normal requirement that RNA molecules must be fully processed in order to be exported from the nucleus. This pathway allows for the cytoplasmic expression of viral proteins encoded

Figure 11: Stable nuclear localisation of liganded GR in a digitonin permeabilised cell export assay

(A) Representation of constructs used in this experiment. (B) Cos7 cells were transfected with GFP-GR by Lipofectamine™. Forty-eight hours following transfection, cells were treated with 1 μM cortisol for 1 h and then permeabilised with digitonin. Following permeabilisation, HeLa cell lysate containing GTP, ATP and an energy regenerating system were added and cells were incubated at 20°C for the indicated times. GFP-GR remains nuclear for at least 45 min following the addition of cell lysate (top row). In contrast, a positive control fusion construct containing the PK protein fused to a nuclear export sequence is efficiently cleared from the nucleus within 5 min under the same conditions in a manner that is inhibited by addition of 10 nM LMB (bottom row). Scale bar = 10 μm.


A
GFP-GR
GFP
GR wild type
NES-PK-
GFP-NLS
Flag
NES
PK
GFP
NLS
B
Before
+energy
+lysate
5 min
+energy
+lysate + LMB
5 min
NES-PK-
GFP-NLS
Before
+energy
+lysate
15 min
+energy
+lysate
45 min
GFP-GR
1h
cortisol

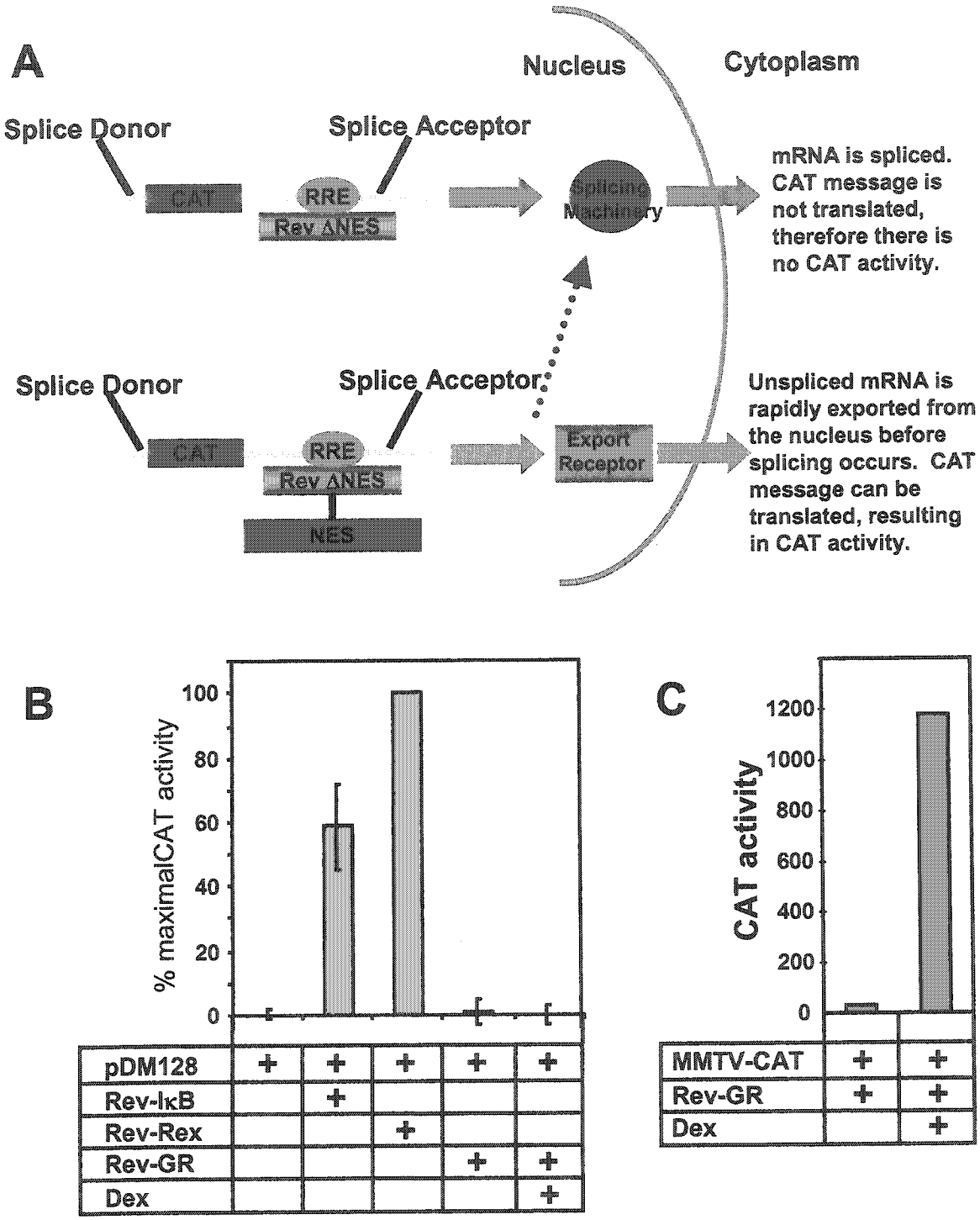
by partially spliced or unspliced transcripts.

The principle of the Rev complementation assay is shown in Figure 12a. This assay system utilises the pDM128 reporter construct, which contains the chloramphenicol acetyl transferase (CAT) cDNA sequence and a Rev recognition element (RRE) flanked by splice donor and splice acceptor sequences. When co-transfected with a Rev construct that lacks its NES, the CAT message is spliced from the mRNA transcript and as a result no CAT activity is observed. However, if the reporter plasmid is co-transfected with a Rev fusion construct that contains an NES, the unspliced RNA molecule will be exported to the cytoplasm. The CAT message can then be translated and CAT activity is observed.

As shown in Figure 12b, minimal CAT activity was observed following transfection of 293T cells with the pDM128 reporter construct. Co-transfection with positive control constructs containing either the N-terminus of IκBα or the NES motif of the HIV protein Rex fused to the RevΔNES resulted in strong CAT activity indicating active nuclear export in agreement with previous results (214). In contrast, co-transfection of the Rev-GR construct resulted in total CAT activity of less than 1% of the maximal CAT activity observed for the HIV Rex NES-containing positive control protein. This lack of significant nuclear export activity was observed in both the absence of steroid agonist and following 24 h of treatment with dexamethasone (dex). This lack of export activity was not due to a defect of the Rev-GR fusion construct as Western blotting confirmed that the protein was of the expected size. Conventional reporter gene analysis using the Rev-GR expression construct and the mouse mammary tumor virus (MMTV) CAT reporter construct, which is strongly inducible by GR, showed strong dex-dependent CAT activity. This finding indicates that the Rev-GR protein is

Figure 12: GR remains localised to the nucleus in a Rev complementation assay

(A) Outline of the Rev complementation assay. The reporter plasmid contains the CAT sequence and a Rev recognition element (RRE) flanked by splice donor and splice acceptor sites. In the absence of a rapid nuclear export sequence (NES), the CAT message is excised from the transcript. In the presence of a rapid export activity the Rev protein binds the unspliced RNA transcript and is exported from the nucleus where the CAT message is then translated. (B) The strong minimal export sequence of the HIV-Rex protein or the NES within IκB both promote the rapid export of CAT RNA prior to splicing. By contrast GR-Rev fusions are exported too slowly to escape splicing. The constructs summarised to the left were transfected into 293T cells by calcium phosphate along with the pDM128 reporter plasmid and pRSVβ-gal. 1 μM dexamethasone was added 24 h prior to harvest as indicated. 48 h following transfection the cells were harvested and CAT activity measured and corrected for β-gal activity. CAT activity values for all constructs are expressed as a percentage of the maximal recorded CAT activity. (C) The Rev-GR construct is transcriptionally active. The Rev-GR construct was co-transfected into 293T cells with the pMMTV-CAT reporter plasmid and pRSV-βgal. 1 μM dexamethasone was added 24 h prior to harvest as indicated. 48 h following transfection the cells were harvested and CAT activity measured and corrected for β-gal activity.


A
Nucleus
Cytoplasm
Splice Donor
Splice Acceptor
CAT
RRE
Rev ANES
Splicing Machinery
mRNA is spliced. CAT message is not translated, therefore there is no CAT activity.
Splice Donor
Splice Acceptor
CAT
RRE
Rev ANES
NES
Export Receptor
Unspliced mRNA is rapidly exported from the nucleus before splicing occurs. CAT message can be translated, resulting in CAT activity.
B
% maximalCAT activity
100
80
60
40
20
0
pDM128 + + + + +
Rev-IκB + +
Rev-Rex +
Rev-GR + +
Dex +
C
CAT activity
1200
1000
800
600
400
200
0
MMTV-CAT + +
Rev-GR + +
Dex +

transcriptionally active (Fig 12c). Together, the results of the Rev complementation assay indicate that GR nuclear export is a slow process. Moreover, this finding and the results of the digitonin permeabilisation export assay provide two independent means of confirming the results obtained using FRAP analysis showing that in the absence of cell fusion, GR nuclear export is a relatively slow process.

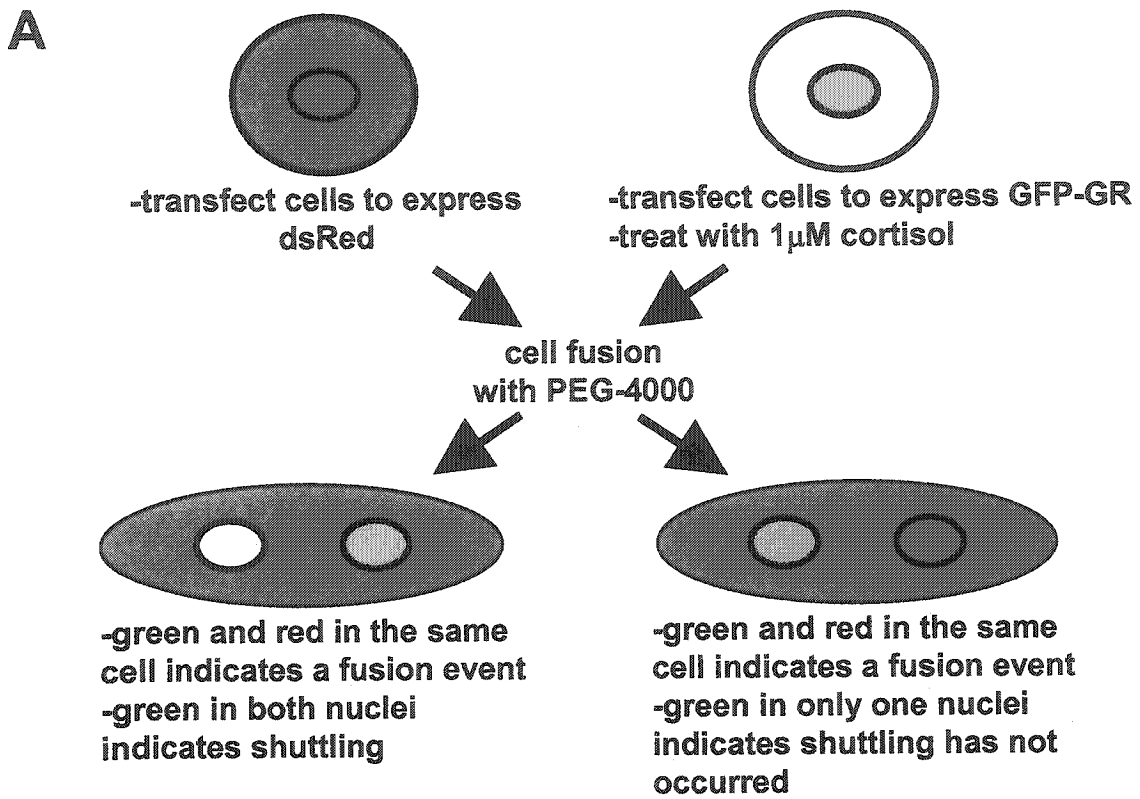
Accelerated GR Nuclear Export Following Cell Fusion is Dependent upon Calreticulin

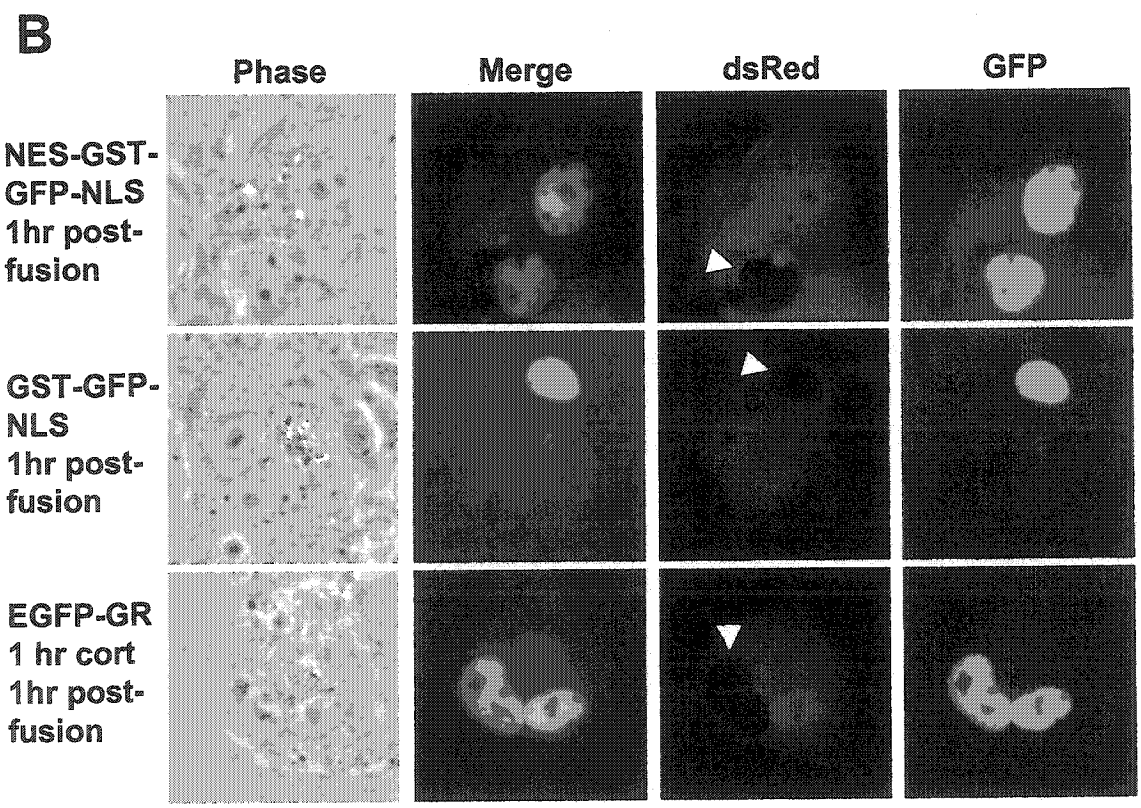
The observation that the liganded GR is exported slowly from the nucleus was unexpected given the results of previous reports that indicate that GR shuttles rapidly between nucleus and cytoplasm. To directly explore the basis for this discrepancy, I developed a homokaryon cell fusion assay to study nucleocytoplasmic trafficking following cell fusion. This assay is based in large part on the heterokaryon cell fusion assay previously used to study NHR nuclear export (39,351-355) but relies on the fusion of Cos7 cells exclusively. This experimental approach was chosen over a traditional heterokaryon fusion assay in order to eliminate the possibility that rapid nuclear export of GR might be mediated by a factor expressed in either the 3T3-L1 or HeLa cell lines used for heterokaryon analysis but not the Cos7 cells used primarily in this study. Further, the use of different fluorophores to mark separate populations of cells eliminates any potential ambiguity in identifying fused cells that may arise due to the presence of naturally occurring multinucleated cells within the population.

Figure 13a outlines the principle of the homokaryon cell fusion assay employed in this study. Cos7 cells are separately transfected with either dsRed or a GFP-tagged construct. Following transfection the cells are harvested and co-plated onto a coverslip in

Figure 13: Homokaryon cell fusion experiment demonstrating the fusion-dependent accelerated shuttling of GR

(A) Outline of the homokaryon cell fusion assay. Cos7 cells are transiently transfected with either dsRed or a vector encoding a GFP-labelled protein of interest. Following transfection the cells are co-plated and then treated with PEG-4000 to induce fusion. A fusion event is detected by the presence of both green and red fluorescent signal. Active shuttling between nuclei is indicated if green fluorescence is detected in all nuclei of the fused cell. In the absence of shuttling at least one nuclei of the fused cell will not contain green fluorescent signal. (B) The rate of GR nuclear export is enhanced following cell fusion. Cos7 cells transiently transfected to express dsRed2 were plated together on coverslips with Cos7 cells expressing the indicated GFP fusion construct. Cells were treated with 1mM cortisol for 1 h and then fused by incubation in 50% w/v PEG-4000 for 2 min at 37°C. One hour following fusion, the cells were observed by fluorescence microscopy with separate filter sets to observe dsRed2 and GFP. Shuttling of the GFP-labelled construct is observed by the appearance of GFP labelled protein in both of the two nuclei of the fused cell that were derived separately from each starting cell (top and bottom panels), whereas the lack of nucleocytoplasmic shuttling is apparent by the presence of at least one nuclei lacking GFP signal (middle panel). To avoid ambiguity in interpreting the results of these experiments, each experiment was subjected to double-blind encryption following plating to coverslips.


A
-transfect cells to express dsRed
-transfect cells to express GFP-GR
-treat with 1μM cortisol
cell fusion with PEG-4000
-green and red in the same cell indicates a fusion event
-green in both nuclei indicates shuttling
-green and red in the same cell indicates a fusion event
-green in only one nuclei indicates shuttling has not occurred


B
Phase
Merge
dsRed
GFP
NES-GST-GFP-NLS 1hr post-fusion
GST-GFP-NLS 1hr post-fusion
EGFP-GR 1 hr cort 1hr post-fusion

equal proportions. The cells are then treated with ligand as appropriate and cell fusion is induced by PEG-4000 treatment. One hour after PEG treatment the cells are visualised by direct fluorescence microscopy. Fusion events are detected by the presence of both red and green signal in the same cell. If the GFP-tagged protein is actively exported, all nuclei of a fused cell will appear green. However, if the GFP-tagged protein is exported slowly or not at all, at least one nuclei of a fused cell will not have any GFP signal.

To demonstrate that the assay is functioning as expected, the trafficking behaviour of two control constructs was monitored. As a positive control I utilised the pNES-GST-GFP-NLS construct, which was previously used to verify the FRAP-based nuclear export assay. As shown in Figure 13b (top panel), phase contrast and multicolour fluorescence imaging both confirm that the observed cell has been fused. Because the dsRed tetramer is too large to passively diffuse into the nucleus, the nucleus originating from the GFP transfected cells is not expected to contain any dsRed protein. Using a filter specific for dsRed, these donor nuclei can be identified, as they do not contain any red signal. To detect trafficking of the GFP-labelled protein between nuclei, this same cell is visualised using a filter specific for GFP. One hour following the induction of cell fusion, complete equilibration of GFP signal between nuclei is observed for a positive-control construct bearing the Rev NES (Fig.13b, top panel). The trafficking behaviour of this fusion protein is dependent on the presence of an NES, as a construct lacking this motif showed minimal transfer between nuclei 1 h post-fusion (Fig 13b, middle panel).

Having established that the trafficking behaviour of control constructs is recapitulated as anticipated in this assay, the trafficking behaviour of GR was examined (Fig. 13b, bottom panel). Cell fusion is evidenced by examination of the phase contrast

image and confirmed by examination of the dsRed image. Trafficking of the receptor is indeed accelerated in these fused cells as visualisation of the homokaryon cell using a GFP-specific filter shows that 1 h following cell fusion the liganded receptor is completely equilibrated between nuclei (Fig. 13b, bottom panel). This result is in agreement with previously published reports (353,354) and indicates that that rapid GR nuclear export is specifically induced following cell fusion.

The accelerated export of GR observed following cell fusion contrasts with the slow rate of nuclear export observed in non-fused cells. The reason for this discrepancy is likely to involve the different experimental manipulations employed in each technique. It seems highly probable that PEG treatment followed by cell fusion could temporarily impact the subcellular architecture of the fused cell. Consequently, the integrity of membrane-bound organelles within the fused cell may be compromised, resulting in the aberrant exposure of proteins normally resident within intracellular organelles. If cell fusion does result in the temporary disruption of membrane integrity, then proteins that are normally unable to directly influence each other's function in an intact cell due to their sequestration in separate subcellular compartments may gain the ability to do so following cell fusion. In the case of GR trafficking, it is possible that disruption of the endoplasmic reticulum could result in the exposure of calreticulin (CRT), a protein that has been proposed to mediate GR nuclear export (228,229). It remains unclear how a protein such as CRT, which is localised to the lumen of the endoplasmic reticulum (313), could have a direct effect on nucleocytoplasmic trafficking under normal physiological conditions. As the experimental support for the role of CRT in GR nuclear export comes primarily from cell fusion based assays, or assays in which CRT is artificially localised to

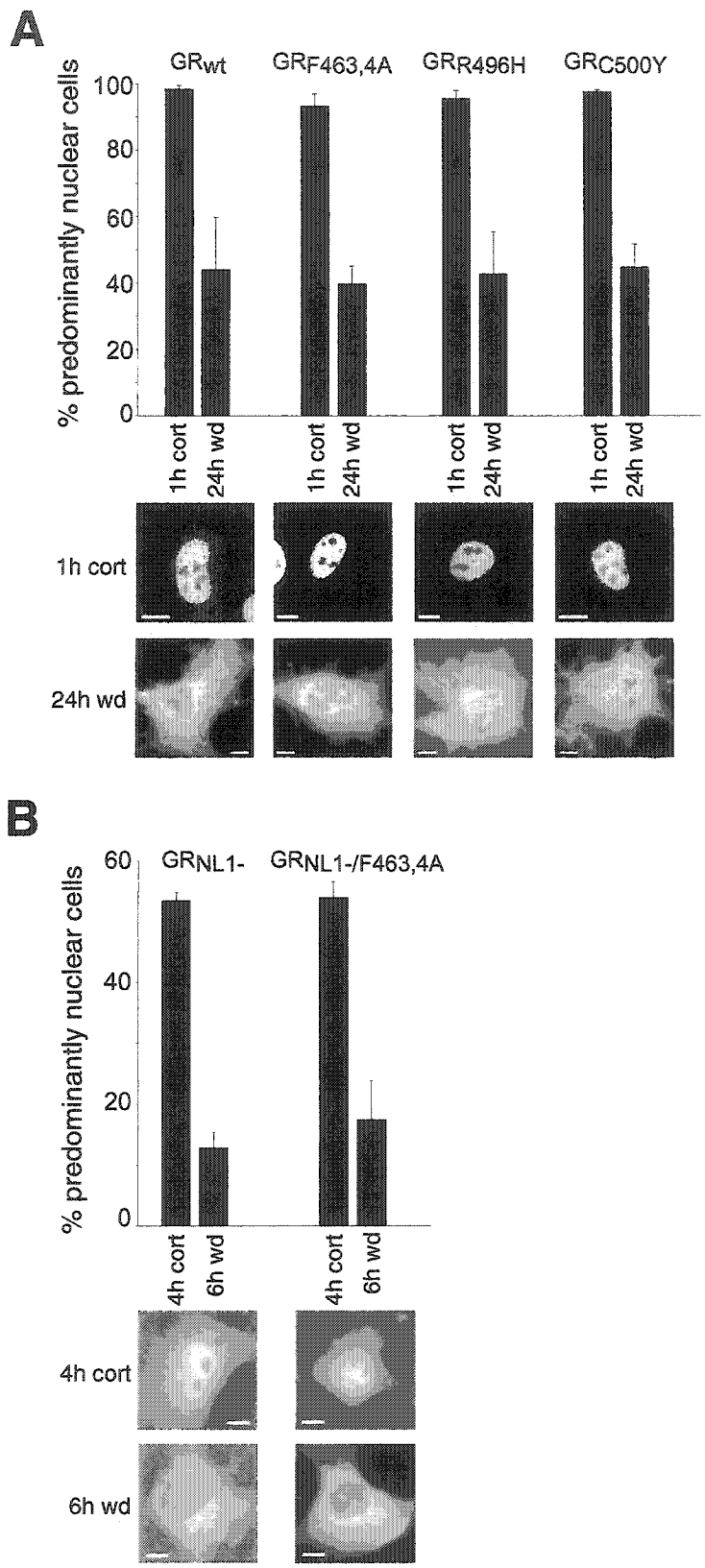
the cytoplasm, I examined the effect of disrupting GR-CRT binding on GR trafficking in the absence of cell fusion. In these studies a mutant form of GR, $GR_{F463,464A}$, was used as this substitution has been shown to disrupt GR-CRT binding *in vitro* (323).

Using the $GR_{F463,464A}$ double mutant, I examined the effect of CRT on GR nuclear export following ligand withdrawal. It has been well documented that following removal of ligand, GR is slowly redistributed to the cytoplsm with a $t_{1/2}$ of 12-24 h (47,130,285,286). As shown in Figure 14a, 24 h following ligand withdrawal, the majority of the receptor is no longer localised exclusively to the nucleus in 40-50% of cells expressing wildtype GR. This clearance of GR from the nucleus is unaffected by the $GR_{F463,464A}$ substitution, indicating that nuclear export of the receptor following ligand withdrawal is independent of direct binding to CRT. As the $GR_{F463,464A}$ substitution also disrupts DNA binding, two additional GR DNA binding mutants GR_{R496H} and GR_{C500Y} were included to control for any non-specific effects due to the loss of DNA binding ability. The GR_{R496H} and GR_{C500Y} mutations are not expected to have any effect on CRT binding. Again, in 40-50% of cells expressing either GR_{R496H} or GR_{C500Y} the receptor was no longer predominantly localised to the nucleus. These results indicate that nuclear export of GR is unaffected by the loss of direct binding to calreticulin.

To extend this finding, the effect of the $GR_{F463,464A}$ substitution was studied in combination with the GR_{NL1-} mutation. As discussed later in this work, GR_{NL1-} is exported more rapidly from the nucleus compared to the wildtype receptor, with its redistribution to the cytoplasm nearing completion 4 h following ligand withdrawal (Fig. 14b). If the loss of direct binding to CRT has only a subtle effect on GR nuclear export,

Figure 14: GR redistribution to the cytoplasm following ligand withdrawal is independent of calreticulin binding

(A) Redistribution of GR to the cytoplasm following ligand withdrawal is not affected by the loss of direct binding to calreticulin. Cos7 cells were transiently transfected with the indicated GFP-GR constructs by Lipofectamine™. Following transfection, the cells were plated onto glass coverslips and synchronised in G_0 by withdrawing serum. After 16-24 h, the cells were treated with 1μM cortisol for the indicated times followed by hormone withdrawal for the indicated times. The localisation of GR was scored by indirect immunofluorescence using a Nikon TE300 microscope. The percentage of cells scored as exclusively nuclear or mainly nuclear were added together to obtain the displayed values. Representative micrographs are shown below each data set. Error bars represent the standard error of the means of three independent experiments performed in duplicate. (B) The enhanced rate of redistribution of the GR_{NL1}- mutant is not affected by loss of direct binding to calreticulin. Cos7 cells were transiently transfected with the indicated GFP-GR constructs and treated as described in panel A.

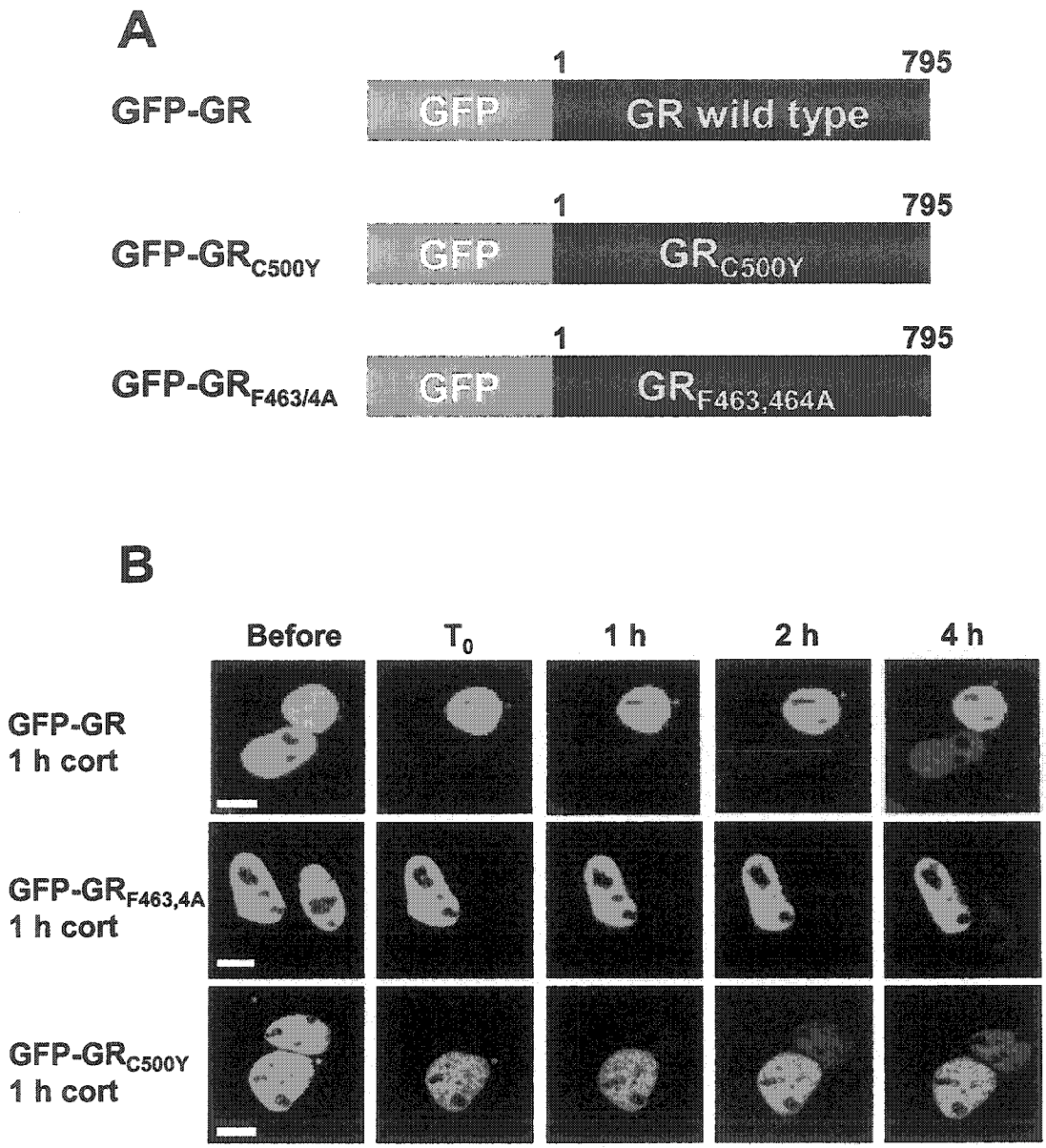

A
GRwt
GRF463,4A
GRR496H
GRC500Y
% predominantly nuclear cells
100
80
60
40
20
0
1h cort
24h wd
1h cort
24h wd
1h cort
24h wd
1h cort
24h wd
1h cort
24h wd
B
GRNL1-
GRNL1-/F463,4A
% predominantly nuclear cells
60
40
20
0
4h cort
6h wd
4h cort
6h wd
4h cort
6h wd

then studying the effect of the $GR_{F463,464A}$ mutation on GR trafficking in the context of a mutated receptor that is more rapidly exported from the nucleus would be more likely to reveal a modest change in trafficking behaviour. In close agreement with previous results (47,130,285,286), GR_{NL1-} becomes localised predominantly to the nucleus in approximately 50% of cells observed following a 4 h treatment with cortisol. Complete nuclear localisation is not observed as the GR_{NL1-} substitution has been shown to inhibit GR nuclear import (130). After only 6 h following ligand withdrawal, nuclear export of GR_{NL1-} is nearly complete, as GR remained predominantly nuclear in approximately 15% of cells scored. This accelerated rate of nuclear export is unaffected by the loss of direct binding to calreticulin. As shown in Figure 14b, the $GR_{NL1-/F464,464A}$ double substitution relocalised to the cytoplasm following ligand withdrawal with similar kinetics as the wildtype receptor. This result confirms that GR nuclear export following ligand withdrawal is unaffected by direct binding to CRT.

To study the effect of CRT on trafficking of the liganded receptor, I performed FRAP analysis using the GFP-$GR_{F463,464A}$ construct. The GFP-GR_{C500Y} was also included to control for any non-specific effects due to the loss of DNA binding ability (Fig. 15a). Similar to the result described in Figure 8b, FRAP analysis reveals that there is a modest transfer of wildtype GFP-GR between nuclei following photobleaching, indicative of a slow rate of nuclear export (Fig. 15b, top panel). Similarly, GFP-$GR_{F463,464A}$ transferred slowly between nuclei, indicating that the slow nuclear export of the liganded receptor is not directly influenced by CRT (Fig. 15b, middle panel). FRAP analysis of the GFP-GR_{C500Y} construct reveals a slight increase in the rate of nuclear export, indicating that DNA binding through the second zinc finger of the DBD may have a small influence on

Figure 15: GR shuttles slowly between nuclei in the absence of calreticulin binding

(A) Representation of constructs used in this experiment. (B) Cos7 cells were transiently transfected with the indicated constructs by Lipofectamine™ and treated with 1 μM cortisol and 20 μg/mL cycloheximide for 1 h. FRAP assays were performed as described previously. Minimal recovery of signal in the bleached nucleus was observed at 4 h post-bleach using the wildtype GR construct. A slight increase in the rate of shuttling was observed with the $GR_{F463,464}$ construct. To control for the effect of DNA binding, the assay was repeated with the GR_{C500Y} construct. Scale bar = 10 μm.

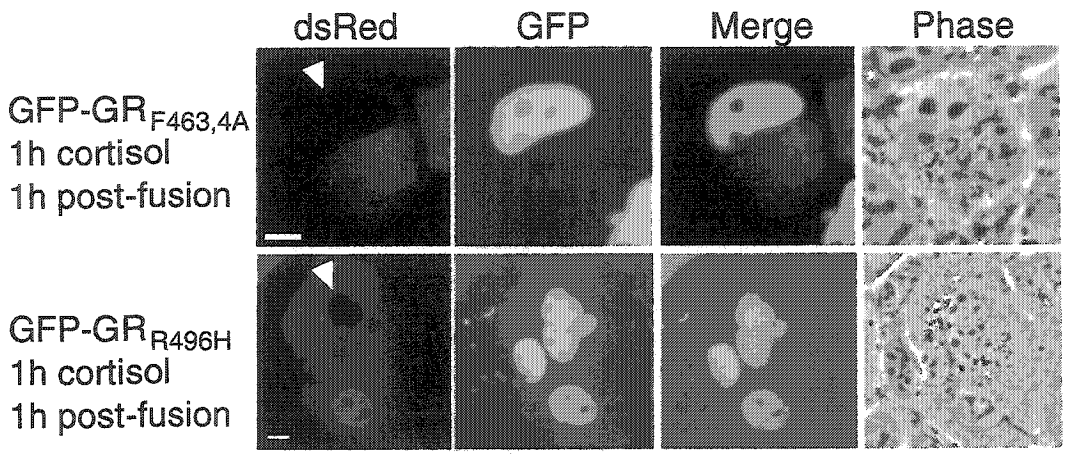

A
1
795
GFP-GR
GFP
GR wild type
GFP-GR$_{C500Y}$
GR$_{C500Y}$
GFP-GR$_{F463/4A}$
GR$_{F463,464A}$
B
Before
T_0
1 h
2 h
4 h
GFP-GR
1 h cort
GFP-GR$_{F463,4A}$
1 h cort
GFP-GR$_{C500Y}$
1 h cort

the trafficking behaviour of the liganded receptor (Fig. 15b, bottom panel). Fluorescence recovery values for GFP-$GR_{F463,464A}$ and GFP-GR_{C500Y} were calculated at 30 % ± 6% and 36% ± 6% respectively compared to 19% ± 6% for GFP-GR_{wt}. This result indicates that direct binding to CRT does not directly influence the slow rate of nuclear export observed for the liganded receptor. Together with the observation that the rate of nuclear export following ligand withdrawal is unaffected by disruption of CRT binding described in Figure 14, my results indicate that calreticulin has no direct effect on GR nuclear export in the absence of cell fusion.

To this point, my results indicate that nuclear export of wildtype GR is dramatically accelerated following cell fusion compared to the relatively slow rate of GR nuclear export observed in live cells using FRAP. As previously discussed this discrepancy could be the result of an alteration in CRT localisation following cell fusion. In this case, the accelerated rate of GR nuclear export observed in fused cells would be sensitive to the loss of direct binding to CRT. To investigate this possibility, the homokaryon cell fusion assay was repeated using the CRT binding mutant $GR_{F463,464A}$. Again, to control for any non-specific effects due to the loss of DNA binding, the GR DNA-binding mutant GR_{R496H} was also studied. Beginning with the GR_{R496H} construct, cell fusion is evident by examination of the phase contrast image and confirmed by examination of the dsRed image (Fig. 16, top panel). Visualisation of GFP-GR_{R496H} 1 h following cell fusion shows that like GR_{wt}, the GR_{R496H} construct is distributed between all nuclei. This confirms that GR nuclear export is accelerated following cell fusion and indicates that DNA binding does not influence the acceleration of GR export in fused cells. In contrast, 1 h following cell fusion $GR_{F463,464A}$ is not equally distributed between

Figure 16: GR shuttling in a homokaryon cell fusion assay is dependent on binding to calreticulin

Cos7 cells transiently transfected to express dsRed2 were plated together on coverslips with Cos7 cells expressing GFP-GR following transient transfection and fused using PEG-4000 as described in Figure 13. One hour following fusion, the cells were observed by fluorescence microscopy using separate filter sets to observe dsRed2 and GFP. The $GR_{F463,464A}$ mutation, which disrupts the binding or GR to calreticulin, abrogates shuttling between nuclei of a homokaryon cell fusion. To control for effects due to disruption of DNA binding, the assay was repeated with the GR_{R496H} DNA binding mutant.


dsRed
GFP
Merge
Phase
GFP-GR F463,4A
1h cortisol
1h post-fusion
GFP-GR R496H
1h cortisol
1h post-fusion

nuclei within a fused cell, indicating that in the absence of direct binding to CRT nuclear export of GR is no longer accelerated following cell fusion (Fig. 16, bottom panel). The lack of equilibration of GFP-$GR_{F463,464A}$ between nuclei was noted in 78% of the homokaryons visualised. This result suggests that accelerated nuclear export of GR following cell fusion is dependent upon binding to calreticulin. As direct binding of GR to CRT did not affect GR export in the absence of cell fusion, these results suggested that CRT is exposed as a result of cell fusion and can then influence the observed rate of GR nuclear export.

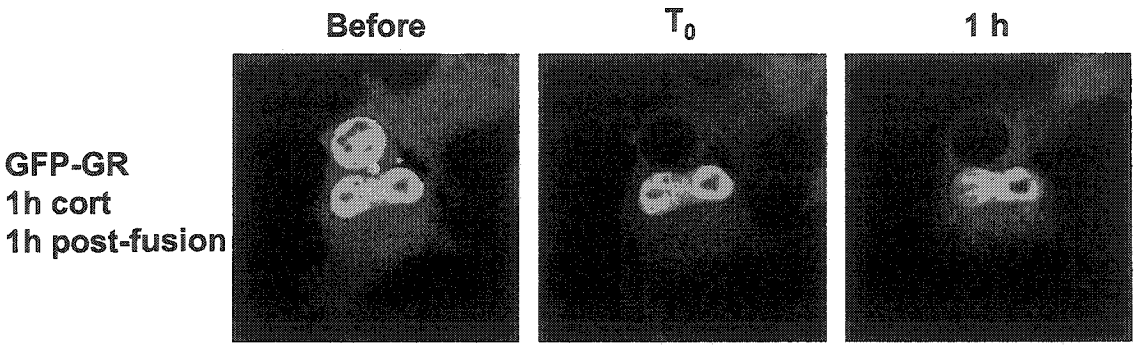
Polyethylene Glycol Exposure Results in Transient Exposure of Calreticulin

Having established that cell fusion accelerates calreticulin-dependent GR nuclear export, I examined whether this enhanced rate of GR nuclear export following PEG treatment is sustained following cell fusion. To address this issue, FRAP analysis was performed subsequent to cell fusion. As shown in Figure 17, 1 h following cell fusion complete equilibration of GR_{wt} is observed between nuclei of a homokaryon cell. However, following ablation of GFP-GR_{wt} in one nucleus of a fused cell by photobleaching, no significant transfer of GFP signal between nuclei is observed within a further 1 h time period. This finding indicates that the acceleration of GR nuclear export following cell fusion is transient.

To this point my data indicate that the accelerated rate of GR nuclear export observed subsequent to cell fusion is a transient event that is sensitive to the loss of direct binding to calreticulin. This suggested that upon cell fusion CRT might be transiently released into the cytoplasm via disruption of the integrity of the endoplasmic reticulum.

Figure 17: Accelerated trafficking of GR following PEG-mediated cell fusion is transient

Cos7 cells transiently transfected to express dsRed2 were plated together on coverslips with Cos7 cells expressing GFP-GR following transient transfection and fused using PEG-4000 as previously described. One hour following fusion, the cells were observed by confocal microscopy using separate filter sets to observe dsRed2 and GFP. One nuclei of a fused cell was subjected to FRAP treatment. Minimal recovery of signal in the bleached nucleus was observed 1 h post-bleach.

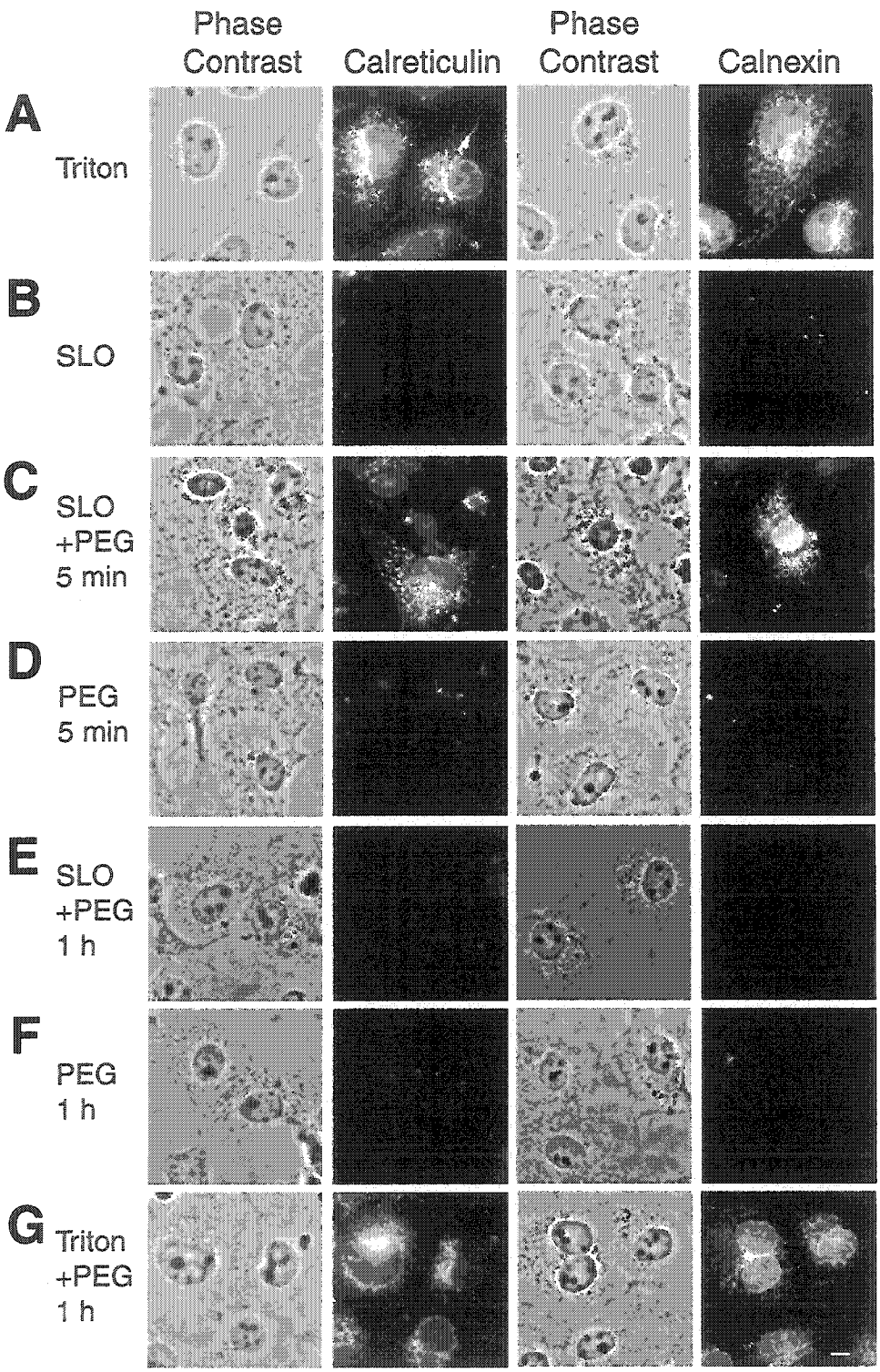

Before
T_0
1 h
GFP-GR
1h cort
1h post-fusion

To identify changes in CRT exposure following cell fusion, indirect immunofluorescence (IIF) utilising selective permeabilisation methods was performed. Triton X-100 is a permeabilising agent that permeabilises all subcellular membranes. Treatment with Triton X-100 allows for visualisation of calreticulin that is localised to all subcellular structures. Streptolysin O (SLO) specifically permeabilises the plasma membrane leaving the endoplasmic reticulum and the nuclear envelope intact. Thus, SLO permeabilisation will only allow for detection of cytosolically exposed CRT. In these experiments visualisation of calnexin (CLX), which is anchored to the inner face of the endoplasmic reticulum, was used as a control for disruption of the endoplasmic reticulum.

As shown in Figure 18a, when permeabilised using Triton X-100 both CRT and CLX display a punctate staining pattern consistent with previous observations (313,327). Both proteins are localised within the endoplasmic reticulum, as SLO permeabilisation followed by IIF yielded no significant fluorescent signal above background (Fig 18b). However, in a sub-population of cells treated with PEG and allowed to recover for only 5 min prior to being subjected to IIF using SLO permeabilisation, there is a distinct increase in the level of CRT and CLX observed by IIF (Fig. 18c). This indicates that PEG treatment affects the integrity of the endoplasmic reticulum in a manner that alters the subcellular localisation of CRT. Treatment with PEG followed by a 5 min recovery period followed by IIF using no method of permeabilisation results in no significant CRT or CLX-specific signal above background levels (Fig. 18d). In agreement with the results shown in Figure 17, this disruption in the integrity if the endoplasmic reticulum is transient as no significant staining of calreticulin or calnexin is observed if the cells are

Figure 18: PEG-mediated disruption of the endoplasmic reticulum is transient

Cos7 cells were treated with 50% (w/v) PEG-4000 where noted and fixed following incubation at 37°C for the indicated times. Selective permeabilisation was performed with either triton X-100 (A, G), streptolysin O (B, C, E) or without permeabilisation (D, F) followed by indirect immunofluorescence using antibodies specific for either calreticulin or calnexin as described in Materials and Methods. For each antibody the optimal exposure time required for visualisation of signal following triton X-100 permeabilisation was determined, and this exposure time was used to capture all subsequent images within that experiment. Scale bar = 10 μm.


Phase Contrast
Calreticulin
Phase Contrast
Calnexin
A
Triton
B
SLO
C
SLO +PEG 5 min
D
PEG 5 min
E
SLO +PEG 1 h
F
PEG 1 h
G
Triton +PEG 1 h

allowed to recover for 1 h after PEG exposure before proceeding to IIF using SLO permeabilisation (Fig. 18e). Additionally, no CRT and CLX signals were observed 1 h following PEG treatment without any means of permeabilisation (Fig. 18f). This lack of signal does not appear to be attributable to a significant change in the expression levels of these proteins as CRT and CLX staining is readily observed using an exposure time equal to that used to obtain the images presented in Figure 18a when treated with PEG and subjected to IIF using triton X-100 following a 1 h recovery period (Fig. 18g). These results indicate that PEG treatment results in a transient disruption of the endoplasmic reticulum and subsequent release of calreticulin, which in turn influences GR nuclear export.

To determine whether PEG treatment in the absence of cell fusion was sufficient to induce accelerated GR nuclear export, the trafficking behaviour of GR was studied using FRAP analysis following PEG treatment. Following PEG treatment, cells were rinsed and then immediately placed in a live-cell chamber for FRAP analysis. In this experiment, cells chosen for FRAP analysis did not necessarily show evidence of cell fusion. While it is possible that some of the cells chosen for analysis had actually undergone cell fusion, cell fusion occurs with a relatively low frequency of between 0-5 cells per coverslip. In contrast bi-nucleated cells represent approximately 5-10% of the cell population and are relatively easy to locate. Due to the relative abundance of naturally occurring bi-nucleated cells compared to the rarity of fused cells it is reasonably probable that the cells observed in this experiment had been exposed to PEG without subsequent cell fusion. Additionally, for this experiment the cells used were approximately 70% confluent, decreasing the likelihood that the cells could be in close

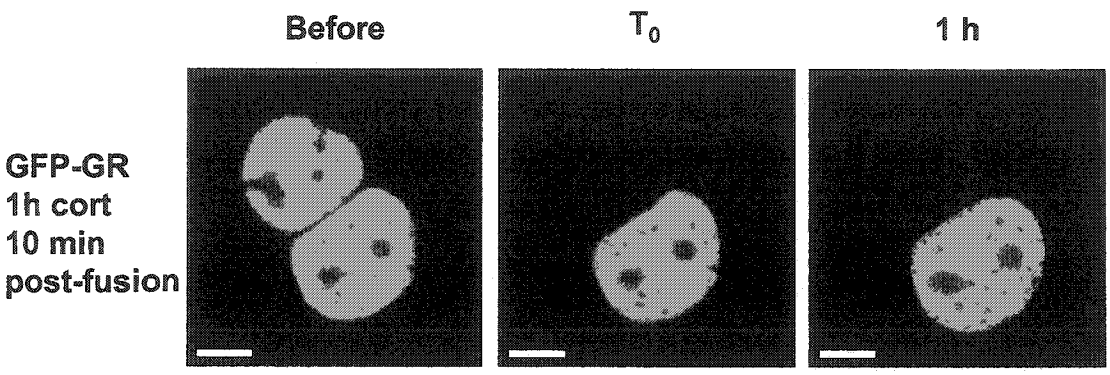
enough proximity to one another to undergo cell fusion. As shown in Figure 19, FRAP analysis of GFP-GR_{wt} trafficking in bi-nucleated cells treated with PEG revealed no significant transfer of GFP signal between nuclei within 1 h when FRAP was performed subsequent to PEG treatment. By comparison, in the majority of homokaryon cells observed, GFP-GR_{wt} equilibrated between nuclei within 1 h following fusion (Fig. 13). This result indicates that PEG treatment alone in the absence of cell fusion is not sufficient to induce calreticulin-dependent accelerated export of GR and suggests that in addition to PEG-induced exposure of CRT, further events specific to the process of cell fusion may be required to fully accelerate GR nuclear export.

Two Distinct Pathways Mediate Steady State Nuclear Export of Liganded GR and Ligand Withdrawn GR

The work presented in this thesis thus far indicates that GR nuclear export proceeds at a slower rate than initially believed. Nonetheless it remains apparent that both liganded GR and ligand-withdrawn GR are influenced to some extent by nuclear export pathways. The most extensively characterised nuclear export pathway utilises the CRM1 nuclear export receptor. CRM1 directly mediates the nuclear export of many shuttling proteins and is specifically inhibited by LMB (199-202). To begin to elucidate the nuclear export pathways that might be utilised by GR, I examined the effect of inhibiting CRM1-mediated export on GR nucleocytoplasmic localisation. In these studies the effect of inhibiting the CRM1 export pathway on GR subcellular localisation through the use of LMB was studied within the context of the GR_{NL1-} mutation for two reasons Firstly, since the NL1 mutation results in decreased nuclear accumulation

Figure 19: PEG treatment alone is not sufficient to fully accelerate GR nuclear export

Cos7 cells were transiently transfected with GFP-GR as described previously. Following serum withdrawal and treatment with 1μM cortisol for 1 h, cells were treated with 50% (w/v) PEG-4000. Cells were extensively rinsed with HBSS and were immediately subjected to FRAP analysis as described previously. Scale bar = 10 μm.


Before
T_0
1 h
GFP-GR
1h cort
10 min
post-fusion

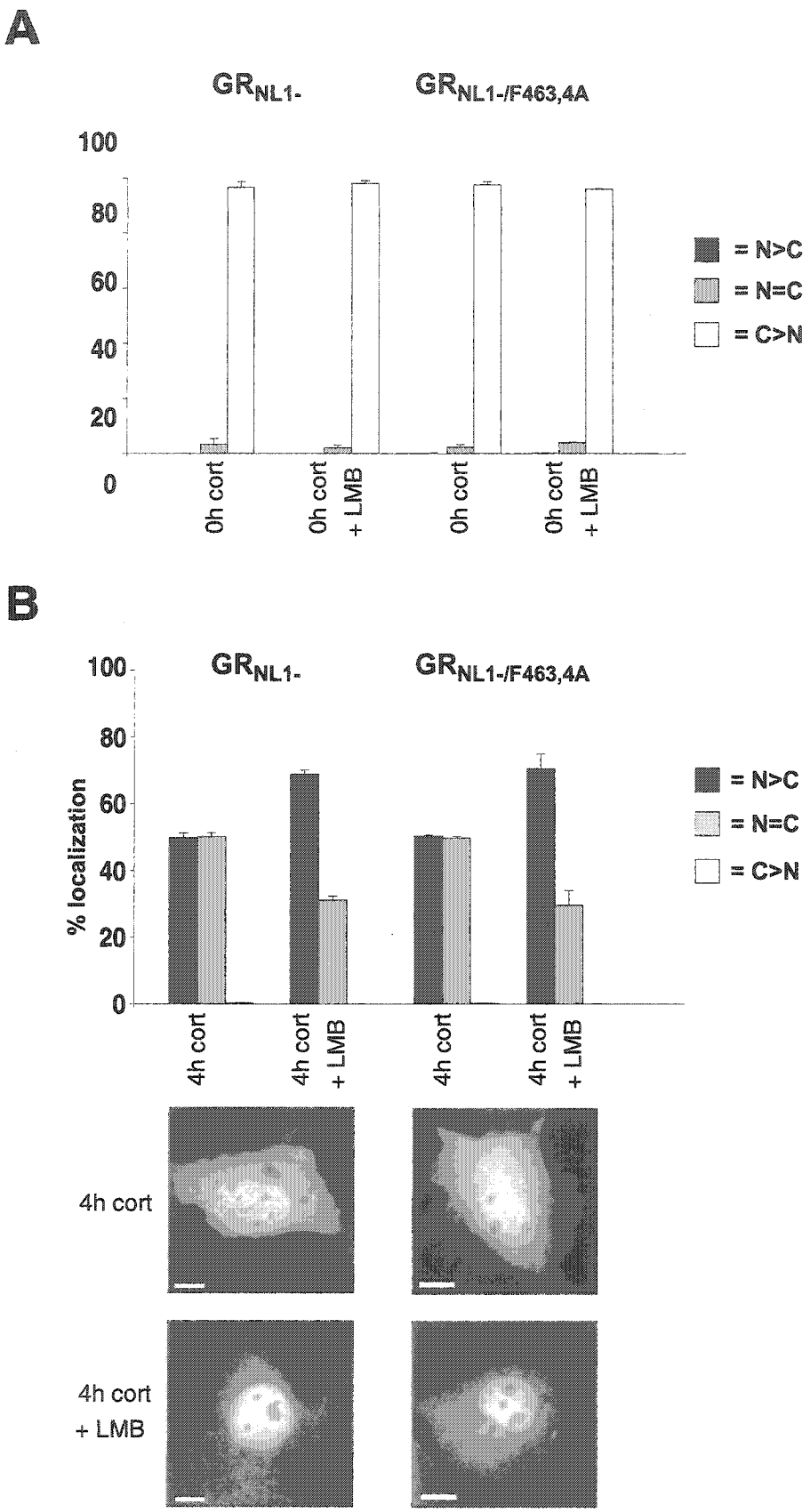
following ligand binding, GR_{NL1-} would be more likely to reveal a LMB-dependent shift to the nucleus than the wildtype receptor. Secondly, as GR_{NL1-} redistributes more rapidly to the cytoplasm following ligand withdrawal (130) (Fig. 14), shorter incubations with LMB are required to determine the effect of the CRM1 pathway on nuclear export of the ligand-withdrawn receptor.

Previous results are unclear as to whether the naïve receptor continuously shuttles between nucleus and cytoplasm. Studies in our own laboratory suggest that the CRM1 pathway may exert a slight effect on GR subcellular localisation prior to ligand binding (130) whereas other reports suggest that inhibiting CRM1-mediated export does not affect localisation of the naïve receptor (285). Additionally, localisation of naïve MR is unaffected by addition of LMB (Appendix C, Fig. 35). To further clarify the extent to which the CRM1 pathway influences the subcellular localisation of GR prior to ligand binding, GFP-GR_{NL1-} was incubated with or without LMB for 4 h prior to direct visualisation of GFP-GR_{NL1-} localisation by indirect immunofluorescence. As shown in Figure 20a, addition of LMB does not alter the subcellular distribution of naïve GR_{NL1-} or GFP-$GR_{NL1-/F463,464A}$. This result indicates that the cytoplasmic localisation of unliganded GR is not maintained by CRM1-mediated nuclear export.

FRAP analysis of GR nuclear export (Figures 8 and 15) suggests that liganded GR slowly exports between nucleus and cytoplasm. To determine if the CRM1 pathway mediates this slow nuclear export, GFP-GR_{NL1-} was incubated with 1 μM cortisol in the presence or absence of LMB prior to direct visualisation. As shown in Figure 20b incubation with 1 μM cortisol alone for 4 h resulted in partial nuclear localisation. Addition of LMB during this incubation period resulted in a notable increase in the

Figure 20: Steady state nuclear export of liganded GR, but not naïve receptor, is influenced by the CRM1 nuclear export pathway

(A) Subcellular localisation of naïve GR is unaffected by the CRM1 pathway. Cos7 cells were transfected with GFP-GR_{NL1-} by Lipofectamine™. Following transfection, the cells were plated onto glass coverslips and synchronised in G_0 by withdrawing serum. After 16-24 h, the cells were incubated in the presence or absence of 10 nM LMB for 4 h. The localisation of GR_{NL1-} was scored into categories as previously described by indirect immunofluorescence using a Nikon TE300 microscope. The percentage of cells scored as exclusively nuclear or mainly nuclear were added together to obtain the displayed values, as were the percentage of cells scored as exclusively cytoplasmic or mainly cytoplasmic. Error bars represent the standard error of the means of three independent experiments performed in duplicate. (B) Steady state export of liganded GR is affected by CRM1. Cos7 cells were transfected GFP-GR_{NL1-} or GFP-$GR_{NL1-/F463,464A}$ and treated as described in (A). Following incubation in serum-free media the cells were incubated with 1 μM cortisol for 4 h in the presence or absence of 10 nM LMB. The localisation of GR_{NL1-} was scored as described in (A). Representative micrographs are shown below each data set.


A
GR NL1-
GR NL1-/F463,4A
100
80
60
40
20
0
0h cort
0h cort + LMB
0h cort
0h cort + LMB
= N>C
= N=C
= C>N
B
GR NL1-
GR NL1-/F463,4A
% localization
4h cort
4h cort + LMB
4h cort
4h cort + LMB
4h cort
4h cort + LMB

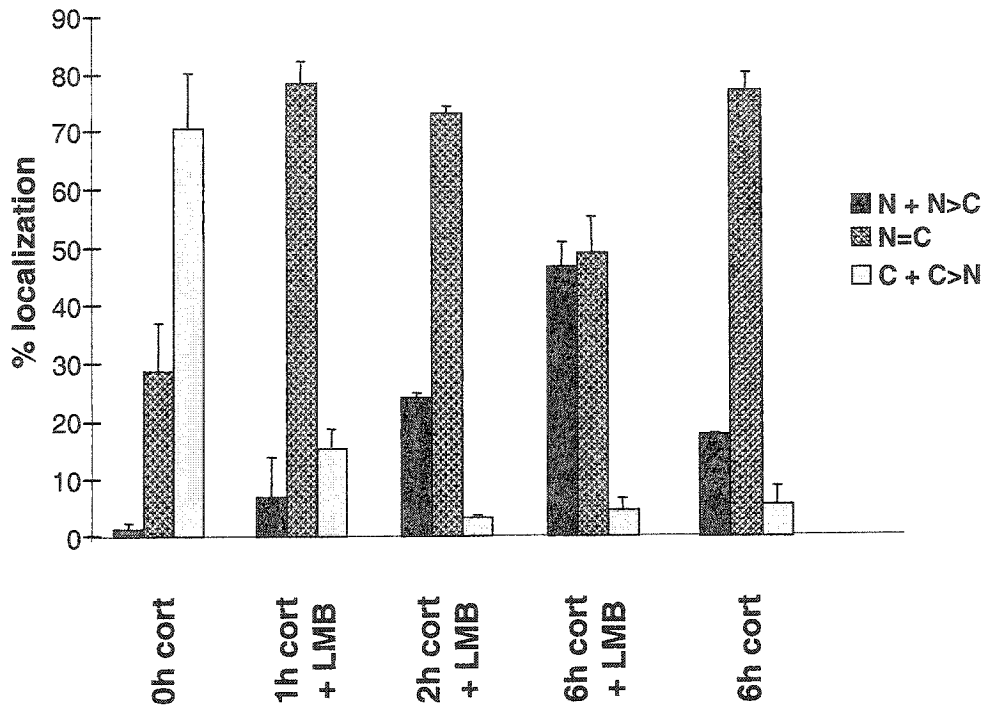
proportion of cells scored as predominantly nuclear. This increased CRM1-dependent nuclear localisation is not directly affected by calreticulin as the GFP-$GR_{NL1-/F463,464A}$ construct accumulates in the nucleus to the same extent as GFP-GR_{NL1-}. This result suggests that the CRM1 nuclear export pathway mediates steady state nuclear export of liganded GR in a manner that is not directly influenced by calreticulin.

To determine the kinetics with which LMB causes increased nuclear accumulation of GR_{NL1-} a time course was performed. Cells expressing GFP-GR_{NL1-} were incubated with cortisol in the presence or absence of LMB for periods of 1, 2 and 6 h prior to fixation. As shown in Figure 21, after 1 h of incubation with cortisol and LMB there is a notable increase in the proportion of cells expressing GFP-GR_{NL1-} that are scored as evenly distributed between nucleus and cytoplasm. After 2 h of incubation with cortisol and LMB, the increase in the proportion of cells scored as nuclear in the presence of LMB becomes more pronounced, and by 6 h there is a significantly greater proportion of cells scored as nuclear in the presence of LMB when compared to cortisol alone. This finding suggests that increased nuclear accumulation in the presence of LMB occurs gradually over a period of 6 h. Although the cortisol-dependent nuclear translocation of GR in this experiment was somewhat less pronounced than previously observed (i.e. 18% of cells were scored as predominantly nuclear following 6 h cortisol treatment in this experiment versus 50% in Figures 14 and 20), this result is in agreement with our finding that nuclear export of GR is a relatively slow process when compared to the observed rate of export of a protein bearing the HIV Rev NES.

Slow nuclear export of GR occurs both in the presence of ligand and following ligand withdrawal. To determine whether nuclear export of the ligand-withdrawn

Figure 21: Increased nuclear localisation of GR in response to LMB occurs gradually

Cos7 cells were transfected with GFP-GR_{NL1-} as described in Figure 21a. Following incubation in serum-free media the cells were incubated with 1 μM cortisol in the presence or absence of 10nM LMB for the indicated period of time. The localisation of GFP-GR_{NL1-} was scored as described in Figure 21a. Error bars represent the average deviation of duplicate samples.


90
80
70
60
50
40
30
20
10
0
% localization
N + N>C
N=C
C + C>N
0h cort
1h cort + LMB
2h cort + LMB
6h cort + LMB
6h cort

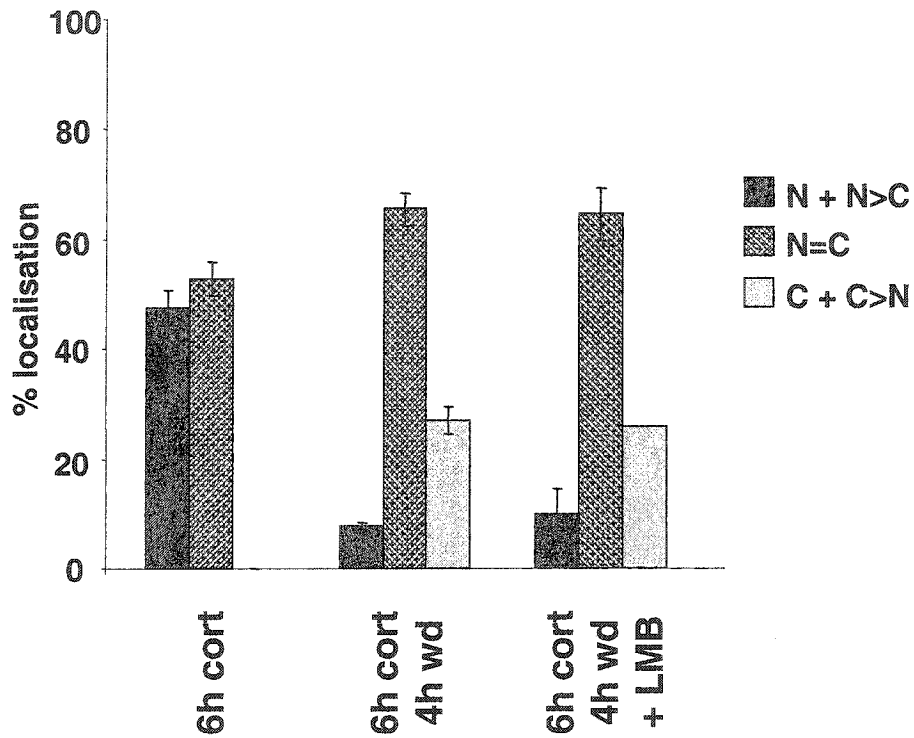
receptor was also affected by inhibition of CRM1-mediated export, LMB was added to cells expressing GFP-GR_{NL1-} at the point of ligand withdrawal. As shown in Figure 22, incubation with 1 μM cortisol alone for 4 h resulted in partial nuclear localization of GFP-GR_{NL1-}. 6 h following ligand withdrawal there is an increase in the population of cells expressing GFP-GR_{NL1-}that are scored as predominantly cytoplasmic. Addition of LMB during the withdrawal period does not appear to change the rate of redistribution to the cytoplasm following ligand withdrawal. This result indicates that GR nuclear export following ligand withdrawal is not influenced by CRM1. Further, this provides evidence that although nuclear export of liganded and ligand-withdrawn GR appears to occur with similar kinetics, two separate nuclear export pathways mediate these processes.

Nuclear Retention of GR is Mediated by the NL1 Motif

Nuclear import of GR is mediated through two nuclear import activities, NL1 and NL2. Previous results from our laboratory indicate that in addition to partially mediating nuclear import, the GR NL1 sequence also affects the rate of nuclear export following ligand withdrawal (130). The core NL1 sequence is found within the hinge region of the receptor. It has been shown that mutation of lysine residues 513-515 to asparagine (GR_{NL1-}) results in decreased ligand-dependent nuclear import. Additionally, upon ligand withdrawal, GR_{NL1-} redistributes to the cytoplasm with a half time of approximately 4 h whereas the wildtype receptor returns to the cytoplasm more slowly with a half time of 12-24 h [(130), see also Figure 14]. This finding suggested that the region of GR containing the NL1 core sequence might also overlap with an activity that directly inhibits nuclear export or specifically promotes nuclear retention.

Figure 22: Nuclear export of ligand withdrawn GR is independent of the CRM1 pathway

Cos7 cells were transiently transfected with GFP-GR_{NL1-} and treated as described in Figure 21. Following incubation in serum-free media the cells were incubated with 1 μM cortisol for 4 h and then withdrawn from ligand for 4 h in the presence or absence of 10 nM LMB. The localisation of GR_{NL1-} was scored as described in Figure 21. Error bars represent the standard error of the means of three independent experiments performed in duplicate.


100
80
60
40
20
0
% localisation
N + N>C
N=C
C + C>N
6h cort
6h cort 4h wd
6h cort 4h wd + LMB

To examine the possibility that the hinge region of GR containing the NL1 sequence might mediate nuclear retention of GR, FRAP analysis of GR trafficking in multinucleated cells was performed on the GFP-GR_{NL1-} mutant (Fig. 23). Following 4 h incubation with cortisol, GFP-GR_{NL1-} is partially localised to the nucleus. This partial nuclear localisation is expected, as the NL2-mediated nuclear import is less efficient than NL1-mediated import of GR. To eliminate the possibility that the recovery of GFP signal could occur through the import of receptor initially localised to the cytoplasm, GFP-GR_{NL1-} was photobleached from both the acceptor nucleus and the cytoplasm. Two hours following photobleaching, GFP-GR_{NL1-} export from the unbleached nucleus was observed by the redistribution of GFP signal to the acceptor nucleus and the cytoplasm. Thus both GR_{NL1-} nuclear export and re-import were observed. The rate of GR_{NL1-} export was accelerated compared to the wild type receptor, which was previously shown to remain predominantly localised to the donor nucleus at 2 h post-bleach (Fig. 8 and Fig. 15). This observation confirms that in addition to mediating GR nuclear import, the region of GR containing the NL1 motif may also function to retain GR within the nucleus.

To further explore the possibility that the hinge region of GR has a retentive function that overlaps with NL1, I hypothesised that the addition of the GR hinge-containing region to an actively exported protein would result in a decreased rate of nuclear export. To investigate this proposition, the control proteins that were initially constructed to establish the functionality of the FRAP-based nuclear export assay system were utilised. As outlined in Figure 24a, the GST moiety of the pNES-GST-GFP-NLS positive control construct was replaced with a region of GR spanning amino acid residues

Figure 23: Deletion of the NL1 sequence enhances the rate of nuclear export of liganded GR in a FRAP assay

(A) Representation of the GFP-GR_{NL1-} construct used in this experiment. (B) Cos7 cells were plated onto 40mm round coverslips transiently transfected with GFP-GR_{NL1-} and treated as indicated in Figure 7. Following transfection cells were treated with 1 μM cortisol for 4 h to induce partial nuclear import. Nuclear export in the continued presence of ligand was monitored following FRAP over a period of 2 h as described previously.

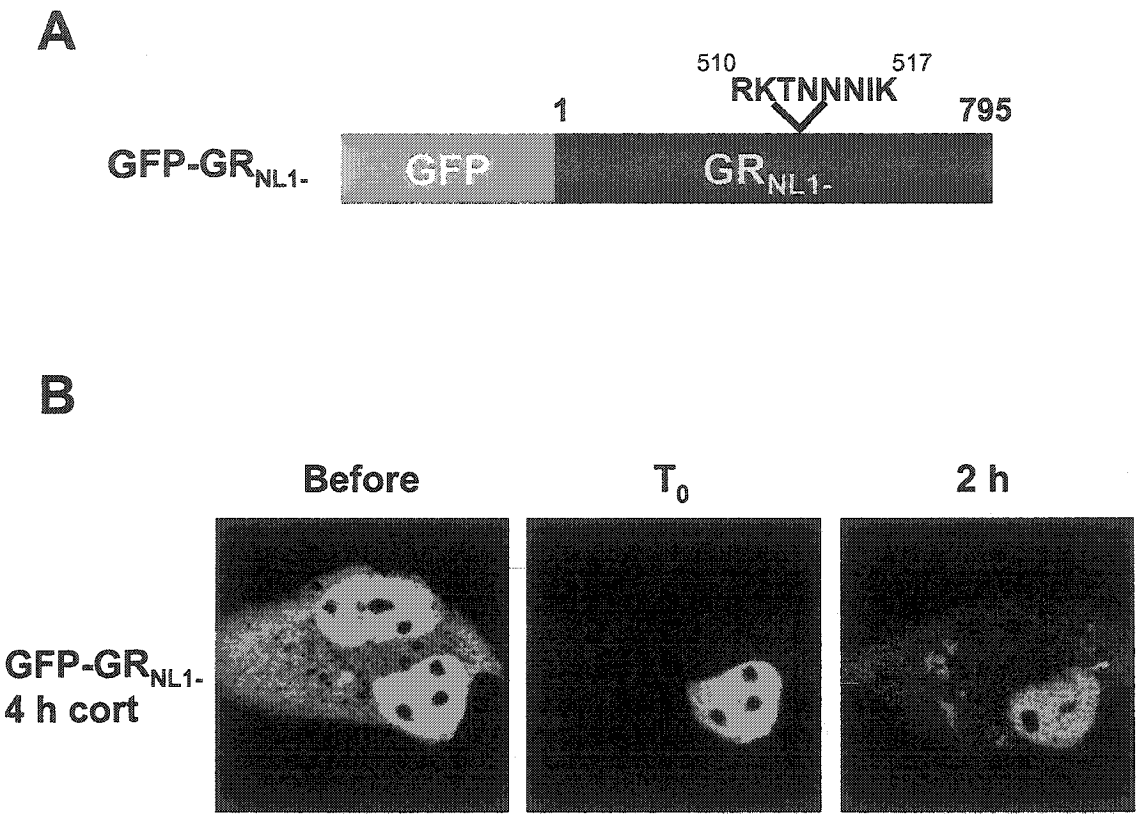
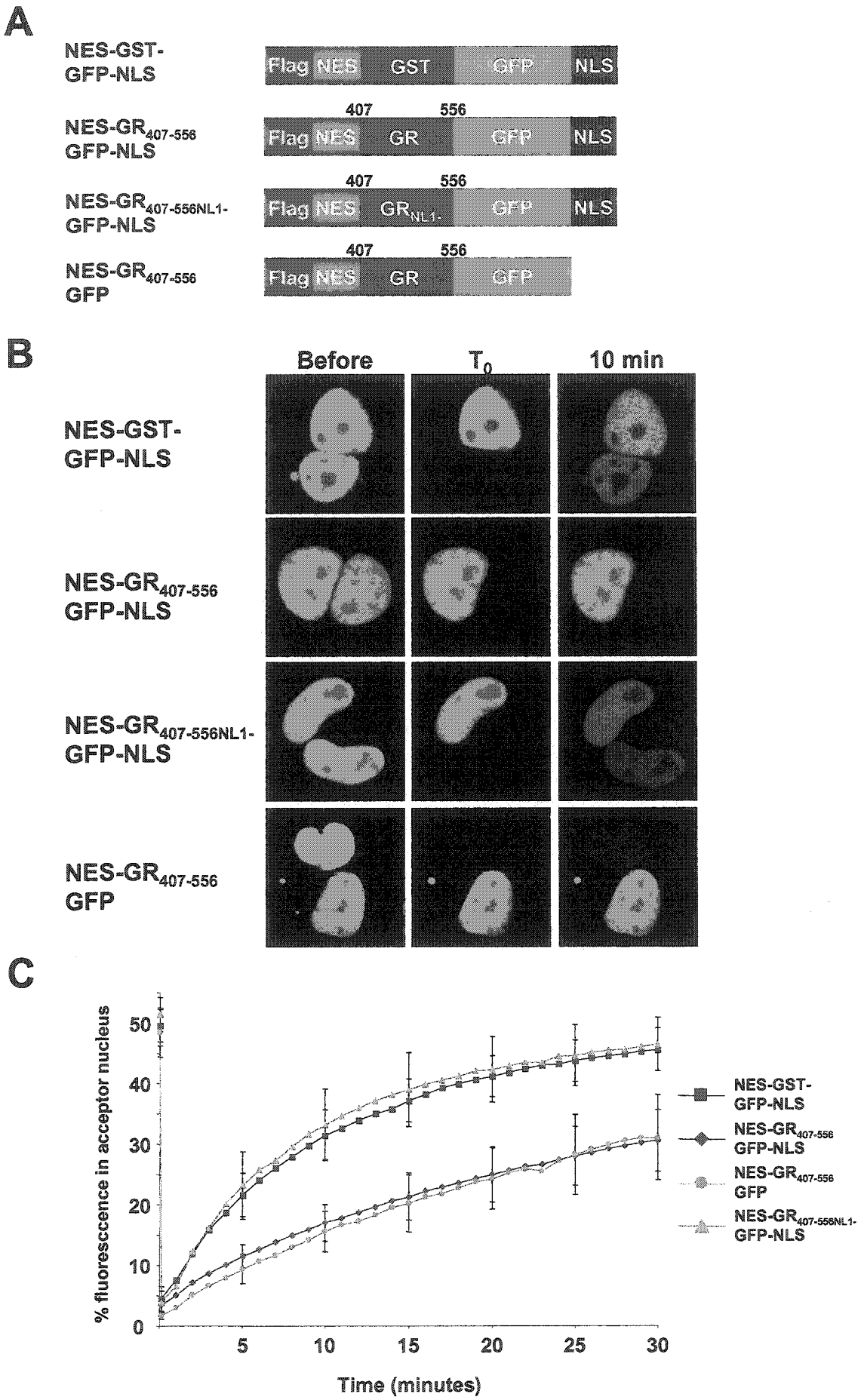

A
510
517
RKTNNNIK
1
795
GFP-GR$_{NL1-}$
GFP
GR$_{NL1-}$
B
Before
T$_0$
2 h
GFP-GR$_{NL1-}$
4 h cort

Figure 24: The GR NL1 sequence, but not an ectopic SV40 NLS blocks CRM1-mediated nuclear export

(A) Outline of constructs used in this experiment. (B) Cos7 cells were plated onto 40 mm round coverslips and transfected with the indicated GFP fusion constructs and analysed by confocal microscopy as described in Figure 7. Nuclear transfer of a synthetic NES containing construct is nearly complete 10 min following bleaching (top row). The rate of export of a construct containing GR residues 407-556 bearing an NES is slowed (second row). Mutation of the GR NL1 sequence results in an increased rate of export that approaches the rate seen for the control (third row). The removal of the SV40 NLS does not result in significant acceleration of the rate of export (bottom row). (C) Quantitative analysis of FRAP data. The percentage of total fluorescence present in the acceptor nucleus following photobleaching was calculated and plotted as a function of time for each of the indicated constructs. Each data point represents the average of at least 8 independent experiments.


A
NES-GST-GFP-NLS
Flag NES GST GFP NLS
NES-GR407-556 GFP-NLS
407 556
Flag NES GR GFP NLS
NES-GR407-556NL1-GFP-NLS
407 556
Flag NES GRNL1- GFP NLS
NES-GR407-556 GFP
407 556
Flag NES GR GFP
B
Before
T0
10 min
NES-GST-GFP-NLS
NES-GR407-556 GFP-NLS
NES-GR407-556NL1-GFP-NLS
NES-GR407-556 GFP
C
% fluoresccence in acceptor nucleus
0
10
20
30
40
50
5
10
15
20
25
30
Time (minutes)
NES-GST-GFP-NLS
NES-GR407-556 GFP-NLS
NES-GR407-556 GFP
NES-GR407-556NL1-GFP-NLS

407-556. This region contains both the DNA binding domain (DBD) and the NL1 motif. Both the wildtype insert, pNES-$GR_{407\text{-}556}$-GFP-NLS, and an insert containing the mutated NL1 sequence, pNES-$GR_{407\text{-}556NL1\text{-}}$-GFP-NLS, were constructed. Since both of these constructs contain an SV40 NLS that could also have a nuclear retention activity, the pNES-$GR_{407\text{-}556}$-GFP construct was created as a further control for specificity. This construct is identical to pNES-$GR_{407\text{-}556}$-GFP-NLS except that it lacks the SV40 NLS.

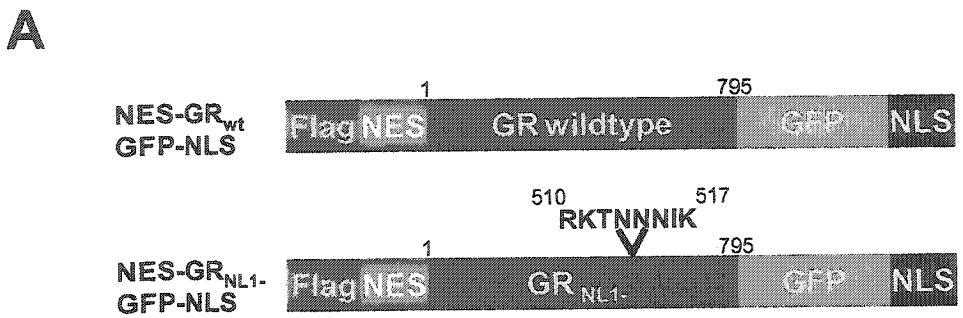
As shown in Figure 24b (top panel), FRAP analysis using the pNES-GST-GFP-NLS confirmed earlier observations (Fig. 7b) that this positive control protein equilibrates rapidly between nuclei with a $t_{1/2}$ of approximately 6 min (Fig. 24c). At 30 minutes post bleach, 45.6% ± 3.6% of the GFP signal had transferred between nuclei. When the GST moiety is replaced by the GR DBD and hinge region there is a clear reduction in the rate of NES-mediated nuclear export (Fig. 24b, second panel) as only 30.5% ± 5.1% of the GFP signal transferred between nuclei at 30 min following photobleaching (Fig. 24c). To determine whether this reduction in the rate of nuclear export is due to the presence of the GR NL1 sequence, FRAP analysis was performed using the pNES-$GR_{407\text{-}556NL1\text{-}}$-GFP-NLS construct. As shown in the third panel of Figure 24b and quantified in Figure 24c, mutation of the GR NL1 sequence within this construct restores nuclear transfer to a rate similar to that observed for the NES-GST-GFP-NLS positive control construct, with 46.4% ± 4.4% recovery of GFP signal observed in the bleached nucleus following photobleaching. To show that this inhibitory effect on nuclear export is specific to the GR NL1-containing region and not shared by the SV40 NLS, the trafficking behaviour of the NES-$GR_{407\text{-}556}$-GFP construct, which lacks the SV40 NLS, was studied. As shown in Figure 24b, removal of the SV40 NLS does not

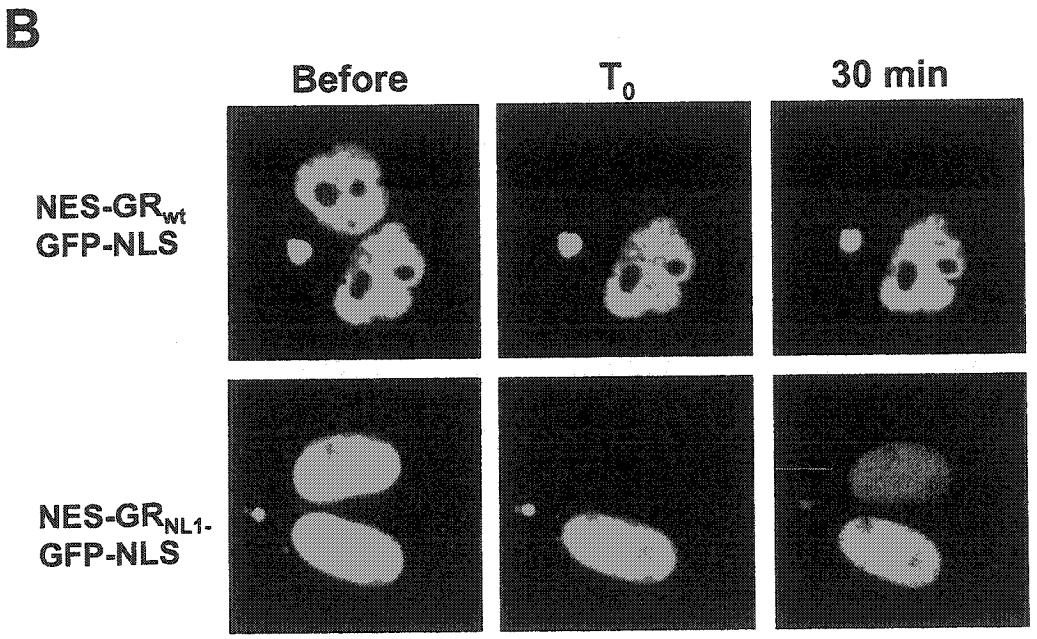
significantly alleviate the inhibitory effect of the $GR_{407-556}$ region on nuclear export mediated through the Rev NES. At 30 min post-bleach 31.0% ± 7.0% recovery of GFP signal was observed in the bleached nucleus (Fig. 24c). These results suggest that a sequence motif dependent upon the GR hinge region overlapping with NL1 can act as a nuclear retention signal in a manner that exerts a transdominant negative effect on export mediated through an active NES.

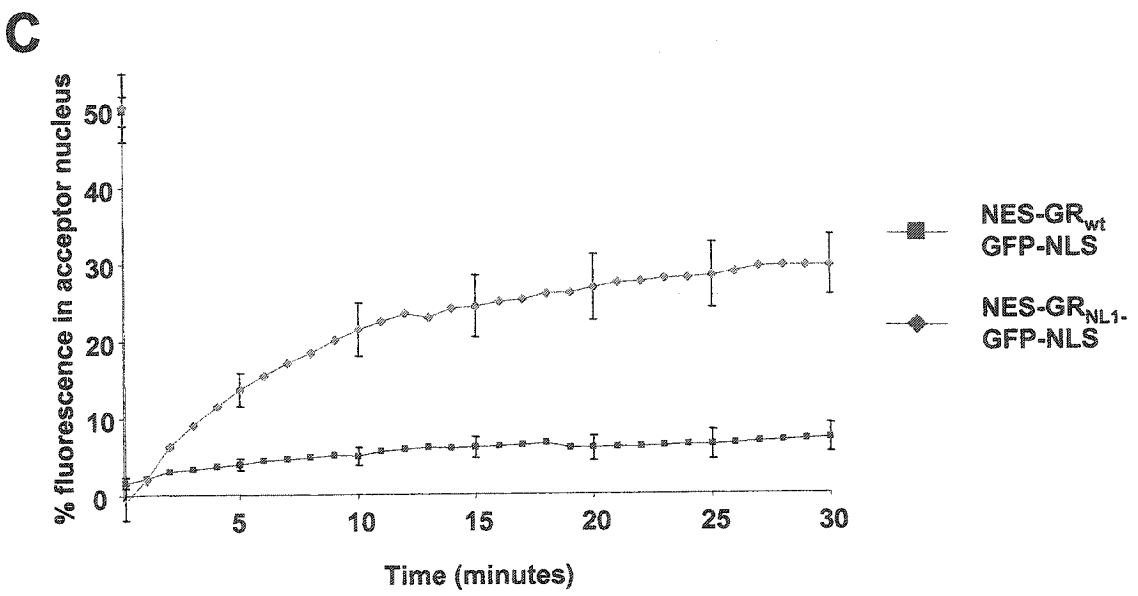
To determine whether the hinge region of GR containing the NL1 sequence functions as a transdominant nuclear retention sequence within the context of the full-length receptor, constructs were made in which the full length GR_{wt} and GR_{NL1-} sequences are fused to the Rev NES (Fig. 25a). As shown in Figure 25b (top panel) and quantified in Figure 25c, FRAP analysis of the NES-GR_{wt}-GFP-NLS construct shows there is little recovery of GFP signal in the bleached nucleus up to 30 min following photobleaching. The amount of GFP-signal transferred between nuclei at 30 minutes post-bleach was quantified at 7.2% ± 1.6% (Fig. 25c). This result indicates that the full-length receptor effectively inhibits nuclear export mediated by the Rev NES. When the GR_{wt} sequence is replaced by the GR_{NL1-} sequence there is a significant, but not complete, increase in the amount of NES-GR_{NL1-}-GFP-NLS signal of 29.6% ± 3.9% observed in the bleached nucleus at 30 min post-bleach (Fig. 25b, bottom panel and Fig. 25c). This result suggests that in the context of full length GR, mutation of the NL1 core sequence abrogates the ability of the hinge region to effectively repress active export mediated through a NES. However, in the context of the full-length receptor the NL1 mutation does not fully restore NES-mediated export to the rate observed for the NES-GST-GFP-NLS positive control construct. While this may be a reflection of the increased size of

Figure 25: The GR NL1 blocks CRM1 mediated nuclear export in the context of the full-length protein

(A) Outline of constructs used in this experiment. (B) Cos7 cells were plated onto 40 mm round coverslips and transfected with the indicated GFP fusion constructs and analysed by confocal microscopy as described in Figure 7. Cells were treated with 1 μM cortisol for 1 h prior to FRAP. At 30 min post-bleach there is minimal nuclear transfer of the full-length receptor fused to a synthetic NES (top row). The rate of export of the full-length receptor fused to a synthetic NES is significantly increased in the presence of the NL1 mutation (bottom row). (C) Quantitative analysis of FRAP data. The percentage of total fluorescence present in the acceptor nucleus following photobleaching was calculated and plotted as a function of time for each of the indicated constructs. Each data point represents the average of at least five independent experiments.


A
NES-GR$_{wt}$
GFP-NLS
1
795
Flag NES
GR wildtype
GFP
NLS
510
517
RKTNNNIK
NES-GR$_{NL1-}$
GFP-NLS
1
795
Flag NES
GR $_{NL1-}$
GFP
NLS


B
Before
T_0
30 min
NES-GR$_{wt}$
GFP-NLS
NES-GR$_{NL1-}$
GFP-NLS


C
% fluorescence in acceptor nucleus
0
10
20
30
40
50
5
10
15
20
25
30
Time (minutes)
NES-GR$_{wt}$
GFP-NLS
NES-GR$_{NL1-}$
GFP-NLS

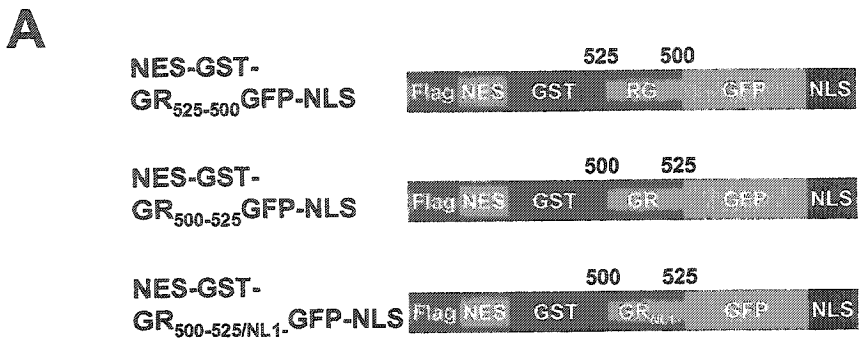
the NES-GR-GFP-NLS construct, this result may indicate that sequences in addition to the NL1 motif may function to retain full-length GR within the nucleus.

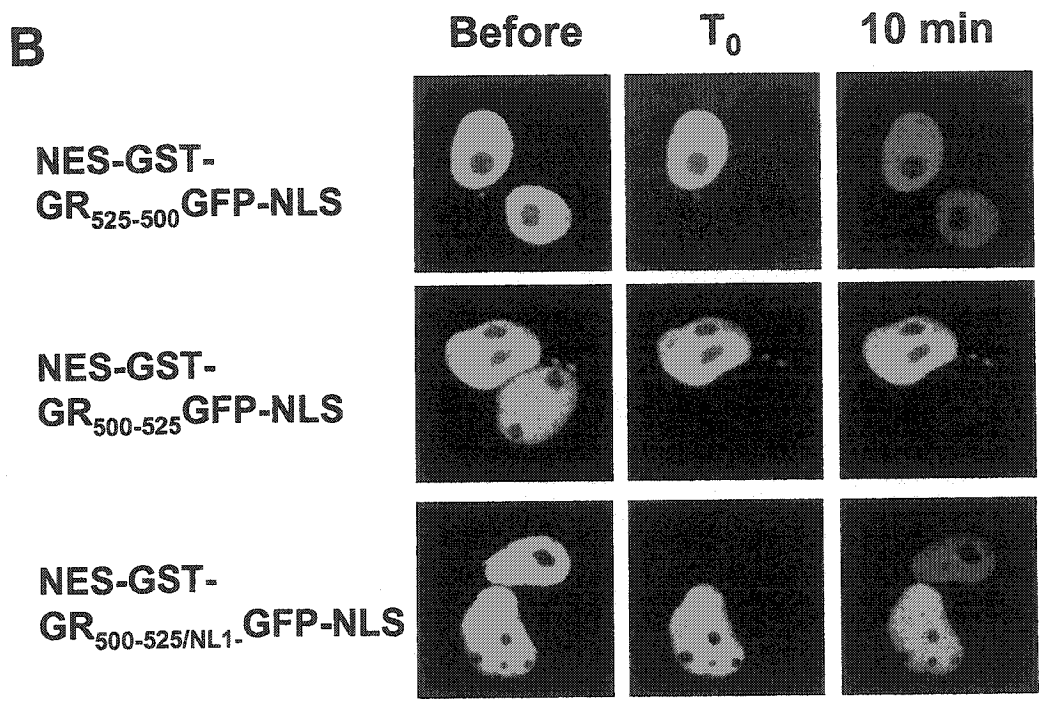
While ligand-dependent nuclear localisation of full length GR through the NL1 motif is significantly disrupted through mutation of three core lysine residues, additional sequences within the DBD (as outlined in Figure 4) have also been shown to affect NL1-mediated nuclear accumulation. To define a minimal sequence that was sufficient to inhibit nuclear export, a GR peptide encompassing amino acid residues 500-525 was cloned onto the NES-GST-GFP-NLS positive control construct (Fig. 26a). The $GR_{500\text{-}525}$ peptide includes the NL1 core region but does not include the two basic clusters that are thought to enhance nuclear accumulation. As an additional control the $GR_{500\text{-}525}$ peptide was cloned into the NES-GST-GFP-NLS construct in the antisense orientation, yielding a protein product that is the same size as NES-GST-$GR_{500\text{-}525}$-GFP-NLS. To determine if nuclear retention of the NES-GST-$GR_{500\text{-}525}$-GFP-NLS fusion protein is sensitive to the NL1 mutation, a third construct bearing the $GR_{500\text{-}525/NL1\text{-}}$ peptide was constructed.

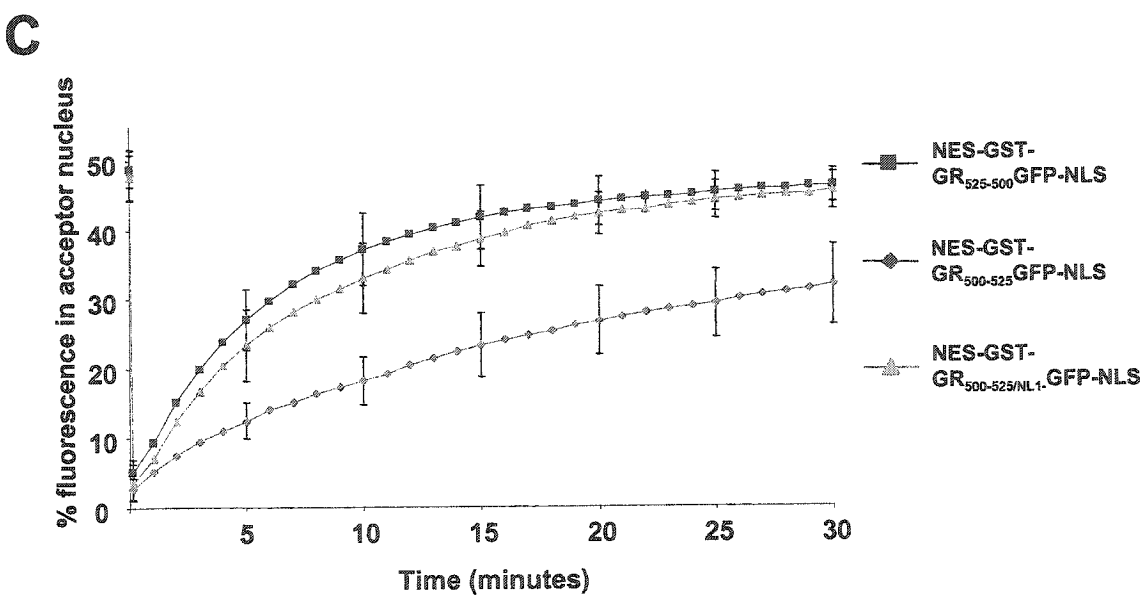
As shown in Figure 26b, and quantified in Figure 26c, FRAP analysis revealed that addition of the $GR_{500\text{-}525}$ peptide to the NES-GST-GFP-NLS construct results in a notable decrease in the rate of NES-dependent nuclear export (top panel). The overall rate of export of the NES-GST-$GR_{500\text{-}525}$-GFP-NLS construct was measured at 31.9% ± 5.8% at 30 minutes post-bleach. This rate of export is directly comparable to that of the NES-$GR_{407\text{-}556}$-GFP-NLS construct, which was measured at 30.6% ± 5.1% at the same time point (see Figure 24c). Insertion of this peptide in the antisense orientation does not appear to affect the rate of nuclear export as compared to the previously observed NES-GST-GFP-NLS construct (compare Fig. 26b, middle panel to Figs. 7 and 24). The

Figure 26: The GR hinge region from amino acid residues 500-525 is sufficient to mediate NL1-dependent nuclear retention

(A) Outline of constructs used in this experiment. (B) Cos7 cells were plated onto 40 mm round coverslips and transfected with the indicated GFP fusion constructs and analysed by confocal microscopy as described in Figure 7. Nuclear transfer of a synthetic NES containing construct bearing the GR 500-525 peptide in the antisense orientation is nearly complete 10 min following bleaching (top row). The rate of export of a NES-bearing construct that contains the GR 500-525 peptide bearing an NES is slowed (middle row). Mutation of the NL1 core within the GR 500-525 peptide results in an increase in the rate of nuclear export compared to the wildtype sequence (bottom row). (C) Quantitative analysis of FRAP data. The percentage of total fluorescence present in the acceptor nucleus following photobleaching was calculated and plotted as a function of time for each of the indicated constructs. Each data point represents the average of at least eight independent experiments.


A
NES-GST-
GR525-500GFP-NLS
525 500
Flag NES GST RG GFP NLS
NES-GST-
GR500-525GFP-NLS
500 525
Flag NES GST GR GFP NLS
NES-GST-
GR500-525/NL1-GFP-NLS
500 525
Flag NES GST GR GFP NLS


B
Before
T0
10 min
NES-GST-
GR525-500GFP-NLS
NES-GST-
GR500-525GFP-NLS
NES-GST-
GR500-525/NL1-GFP-NLS


C
% fluorescence in acceptor nucleus
0
10
20
30
40
50
5
10
15
20
25
30
Time (minutes)
NES-GST-
GR525-500GFP-NLS
NES-GST-
GR500-525GFP-NLS
NES-GST-
GR500-525/NL1-GFP-NLS

overall rate of transfer of the NES-GST-$GR_{525-500}$-GFP-NLS construct was measured at 46.2% ± 2.4% at 30 minutes post-bleach (Fig 26c), which is directly comparable to the rate of transfer of the NES-GST-GFP-NLS construct of 45.5 ± 3.6% measured at the same time point (see Figure 24c). This suggests that the decrease in the observed rate of nuclear export is dependent on the $GR_{500-525}$ peptide. Mutation of the NL1 sequence within the $GR_{500-525}$ peptide results in the recovery of NES-mediated nuclear export (Fig. 26b, bottom panel) with 45.5% ± 2.7 recovery measured at 30 minutes post-bleach (Fig. 26c). This result indicates that while disruption of the NL1 core sequence abrogates nuclear retention, residues in addition to the core ^{513}KKK^{515} residues contribute to nuclear retention in this context. Together, the results of Figures 23 through 26 indicate that the GR hinge region can act as a dominant nuclear retention signal over export mediated through an active NES in a manner that is at least partially sensitive to mutation of the NL1 core sequence.

Discussion

It has been known for many years that GR undergoes nuclear export following ligand withdrawal, yet a satisfactory explanation regarding the mechanism and regulation of GR relocalisation to the cytoplasm remains elusive. The key to understanding this process hinges on explaining the dichotomy between the slow relocalisation of GR to the cytoplasm following ligand withdrawal and the apparently rapid nucleocytoplasmic shuttling of the receptor that has between observed in cell fusion-based assays. Work presented in this thesis provides a new perspective on GR trafficking obtained through the use of live cell imaging techniques. This methodology allows for direct visualisation of receptor localisation without relying on invasive experimental manipulations. Through the application of these methods I have found that in the absence of cell fusion nucleocytoplasmic shuttling of liganded GR proceeds at a much slower rate than previously suggested. Accelerated GR shuttling observed in cell fusion-based assays appears to be a consequence of the mis-localisation of calreticulin that results from transient disruption of the endoplasmic reticulum. Under resting cellular conditions calreticulin is sequestered within the endoplasmic reticulum and has no direct influence over GR trafficking.

The slow rate of GR nuclear export observed in the absence of cell fusion is due in part to active nuclear retention. Nuclear retention of GR occurs through a sequence motif that overlaps with the GR NL1 sequence. The observation that an NLS can also participate in nuclear retention is a unique finding and suggests that two mechanisms that control GR subcellular localisation -- nuclear import and nuclear retention -- converge through a single motif. My findings imply that liganded GR is less accessible to the

cytoplasm than previously anticipated and suggests that the view that steroid hormone receptors have the potential to rapidly respond to cytosolic signalling pathways should be more generally re-evaluated. Importantly, my results highlight a potential drawback of cell-fusion-based nuclear export assays and emphasise the need to re-evaluate reports of nucleocytoplasmic shuttling that are based only on cell-fusion based assays.

Consequences of Nucleocytoplasmic Shuttling on Protein Function

Through recent advances in imaging technology it has become apparent that protein subcellular localisation has the potential to be a much more dynamic process than previously envisioned. This emerging viewpoint has led to a general shift away from the view that proteins are restricted to a defined subcellular location and increases the possibilities for interplay between signalling pathways found within different areas of the cell, allowing for rapid and direct communication between separated pathways [reviewed in (356)]. Indeed, for a number of proteins, continuous shuttling between compartments has been shown to allow for regulation of nuclear activity through modification in the cytoplasm or regulated sequestration of the factors from the nucleus.

This principle has been suitably demonstrated for a number of signalling pathways. For example, Smad2 and Smad3 are known to accumulate in the nucleus upon activation of TGF-β signalling. It has been shown recently that levels of activated Smad protein in the nucleus are regulated through communication with components of the TGF-β signalling pathway localised to cytoplasm. This regulation is dependent upon the continuous shuttling of activated Smads between nucleus and cytoplasm (369,370).

Additionally, nucleocytoplasmic shuttling of Swi6p, a yeast transcription factor involved in the initiation of cell division, is necessary for the function of Swi6p (371).

Proteins localised to the cytoplasm have also been shown to continuously shuttle in and out of the nucleus. IκB is a regulatory subunit of the NFκB transcription factor. The IκB and NFκB heterodimer is found predominantly in the cytoplasm as an inactive complex. Various stimuli can activate NFκB signalling through the phosphorylation and degradation of IκB [reviewed in (372)]. The formation of a heterodimer between IκB and NFκB was originally proposed to anchor NFκB within the cytoplasm by masking its NLS motif (373). It has since been demonstrated that the IκB-NFκB complex continuously shuttles between nucleus and cytoplasm via an NLS and a CRM1-dependent NES motif resident within the IκB subunit (214,374,375). The purpose of this continuous shuttling prior to NFκB activation remains unclear, however following induction, shuttling of nuclear NFκB bound to IκBα may be required to clear activated NFκB from the nucleus upon removal of stimulation. This nuclear clearance mechanism may be required to rapidly re-establish levels of cytoplasmic NFκB that are primed for subsequent rounds of signalling (376). Similarly, Stat1 is a predominantly cytoplasmic protein that translocates to the nucleus in response to interferon-γ. Interestingly, Stat1 can also constitutively regulate the activity of target genes. It has recently been shown that uninduced STAT1 constantly shuttles between cytoplasm and nucleus, thereby allowing for constitutive gene regulation in the absence of interferon-γ signalling (377).

An interesting example of oscillatory shuttling between nucleus and cytoplasm has been recently described (378). Msn2 and Msn4 are yeast transcription factors that are localised to the cytoplasm in the absence of cell stress and become predominantly

nuclear in response to severe stress. Under conditions of moderate stress, the localisation of Msn2 and Msn4 within an individual cell varies between predominantly nuclear and predominantly cytoplasmic with a period of a few minutes. This oscillating shuttling mechanism is thought to allow for a finer degree of control over the level of gene expression in response to stress by allowing for brief periods of maximal gene activity.

The observation that liganded GR and other steroid hormone receptors could potentially shuttle between nucleus and cytoplasm gave rise to the view that receptor function might be influenced by factors resident in the cytoplasm. If shuttling did indeed represent the default state for nuclear receptors, then it was conceivable that shuttling would allow for interaction with cytosolic factors that could either mediate specific modifications or engage in specific co-transport of other cellular factors that could modify receptor function. The inconsistency between rapid nuclear import, slow nuclear export and continuous shuttling led to the hypothesis that GR subcellular localisation represents a dynamic balance between nuclear import and nuclear export, with the relative rate of each process combining to determine the net subcellular localisation of the receptor (379). The work presented in this thesis significantly alters this notion of receptor function. Based on my findings, it appears that while GR does appear to be continuously exported from the nucleus to some extent, the rate of export is not as rapid as previously believed. In fact, the export rate of the receptor appears to be consistent with the slow rate of return to the cytoplasm observed following ligand withdrawal. This implies that cytosolic signalling pathways may have less opportunity to interact with and influence the liganded receptor than previously thought.

My findings suggest that the degree to which signalling proteins are able to shuttle between nucleus and cytoplasm varies considerably. While it is apparent that active shuttling may be essential to the proper function and regulation of particular signalling pathways, this mode of regulation may not necessarily apply to all proteins. The concept that signalling proteins are in continuous movement across membrane barriers appears to be a rather fashionable notion. While shuttling provides an attractive mechanism for rapid regulation of protein function, this pathway may not apply equally to all signalling proteins. My results indicate that this may be the case for GR. While the subcellular localisation of GR is clearly influenced by signal-mediated translocation through the nuclear pore, the steady state localisation of the receptor may be more greatly influenced by both nuclear and cytoplasmic retention rather than by continuous transport.

Based on the results of cell fusion-based assay systems, nucleocytoplasmic shuttling has been described for several steroid hormone receptors. Guiochon-Mantel and colleagues first demonstrated that liganded PR, which is primarily localised to the nucleus, rapidly shuttles between nucleus and cytoplasm with equilibrium of receptor distribution between the nuclei of fused cell obtained within 1 h (39,351). This initial finding was then extended to ER (352) and GR (353,354). However, these reports rely primarily on the use of heterokaryon cell fusions to show rapid nuclear export of these receptors in the presence of ligand. Rapid nucleocytoplasmic shuttling has also been reported for the related nuclear hormone receptor TR (355,380). Although shuttling appears to be complete within 1 h following cell fusion (380), when nuclear export is studied through the use of sodium azide to deplete GTP levels, shuttling appears to occur rather slowly with kinetics that more closely resemble those observed for GR in this

thesis (355). While the significance of this finding may be questionable as CRM1-mediated nuclear export requires GTP and because sodium azide has numerous effects on cell function, this finding indicates that the rate of nuclear export of nuclear hormone receptors may be slower than anticipated based on the results of cell fusion-based assay systems.

Through use of a FRAP-based nuclear export assay similar to that used in this work that utilises multi-nucleated cells, Prufer and colleagues have shown that the vitamin D receptor (VDR) and RXR may potentially shuttle between nucleus and cytoplasm in both the presence and absence of ligand (381). Interestingly, VDR is also thought to be localised to the cytoplasm prior to ligand binding (382). The kinetics of shuttling observed by Prufer *et al.* appears to be more rapid than that observed for GR this thesis. Prufer and colleagues have reported that unliganded VDR equilibrates between nuclei within 15 to 30 minutes of photobleaching. In this same study, export of liganded VDR was reported to occur more rapidly with equilibration between nuclei reached within 5 to 15 minutes following photobleaching (381). Shuttling of RXR was observed to occur more slowly with a half time of about 20 to 30 minutes (381), although it is not clear whether these experiments were performed with or without ligand. This finding regarding the rate of RXR nuclear export is in closer agreement to preliminary results obtained in the course of my own studies for shuttling of liganded RXR than to the rate of export described for either liganded or unliganded GR. Preliminary results indicate that nuclear export of RXR treated with 9-cis retinoic acid occurs with a half time of about 40 minutes (R. Walther and R.J.G. Haché, unpublished observation). By contrast, both liganded and ligand-withdrawn GR were shown to export from the nucleus

at a relatively slow rate, with a modest transfer of GFP fluorescence of approximately 5% per hour over the 4 h period following photobleaching of the liganded receptor, and only a 12% ± 3% increase in cytoplasmic GFP-fluorescence observed over the same period following photobleaching of ligand-withdrawn GR (see Figure 8). Additionally, preliminary results suggest that MR is also exported from the nucleus slowly over a period of several hours (see Figure 31, Appendix C).

While it is unclear whether the differences in the rates of nuclear hormone receptor nuclear export observed between my own studies and those determined by Prufer *et al.* are merely a reflection of subtle differences in the experimental systems used in each study, it is possible that there are indeed measurable differences in the rate of nuclear export for each of these receptors. The variation in the net rate of nuclear export for each receptor may be due to disparities in the inherent rate of nuclear export. Alternatively, the extent to which nuclear retention influences export may vary among receptors. It is likely that in determining the net rate of nuclear hormone receptor export from the nucleus both retention and direct nuclear export exert an influence to varying degrees depending on the protein.

The reason as to why GR and other nuclear hormone receptors may shuttle to varying degrees between nucleus and cytoplasm remains unclear. While a physiological function for GR shuttling has yet to be determined, it has been suggested by Liu and DeFranco that GR protein stability is influenced by its subcellular localisation (285). Fusion of an NES motif to GR resulted in an overall decrease in the stability of the protein, suggesting that GR is preferentially degraded in the cytoplasm (285). This would suggest that the liganded receptor is stabilised through nuclear retention. The

observations of Liu and DeFranco are consistent with my own preliminary observation that the NES-GR_{NL1}-GFP-NLS fusion construct, which was used to establish that the NL1 motif functions as a retention sequence in the context of full length GR (Figure 25), accumulates to a lower level than the NES-GR_{wt}-GFP-NLS construct (R. Walther and R. J. G. Haché, unpublished observation). While the physiological rationale underlying the preferential degradation of GR in the cytoplasm is unknown, retaining GR in the nucleus for a prolonged period of time following withdrawal of hormone stimuli could offer partial protection from degradation. Nuclear retention of GR could potentially allow for extended sensitivity to subsequent rounds of glucocorticoid signalling pending synthesis of new receptor. However, it is important to stress that the increase in protein stability that might potentially result from prolonged nuclear occupancy of liganded GR cannot be complete as ligand-induced degradation of GR has been observed (312,383). Further studies are currently underway to investigate the apparently complex relationship between ligand-dependent GR nuclear localisation and stability.

Calreticulin and its Role in GR Nuclear Export

A number of signalling proteins have been implicated in the control of GR nuclear export. Of these candidates, the observation that GR nuclear export can be mediated through calreticulin is most puzzling. Although CRT has been shown to localise at the cell surface in specialised cell types (384-386), in most cell types CRT is sequestered within the endoplasmic reticulum under resting conditions. Thus, CRT has limited opportunity to directly influence GR nuclear export. My studies show that under conditions used to study protein trafficking through cell fusion, disruption of the

endoplasmic reticulum can occur. As a result CRT becomes temporarily mislocalised to the cytoplasm, causing GR nuclear export to be transiently accelerated in a manner that is sensitive to a mutation that interrupts GR-CRT binding (See Figure 16). However, in the absence of experimentally induced CRT mislocalisation, disruption of the potential GR-CRT interaction does not appear to affect the rate of GR nuclear export, indicating that under resting cellular conditions CRT is very unlikely to directly influence the subcellular trafficking behaviour of either the liganded or unliganded receptor (See Figure 17).

In reconciling the different conclusions made in this thesis and in previously published reports regarding the potential of CRT to mediate GR nuclear export, the methodology used in each study must be considered. The initial finding that calreticulin can potentially act as an export factor stemmed from a report describing a cytosolic factor other than CRM1 that can mediate nuclear export of PKI (328). This report was based on observations obtained through digitonin permeabilisation experiments. Treatment with digitonin results in the permeabilisation of cholesterol-rich membranes such as the plasma membrane (387). This causes a loss of plasma membrane integrity resulting in the removal of cytosolic factors that are required for nuclear export. In effect this technique results in the preparation of purified nuclei that are no longer surrounded by the cytoplasm. As nuclear export requires factors resident within the cytosol, the addition of cytosolic extracts to permeabilised cells is required to initiate nuclear export in this assay (387). In the preparation of these extracts no distinction is made between a purely cytoplasmic extract and an extract that also contains proteins resident in organelles found within the cytoplasm. Thus, it is quite likely that in a HeLa cell extract, significant

amounts of calreticulin are present, which could in turn mediate nuclear export in a permeabilised cell assay.

Digitonin permeabilisation experiments were initially used in order to establish a link between CRT and GR nuclear export (228). Rather than using HeLa cytosolic extracts as a source of cytoplasmic factors needed to support nuclear export, these experiments were performed using bacterially expressed CRT to stimulate export. Under these circumstances the concentration of CRT used in this assay was considerably higher than would be expected in a cytosolic extract. In a separately published study, GR nuclear export was studied by digitonin permeabilisation experiments using HeLa cytosol as a source of cytosolic export factors (286). The conditions used in this study closely resemble those used in this thesis. Rapid nuclear export of GR was not observed under these experimental conditions (286), in agreement with my observations (See Figure 11). Thus, it appears that in a digitonin permeabilisation assay, GR nuclear export can be stimulated by elevated levels of CRT that are in effect mislocalised to the cytoplasm.

The role of CRT in GR nuclear export has also been studied by cytoplasmic microinjection of purified CRT (228,229). While microinjection is suitably precise to target proteins to either the nucleus or cytosol, it is not technically possible to directly microinject proteins into the endoplasmic reticulum using current technology. This is due in part to the limits of the maximum resolving power of a light microscope and to the size of the injection capillaries used in these microinjection systems. Thus, microinjection will certainly result in the mislocalisation of CRT to the cytoplasm where it can potentially exert a greater influence over the trafficking behaviour of proteins such as GR than would be observed under physiological conditions.

Interestingly, the relocalisation of GR to the cytoplasm following ligand withdrawal is inhibited in cells lacking CRT (228). While this finding arguably provides the most convincing evidence of the potential involvement of calreticulin in GR nuclear export, it is possible that this observation is an indirect reflection of the diverse cellular processes that require CRT (388). The nuclear import of MEF2C and NF-AT3 is impaired in CRT deficient cells (319,321). In the case of MEF2C, import can be restored by increasing the concentration of Ca^{2+} in the cytoplasm, indicating that the effect of CRT on MEF2C localisation is indirect (321). This finding is consistent with the previous demonstration that the depletion of Ca^{2+} levels within the endoplasmic reticulum inhibits both passive diffusion and signal-mediated nuclear import (322). While Ca^{2+} levels in the endoplasmic reticulum are not completely depleted in $CRT^{-/-}$ cells, there is a decrease in the overall Ca^{2+} storage capacity in these cells (315). As calcium-dependent pathways are sensitive to small variations in Ca^{2+} levels, a relatively small change in Ca^{2+} homeostasis may have many downstream effects on cell function. Further, calcium signalling within the nucleus has been shown to influence protein subcellular localisation (389). Regulation of nuclear Ca^{2+} signalling occurs through a recently described nucleoplasmic reticulum that is continuous with the endoplasmic reticulum. Release of Ca^{2+} into the nucleus results in the translocation of nuclear protein kinase C (PKC) to the nuclear envelope, while release of Ca^{2+} into the cytoplasm results in the relocalisation of cytoplasmic PKC to the plasma membrane (389). Together these findings demonstrate that the extent to which Ca^{2+} signalling can influence protein localisation and suggest that the inability of GR to undergo nuclear export in cells lacking CRT may not necessarily reflect a direct influence of CRT on GR trafficking.

Alternatively, the lack of redistribution to the cytosol may be caused by an alteration in the conformation adopted by GR following ligand withdrawal in $CRT^{-/-}$ cells. This possibility is not without precedent, as GR does not redistribute to the cytoplasm following withdrawal from the hormone antagonist RU486 (354). This suggests that an agonist-dependent event may be required for nuclear export of the ligand-withdrawn receptor and is somehow compromised in $CRT^{-/-}$ cells. Possibilities include post-translational modification of the receptor or an alteration in the reassociation of GR with its molecular chaperones following ligand withdrawal.

Cell fusion-based assays were also used to study the function of CRT in GR nuclear export. It is important to note that the work presented in this thesis that utilises cell fusion-based assays directly replicates previous findings using similar assay systems. Accelerated CRT-dependent nuclear export of GR is clearly observed following cell fusion (see Figures 13 and 16). However, this finding clearly contrasts with those obtained when GR export is studied in the absence of cell fusion, as in the absence of cell fusion CRT does not appear to influence GR nuclear export. To resolve this discrepancy, I have investigated the changes in subcellular morphology that accompany PEG treatment. I have found that treatment with PEG affects the cell beyond simple membrane fusion and results in the transient loss of integrity of the endoplasmic reticulum. The loss of intracellular membrane integrity alters the exposure of proteins normally resident in the endoplasmic reticulum including CRT. Accelerated GR nuclear export following cell fusion directly correlates with the mislocalisation of CRT to the cytoplasm following treatment with PEG. This suggests that in a cell fusion-based assay, accelerated nuclear export of GR is a consequence of the mislocalisation of CRT. Under

the resting cellular conditions that I have studied where CRT resides within the lumen of the endoplasmic reticulum, CRT does not appear to exert a direct influence over GR subcellular trafficking.

The work presented in this thesis suggests that the export of GR across the nuclear membrane to the cytoplasm occurs rather slowly and is mediated through a process that does not involve the direct interaction of GR with CRT. It remains possible that the CRT-mediated rapid export of nuclear hormone receptors from the nucleus could be an inducible response to some acute forms of cellular stress. The physiological pathways that could potentially result in the transient release of CRT from the endoplasmic reticulum remain elusive. In macrophages, the formation of phagocytotic vesicles involves the fusion of the endoplasmic reticulum with the plasma membrane (390). It is possible that lumenal components of the endoplasmic reticulum could be released into the cytoplasm during the membrane rearrangements that accompany phagocytosis. However, preliminary experiments revealed that in macrophages GR is constitutively localised to the cytoplasm even in the presence of hormone agonist and antagonist (R. Walther and R.J.G. Haché, unpublished observation). The mechanism through which GR becomes sequestered in the cytoplasm in this cell type has not yet been determined. However, it is interesting to note that in macrophages CRT has been shown to localise to the cell surface through association with the low density lipoprotein receptor-related protein (LRP) (384,385). An alternative possibility is that CRT might mediate nuclear export of GR prior to mitosis. Immediately preceding mitosis, cytoplasmic relocalisation of several transcription factors including heat shock factor 1 (HSF1), Oct-1 and 2, and c-Fos has been observed (391). While it is tempting to speculate that CRT could play a

role in this process, the extent to which CRT may be released from the endoplasmic reticulum during mitosis is uncertain as the endoplasmic reticulum remains largely intact through mitosis [reviewed in (392)]. That said, during mitosis disassembly of the nuclear envelope, which is continuous with the endoplasmic reticulum, has been well documented [for review see (393)]. It remains possible that upon breakdown of the nuclear envelope preceding mitosis, CRT could be released resulting in increased export of GR from the nucleus. Finally, CRT could also be released during apoptosis following the breakdown of the endoplasmic reticulum. However, preliminary experiments designed to investigate this possibility were unsuccessful as induction of apoptosis through stimulation with TNFα and cycloheximide for 6 h (336) caused the cells to enter apoptosis too rapidly to see any effect on GR relocalisation following ligand withdrawal (R. Walther and R.J.G. Haché, unpublished observation).

Alternative GR Nuclear Export Pathways

As of yet there is little consensus as to pathway through which GR is exported from the nucleus. The results presented in this thesis offer the exciting possibility that two separate pathways mediate GR nuclear export. While associated with ligand, GR is exported from the nucleus slowly through the CRM1 pathway as indicated by the observed increase in nuclear occupancy of the GR_{NL1-} construct in the presence of the CRM1 inhibitor LMB (see Figure 20). Whether CRM1-dependent nuclear export of GR is mediated by the direct interaction of the receptor with CRM1 or indirectly through the association of GR with another protein bearing a CRM1-dependent NES has yet to be determined. Following ligand withdrawal, relocalisation of GR to the cytoplasm appears

to be achieved through the action of a separate CRM1-independent pathway, as the addition of the CRM1 inhibitor LMB had no apparent effect on the rate of redistribution of GR_{NL1-} to the cytoplasm following ligand withdrawal. The nature of this export pathway remains unknown. The identification of the receptor involved in the export of ligand-withdrawn receptor will provide important insight into how GR subcellular localisation is regulated.

The involvement of CRM1 in GR nuclear export has been controversial. While a potential role for CRM1 in GR nuclear export has been proposed (130), other reports have indicated that the CRM1 pathway does not influence GR nuclear export (285). In both of these reports LMB was used to assess the potential involvement of CRM1 in GR nuclear export. CRM1 has also been differentially implicated in the nuclear export of VDR. Nuclear export of unliganded VDR was found to be dependent on CRM1, but CRM1 was shown to have no impact on VDR export in the presence of ligand (381). Conversely, unliganded TR has been shown to export in a manner that is independent of CRM1 (380) however, these results may be inconclusive as heterokaryon fusion experiments were used in this study to demonstrate export. Interestingly, my results are consistent with both of the seemingly contradictory reports concerning the role of CRM1 in GR nuclear export. The data presented by Savory and colleagues indicates that CRM1 participates in the nuclear export of the ligand-bound GR (130) while data presented by Liu and DeFranco focused entirely on the ligand-withdrawn receptor (285). This offers the exciting possibility that GR nuclear export can proceed through alternate pathways. As ligand withdrawal is accompanied by the re-association of GR with hsps, perhaps the

differential use of various export pathways is influenced by ligand-induced changes in GR protein-protein association.

The identity of the non-CRM1 export receptor that mediates nuclear export of GR remains elusive. While several proteins other than CRM1 have been implicated in GR export, all of these pathways appear to operate within a narrowly defined set of cellular conditions. For example, overexpression of p53 has been clearly shown to result in the relocalisation of GR to the cytoplasm (311,312). However, in the absence of p53 activation, cellular levels of p53 are maintained at rather low levels. Rapid turnover of p53 occurs through MDM2-mediated degradation, which maintains a relatively low level of p53 protein expression under resting conditions [for reviews see (305,394)]. Due to the relatively low levels of p53, it seems unlikely that p53 could mediate general GR nuclear export under resting conditions in most cell types. Similarly, the JNK signalling pathway has been proposed to mediate phosphorylation of GR and stimulate its nuclear export. Again, it seems unlikely that the JNK signalling pathway could significantly influence the general nuclear export of GR as JNK is normally inactive under resting cellular conditions (303). This suggests that the effect of activated JNK on GR subcellular trafficking could represent a specialised response to UV-induced DNA damage.

Recent reports have shown that ER, which normally resides in the nucleus, can be found in the cytoplasm upon co-expression of a naturally occurring metastatic tumour antigen 1 (MTA1) variant. MTA1 is up-regulated in several forms of cancer and can function as an ER co-repressor (395). A shortened variant of MTA1, MTA1s, binds ER and sequesters it in the cytoplasm. This relocalisation of ER to the cytoplasm prevents

ER signalling and is associated with increased malignancy. Expression of MTA1s also results in the cytoplasmic relocalisation of liganded GR and appears to affect signalling through PR but not RAR (71). The mechanism through which MTA1s induces cytoplasmic accumulation of ER and GR is unclear, although perhaps binding of MTA1s may influence SHR localisation in several possible ways. Firstly, binding of MTA1s may inhibit nuclear import by masking an NLS. Secondly, MTA1s may either expose or activate an export signal resident within the receptor, or it may possess its own export signal that then piggybacks the receptor into the cytoplasm. A third possibility would be that MTA1s contains a cytoplasmic retention signal. In this case, binding of a steroid hormone receptor with MTA1s might result in the increased cytoplasmic retention of the SHR, leading to increased accumulation of the receptor in the cytoplasm. Finally, MTA1 may promote cytoplasmic accumulation of a steroid hormone receptor by blocking a nuclear retention signal. In any case, the limited expression pattern of MTA1s makes it unlikely that MTA1s modulates subcellular localisation of ER and other SHRs in the majority of cell types (71). However, the finding that ER signalling is prevented by expression of MTA1s underscores how changes in subcellular localisation can significantly impact receptor function.

One potential candidate GR export receptor is 14-3-3. The 14-3-3η isoform was identified as a possible regulator of GR function through two-hybrid screening (396). Co-expression of GR and 14-3-3η results in an increase in GR transcriptional activity (396). This effect may be indirect, as 14-3-3η has been shown to mediate nuclear export of the RIP140 corepressor. This finding implies that the increased transcriptional activity of GR observed upon co-expression of 14-3-3η is due to the influence of 14-3-3η on

RIP140 subcellular localisation rather than an effect on GR trafficking (397). Recently, the 14-3-3σ isoform has been directly implicated in export of ligand-withdrawn GR and was found to negatively regulate GR transcription (234). Interestingly, the effect of 14-3-3σ on GR export is specific to the GRα isoform and does not appear to influence GRβ. GRβ is a variant produced by alternative splicing of the human GR transcript. Its sequence differs from GRα at the extreme C-terminus of the LBD. GRβ contains a unique 15 amino acid sequence in place of the penultimate helix of the LBD and entirely lacks the AF-2 helix (24). GRβ is unable to bind hormone and functions as a dominant negative inhibitor of GRα function (398). The observation that the subcellular localisation of GRβ is not influenced by 14-3-3σ suggests that binding of GR to 14-3-3σ is ligand-dependent. 14-3-3σ may promote cytoplasmic accumulation of GR through a piggyback mechanism, as a 14-3-3σ variant carrying a mutation within its NES motif was no longer able to enhance GR nuclear export. However, even in cells lacking 14-3-3σ, GR was still exported from the nucleus following ligand withdrawal (234). Whether this represents the influence of another 14-3-3 isoform on GR export or the action of an entirely different protein has yet to be determined.

Despite the uncertainty regarding the identity of the non-CRM1 receptor that mediates nuclear export of GR following ligand withdrawal, my results indicate that liganded GR is slowly exported through the CRM1 pathway. Having identified CRM1 as a mediator of GR export, determining the significance of CRM1-mediated export on GR function will be the subject of future investigation. Analysis of the primary amino acid sequence reveals that GR contains four leucine rich sequences that loosely resemble a classical NES motif. Current studies are underway to determine which, if any, of these

sequences is responsible for CRM1-mediated export of the liganded receptor. If a CRM1-dependent NES were to be identified, then assessing the role nuclear export plays in the function of the liganded receptor would be a relatively accessible task. One potential pathway where CRM1-mediated export could impact on receptor function is in the degradation of GR. If GR is preferentially degraded in the cytoplasm as has been previously suggested (285), then blocking nuclear export by mutation of the GR export signal would be expected to result in increased stability of the receptor and possible an increase in the transactivation potential of GR.

An alternative possibility is that GR is exported from the nucleus via association with another protein bearing a CRM1-dependent NES. Interestingly, recent reports indicate that SRC-1, a coactivator protein that associates with GR in a ligand-dependent manner, is exported in an LMB-dependent manner through an atypical CRM1-dependent NES (208). Preliminary reports indicate that the closely related SRC-3 is also exported through the CRM1 pathway (J. Torchia, personal communication). Given that the GR-coactivator complex has been reported to consist of many diverse members [reviewed in (126)], there are a number of proteins that interact with GR that could potentially piggyback GR out of the nucleus. If GR export is mediated through interaction with a co-activator protein, then assessing how export impacts GR function would be complicated by the fact that interrupting a GR/co-activator interaction would affect GR function on multiple levels. Nonetheless, such a finding would possibly provide an additional explanation as to why GR nuclear export proceeds at a relatively slow rate. The interactions between GR and members of the co-activator complex are transient (126). If export were dependent on transient interactions, then it is possible that GR

would associate with another protein capable of mediating its export for only a limited period of time and thus the potential for GR export would be limited. Additionally, the composition of these co-activator complexes appears to vary among different cell types (126). In cell types that express low levels of a coactivator required for export, GR nuclear export would be expected to proceed more slowly than in a cell type that expresses more abundant levels of a coactivator protein involved in export. If GR export were dependent on such variable interactions, then small differences in the measured rate of GR nuclear export would be expected. Indeed, the half time of GR relocalisation to the cytoplasm following ligand withdrawal has been reported to range from approximately 8 h (285) to between 12 and 24 h [(47,130), also see Figure 14]. This variation in the rate of nuclear export observed by different groups studying GR trafficking in different cell types would be understandable if GR export were dependent on complex interactions with other proteins that are expressed to varying degrees under slightly different experimental conditions.

Nuclear Retention and its Role in GR Function

My studies have shown that GR nuclear export proceeds at a rate that is slower than anticipated based on previous data. However, it has also been shown that GR is relatively mobile within the nucleus (344,349) (also see Figure 10). These two observations suggested that GR nuclear export might be inhibited by nuclear retention through a transient interaction. The finding that mutation of the NL1 core sequence results in the acceleration of the rate of export of both the liganded and ligand-withdrawn receptor led me to investigate the possibility that GR nuclear retention is at least partially

mediated through the NL1 motif. These studies led to the discovery that a region of GR overlapping with the NL1 motif can act to suppress active nuclear export mediated by the HIV Rev NES. Thus, in addition to binding importin α to partially mediate nuclear import of GR, the NL1 motif may also function as a nuclear retention sequence. This finding provides insight into a potential mechanism for GR nuclear retention.

Data presented in the later part of this thesis (see Figures 23-26) suggest that the NL1 sequence acts in a transdominant manner to repress nuclear export mediated through a classical NES motif. Interestingly, the FRAP experiments that demonstrated this potential function of the GR NL1 were performed with fusion constructs bearing an SV40 NLS. This suggests that nuclear retention is a function of the GR NL1 sequence that is not shared by all NLS motifs. This is a unique finding and suggests that the GR NL1 motif serves multiple functions in determining GR subcellular distribution.

The relative contribution of nuclear import and nuclear export to the net localisation of a particular protein may be affected by the extent to which the protein is retained in either the nucleus or the cytoplasm. It has been previously shown that the nucleocytoplasmic distribution of a synthetic fusion protein containing both the NLS of the SV40 large T antigen and the PKI NES favours nuclear localisation (204). In the case of GR, the complete nuclear accumulation observed following ligand binding might be initiated by nuclear import through NL1 and NL2 and maintained in part by NL1-dependent nuclear retention. If nuclear retention by NL1 were required to maintain GR in the nucleus, this would provide a potential explanation as to why the GR_{NL1-} mutant does not entirely accumulate in the nucleus even after prolonged hormone treatment.

Mutation of the NL1 motif followed by replacement with another NLS such as that of the SV40 large T antigen may help to address this possibility.

The extent to which nuclear retention through NL1 affects subnuclear trafficking of GR is as yet unclear. It is apparent that wildtype GR is relatively mobile within the nucleus (344,349) (also see Figure 10). This implies that nuclear retention is mediated through a transient interaction rather than sequestration within a defined subnuclear domain. However, the effect of the NL1 mutation on subnuclear trafficking has not yet been addressed in a quantitative manner. If retention is mediated through the NL1 region, then it is likely that mutation of the NL1 core sequence would result in an increase in the rate of intranuclear trafficking.

It is interesting to note that the NES-$GR_{407\text{-}556}$-GFP-NLS fusion protein adopts a unique subnuclear distribution pattern in comparison to the NES-GST-GFP-NLS control construct and the NES-$GR_{407\text{-}556NL1\text{-}}$-GFP-NLS mutant. Expression of the NES-$GR_{407\text{-}556}$-GFP-NLS fusion protein results in the formation of large areas of intense GFP signal throughout the nucleus (See Figure 24). Additionally, the NES-$GR_{407\text{-}556}$-GFP-NLS fusion protein is the only construct used in this study that is not excluded from the nucleolus. This observation, together with previous observations that particular GR truncation mutants accumulate in nuclear foci that co-localise with hsp90 (131), hints that perhaps the NL1 is involved in a process that transiently targets GR to subnuclear foci where the folding of the receptor could be monitored and/or altered. Interestingly, GR has been shown to associate with hsp70 through the NL1 motif (289), adding additional support to this possibility. By studying the mechanism through which the NL1 sequence

mediates nuclear retention I hope to gain more insight into the function of GR nuclear retention.

The mechanism through which NL1 promotes GR nuclear retention is the focus of my ongoing studies. To explain the mechanism behind this process I have considered two possibilities. Nuclear receptors have been reported to interact with components of the nuclear matrix and the nuclear lamina in an NL1-dependent manner (J. Savory, F. Sackey and Y. Lefebvre, unpublished results). To address the possibility that interaction of GR with the nuclear lamina through the NL1 motif mediates GR nuclear retention, one could selectively disrupt the structure of the nuclear lamina by transient expression of a variant form of lamin A that lacks its N-terminal head domain (141,399). The N-terminal head domain mediates the formation of a stable network of lamin oligomers that give structure and support to the nuclear lamins [for review see (140)]. Expression of a variant lamin A lacking the head domain results in the destabilisation of the nuclear lamina accompanied by a gross morphological change in structure of the nucleus (141,399). Surprisingly though, many nuclear functions including Pol II-mediated transcription remain unaffected (141). If nuclear retention of GR through the NL1 motif were mediated via interaction with the nuclear lamina, then destabilisation of the nuclear lamina would be expected to alleviate nuclear retention. This would be expected to result in an increase in the rate of GR nuclear export in a manner similar to that observed upon direct mutation of the NL1 sequence.

A second possible mechanism through which the NL1 sequence may mediate GR nuclear retention is through association with hsp70. Hsp70 interacts with GR through a region co-incident with the NL1 core sequence. This interaction has been shown to

influence hsp70 nucleocytoplasmic distribution (289) and GR function (288). As association of GR with hsps has been proposed to influence GR trafficking (286), hsp70 is an interesting potential candidate as a possible mediator of nuclear retention. As alluded to previously, nuclear retention through heat shock proteins may indicate that nuclear retention through NL1 transiently targets GR to subnuclear foci where GR folding is either monitored or altered. Initial studies addressing whether the GR NL1 sequence preferentially interacts with hsp70 are currently underway.

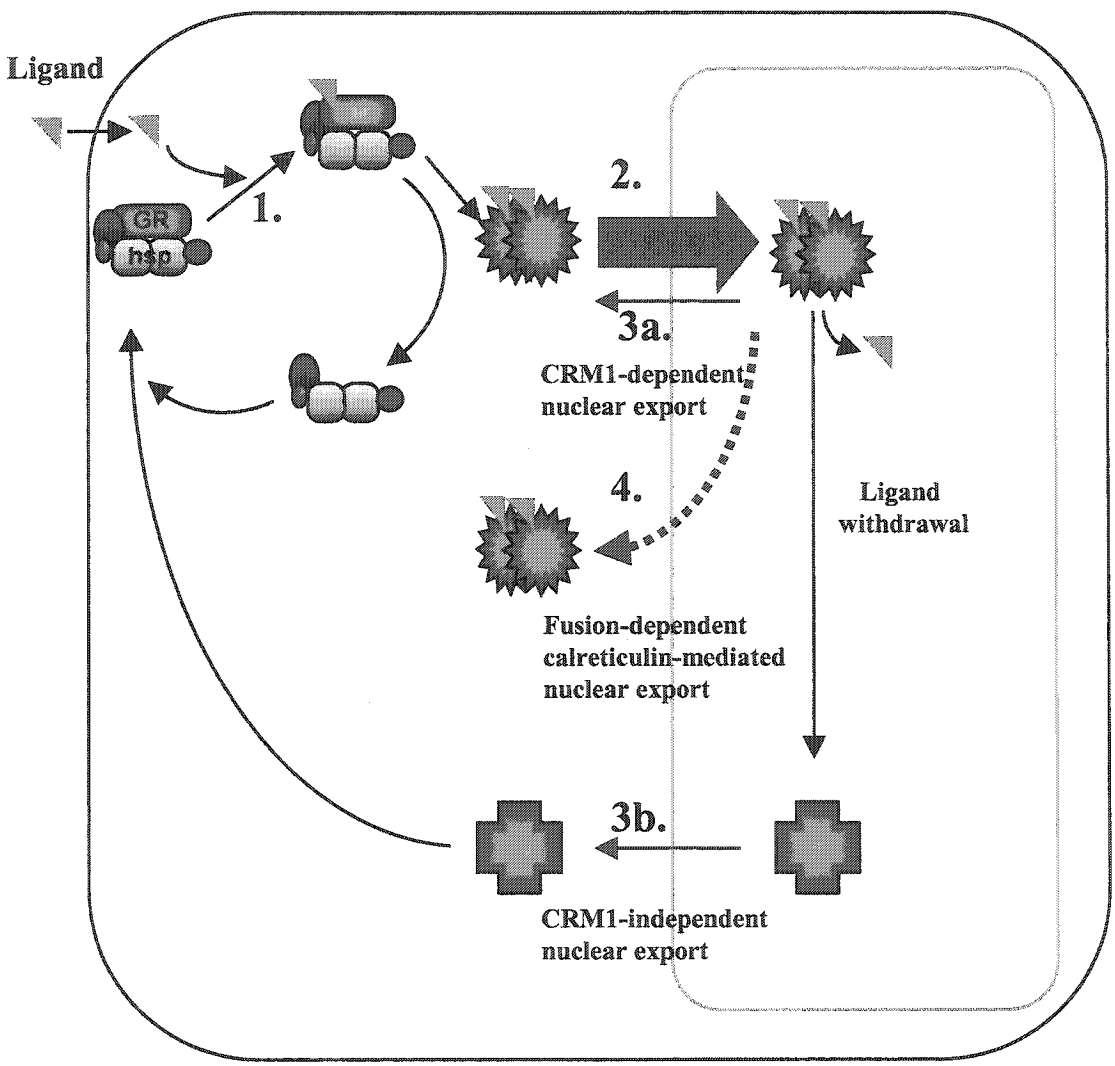
The determination of how the NL1 sequence participates in mediating nuclear retention will provide important insight into how retention impacts receptor function. Preliminary results indicate that increased shuttling of full length GR through the addition of an exogenous NES motif and mutation of the NL1 core sequence has a profound effect on GR stability. The level of expression of the NES-GR_{NL1}-GFP-NLS fusion protein appears to be greatly reduced in comparison to its wildtype counterpart as increased exposure times were required in order to visualise the NES-GR_{NL1}-GFP-NLS fusion product by confocal microscopy (Figure 25b). While a more in depth analysis of the relationship between nuclear retention of GR through NL1 and GR stability is required, my observations are in agreement with previous findings implicating increased cytoplasmic localisation with a decrease in GR stability (285).

Summary and Conclusions

The results presented in this thesis offer a new perspective on GR trafficking. In Figure 27 I have presented a revised view of GR trafficking based on my findings. I have shown that following ligand-induced rapid nuclear import of GR, nucleocytoplasmic

Figure 27: Revised model for GR subcellular trafficking

Following exposure with hormone ligand, the GR-hsp complex is dissociated (1) and is rapidly imported into the nucleus (2). Shuttling of the liganded receptor between nucleus and cytoplasm occurs slowly (3a). The rate of this shuttling mirrors the previously observed rate of redistribution of the receptor to the cytoplasm following ligand withdrawal (3b). Accelerated export of GR mediated by calreticulin occurs in a cell-fusion dependent manner (4).


Ligand
GR
hsp
1.
2.
3a.
CRM1-dependent
nuclear export
4.
Ligand
withdrawal
Fusion-dependent
calreticulin-mediated
nuclear export
3b.
CRM1-independent
nuclear export

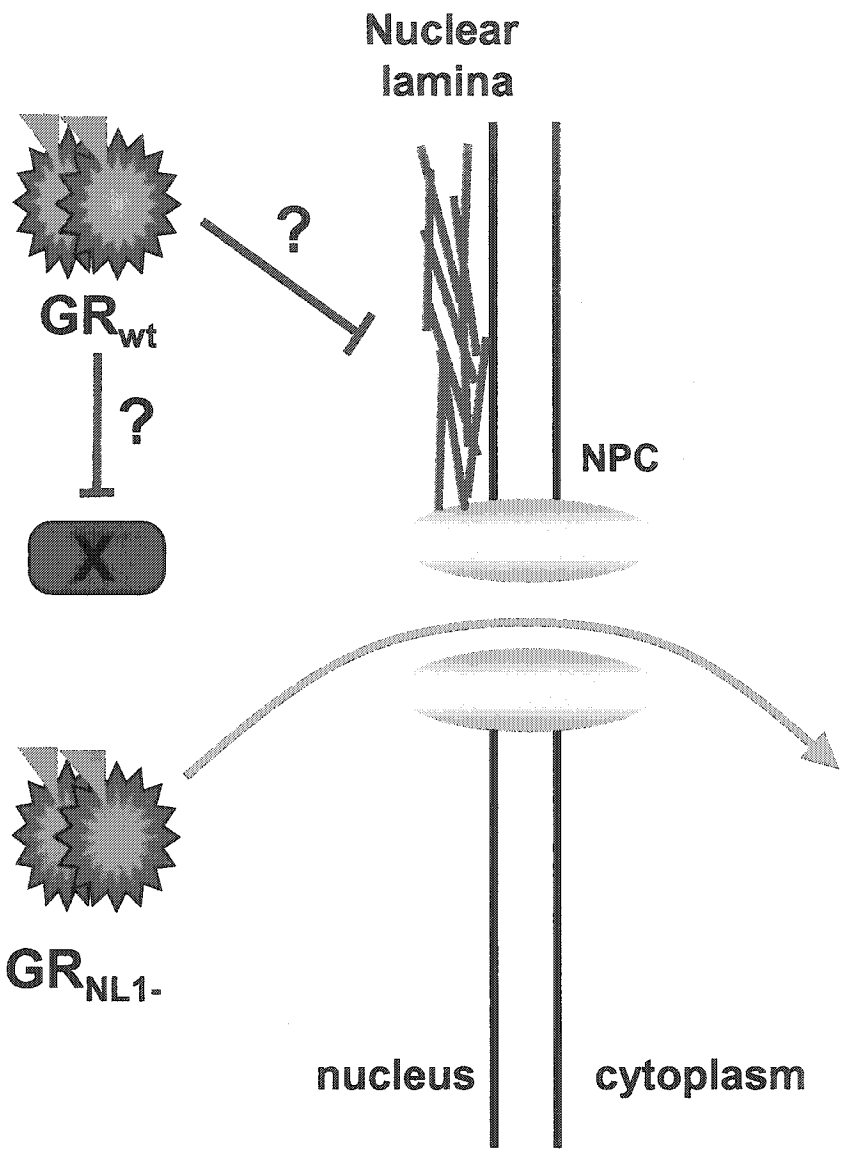
shuttling of the liganded receptor occurs much more slowly than previously believed. This slow shuttling is due to the slow rate of GR nuclear export. Nuclear export of liganded GR occurs at a rate similar to the export of the receptor following ligand withdrawal. The process of cell fusion results in an apparent increase in the rate of GR nuclear export. Accelerated nuclear export of GR in this experimental system is a consequence of the transient release of calreticulin from the endoplasmic reticulum. Under resting cellular conditions, CRT is sequestered within the lumen of the endoplasmic reticulum and is thus unable to mediate GR nuclear export.

My results reveal a limitation of the heterokaryon fusion assay for studying nucleocytoplasmic trafficking. Cell fusion-based assays rely on treatment with high concentrations of PEG to induce membrane fusion of neighbouring cells. My data suggest that PEG treatment has effects beyond simple membrane fusion and may result in the disruption of membrane-bound subcellular structures including the endoplasmic reticulum. The disruption of internal membrane structure can lead to the transient exposure of proteins normally resident within these organelles. In specialised cases this can activate additional modes of protein function that are not apparent in the untreated cell. My findings show that a cell fusion-based export assay can be used to reliably predict the trafficking behaviour of proteins exported through a traditional NES motif. However, my results suggest that findings of nuclear export attributed to export receptors other that CRM-1 may need to be re-confirmed using techniques that are not reliant on cell fusion.

The slow rate of GR nuclear export appears to be due in part to active retention within the nucleus. As depicted in Figure 28, nuclear retention through a sequence

Figure 28: GR nuclear export is accelerated upon mutation of the NL1 motif

Nuclear export of wildtype GR proceeds slowly. The slow rate of export of GR_{wt} appears to be due in part to nuclear retention. Nuclear retention of GR is mediated by an activity that overlaps with the GR NL1 sequence. Though the mechanism has yet to be determined, GR nuclear retention may be mediated through transient association with another nuclear protein (protein X), or through association with the nuclear lamina. Upon mutation of the NL1 sequence, nuclear retention of GR is relieved and export proceeds at an increased rate.


Nuclear
lamina
?
GRwt
?
X
NPC
GRNL1-
nucleus
cytoplasm

overlapping the NL1 motif appears to have an impact on the rate of passage through the nuclear pore. This does not appear to occur through direct sequestration, as the receptor is relatively mobile within the nucleus. It is anticipated that ongoing studies into the mechanism of NL1-mediated nuclear retention will provide further insight into the relevance of this process.

It is apparent that GR subcellular localisation is regulated at many different levels. GR nucleocytoplasmic localisation has been the focus of intense study for some time, yet a clear consensus regarding the mechanisms through which GR localisation is regulated has not been reached. My research will help to clarify this area of research by offering a fresh take on GR export. Through the use of novel imaging techniques that allow for direct visualisation of protein movement I have been able to study GR nuclear export with minimal experimental manipulation. I have found that previous findings derived from cell fusion-based experiments suggesting that liganded GR exports rapidly between nucleus and cytoplasm do not reflect the behaviour of the receptor in a non-fused cell. My finding that GR remains largely localised to the nucleus and shuttles minimally between nucleus and cytoplasm means that the extent to which cytosolic factors can influence GR function is somewhat limited. This streamlines the pathways that could possibly affect the function of the active, liganded receptor. This will in turn allow for more focused research that is more likely to provide important insight into GR function under physiological conditions.

Appendix A: Oligonucleotide Sequences

The table below lists the sequences of all oligonucleotides used for plasmid cloning. All oligonucleotide sequences are written in the 5' to 3' orientation. Restriction enzyme recognition sequences are underlined. Nucleotides that introduce amino acid mutations are written in red type, while mutations that alter restriction enzyme sequences are written in bold type. Where appropriate the protein sequence of the expression product is listed above the nucleotide sequence of the forward primer in standard one-letter code.

Plasmid	Primer Name	Primer Sequence
pGFP-NLS	polylinker ApaI upper	Not I HpaI EcoRV XbaI CGC GGC CGC GTT AAC GGA TAT CTC TAG AC
	polylinker ApaI lower	Xho I XbaI EcoRV HpaI NotI TCG AGT CTA GAG ATA TCC GTT AAC GCG GCC ApaI GCG GGC C
pGST-GFP-NLS	GST-EcoRV$_{forward}$	EcoRV M S P I L G GTG TGT GAT ATC ATG TCC CCT ATA CTA GGT Y W TAT TGG
	GST-XhoI$_{reverse}$	XhoI GTG TGT CTC GAG ATC CGA TTT TGG AGG ATG GTC G
pNES-GST-GFP-NLS	NES$_{upper}$	L P P L E R L T XbaI C TTA CCA CCT CTG GAA AGA CTT ACT CTA GAT ApaI GGG CC
	NES$_{lower}$	CA TCT AGA GTA AGT CTT TCC AGA GGT GGT ApaI AAG GGC C

pGFP-GR$_{Full}$	RW-GR$_{fullF}$	XhoI M D S K E AC ACA CCT CGA GGC ATG GAC TCC AAA GAA S TCC
	RW-GR$_{159R}$	SalI GTT CTC TGG AAC GCT GGT CGA CC
pGFP-GR$_{F463,4A}$	F463,4A +	G S C K V A A K R A GGA AGC TGC AAA GTA GCC GCT AAA AGA GCA V E GTG GAA GG
	F463,4A -	CC TTC CAC TGC TCT TTT AGC GGC TAC TTT GCA GCT TCC
pGFP-GR$_{F463,4A/NL1-}$		Used same primer set as pGFP-GR$_{F463,4A}$.
pGFP-GR$_{R496H}$	R496H +	K N C P A C H R Y GG AAA AAC TGC CCA GCA TGT CAC TAT CGG K C L Q AAA TGT CTT CAG G
	R496H -	C CTG AAG ACA TTT CCG ATA GTG ACA TGC TGG GCA GTT TTT CC
pGFP-GR$_{C500Y}$	C500Y +	P A C R Y R K Y L GC CCA GCA TGT CGC TAT CGG AAA TAT CTT Q A G M N CAG GCT GGA ATG AAC C
	C500Y -	G GTT CAT TCC AGC CTG AAG ATA TTT CCG ATA GCG ACA TGC TGG GC
pREV-GR	REVGR1-5'	BamHI M D S K E S ACA CAG GAT CCT ATG GAC TCC AAA GAA TCC L A P TTA GCT CCC
	REVGR795-3'	XbaI ACA CAC TCT AGA TCA TTT TTG ATG AAA CAG AAG CTT TTT GAT ATT TCC
pNES-GR$_{407-556}$-GFP-NLS	GR407$_{Forward}$	EcoRV S V F S N G GTG TGT GAT ATC TCA GTG TTT TCT AAT GGG Y S S TAC TCA AGC
	GR556$_{Reverse}$	XhoI GTG TGT CTC GAG CAC CTC CAG CAG TGA CAC CAA GG

pNES-$GR_{407-556NL1}$-GFP-NLS		Used same primer set as for pNES-$GR_{407-556}$-GFP-NLS.
pNES-GR_{WT}-GFP-NLS	$GR1_{Forward}$	EcoRV M D S K E S L A GAG ATG GAC TCC AAA GAA TCC TTA GCT CC
	$GR795_{Reverse}$	XhoI TGC TCG AGT TTT TGA TGA AAC AGA AGC TTT TTG ATA TTT CC
pNES-GR_{NL1}-GFP-NLS		Used same primer set as for pNES-GR_{WT}-GFP-NLS.
pNES-GST-$GR_{500-525}$-GFP-NLS	$GR500_{Forward}$	XhoI C L Q A G M CAC ACA CTC GAG TGT CTT CAG GCT GGA ATG N AAC C
	$GR525_{Reverse}$	XhoI CAC ACA CTC GAG TCC TGC AGT GGC TTG CTG AAT CC
pNES-GST-$GR_{500-525/NL1}$-GFP-NLS		Used same primer set as for pNES-GST-$GR_{500-525}$-GFP-NLS.

Appendix B: Sources of Chemicals, Reagents and Other Materials

Chemical or Reagent	Source
Aldosterone	Sigma, Mississauga, ON
Bio-Rac Protein Assay Dye Reagent	BioRad, Mississauga, ON
BuGR primary antibody	Affinity Bioreagents, Golden CO, USA
Calnexin H-70 primary antibody	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Calreticulin C-17 primary antibody	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Charcoal Stripped Fetal Bovine Serum (SFBS)	HyClone, Logan, UT, USA (Lot #100201)
Cortisol	Sigma, Mississauga, ON
Dexamethasone	Sigma, Mississauga, ON
Donkey anti-mouse secondary antibody conjugated to cy2	Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA
Donkey anti-mouse secondary antibody conjugated to rhodamine red X	Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA
Donkey anti-goat secondary antibody conjugated to rhodamine red X	Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA
Dulbecco's Modified Eagle's Medium (DMEM)	Invitrogen, Burlington, ON
ExGen transfection reagent	Fermentas, Burlington, ON
Fetal Bovine Serum (FBS) (for Cos7 cells)	HyClone, Logan, UT, USA (Lot #100201)
Fetal Bovine Serum (FBS) (for 293T cells)	Gibco BRL, Burlington, ON (Lot #1094621)
GFP JL-8 primary antibody	Clontech, Mississauga, ON
Leptomycin B (LMB)	Gift of Barbara Wolffe, Novartis
Lipofectamine	Invitrogen, Burlington, ON
Paraformaldehyde	EM Sciences, Gibbstown, NJ, USA
Poly-ethylene glycol	Sigma, Mississauga, ON
PVDF membrane	BioRad, Mississauga, ON

Chemical or Reagent	Source
RU-486	Roussel-Uclaf, France
Sheep anti-mouse secondary antibody conjugated to horse radish peroxidase	Amersham Biosciences, Baie d'Urfe, QC
Streptolysin O	Sigma, Mississauga, ON
Triton X-100	EM Sciences, Gibbstown, NJ, USA
Vent polymerase	New England Biolabs, Beverly, MA, USA
Western Lightening Chemiluminescence Reagent (Enhanced Luminol)	PerkinElmer Life Sciences Inc, Boston MA, USA

Appendix C: Nuclear Localisation of the Mineralocorticoid Receptor

Abstract

The mineralocorticoid receptor (MR) is a nuclear hormone receptor that plays a critical role in the maintenance of blood pressure through the regulation of sodium and potassium homeostasis and in memory consolidation. It is highly homologous to other nuclear hormone receptors, particularly the glucocorticoid receptor (GR). Both MR and GR are responsive to the hormone cortisol, whereas only MR is activated by aldosterone. This offers the potential for multiple modes of regulation in tissues expressing both receptors. In the absence of hormone, MR is equally distributed between the nucleus and cytoplasm whereas GR is predominantly cytoplasmic in the absence of ligand. GR is known to contain a basic nuclear localisation signal (NLS) in its hinge region, termed NL1. Additionally, GR contains a steroid-dependent NLS termed NL2 within its ligand-binding domain (LBD). To begin to delimit the basis for the differential localisation of MR and GR we have analyzed the molecular determinants for the nuclear localisation of MR. Mutation within the basic region analogous to the GR NL1 decreased ligand-dependent nuclear transfer of MR, but had little effect on the subcellular distribution of the unliganded receptor, suggesting that MR contained both an NL2-like activity and an additional NLS responsible for the partial nuclear localisation of the naïve receptor (NL_0). The NL_0 activity maps to a region of the MR N-terminus adjacent to the DNA-binding domain. The NL_0 motif is an atypical NLS that functions as a transferable nuclear localisation signal and provides a potential explanation for the differential localisation of GR and MR prior to ligand binding.

Introduction

The mineralocorticoid receptor (MR) is a steroid hormone receptor that plays an important role in maintaining homeostasis through the regulation of plasma sodium and potassium levels (400). MR is also involved in memory consolidation and learning as its activation promotes neuronal survival within the hippocampus (7,8). As maintenance of Na^+/K^+ homeostasis has important physiological consequences in the management of hypertension and heart disease (401), understanding the regulation of MR activity is of particular interest.

While MR is specifically activated by ligands such as aldosterone, it also shares overlapping affinity for glucocorticoid agonists including cortisol. In fact, MR binds to glucocorticoids with higher affinity than GR (402,403), while GR is not activated by MR-specific ligands. Because MR is activated by the same hormonal signals and activates transcription through the same DNA response element as GR with equal affinity (89), it is important that the specificity of MR signalling is maintained. In epithelial tissues such as the tubules of the kidney and the distal colon, specificity of MR signalling is accomplished through the enzymatic inactivation of glucocorticoid hormones (404). In these tissues, inappropriate activation of MR by glucocorticoids is prevented by the action of 11β-hydroxysteroid dehydrogenase, which converts cortisol or corticosterone to inactive keto-metabolites [for review see (405)]. In tissues that express both GR and MR but do not express 11β-hydroxysteroid dehydrogenase such as the hippocampus, MR is activated by circulating glucocorticoids. Where both MR and GR are co-expressed, glucocorticoids may have differential effects on cell activity depending on which receptor is activated. For example, in the hippocampus low levels of circulating glucocorticoids activate MR and promote cell survival while high levels glucocorticoids activate GR and

lead to cell death [reviewed in (7,8)]. The mechanism by which specificity of MR and GR signalling is maintained in these tissues is not entirely understood, although it appears that spacing of the receptor recognition sites and non-receptor components of the regulatory complex such as co-activator proteins have an important influence in determining promoter-specific activity (89,406).

Interestingly, co-operative regulation of promoter activity through the formation of GR-MR heterodimers has been described (407-409). Heterodimerisation of GR and MR has been shown to occur on the tyrosine amino transferase promoter (407), and binding of GR-MR heterodimers to the negative response element of the neuronal serotonin gene appears to occur with greater affinity than binding of either GR or MR alone (408). GR and MR have also been shown to form heterodimers in solution through a region of GR that is unique from the GR solution homodimerisation interface (128). Further, co-localisation of GR and MR has been demonstrated in the hippocampus (410). The observation that MR and GR form heterodimers adds an additional level of complexity to the regulation of GR and MR signalling.

While naïve GR is characterised by a predominantly cytoplasmic localisation, the subcellular localisation of MR prior to ligand binding is somewhat controversial. Naïve MR has been reported to localise primarily to the cytoplasm prior to ligand binding (59-62) , however the majority of published reports describe the unliganded MR as being equally distributed between nucleus and cytoplasm (63-70). These reported differences appear to be due in part to cell-type specific effects and thus might be due to the differential expression of factors that mediate cytoplasmic sequestration of MR. Alternatively, these differences may be a consequence of overexpression of the receptor (48,49). It has been reported that association with 11β-hydroxysteroid dehydrogenase at

the surface of the endoplasmic reticulum influences MR localisation prior to ligand binding (68). This association may provide a means to prevent MR activation by plasma glucocorticoids.

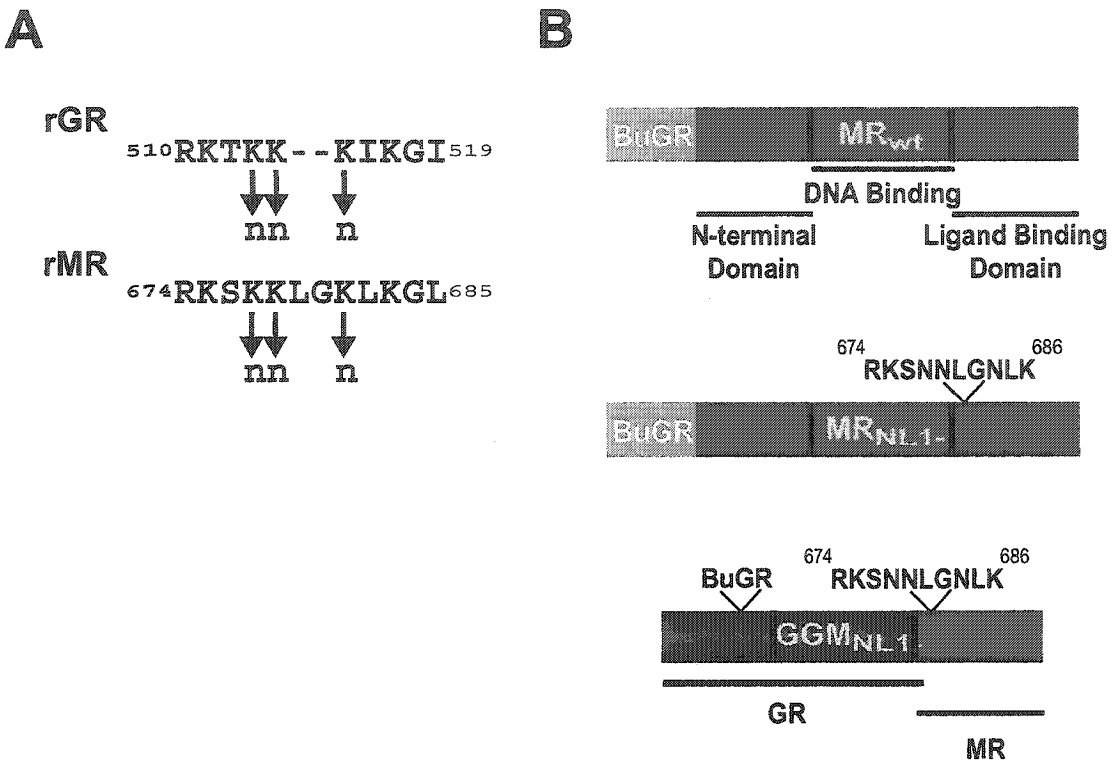
The sequences that mediate nuclear import of GR have been well studied, however little is known regarding the process that regulates MR subcellular localisation. To begin to study the regulation of MR nucleocytoplasmic localisation, we have examined the potential NLS activity of various MR sequence motifs that share homology with the GR NLS signals. We have found that MR contains two NLS motifs that bear resemblance to the GR NL1 and NL2 activities. Additionally, we have discovered that MR contains an additional NLS within its N-terminal region, which we have termed NL_0. The NL_0 activity is a transferable NLS that appears to play an important role in mediating the partial nuclear localisation of MR prior to ligand binding. Like GR, liganded MR does not appear to rapidly shuttle between nucleus and cytoplasm, indicative of a slow rate of nuclear export. Further, the initial subcellular localisation of the unliganded receptor was found to be unaffected by inhibition of the CRM1 nuclear export pathway. These findings provide insight into how MR subcellular localisation is regulated and will contribute to the understanding of differential regulatory potential of GR and MR.

Results

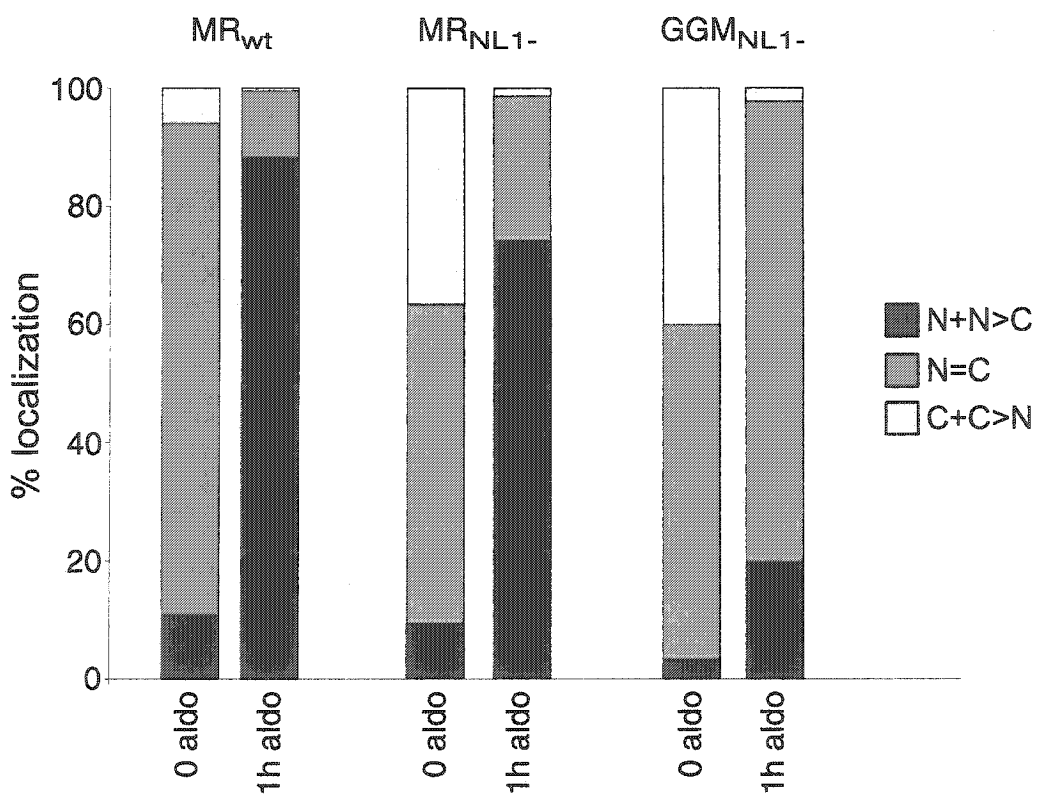
To begin to identify the sequence motifs that determine MR nuclear localisation, the subcellular distribution of two MR constructs and a hybrid MR/GR construct, depicted in Figure 29b, was examined. The MR_{wt} expression plasmid contains full length rat MR and was constructed by Ms. Claudia Bayer. The MR_{NL1-} construct, also generated

Figure 29: Several regions of MR influence its subcellular localisation.

(A) Sequence of the GR NL1 motif and the homologous sequence within the MR hinge region. Mutation of three key lysine residues within the GR NL1 core results in the abrogation of ligand induced GR nuclear import. The hinge region of MR contains a cluster of basic residues that loosely resembles the GR NL1 sequence. To determine if this sequence of MR is a potential NLS, the indicated mutations were introduced. (B) Representation of the constructs used in this experiment. (C) MR contains three potential nuclear localisation signals. Cos7 cells were transiently transfected with the indicated pTL-MR constructs by Lipofectamine™. Following transfection, the cells were plated onto glass coverslips and synchronised in G_0 by withdrawing serum. After 16-24 h, the cells were treated with 1 μM aldosterone for the indicated times. The localisation of MR was scored by indirect immunofluorescence using double-blind encryption. The values displayed represent the percentage of cell scored as exclusively nuclear or mainly nuclear (N + N>C), evenly distributed between nucleus and cytoplasm (N=C) and mainly cytoplasmic or exclusively cytoplasmic (C + C>N). Error bars represent the standard error of the means of three independent experiments performed in duplicate. This experiment was performed by Ms. Allison Edgecombe and Ms. Yanouchka Rouleau.


A
rGR
510RKTKK--KIKGI519
nn n
rMR
674RKSKKLGKLKGL685
nn n
B
BuGR
MRwt
DNA Binding
N-terminal Domain
Ligand Binding Domain
674
686
RKSNNLGNLK
BuGR
MRNL1-
674
686
BuGR
RKSNNLGNLK
GGMNL1
GR
MR

C


MRwt
MRNL1-
GGMNL1-
100
80
60
40
20
0
% localization
0 aldo
1h aldo
0 aldo
1h aldo
0 aldo
1h aldo
N+N>C
N=C
C+C>N

by Ms. Claudia Bayer, contains specific mutations as outlined in Figure 29a within the potential NL1 sequence. This mutation is similar to the GR_{NL1-} mutation that has been previously shown to interrupt binding of the GR NL1 sequence to importin α (130). To determine whether MR contains an NL2 activity, the GGMNL1- construct, which consists of the GR N-terminus and DBD fused to the MR LBD that is mutated within the putative NL1 motif was generated by Ms. Yanouchka Rouleau. To allow for detection of these constructs by indirect immunofluorescence under the same experimental condidtions, the BuGR epitope was cloned onto the N-terminus of the MR_{wt} and MR_{NL1-} expression vectors.

To determine whether MR contains an NL1 and an NL2 activity similar to that of GR, the subcellular localisation of the MR constructs described in Figure 29b was studied by indirect immunofluorescence in Cos7 cells. As depicted in Figure 29c, prior to ligand binding, wildtype MR is evenly distributed between nucleus and cytoplasm. Upon addition of aldosterone, MR_{wt} becomes predominantly localised to the nucleus. Mutation of the putative NL1 motif impacts on the localisation of both liganded and unliganded MR. In the absence of hormone, MR_{NL1-} displays a slight shift towards a more cytoplasmic pattern of localisation in comparison to wildtype MR. Likewise, liganded MR_{NL1-} accumulates in the nucleus to a lesser extent than MR_{wt}. This result indicates that MR may contain an NL1-like motif. However, mutation of this putative NL1 sequence exerts less of an impact on ligand-induced nuclear accumulation of MR than it does in the context of GR. Nuclear accumulation of GR_{NL1-} is significantly reduced as the receptor is localised mainly to the nucleus in less than 60% cells expressing GR_{NL1-} [(130), also see Figure 14]. In contrast, almost 90% of cells expressing MR_{NL1-} are scored as

predominantly nuclear. This suggests that the NL1 motif may play less of a role inmediating MR import. Alternatively, this may suggest that MR contains an NL2 activity that is relatively stronger than the NL2 activity of GR.

To further examine the potential NL2 activity of MR, the subcellular distribution of the GGM_{NL1-} construct was studied. The GGM_{NL1-} construct contains the GR N-terminus and DBD fused to the MR LBD in the context of the MR_{NL1-} mutation. As GR does not contain an NLS activity within its N-terminus or DBD (42), by substituting the N-terminus and DBD of MR with that of GR, the possibility that additional sequence motifs in the MR N-terminus or DBD could influence subcellular distribution is eliminated. The subcellular localisation of GGM_{NL1-} was investigated by Ms. Yanouchka Rouleau. As shown in the final panel of Figure 29c, a greater proportion of cells expressing GGM_{NL1-} were scored as predominantly cytoplasmic in the absence of ligand compared to the wildtype MR sequence. Addition of ligand results in a partial shift towards increased nuclear localisation, indicating that the MR LBD contains an NL2-like activity. However, the GGM_{NL1-} construct does not localise to the nucleus to the same extent as MR_{NL1-}. Only approximately 20% of cells expressing GGM_{NL1-} were scored as predominantly nuclear. By comparison, approximately 75% of cells expressing MR_{NL1-} were scored as predominantly nuclear. This finding suggests that MR contains a potential NLS activity within its MR N-terminus or DBD.

It has been previously shown that upon removal of hormone ligand, GR slowly relocates from the nucleus to the cytoplasm (47,130,285,286) (see also Fig. 14). The redistribution of GR to the cytoplasm following ligand withdrawal is accelerated upon mutation of the GR NL1 sequence (130). This observation, together with other results

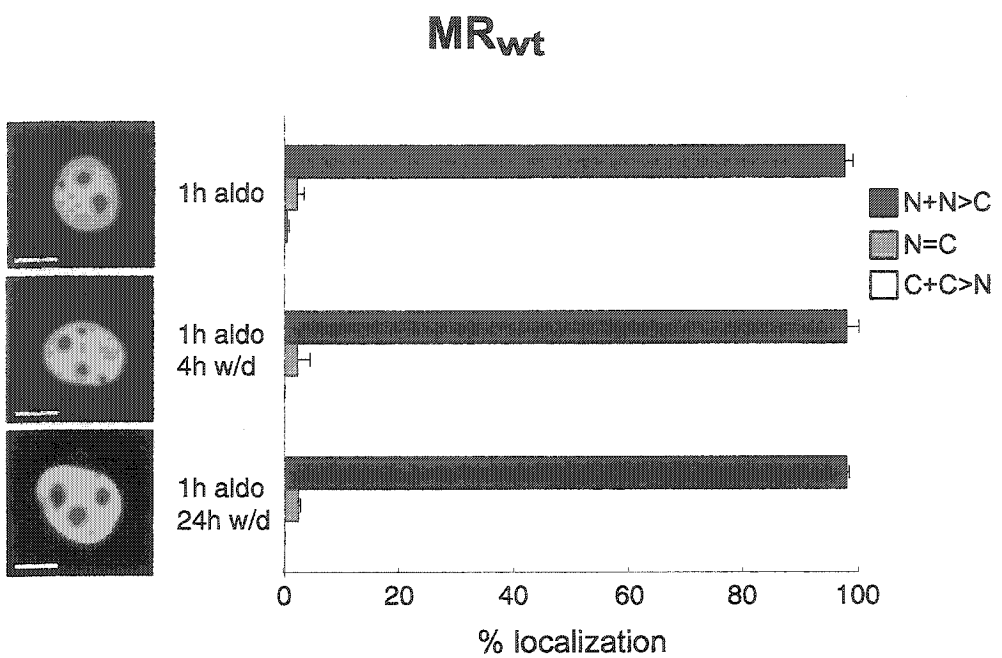
presented in this thesis, suggests that the GR NL1 sequence functions to retain GR in the nucleus. To determine if the MR NL1 sequence has a similar function, the subcellular localisation of MR_{wt} and MR_{NL1-} following ligand withdrawal was examined. These experiments, presented in Figure 30, were performed by Ms. Allison Edgecombe. Cos7 cells were transiently transfected with either MR_{wt} or MR_{NL1-} and treated with aldosterone for 1 h or 4 h respectively to induce nuclear accumulation. Ligand was withdrawn for a 4 h or 24 h period and subcellular distribution was examined by indirect immunofluorescence. As shown in Figure 30a, MR_{wt} remains mainly localised to the nucleus for up to 24 h following ligand withdrawal. This was a surprising finding as the closely related GR has been repeatedly shown to relocalise to the cytoplasm following ligand withdrawal with a $t_{1/2}$ of between 12 and 24 hours (47,130,285,286). This observation indicates that MR is constitutively localised to the nucleus following ligand withdrawal. The MR_{NL1-} receptor also remains predominantly nuclear at up to 24 h following ligand withdrawal (Fig. 30b). This observation is similar to the constitutive nuclear occupancy of GR observed following withdrawal form the hormone antagonist RU-486 (354). The observation that mutation of the putative MR NL1 sequence does not result in an enhanced rate of nuclear export following ligand withdrawal indicates that although MR appears to have an NL1 activity within its hinge region, the function of the MR NL1 sequence is not entirely analogous to the GR NL1 activity as it may not function as a nuclear retention sequence.

Data presented in this thesis indicates that liganded GR is exported from the nucleus at a much slower rate than anticipated based on the conclusions of previous cell-fusion based studies. To determine if MR shares this property, Cos7 cells were

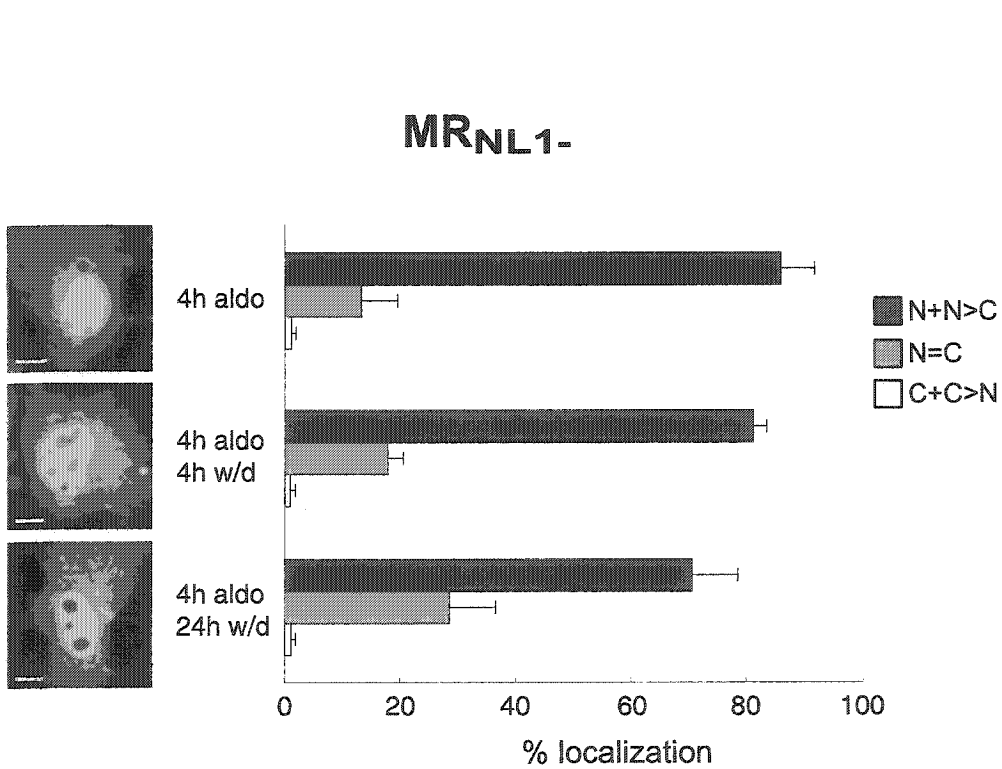
Figure 30: MR remains predominantly nuclear following ligand withdrawal.

(A) Wildtype MR remains nuclear following ligand withdrawal. Cos7 cells were transiently transfected with pTL-MR_{wt} by Lipofectamine™. Following transfection, the cells were plated onto glass coverslips and synchronised in G_0 by withdrawing serum. After 16-24 h, the cells were treated with 1μM aldosterone for 1 h and then withdrawn from ligand for indicated times. The localisation of MR was scored by indirect immunofluorescence using double-blind encryption as described in Figure 29. Error bars represent the standard error of the means of three independent experiments performed in duplicate. Representative micrographs are shown to the left of each data set. Scale bar = 10 μm. (B) Deletion of the potential MR NL1 sequence does not stimulate relocalisation of MR to the cytoplasm following ligand withdrawal. Cos7 cells were transiently transfected with the pTL-MR_{NL1-} construct and treated as described in panel A. This experiment was performed by Ms. Allison Edgecombe.

A


MRwt
1h aldo
1h aldo
4h w/d
1h aldo
24h w/d
N+N>C
N=C
C+C>N
0
20
40
60
80
100
% localization

B


MRNL1-
4h aldo
4h aldo
4h w/d
4h aldo
24h w/d
N+N>C
N=C
C+C>N
0
20
40
60
80
100
% localization

transiently transfected with GFP-MR_{wt} and analysed using the FRAP-based nuclear export assay described previously (See Figure 7). As presented in Figure 31, at 1 h after photobleaching, minimal recovery of GFP-MR_{wt} was observed in the bleached nucleu, indicating that like GR, MR is exported from the nucleus at a relatively slow rate. This finding implies that slow nuclear export in the presence of ligand is not a unique property of GR, and may be shared by other steroid hormone receptors.

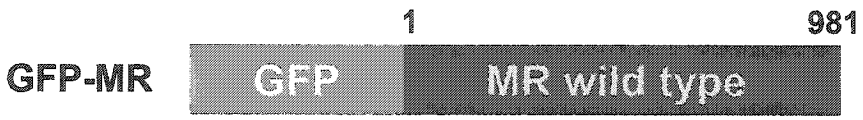
The observation that the GGM_{NL1-} construct does not localise to the nucleus to the same extent as MR_{NL1-} suggests that a sequence within the MR N-terminus or DBD may function as an NLS. While it is possible that a sequence within the DBD might have NLS activity, this seemed unlikely given that the GR DBD does not contain an NLS motif (42) and that the DBDs of GR and MR are very highly conserved with 93% amino acid identity shared between rat GR and rat MR. As the sequence of the N-terminal regions of GR and MR are significantly divergent with only approximately a 15% identity between rat GR and rat MR, it seemed most likely that an additional NLS activity might be found within the MR N-terminus. To address this possibility, the localisation of the MR N-terminus was examined by Ms. Laura Visentin. As shown in Figure 32a, the MR N-terminus does not contain a sequence motif resembling a classic mono or bi-partite NLS. However, there are two weak clusters of basic amino acids at amino acid residues 142-150 and 561-570. The latter sequence bears a striking resemblance to the NLS of the Borna disease virus (BDV) protein p10 (411).

To determine if the MR N-terminus contains an NLS activity, this region of MR spanning amino acid residues 1-602 was fused to GFP. The subcellular localisation of this construct was monitored by direct visualisation of GFP fluorescence. As shown in

Figure 31: MR is slowly exported from the nucleus.

(A) Outline of the GFP-MR construct utilised in this experiment. (B) Cos7 cells transiently transfected to express GFP-MR were treated with 1 μM aldosterone for 1 h prior to placing the cells in a Bioptechs FCS2 environmental chamber maintained at 37°C as previously described. FRAP analysis was performed as previously described. All experiments were performed in the presence of 20 μg/mL cycloheximide to prevent *de novo* protein synthesis.

A


1
981
GFP-MR
GFP
MR wild type

B

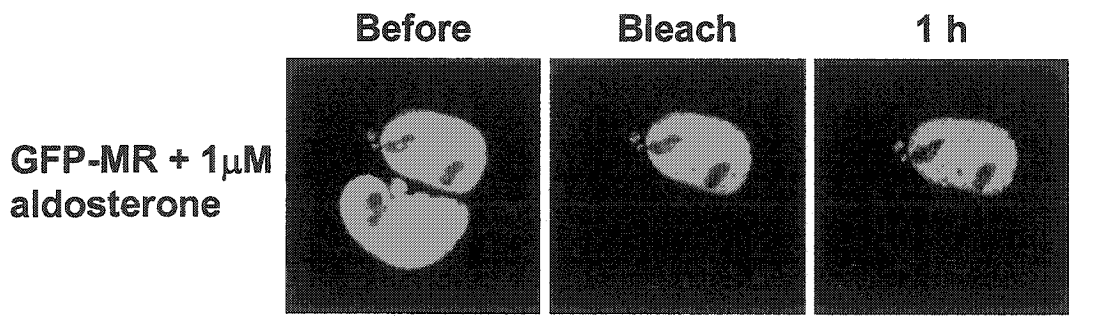

Before
Bleach
1 h
GFP-MR + 1μM aldosterone

Figure 32: The MR N-terminus contains a potential NLS activity, NL0.

(A) rMR sequence from amino acid residues 1-602. The MR N-terminus contains no classical mono or bipartite signals, however there are two weak clusters of basic amino acids. (B) The NL_0 sequence maps to rMR amino acid residues 550-602. Cos7 cells were transfected with the indicated GFP-MR fusion constructs and treated as described in Figure 1. The localisation of MR in the absence of ligand was scored by direct visualisation of GFP fluorescence using double-blind encryption as described in Figure 1. Error bars represent the standard error of the means of three independent experiments performed in duplicate. Representative micrographs are shown to the left of each data set. Scale bar = 10 μm. This experiment was performed by Ms. Laura Visentin.

A

metkgyhslp egldmerrws qvsqtlerss lgpaertten nymeivnvsc
vsgaipnnst qgsskekhel lpyiqqdnsr sgilpsdikt eleskelsat
vaesmglymd svrdaeytyd qqnqqgslsp tkiyqnmeql vkfykenghr
sstlsamsrp lrsfmpdsaa smnggalrai vkspiichek sssvssplnm
assvcspvgi nsmsssttsf gsfpvhspit qgtsltcsps venrgsrshs
pthasnvgsp lssplssmks pissppshcs vkspvsspnn vplrssvssp
anlnnsrcsv sspsnntnnr stlssptast vgsigspisn afsyatsgas
agagaiqdvv pspdthekga hdvpfpktee vekaisngvt gplnivqyik
sepdgafsss clggnskisp sspfsvpikq esskhscsga sfkgnptvnp
fpfmdgsyfs fmddkdyysl sgilgppvpg fdgscedsaf pvgikqepdd
gsyypeasip ssaivgvnsg gqsfhyriga qgtislsrsp rdqsfqhlss
fppvntlves wkphgdlssr rsdgypvley ipenvssstl rsvstgssrp
sk

B

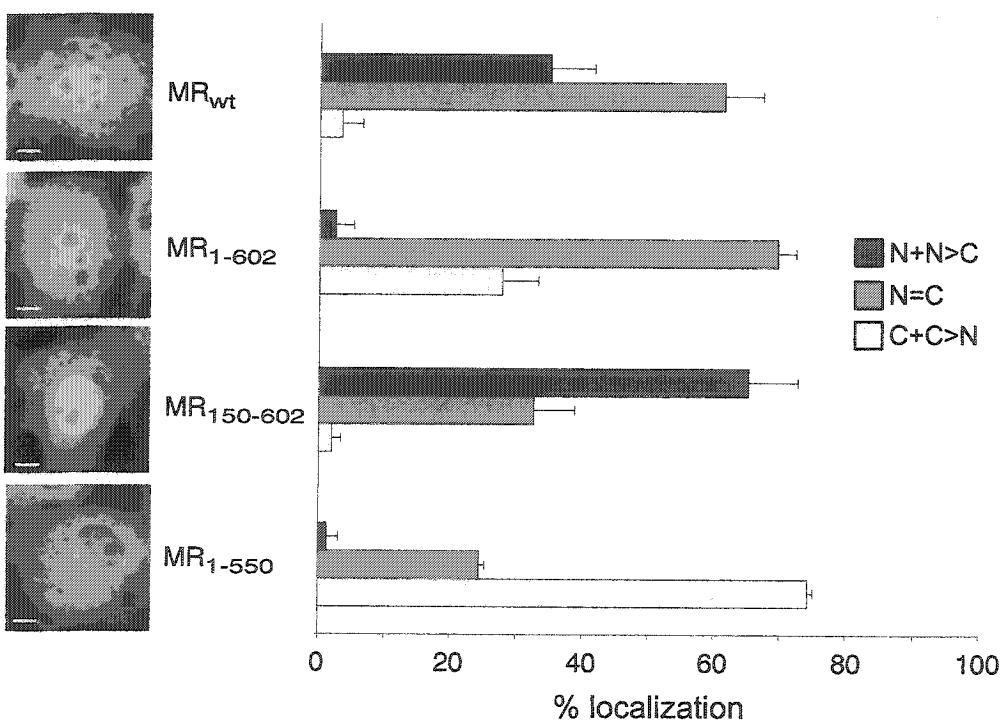


Figure 32b, GFP-MR_{1-602} is found evenly distributed between nucleus and cytoplasm. Since the GFP-MR_{1-602} construct has an expected size of approximately 70 kDa, this partial nuclear localisation is most likely mediated by direct nuclear import as 70kDa is beyond the upper limit for passive diffusion through the NPC (132). This finding implies that the MR N-terminus contains an additional NLS activity, which has been termed NL_0. As depicted in Figure 32a, the MR N-terminus contains two weakly basic clusters of amino acids. To more precisely delimit the sequence motif within MR that contains the NL_0 activity, deletion constructs consisting of $MR_{150-602}$ and MR_{1-550} fused to GFP were made. The subcellular localisation of these fusion proteins was monitored by direct visualisation of fluorescence in live cells. As seen in Figure 32b, the majority of cells expressing $MR_{150-602}$ were scored as predominantly nuclear. This result suggests that the MR NL_0 sequence is not located within amino acids 1-150, as deletion of these residues does not result in a shift in the localisation of the MR N-terminus towards the cytoplasm. However, the majority of cells expressing MR_{1-550} were scored as predominantly cytoplasmic. This finding suggests that the NL_0 activity resides within amino acid residues 550-602.

While MR does not contain a sequence resembling a classical NLS within amino acid residues 550-602, there is a weakly basic cluster at residues 561-570 that closely resembles the BDV p10 NLS motif. The BDV p10 NLS motif is an atypical NLS that mediates nuclear import through association with importin α (411). As depicted in Figure 33a, the BDV p10 NLS consists of a single lysine or arginine residue and a pair of arginine residues separated by a linker consisting of seven amino acids. To determine whether the MR NL_0 activity is mediated by this BDV-like sequence within the MR N-

Figure 33: Mutation within a region of MR that resembles the Borna disease virus protein NLS does not affect the MR NL0 activity.

(A) Sequence of the Borna disease virus protein (BDV) NLS motif and the homologous sequence within the MR N-terminus. Three key lysine residues are required for activity of the BDV NLS. (B) Mutation of the MR sequence that resembles the BDV NLS does not affect the subcellular localisation of the MR N-terminus. Cos7 cells were transfected with the indicated GFP-MR fusion constructs and treated as described in Figure 29. The localisation of MR in the absence of ligand was scored by direct visualisation of GFP fluorescence using double-blind encryption as described in Figure 29. Error bars represent the standard error of the means of three independent experiments performed in duplicate. Representative micrographs are shown to the left of each data set. Scale bar = 10 μm. This experiment was performed by Ms. Allison Edgecombe.

A

BDV

^{1}MSSDLRLTLLELVRRLNGNATIES^{24}

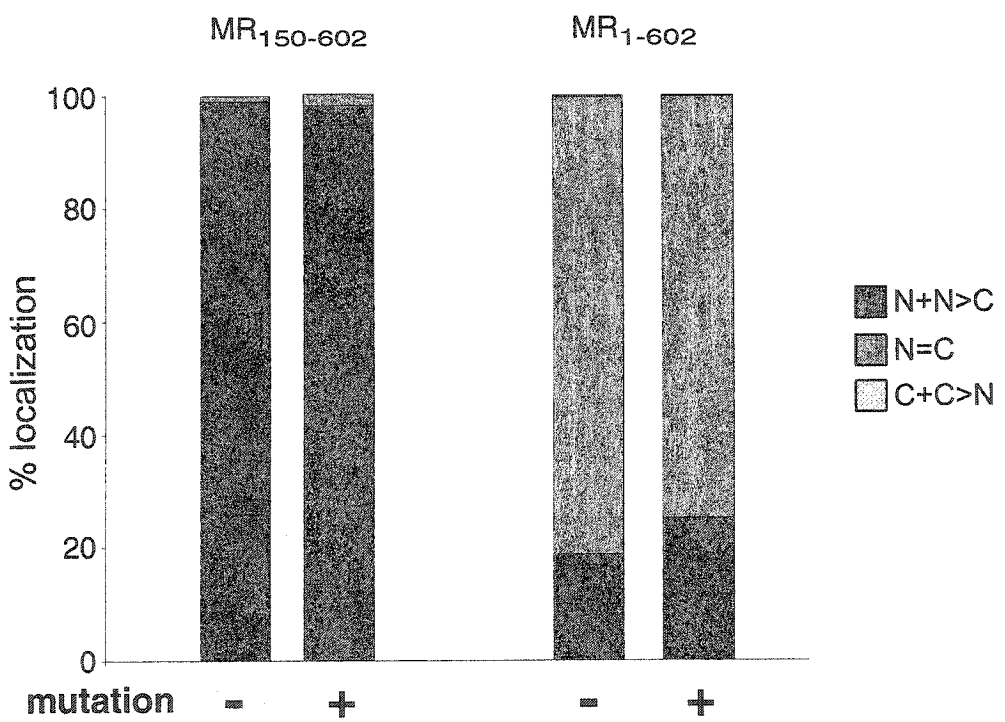
rMR

^{550}FPPVNTLVESWKPHGDLSSRRSDGVPVLEY^{570}

nn

Consensus: K/R(X)$_7$RR

B

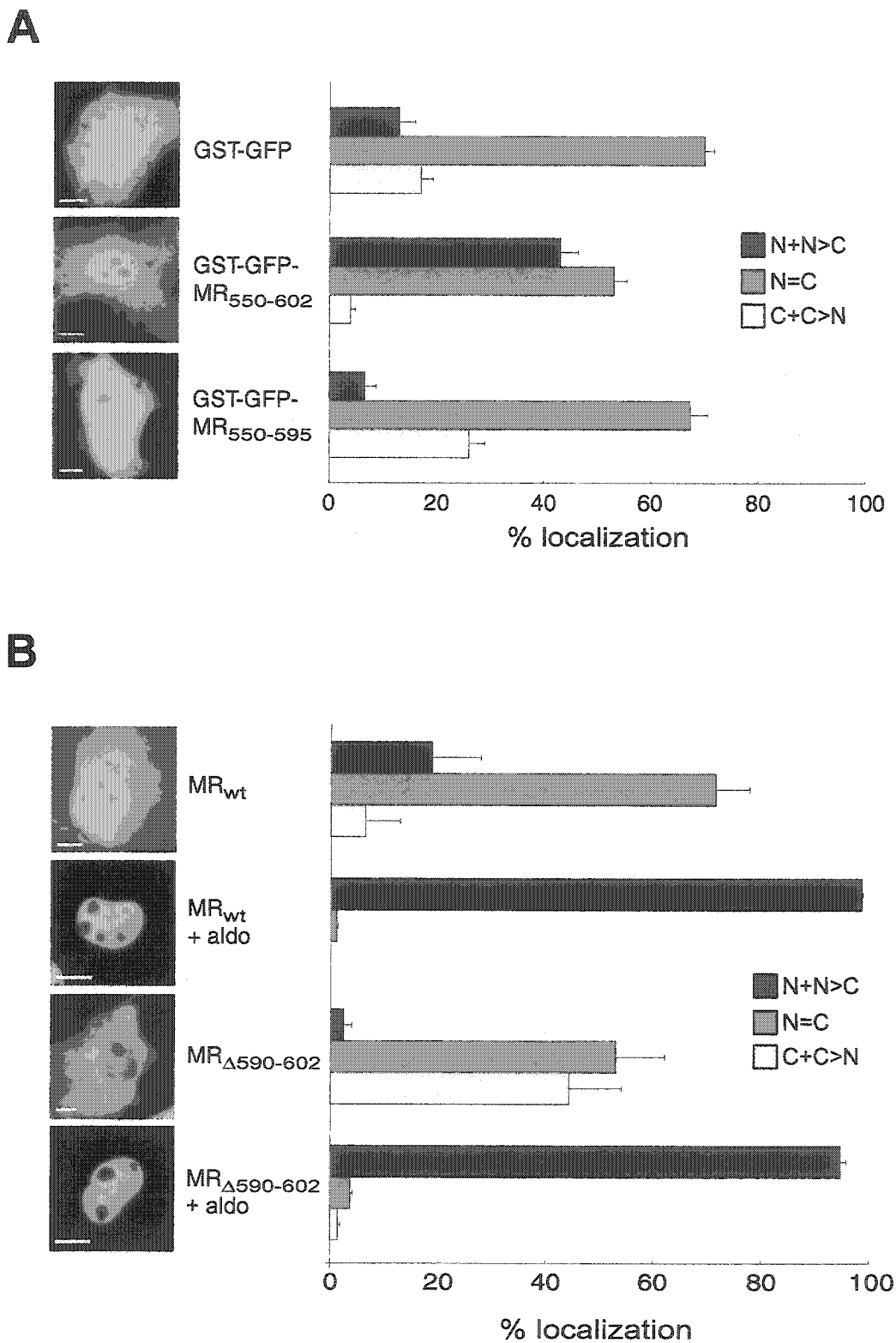


terminus, two key arginine residues within this sequence were mutated to alanines by Ms. Allison Edgecombe. Analysis of the subcellular distribution of the GFP-$MR_{1\text{-}602mt}$ construct compared to wildtype GFP-$MR_{1\text{-}602}$ revealed that mutation of this BDV-like sequence had no effect on the subcellular localisation of the MR N-terminus (Fig. 33b). This indicates that the BDV-like motif present at MR residues 561-570 does not function as an NLS within the context of the MR N-terminus. However, since the NL_0 activity mediates only partial nuclear localisation, it is possible that mutating this sequence would have only a modest effect on nuclear import that is not detectable in this experimental system. To address this possibility, the BDV-like motif of MR was mutated in the context of the GFP-$MR_{150\text{-}602}$ construct. $MR_{150\text{-}602}$ is more strongly nuclear than $MR_{1\text{-}602}$ and thus if this sequence of MR resembling the BDV NLS does mediate nuclear import, then the effect of its mutation would likely be more apparent in this context. However, as presented in Figure 33b, mutation of the BDV-like sequence had no effect in the context of the $MR_{150\text{-}602}$ construct as the same proportion of cells expressing GFP-$MR_{150\text{-}602}$ were scored as predominantly nuclear with or without the BDV mutation. Together, these results indicate that the MR NL_0 activity present within amino acid residues 550-602 does not reside in the BDV-like motif present at residues 561-570.

To assess whether the NL_0 activity resident between MR residues 550-602 is a transferable NLS, this region of MR was fused to both GST and GFP. It has been previously demonstrated that the GST-GFP fusion protein does not contain an active nuclear import signal (412). As depicted in Figure 34a, analysis of the subcellular localisation of GST-GFP by direct visualisation of GFP fluorescence revealed that GST-GFP is equally distributed between nucleus and cytoplasm. When MR residues 550-602

Figure 34: The MR NL_0 sequence maps to amino acid residues 590-602.

(A) The MR NL_0 activity functions as nuclear localisation sequence out of context of full length MR. Cos7 cells were transfected with the indicated GFP-MR fusion constructs and treated as described in Figure 29. The localisation of MR in the absence of ligand was scored by direct visualisation of GFP fluorescence using double-blind encryption as described in Figure 29. (B) Deletion of the NL_0 sequence results in decreased nuclear localisation of MR in the absence of ligand. Cos7 cells were transfected with the indicated pTL-MR constructs and treated as described in Figure 29. The localisation of MR in the absence of ligand was scored by indirect immunofluorescence using double blind encryption as described in Figure 29. Error bars represent the standard error of the means of three independent experiments performed in duplicate. Representative micrographs are shown to the left of each data set. Scale bar = 10 μm. This experiment was performed by Ms. Allison Edgecombe.


A
GST-GFP
GST-GFP-
MR550-602
GST-GFP-
MR550-595
N+N>C
N=C
C+C>N
0
20
40
60
80
100
% localization
B
MRwt
MRwt
+ aldo
MRΔ590-602
MRΔ590-602
+ aldo
N+N>C
N=C
C+C>N
0
20
40
60
80
100
% localization

are fused to GST-GFP, there is a shift in localisation towards the nucleus, indicating that the NL_0 activity resident within $MR_{550-602}$ is a transferable NLS. To begin to further delimit the location of the NL_0 sequence, a MR peptide encompassing amino acids 550-595 was fused to GST-GFP. As shown in Figure 34a, the GST-GFP-$MR_{550-595}$ construct did not show any change in nucleocytoplasmic distribution as compared GST-GFP alone. This result indicates that amino acid residues 595-602 are required to maintain the activity of the MR NL_0 sequence.

As MR residues 595-602 are located at the extreme C-terminus of all of the MR constructs used thus far to define NL_0, it was quite possible that amino acid residues within the linker region of the expression vector employed in these studies could contribute to the NL_0 activity. To eliminate this possibility, a deletion construct lacking amino acids 590-602 was made in the context of full length MR. To determine whether the NL_0 sequence is functional in the context of the full length protein, the subcellular localisation of $MR_{\Delta 590-602}$ was examined. Deletion of amino acid residues 590-602 results in a shift in the distribution of the naïve receptor from evenly distributed between nucleus and cytoplasm towards a more cytoplasmic localisation (Fig. 34b). This result suggests that the NL_0 motif mediates the partial nuclear localisation of MR prior to ligand binding. However, upon addition of hormone, a similar proportion of cells expressing either MR_{wt} or $MR_{\Delta 590-602}$ were scored as predominantly nuclear. These findings suggest that while the NL_0 sequence has a clear impact on the localisation of unliganded MR, it appears to be dispensable for ligand-induced nuclear accumulation.

Previous reports have suggested that nuclear export through the CRM1 pathway may influence nuclear localisation of naïve GR (130). Additionally, it has been

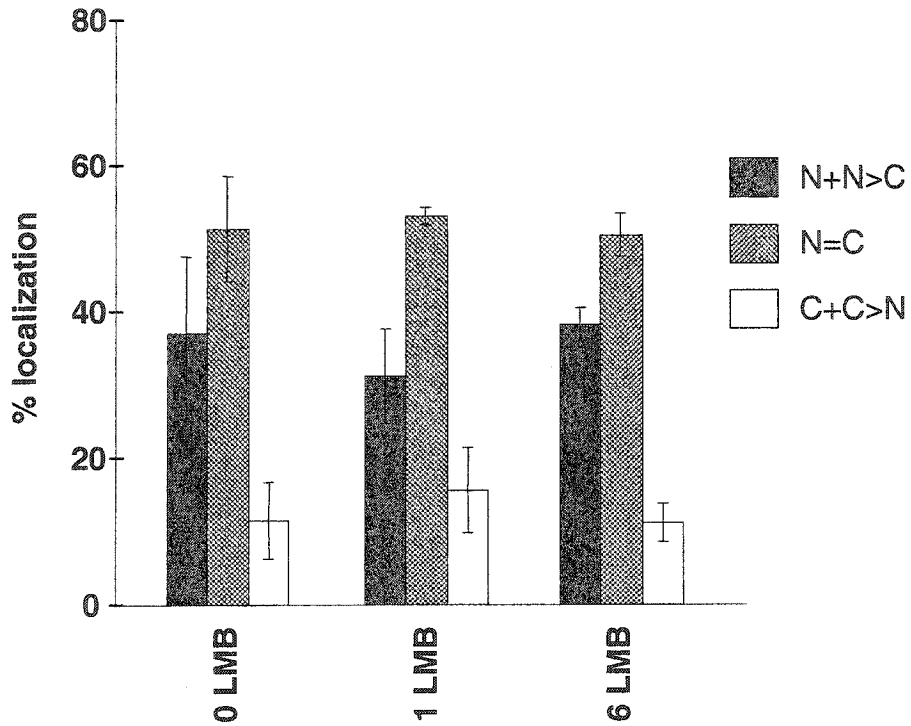
suggested that unliganded VDR is exported from the nucleus in a CRM1-dependent manner (381). To determine if the localisation of naïve MR is influenced by CRM1-dependent nuclear export, I studied the effect of the CRM1-specific inhibitor LMB on MR subcellular localisation in the absence of ligand. As shown in Figure 35, treatment with LMB for 1 h had no significant influence on the localisation of unliganded MR as an equivalent proportion of cells expressing MR were scored as evenly distributed between nucleus and cytoplasm in the presence or absence of LMB. To ensure that this lack of effect was not due to insufficient incubation time with LMB, the incubation period was extended to 6 h. Again, inhibition of the CRM1 export pathway had no significant effect on MR localisation prior to ligand binding. While this finding does not eliminate the possibility that unliganded MR is indeed constitutively exported and then re-imported from the nucleus by a separate, non-CRM1 pathway, this result suggests that localisation of unliganded MR is not influenced by CRM1.

Discussion

Our results indicate that nuclear localisation of MR is determined by multiple dispersed signals. We have shown that MR contains NLS motifs that bear resemblance to the GR NL1 and NL2 activities and also an additional NLS within the N-terminal region of the receptor, which we have termed NL_0. This nuclear localisation sequence maps to amino acids 590-602 of the rat MR sequence and appears to be quite atypical in nature. NL_0 is not rich in basic amino acids as is typical of NLS motifs that mediate import through binding to importin α. Rather, the NL_0 sequence contains several

Figure 35: Subcellular localisation of naïve MR is unaffected by inhibition of Crm-1

Cos7 cells were transiently transfected with 1 μg GFP-MR by Lipofectamine™. Following transfection, the cells were plated onto glass coverslips and synchronised in G_0 by withdrawing serum. After 16-24 h, the cells were treated with 10 nM LMB for the indicated times. The localisation of GFP-MR was scored into categories as previously described by indirect immunofluorescence using a Nikon TE300 microscope. The percentage of cells scored as exclusively nuclear or mainly nuclear were added together to obtain the displayed values, as were the percentage of cells scored as exclusively cytoplasmic or mainly cytoplasmic. Error bars represent the standard error of the means of three independent experiments performed in duplicate.


80
60
40
20
0
% localization
N+N>C
N=C
C+C>N
0 LMB
1 LMB
6 LMB

serine and threonine residues. The identification of this unique NLS motif indicates that MR subcellular localisation is subject to regulation on multiple levels.

Import of proteins carrying typical mono or bi-partite basic nuclear localisation sequences is usually mediated through interaction with importin α (162,163). Nuclear import of proteins through atypical NLS motifs is less well understood. In many cases, import through atypical NLS motifs occurs through direct interaction with importin β (197). While the number of importin α receptors is somewhat limited, many more unique importin β isoforms have been identified in both yeast and in vertebrates. As importin β isoforms share a lesser degree of homology in their C-terminal cargo binding domains than compared to the N-terminal Ran binding domain, it is conceivable that a wide variety of protein cargos could be imported into the nucleus through an importin β-dependent mechanism, including the highly divergent NL_0 motif that we have chararcterised.

The NL_0 sequence contains serine and threonine residues that could potentially be phosphorylated. Phosphorylation is known to influence the activity of nuclear import signals. For example, phosphorylation of the cell cycle regulator p27 by PKB/Akt within the p27 NLS inactivates p27 NLS leading to increased accumulation in the cytoplasm and subsequent disruption of the ability of p27 to mediate cell cycle arrest in G1 in response to anti-proliferative signalling through cytokines (413). Conversely, nuclear import of the serine/arginine-rich proteins (SR proteins), which function in the regulation of mRNA splicing and contain a novel type of NLS motif characterised by arginine/serine repeats, is dependent on phosphorylation within the SR nuclear targeting signal (414). Phosphorylation has also been implicated in the regulation of nuclear export. For example, phosphorylation near the HDAC4/5 NES indirectly activates nuclear export by

triggering the dissociation of HDAC4/5 from MEF2 (238). Once dissociated from MEF2, HDAC4/5 is able to form a complex with 14-3-3, which in turn mediates its export from the nucleus (232,233). The possibility that phosphorylation of the MR NL_0 sequence affects MR function is currently under investigation.

The regulation of steroid hormone receptor subcellular localisation, both in the presence and absence of hormone, provides a means of controlling receptor activity. The data presented in this study offer new insights into the regulation of MR subcellular localisation. As could be anticipated based on the high degree of sequence and functional similarity shared between MR and GR, the mechanisms controlling MR subcellular localisation resemble those of GR to some extent. We have shown that the hinge region of MR contains a NLS function that appears to be analogous to the GR NL1 motif. Additionally, the MR LBD contains a ligand inducible nuclear localisation activity that appears to act in much the same capacity as the GR NL2 activity. However, control of MR subcellular localisation is not entirely similar to that of GR. This is most strikingly demonstrated by the presence of an additional MR NLS motif, which we have termed NL_0. Further, while the GR NL1 motif appears to act as a nuclear retention sequence, mutation of the putative MR NL1 sequence does not result in an increased on the rate of return to the cytoplasm following ligand withdrawal (See Figure 30). While it is important to recognize that this finding does not conclusively eliminate the possibility that the MR NL1 sequence might have the potential to mediate nuclear retention, this observation does suggest that continued nuclear occupancy of MR following ligand withdrawal is mediated at least in part through a mechanism that is not shared with GR.

In rationalizing the potential reason as to why MR does not appear to relocalise to the cytoplasm following ligand withdrawal, it should be considered that following exposure to the hormone antagonist RU-486, GR remains constitutively localised to the nucleus for an extended period of time following hormone withdrawal (354). As the three dimensional conformation of the LBD varies considerably when treated with hormone agonist versus antagonist (109,415), the observation that RU-486-withdrawn GR fails to export from the nucleus suggests that nuclear export of GR following hormone withdrawal may require a post-translational event that is dependent on the receptor adopting an initial conformation that only occurs following exposure to hormone agonist and not antagonist (354). It is interesting to note that, despite the high degree of amino acid identity shared between the steroid hormone receptor LBDs, the crystal structure of the GR LBD reveals subtle yet significant variations in the three-dimensional arrangement as compared to the LBDs of both PR and ER (83). Perhaps the ligand-induced conformation of MR is such that addition of ligand does not adequately prime MR to undergo a subsequent conformational change that allows for nuclear export following ligand withdrawal. This would explain the failure of MR to relocalise to the cytoplasm following ligand withdrawal.

The functional significance of MR nuclear localisation prior to ligand binding is the subject of ongoing study. It has been shown that when expressed under the control of the same promoter, MR expression levels are markedly decreased in the absence of hormone when compared to GR. This difference in protein stability is not a consequence of decreased gene expression, as RNA levels of MR and GR appear to be equal (K. Swan, Y. Rouleau and A. Edgecombe, unpublished results). The regulation of MR

degradation as it relates to protein stability and other aspects of MR function is just beginning to emerge. While degradation of GR may preferentially occur in the cytoplasm (285), it may be possible that the regulation of the stability of naïve MR and the regulationg of its subcellular localisation are independent events. Though deletion of the NL_0 sequence does not appear to significantly affect the expression of MR at the protein level, mutation of the core serine residues to alanines drastically reduces protein expression (K. Swan and A. Edgecombe, unpublished results). It is known that that GR is degraded following ligand binding (312,383), which is accompanied by the dissociation of the GR-hsp complex (273). Further, stabilisation of the GR-hsp interaction leads to increased accumulation of GR in the cytoplasm (274,280). Perhaps the two seemingly contradictory observations that removal of the NL_0 sequence has no affect on stability but mutation within NL_0 drastically decreases the level of MR protein indicates that the NL_0 sequence influences subcellular localisation of MR through destabilization of the hsp complex as opposed to directly interfacing with the subcellular trafficking machinery.

While much is known concerning the regulation of GR nucleocytoplasmic trafficking, the regulation of MR subcellular localisation is as yet largely uncharacterised. Our results indicate that the subcellular localisation of MR and GR are determined through a partially intersecting set of pathways. While MR localisation is in part determined through motifs similar to the GR NL1 and NL2 sequences, we have shown that MR possesses a unique nuclear localisation motif that we have termed NL_0. The presence of the MR NL_0 sequence provides a potential explanation for the differences observed in the localisation of GR and MR prior to ligand binding. As signalling

mechanisms through GR and MR share a great potential for overlap, a careful consideration of the subtle differences in GR and MR signalling, including their subcellular localisation, will contribute to a greater understanding of how fidelity of signalling through these closely related receptors is maintained.

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(1) Papers

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