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Nucleocytoplasmic Trafficking and Stability of the Mineralocorticoid Receptor

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Nucleocytoplasmic Trafficking and Stability of the Mineralocorticoid Receptor

By

Yanouchka Rouleau

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology

in partial fulfillment of the requirements for the degree of

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Abstract

The mineralocorticoid receptor (MR) is a ligand inducible sequence specific transcription factor belonging to the nuclear receptor superfamily. It is the least characterized of the steroid hormone receptors but it has a high similarity with the well-characterized glucocorticoid receptor (GR).

Upon binding ligand, steroid receptors such as GR and MR, translocate to the nucleus where they bind hormone response elements to activate transcription. To enter the nucleus, these steroid receptors have been shown to have a nuclear localization signals. Nuclear import of GR is mediated by a well-characterized sequence, called NL1, located in the hinge region and a second uncharacterized motif, called NL2, present in the LBD of the receptor. By sequence homology, our laboratory had previously identified and characterized an NL1 in rat MR, also located in the hinge region of the protein. In this study, I report the presence of a second NLS (NL2) in MR that mediates a slower translocation of MR to the nucleus in an agonist-dependent manner. I also report that the LBD of MR, containing the NL2, could contain a sequence that prolongs nuclear subcellular localization of the receptor or alternatively lacks an export signal.

It is known that steroid receptors such as GR and PR undergo proteasomal degradation. From our observation that independent of the amount of MR cDNA transiently transfected in Cos7 cells, the expression level of MR was always markedly lower than for GR, we hypothesised that the lower level of MR could be due to a more rapid degradation of the receptor. I report that MR is degraded by the proteasome, that

addition of steroid induces further degradation and that the N-terminal portion of MR is responsible for the appearance of higher molecular weight forms of the receptor after aldosterone treatment. Finally, we identified, by using the PESTfind software, a PEST signal starting at position 686 to 711, but confirm that the lysine residue at position 712 is not an acceptor ubiquitin site.

Acknowledgments

I would like to thank my supervisor, Dr Robert J.G. Haché, whose expertise, understanding and vast knowledge added considerably to my graduate experience and contributed to my research.

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Also warmest thanks must go to my lab mates who provided immeasurable help during this project with the techniques I had to learn and also inestimable moral support when it was most needed. Most of all, I thank Claudia Bayer and Laura Visentin for preliminary data on which my project was based.

I recognize that this research would not have been possible without the financial assistance of CIHR, and express my gratitude to them.

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Abbreviations

11β-HSD2	11β-hydroxysteroid dehydrogenase types 2
ACE	angiotensin converting enzyme
ADH	antidiuretic hormone
BSA	bovine serum albumin
cDNA	complementary DNA
DBD	DNA-binding domain
DTT	dithiothreitol
E	glutamic acid
EDTA	ethylenediamine tetraacetic acid
EGFP	enhanced green fluorescent protein
ENaC	epithelial sodium channel
ER	endoplasmic reticulum
F	phenylalanine
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G	glycine
GFP	green fluorescent protein
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	*Homo sapiens*
HRE	hormone response element
HRP	horseradish peroxydase

Hsp90	90kDa heat-shock protein
IBB	importin β-binding domain
K	lysine
kDa	kilodaltons
LBD	ligand binding domain
LBP	ligand binding pocket
MDa	megadalton
MMTV	mouse mammary tumor virus long terminal repeat sequence
MR	mineralocorticoid receptor
MRE	mineralocorticoid response element
NES	nuclear export signal
NLS	nuclear localization signal
nm	nanometer
NP-40	nonidet P-40
NPC	nuclear pore complex
NTF2	nuclear transport factor 2
Nups	nucleoporins
ONPG	O-nitrophenyl β-d-galactopyranoside
P	proline
PBS	phosphate-buffer saline
PEST	Proline (P) Glutamate (E) Serine (S) Threonine (T)
PCR	polymerase chain reaction
PR	progesterone receptor

Q	Glutamine
r	*Rattus norvegicus*
R	arginine
RanGAP	RanGTPase-activating protein
RanGEF	RanGTP-GDP exchange factor
RanGEP	Ran guanine nucleotide exchange factor
RIP-140	receptor-interacting proteins of molecular weights 140
RLU	relative luminescence unit
S	serine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFBS	charcoal-stripped fetal bovine serum
sgk	serum- and glucocorticoid-regulated kinase
SRC-1	steroid receptor coactivator –1
T	threonine
TAT	tyrosine aminotransferase
WCE	whole cell extract

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Introduction

The human body is a complex organism composed of different interrelated systems that have to maintain homeostasis to be functional. If the equilibrium is not conserved within limits, it results in illness. This maintenance of homeostasis is accomplished by communication among these different systems, in part by hormones used as intermediates, which trigger intracellular responses in target organs. Hormones can be divided in two categories according to the molecule from which that they are derived. Most of the hormones are synthesized from amino acids and are hydrophilic. In the second category, steroids and related hormones are synthesized from cholesterol and are hydrophobic (Marieb, 1993). Steroid hormones have an effect on growth, development and homeostasis by interaction with intracellular receptors, members of the nuclear hormone receptor superfamily, that regulate transcription of target genes.

Structure of the receptor

The nuclear hormone receptor superfamily is divided in three: the steroid receptor family, including the progesterone receptor (PR), the androgen receptor (AR), the oestrogen receptor (ER), the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), the thyroid/retinoid/vitamin D receptor family and the orphan receptor family. MR, similar to all other nuclear receptors, has a modular structure with a central highly conserved double zinc fingered DNA-binding domain (DBD) (Fig. 1). The variable amino-terminal domain contains an autonomous activation function (AF-1) (Govindan and Warriar, 1998 and Fuse *et al.*, 2000). The carboxy-terminal contains the

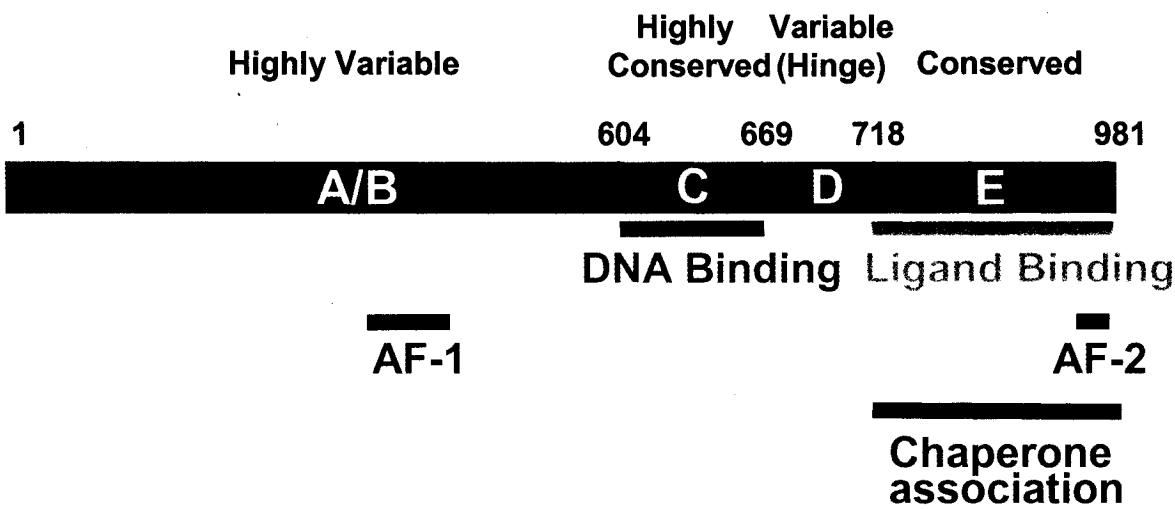

Highly Variable
Highly Conserved
Variable (Hinge)
Conserved
1
604
669
718
981
A/B
C
D
E
DNA Binding
Ligand Binding
AF-1
AF-2
Chaperone association

Fig. 1
Schematic representation of rat MR.
The variable amino-terminal (A/B) domain contains an autonomous activation function (AF-1). The carboxy-terminal contains the centrally located DBD (C), a hinge region (D) and a ligand-binding domain (LBD) (E). The LBD also contains a binding domain for the chaperone proteins and a ligand-dependent activation function (AF-2).

centrally located DBD, a hinge region including a stretch of prolines that is thought to break the structure and provides flexibility (Tsai and O'Malley, 1994) and a ligand-binding domain (LBD). The LBD also contains a binding domain for the chaperone proteins (Rafestin-Oblin *et al.*, 1989, Couette *et al.*, 1998) and a ligand-dependent activation function (AF-2). Of all steroid hormone receptors, MR is the least characterized but its high similarity with the well-characterized glucocorticoid receptor (GR) facilitates study by comparison to what is known about GR. Human MR (hMR) has 94% amino acid identity to the human GR (hGR) DNA-binding domain (DBD), 57% to the hGR ligand-binding domain (LBD) (Arriza *et al.*, 1987) and less than 15% identity to the N-terminal portion of hGR (Fuller *et al.*, 2000).

Physiological ligands for GR and MR are the corticosteroids: glucocorticoids (cortisol), which promote glycogen accumulation in the liver through GR, and mineralocorticoids (aldosterone), which act through MR to control blood pressure by regulation of sodium and potassium homeostasis. MR has also a role in the brain (memory) and an unknown function in the cardiovascular system.

Expression of the mineralocorticoid receptor

Despite the sequence similarity to GR, some characteristics of MR are different from those of GR, including the expression of the receptor and its subcellular localization. MR is expressed at varying levels in the classical aldosterone target tissues such as renal collecting tubules, colon and sweat and salivary glands and in non-epithelial tissues such as the brain and the heart (Arriza *et al.* 1987). The physiological mineralocorticoid is aldosterone, but MR is also highly sensitive to corticosteroids, both glucocorticoids and

mineralocorticoids. To decrease MR response to glucocorticoids in aldosterone target tissues, cells synthesize 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) to oxidize 11β-hydroxyglucocorticoids to their inactive 11-keto forms. The enzymatic activity of 11β-HSD2 transforms cortisol to cortisone by changing the hydroxyl group at position 11 of cortisol to a ketone functional group instead. Aldosterone is not a substrate of 11β -HSD2 activity because of its unique chemistry. In solution, the aldehyde group at C18 cyclizes with the hydroxyl group at C11 to form a hemicetal and consequently protect it from enzymatic attack (Funder, 1996). 11β-HSD2 is also found in non-epithelial tissues such as the placenta, pancreas and female reproductive tracts but its function there is unknown. Deficiency of 11β-HSD2 results in cortisol-mediated sodium retention and hypertension (Ricketts and Stewart, 1999, Vogt *et al.*, 1999 and van Uum *et al.*, 1998).

Reports on subcellular localization of 11β-HSD2 are controversial. Studies done with cells from mineralocorticoid target tissues have shown that 40% of the total immunostaining for 11β-HSD2 was nuclear (Bujalska *et al.*, 1997 and Shimojo *et al.*, 1997) and 11β-HSD2 activity was found in a nuclear fraction (Petrelli *et al.* 1997). It has been postulated that depending on the tissue where 11β-HSD2 is expressed, tissue specific factors are responsible for the subcellular localization of 11β-HSD2. On the other hand, studies done using overexpressed 11β-HSD2 wild-type or 11β-HSD2 fused to the green fluorescent protein (GFP) have shown subcellular localization of the enzyme only at the endoplasmic reticulum (ER) and the nuclear membrane (Naray-Fejes-Toth and Fejes-Toth, 1996 and Odermatt *et al.*, 1999). 11β-HSD2 shown to be

located in the ER has its amino-terminus towards the lumen of the ER and its catalytic domain facing the cytoplasm. MR has been suggested to colocalize with 11β-HSD2 at the ER (Naray-Fejes-Toth and Fejes-Toth, 1996 and Odermatt *et al.* 2001) allowing the proximity of the enzyme to protect MR against 11β-hydroxyglucocorticoids to make it aldosterone specific. The 11-ketoglucocorticoids produced inhibited the activation of MR by aldosterone, which could explain in part why glucocorticoids given at high dosage act as MR antagonists in the kidney and the heart (Funder, 2000). In tissues not expressing 11β-HSD2, glucocorticoids can act through MR as agonists or antagonists, depending on the tissue.

Heat shock proteins and immunophilins

For steroid receptors to be in a high affinity ligand binding conformation, the LBD has to maintain an association with chaperone proteins. This complex is composed in part of heat shock proteins (Hsp90, Hsp70) and immunophilins (Hsp56), which are intracellular proteins that bind immunosuppressive drugs. Steroid receptors (ER (Chambraud *et al.*, 1990), PR (Schowalter *et al.* 1991) and GR (Cadepond *et.al.* 1991)), as opposed to other members of the nuclear receptor superfamily, have been shown to bind the 90kDa heat-shock protein (Hsp90), an ubiquitous, essential and highly conserved protein. Disruption of Hsp90 homologues in yeast expressing GR does not lead to a constitutive activation of the receptor but rather to a significant impairment of hormone induction (Bohen and Yamamoto 1993), suggesting a role in proper folding of the receptor as opposed to a repression function.

There is some evidence that more than one site in MR is involved in the interaction between steroid receptors and Hsp90. MR from chicken has been used for the study of protein interaction with the chaperones and has been shown to exist in a complex including Hsp90 (Rafestin-Oblin *et al.*, 1989). In a separate study, amino acids 729-733 of human MR (hMR) have been shown to be necessary for Hsp interaction and that deletion of the last 4 amino acids of the protein abolishes ligand binding completely (Couette *et al.*, 1998).

The ligand-binding domain

Upon ligand binding to the receptor, the receptor changes conformation, releases the chaperone complex and exposes sequences involved in nuclear localization, receptor dimerization, co-activators interaction and DNA binding (Picard and Yamamoto, 1987, Derfoul *et al.*1998 and Shibata *et al.*, 1997). MR has been shown to bind agonists (aldosterone and cortisol) or antagonists (progesterone and spironolactone) while still in a complex with the Hsps (Couette *et al.* 1996), suggesting that the conformational changes of the LBD precede the release of the Hsps.

The crystal structures of a few steroid receptors such as PR (Williams and Sigler, 1998), TR (Wagner *et al.* 1995) and ER (Brzozowski *et al.* 1997) have been characterized and the conformational changes occurring after ligand binding have been described as the mousetrap model. The LBDs of these steroid receptors display a common fold, with 11 or 12 α-helices and one β-turn located between the 5^{th} and the 6^{th} helix. Upon ligand binding, there is a rearrangement of the helices of which it is thought that the end step is the flipping of helix 12, which seals the ligand-binding pocket (LBP) and further

stabilizes the receptor-ligand interaction (Moras and Gronemeyer 1998). The ligand binding pocket architecture is delimited by H5 (helix 5), H7, H11, H12 and the β-turn.

Despite the fact that the crystal structure of many of the LBD is known, the one of MR has not been resolved. The high sequence identity of MR and GR LBDs explains the overlap in specificity for ligands (cortisol and corticosterone), which they both bind with high affinity, but only MR binds aldosterone with high affinity. A study has been done of the MR LBD to define the structural determinants of aldosterone binding (Rogerson *et al.* 1999). It was shown that amino acids 804-874 from hMR are necessary for specific binding of the receptor with aldosterone. A second domain, from amino acids 932 to 984 also seems to contribute to the binding but is not essential. These two domains, by comparison to the crystal structure of ERα and PR, form the LBP of the receptor. In a similar study, the sequence of AR (amido acids 766-799), corresponding to the amino acids 804-874 of MR, has been shown to be in part responsible for the specificity of testosterone binding to the androgen receptor (Vivat *et al.* 1997).

The LBP of MR contains five predominant hydrophobic residues located at the extremities of the pocket, consistent with the two polar extremities of aldosterone created by the C3-ketone group on the A-ring on one side and the C20-ketone and the C21-hydroxyl groups on the D-ring on the other side. Mutation of the polar residues in the so-called site I (Q776 and R817) by an alanine residue, reduced the affinity for both agonists and antagonists with a C3-ketone group. In contrast, mutation of the N770 abolished the interaction with all C21-hydroxylated agonists, but not the interaction with

ligand lacking this substituent (i.e. progesterone). These results allowed the identification of the orientation of aldosterone in the pocket, with the A-ring oriented towards the site I of the LBP and the D-ring towards the site II (Fagart *et al.*, 1998).

The DNA-binding domain

The primary mechanism of action of steroid receptors is to regulate transcription of target genes. For some steroid receptors such as GR and AR, this process is accomplished after ligand binding and entry of the transformed receptor in the nucleus. In the case of MR, since naïve receptors are equally distributed through the cytoplasm and the nucleus, only those remaining cytoplasmic enter the nucleus. To regulate transcription, steroid receptors bind DNA through their DNA-binding domain made of two highly conserved zinc finger motifs that bind specific palindromic DNA sequences (Beato *et al.*, 1987 and Zandi *et al.*, 1993), also known as hormone response elements (HREs) (Kolla *et al.* 1999, Govindan *et al.*, 1991 and Schweizer-Groyer *et al.*, 1997). The structure of the zinc finger motifs allows the receptor to interact with the major groove of the DNA.

Among steroid receptors, there is recognition of some of the same ligands (i.e. GR and MR bind cortisol, corticosterone and progesterone) and also the same HRE resulting in a cross talk between receptors. Until now, no specific mineralocorticoid response element (MRE), distinct from a response element for GR/PR, has been characterized. To exert its function, GR and MR can bind the same DNA sequence, the glucocorticoid response element (GRE) (Lombes *et al.*, 1993). A "perfect" GRE is composed of two half-sites organized as inverted repeats, separated by three amino acids: NNTACA NNN

TGTTCT, where N represents different amino acids depending on the promoter (i.e. the mouse mammary tumor virus promoter (MMTV) or the tyrosine aminotransferase promoter (TAT) (Beato *et al.*, 1987)).

MR binds as a homodimer on response elements present in the promoter region of target genes and initiates transcription through specific interaction with the transcription machinery. As happens with the other members of the steroid receptor family (Shibata *et al.*, 1997), MR is known to interact with coactivators, such as the steroid receptor coactivator 1 (SRC-1), the receptor-interacting proteins of molecular weights 140 (RIP-140) and TIF1α, in the presence of ligand (Hellal-Levy *et al.* 2000). Binding to coactivators allows the receptor to interact with the basal transcriptional machinery more efficiently and to activate transcription. At the transcriptional level, it is thought that MR regulates genes involved in regulation of sodium homeostasis such as the sodium-potassium ATPase subunits α and β (Na/K-ATPase α1 or β1) (Derfoul *et al.*1998, Kolla *et al.* 1999, 2000 and Summa *et al.* 2001), the epithelial sodium channel (ENaC) and the serum- and glucocorticoid-regulated kinase (sgk) (Shigaev *et al.*, 2000).

Mechanisms of nuclear import

Consequent to ligand binding, the changes in conformation and the release of heat-shock proteins, cytoplasmic MR translocates to the nucleus where it binds hormone response elements to activate transcription. In eukaryotic cells, the nuclear membrane separates the nuclear synthesis of DNA and RNA from cytoplasmic protein synthesis. Ions and small molecules can cross the nuclear membrane by passive diffusion, but single

proteins or protein complexes bigger than 60 kilodaltons (kDa), such as MR, have to be transferred by active transport through the nuclear pore complex (NPC) (Ohno *et al.*, 1998). On the other hand, it should not be assumed that a protein smaller than the cut-off of 60kDa would necessarily be translocated by passive diffusion. Ribbeck and Görlich (2001) have shown that a protein known to interact with the nuclear pore complex was translocated to the nucleus 120 times faster than a protein not interacting with the NPC, even if the size of the protein was only 29kDa.

Nuclear pore complexes are structures of approximately 66 megadaltons (MDa) in *Saccharomyces cerevisiae* and 125MDa in higher eukaryotes, which form aqueous channels where translocation of proteins occurs. They are composed of proteins called the nucleoporins (Nups). NPC's form 8 symmetrical spoke structures span the nuclear membrane to form the core of the NPC. On each side of the channel are different annular structures, called the cytoplasmic and the nuclear rings. Eight filaments of 30 to 50nm project from the cytoplasmic ring in the cytoplasm and the nuclear ring carries 8 longer (~100nm) and thinner filaments joined by a terminal ring, forming a structure called the nuclear basket. The diameter of the channels formed by the NPC appears to be flexible, and can expand from ~10 nanometers (nm) to ~40 nm (Feldherr *et al.*, 1984 and Dworetzky *et al.*, 1988).

To be translocated through the NPC, a cargo has to be recognized by a member of the nuclear transport receptor family, named karyopherins or transportins (importins or exportins). These receptors are large (90-130kDa) acidic proteins able to bind components of the NPC. Multiple classes of cargos contain signals that bind directly to

an import receptor, but proteins with a classical basic NLS (either simple or bipartite) bind to their import receptor (importin β) via an adapter protein. The superfamily of importin β-related factors is the best-characterized class of karyopherins. They have also been studied *in vitro* for their role as cytoplasmic chaperones for exposed basic domains (Jäkel *et al.*, 2002). The adapter protein with which importin β has to interact (i.e. importin α) binds the cargo via the basic NLS binding domain and bind importin β via their N-terminal importin β-binding (IBB) domain (Mattaj and Conti, 1999). The importin β, on its side, contains two specific domains, an N-terminal RanGTP-binding domain and a C-terminal importin α-binding domain.

Active transport of proteins requires energy and the only known source is the small GTP. Since the translocation is not linked to GTP hydrolysis, it is likely that the energy comes from a potential energy gradient established by distinct pools of Ran: GTP-Ran in the nucleus and GDP-Ran in the cytoplasm. The directionality of active transport is considered to be promoted by the Ran GTPase cycle, as it triggers the assembly and disassembly of transport complexes in the correct compartments (Fig.2) (Rout and Aitchison, 2001). Import receptors bind their cargos in a RanGTP-independent manner. Once in the nucleus, RanGTP binding dissociates the import receptor-cargo complex, releasing the cargo in the nucleus. The import receptors return to the cytoplasm as a RanGTP-receptor complex (Hieda *et al.*, 1999). Ran is maintained as RanGTP in the

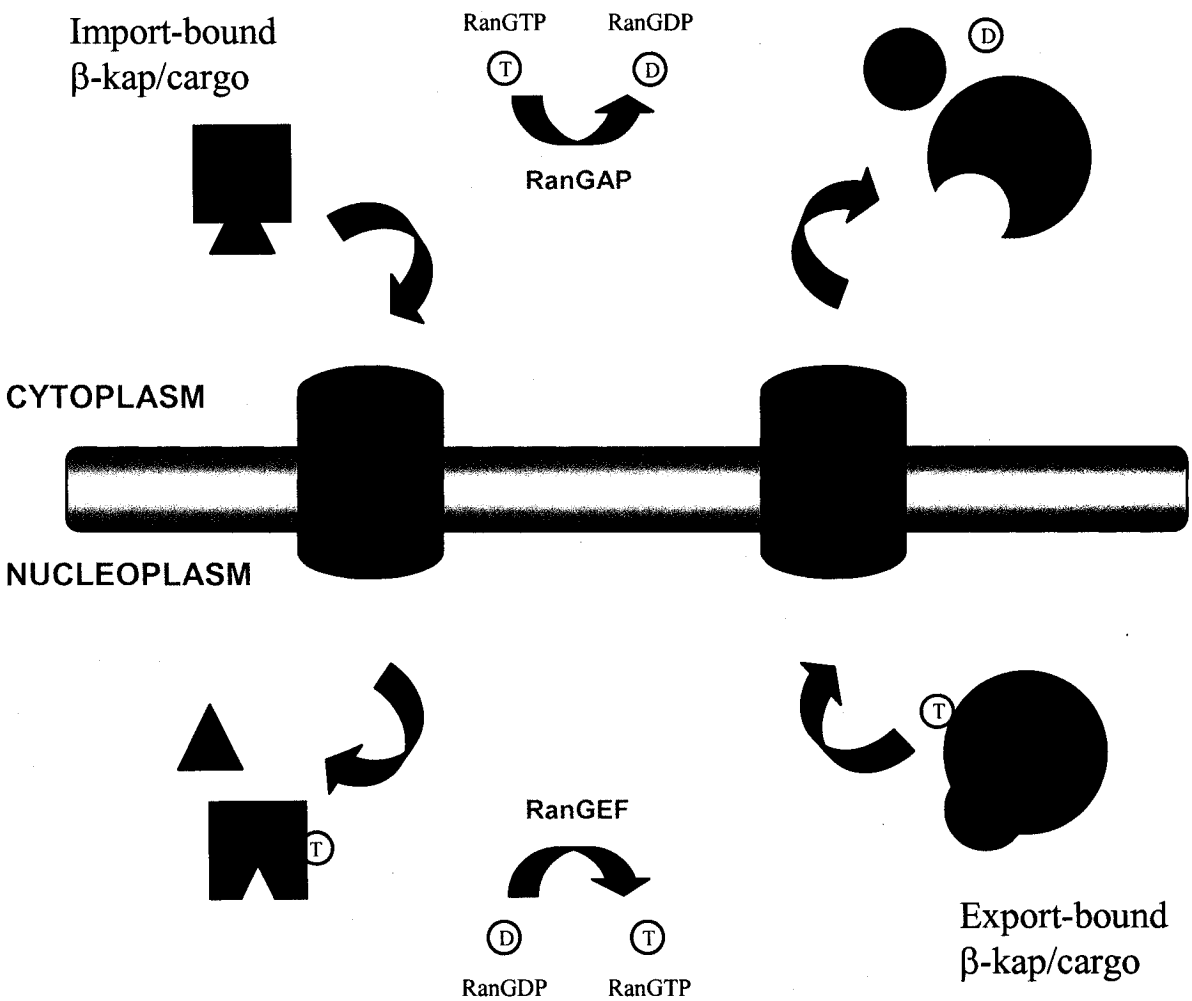

Import-bound
β-kap/cargo
RanGTP
RanGDP
T
D
RanGAP
D
CYTOPLASM
NUCLEOPLASM
T
RanGEF
T
D
RanGDP
RanGTP
Export-bound
β-kap/cargo

Fig. 2
The Ran cycle.
Ran cycles between its GTP- and GDP-bound form depending on its subcellular localization. Import receptors bind their cargos in a RanGTP-independent manner. Once in the nucleus, RanGTP binds the import receptor-cargo complex and dissociates it, releasing the cargo in the nucleus. In the other direction, export receptors form stable complexes with their cargos only in the presence of RanGTP. When the complexes are translocated to the cytoplasm, they are dissociated by the hydrolysis of RanGTP to RanGDP by the GTPase-activating protein (RanGAP).

nucleus by the activity of RanGTP-GDP exchange factor (RanGEF, also called RCC1), which is associated with chromatin (Ohtsubo *et al.*, 1989). In the other direction, export receptors form stable complexes with their cargos only in the presence of RanGTP. When the complexes are translocated in the cytoplasm, they are dissociated by the hydrolysis of RanGTP in RanGDP by the GTPase-activating protein (RanGAP). This cycle would result in an accumulation of Ran in the cytoplasm if it were not of the nuclear transport factor 2 (NTF2) and its ability to bind RanGDP. Its capability to also bind the Nups brings the NTF2-RanGDP back to the nucleus (Ribbeck *et al.*, 1998 Smith *et al.*, 1998) where RanGEF recharges Ran with GTP.

Nuclear import of steroid receptor

After ligand binding, steroid receptors, such as GR (Picard and Yamamoto 1987) and AR (Zhou *et al.* 1994) enter the nucleus. Their sequence contains a basic nuclear localization signal (NLS) considered to be hidden by Hsps when the receptor is not interacting with agonist. That NLS mediates the binding to the proteins of the nucleocytoplasmic trafficking machinery. ER (Picard *et al.* 1990) and PR (Guiochon-Mantel *et al.* 1989) also possess such a basic NLS even though they are constitutively nuclear.

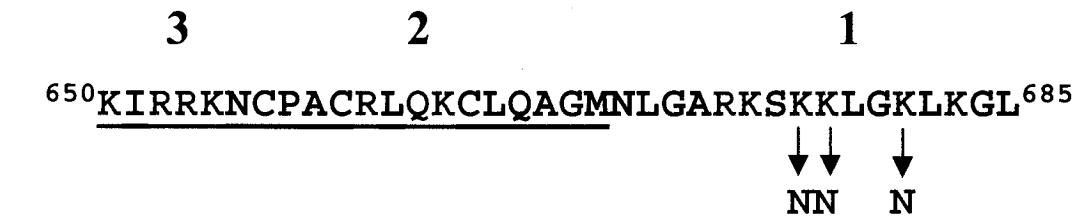
GR has a classical basic NLS (also named NL1) that overlaps with the C-terminal part of the DBD and the beginning of the hinge region (Picard and Yamamoto, 1987). The NLS is composed of three components rich in basic amino acids (i.e. lysine (K) and arginine (R)): the core basic region that is required for NLS function and two smaller clusters of basic amino acids, upstream of the core, which appear to increase the strength of the

NLS without being essential (Ylikomi *et al.*, 1992). The NL1 of naïve GR (GR that has not been exposed to hormone) is constitutively active when separated from the inhibiting function of the hormone-binding domain. In addition to its NL1, rat GR (rGR) (Picard and Yamamoto, 1987), as well as hGR (Cadepond *et al.* 1992), has a second nuclear localization signal named NL2, located in its LBD. This agonist-specific NL2 mediates a weak and incomplete transfer to the nucleus and seems to be using a different pathway than the NL1 (Savory *et al.*1999). This second NLS was thought to be a unique feature of GR, since other steroid receptors lack similar activity in their LBD.

Considering the similarity between MR and GR sequences, our laboratory was first interested in knowing if a similar NL1 located in the hinge region was conserved in MR. Preliminary results obtained in our laboratory identified and knocked-out an analogous NL1 in MR. Sequence alignment permitted the identification of three clusters of basic amino acids which aligned with the three clusters from GR (Fig. 3). Mutations of the three lysines of MR corresponding to the core NLS in GR (lysines at position 677, 678 and 681) (MRNL1-) strongly reduced steroid-dependent translocation to the nucleus (C. Bayer unpublished data). Surprisingly, after steroid treatment, MRNL1- shifted partially to a nuclear distribution, a behaviour similar to that of GRNL1-, shown previously to be due to the NL2 located in the LBD of GR. These results suggested the presence of a second NLS in MR.

The goal of my project was to investigate the possibility of an NL2-like activity in the C-terminal portion of MR. Preliminary localization studies were performed with a construct comprising only the LBD of MR to confirm the presence of an NLS in the

rMR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2
1
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NN
N

rGR

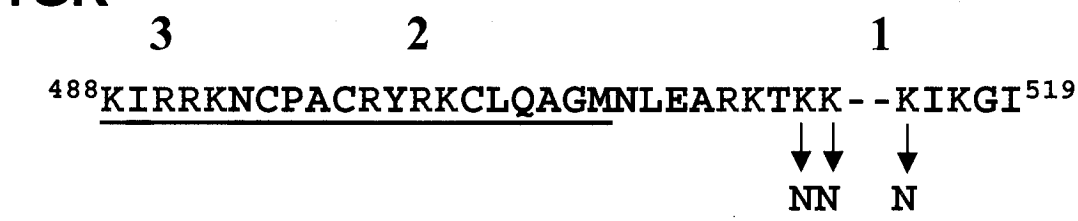

3
2
1
488KIRRKNCPACRYRKCLQAGMNLEARKTKK--KIKGI519
NN
N

Fig. 3
Alignment of the nuclear localization signals in MR and GR.
The basic amino acids of the three clusters are in green and arrows indicate substitutions of lysines to asparagines to create GRNL1- and MRNL1-. The DBD of rMR starts at amino acid 604 and ends at 669 and the DBD of GR is from 440 to 505. The portion of DBD in underlined.

C-terminal portion of the receptor. Further studies were undertaken with a chimera of GRNL1- with its LBD swapped for the LBD of MR. Kinetics studies with agonist (aldosterone) and antagonist (spironolactone) treatment and withdrawal of agonist were completed.

Degradation

Protein degradation is an essential activity of cells to supply amino acids for fresh protein synthesis, to remove excess enzymes and to remove transcription factors that are no longer needed. Eukaryotic cells contain two systems for the degradation of proteins. The first to be discovered and characterized is the lysosomal apparatus, a vacuole containing multiple acid proteases. For a long time, it was thought to be the only protein breakdown system in cells. Studies using inhibitiors of lysosomal acidification (i.e. weak bases) established that this structure only plays a minor role in degradation of cytosolic proteins and is more involved in breakdown of cell-surface membrane proteins or extracellular proteins taken into the cell by endocytosis (Cuervo and Dice, 1998 and Turk *et al.*, 2000).

Endogenous proteins, such as transcription factors (Dennis *et al.* 2001), cyclins (Glotzer *et al.*, 1991), proteins encoded by viruses or misfolded proteins appeared to be primarily degraded through a second system: the proteasome, a controlled pathway that requires ATP (Lam *et al.*, 2002). The proteasome, a 26S complex, is made of a 20S proteolytic component organized as four stacked rings creating a central chamber where the degradation occurs (via chymotrypsin-like, trypsin-like and caspase-like activities (Orlowski and Wilk, 2000) and 19S complexes, located at the extremities of the 20S

unit, containing multiples ATPases and binding sites for ubiquitin chains. The role of the 19S complex is to unfold the proteins and to inject them in the proteasome to be degraded (Lam *et al.*, 2002).

For a protein to be degraded by the proteasome, ubiquitin, a small protein (76 amino acids) practically identical in sequence in all organisms, has to be covalently linked to a lysine in the protein (Pickart, 2001). The protein is conjugated to a chain of ubiquitins in an enzymatic cascade involving an ubiquitin-activating enzyme (E1), an ubiquitin carrier protein (E2) and an ubiquitin-protein ligase (E3). The latter is responsible for the crucial step of the enzymatic cascade: the substrate recognition. Different E3s recognize different degradation signals. The N-terminal residue has been shown to correlate with the life span of a protein (Varshavsky, 1996), suggesting that certain residues accelerate ubiquitination. Other sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST sequences) are recognized by other E_3 enzymes. These PEST signals are generally flanked by clusters of positively charged amino acids (Rogers *et al.* 1986 and Rechsteiner and Rogers, 1996).

Acidic activation domains of transcription factors have been shown to be overlapping with degradation signals for ubiquitin-dependent pathway (Salghetti *et al.*, 2000) and degradation signals from yeast have been shown to activate transcription. Steroid receptor activation domains have not been studied for this characteristic. The degradation of overexpressed GR has been correlated with the presence of a PEST signal slightly before the DBD (amino acids 407 to 426 of the mouse GR (mGR)) (Wallace and Cidlowski, 2001). On proteins destined for proteolysis by the proteasome, ubiquitin is

conjugated to the terminal amino group of a lysine residue. Mutation of the lysine, following the PEST signal, to an alanine, abrogated ligand-dependent down-regulation and induced GR-induced transcriptional activation of gene expression.

In our laboratory, it was observed that, independent of the amount of MR cDNA transiently transfected in Cos7 cells, we could never get the same expression level as for GR on Western blot. The expression level of MR was always much lower. It was hypothesized that the lower level of MR could be due to a more rapid degradation as opposed to transcriptional regulation.

To test this hypothesis, my project was to compare expression levels of MR in the presence or absence of the proteasome inhibitior MG132, Cbz-leu-leu-leucinal. This inhibitor is a member of the peptide aldehyde group, the most widely used category of proteasome inhibitors. MG132 primarily blocks the chymotrypsin-like activity of the proteasome but can also inhibit lysosomal cysteine proteases and calpains, enzymes from other proteolysis pathways. Therefore, it is important to verify that similar biological effects occur with other, more specific, proteasome inhibitors, such as lactacystin (Lee and Goldberg, 1998). Within the context of our studies, only MG132 was used to verify if the degradation of MR could be due, in part, to proteasome-independent proteolysis. Expression levels of the receptor were compared in the presence or absence of MG132. A difference in expression level would implicate the proteasome in the regulation of MR levels.

Another aspect that we investigated was the possible presence of a PEST signal in MR and the role that it could have on degradation of the receptor. Knowing that the presence of a PEST signal in the GR sequence and mutation of the lysine following the signal had consequences on the fate of the protein, we were interested in knowing if the presence of a PEST signal would be responsible for the lower level of expression of MR.

Objectives

In summary, in the first section of my thesis, the objectives were to identify the presence of an NL2 in MR LBD and to characterize the kinetics of nuclear import by this NL2. In the second section, the objectives were to investigate the regulation of MR at the degradation level and the role of a PEST motif in the sequence of MR as a signal for ubiquitination.

Material and Methods

Plasmids

A number of plasmids used in this study have been made previously. The pTL_2BuMR and pTL_2BuMRNL1- constructs used for indirect immunofluorescence contain the wild-type rat MR sequence inserted into the pTL_2 vector (derivative of pSG5 from Stratagene, La Jolla CA, with an expanded multiple cloning site) with a BuGR tag (rat GR epitope for BuGR antibody from amino acid 408 to 422) on the amino (N) terminus of MR (Savory *et al.*, 2001). In the pTL_2, the protein expression was driven under an SV40 promoter. The pTL_2BuMRNL1- construct contains a mutation in the NLS sequence. Specifically, the lysines of the nuclear localization signal (NLS) have been mutated to asparagines (^{677}KKLGK^{681} to ^{677}NNLGN^{681}) to create a NLS mutant of MR (MRNL1-). The pTL_2GR plasmid used to study GR stability contains full-length wild-type rat GR cloned into pTL_2. For transient transfection analysis of reporter gene activation, three previously made constructs were used. p6RGR was a kind gift from K.R. Yamamoto, University of San Francisco. pRSV-βgal contains the β-galactosidase under the control of the Rous sarcoma virus (RSV) promoter. pMMTV-237Luc contains the mouse mammary tumor virus (MMTV) long terminal repeat sequence (-237/+105), followed by the luciferase reporter gene.

The plasmid pEGFPMR_{690C} was constructed by polymerase chain reaction (PCR) amplification (using the PTC-200 Peltier Thermocycler, MJ Research, Reno, CA) of amino acids 690 to 981 of rat MR, using pTL_2BuMR as a template. The primers used

were MR690 forward (see Appendix 1 for primer sequences) and MR981 3' XmaI. All primers were purchased from Invitrogen Corporation (Burlington, ON). The amplification product was gel purified using the Gel Extraction Kit from QiaGEN (Mississauga, ON), digested with KpnI and SmaI and then cloned into the pEGFP-C1 vector (Clontech, Mississauga, ON). All enzymes used for cloning were bought from New England Biolabs (Mississauga, ON).

Plasmids, $pTL_2GGMNL1$- and pTL_2GGM, were constructed using the same 2-step cloning strategy. First, the plasmid pTL_2MR_{698C} was generated. The rat MR ligand-binding domain (LBD) (amino acids 698-981) was amplified using the MR700 SmaI forward primer and the MR BamHI 3' reverse primer. The PCR amplified MR LBD was digested with SmaI and BamHI and cloned in pTL_2. The N-terminal portion of GR (amino acids 22 to 526), including the DNA binding domain and the NL1 sequence was then PCR amplified from either wildtype pTL_2MTG-GR (^{513}KKK^{515}) or pTL_2MTG-GRNL1- (Savory *et al.*, 1999) containing the NL1 mutation (^{513}NNN^{515}). The same set of primers was used for both amplifications: GR22 KpnI forward and GR526 EcoRV reverse. The fragments of GR wild type and mutated NLS were cut with SmaI and KpnI and cloned in pTL_2MR_{698C}. The NL1 mutation was confirmed by DNA sequencing.

The pTL_2GMM was cloned from the T_7T_SGMM. T_7T_SGMM is a chimeric construct containing GR amino acids 1 to 437 and MR amino acids 600 to 981 cloned in the T_7T_S oocyte expression plasmid. T_7T_SGMM was digested with KpnI and BamHI and religated into pTL_2 to produce pTL_2GMM.

To create the plasmid $pTL_2MRK712A$, the lysine at position 712 of wild-type MR was mutated to an alanine, using the *Pfu Turbo*™DNA Polymerase kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions with the primers MRK712A upper and MRK712A lower. The final product was transformed into *Escherichia coli* DH5α by electroporation, and the K712A mutation was confirmed by DNA sequencing.

Cell culture and transient transfections

Cos7 cells (ATCC CRL1651) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Burlington, ON) supplemented with 10% fetal bovine serum (FBS lot#100201, HyClone, Logan, UT) at 37°C with 5% CO_2. Transient transfections of cDNA expressing plasmids were performed using 10µl Lipofectamine™ (Invitrogen) per 60mm dish according to the manufacturer's protocol. Usually, 500ng to 1µg of DNA was transfected. After a 16-hour incubation, transfections were stopped by adding charcoal-stripped fetal bovine serum (SFBS lot#100201, HyClone, Logan, UT) to 10% in phenol-red free DMEM. Cells were washed twice in phosphate-buffered saline (PBS) (137mM NaCl, 27mM KCl, 43mM $Na_2HPO_4 \cdot 7H_2O$ and 18mM KH_2PO_4, pH 7.4), synchronized in G_0 by incubation for 16h in serum-free medium.

Preparation of whole cell extracts

Cells from sixty millimetre plates transfected as described above were washed twice with PBS, harvested using a rubber policeman, then centrifugated at 4000rpm for 5 min and resuspended in WCE buffer (150mM NaCl, 1mM ethylenediamine tetraacetic acid (EDTA, Sigma, Oakville, ON), 50mM Hepes pH 7.4, 10% glycerol, 0.5% Nonidet P40 (NP-40) (EM Science, Cincinnati, OH, USA), 50mM molybdate, 1mM dithiothreitol

(DTT) and 1.25X protease inhibitor cocktail (Roche, Laval, QC)). Cells were left on ice for 10 minutes to allow them to swell and then sonicated for 10s using Branson Sonifier 450 on constant duty cycle (output control: 1). Cellular debris was removed by centrifugation at 13 000 rpm for 5 min at 4°C. Protein concentration of the WCE's was determined using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON).

Western blotting

Protein extract (50μg) was loaded on an 8% denaturing gel and separated by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE). Proteins were transferred to immuno-Blot™ PVDF membranes (Bio-Rad, Mississauga, ON) for 1h at 100V using the BioRad Mini-Protean®II Cell system (Bio-Rad, Mississauga, ON). The membrane was blocked for 1h in 5% skim milk in PBS-T (PBS with 0.1% Tween-20) (EM Science, Cincinnati, OH, USA) at room temperature and incubated with the primary antibody (FiGR 1:400 v/v) in PBS-T overnight at 4°C. Following 16-hour incubation, the membrane was washed 3 times for 5min in PBS-T at room temperature and then incubated with secondary antibody conjugated to horseradish peroxydase (HRP) (Amersham Pharmacia Biotech, Québec, QC) for 1h at room temperature. The blot was then washed 3 times for 5min each in PBS-T before detection of the signal with Western Lightning™ Chemiluminescence Reagent (Enhanced Luminol) (Perkin Elmer life Sciences, Boston, MA, USA).

Antibodies

FiGR antibody was used for detection of protein containing the BuGR epitope (rat GR epitope from amino acid 408 to 422) at a dilution of 1/400 (v/v). The JL-8 antibody (Clontech, Mississauga, ON) was used at a dilution of 1/1000 (v/v) for detection of proteins fused to Enhanced Green Fluorescent Protein (EGFP).

Direct and indirect immunofluorescence

Cos7 cells were transfected with 1μg of the cDNA expression plasmids per 60mm dish as described. Twenty-four hours after starting the transfection, the cells were trypsinized and seeded onto poly-L-lysine coated micro cover glass (VWR, Mississauga, ON) and incubated for 6h to allow the cells to adhere to the coverslip. The cells were then synchronized in G_0. Where indicated, aldosterone, cortisol, spironolactone (Sigma, Oakville, ON) or RU486 (Roussel Uclaf Laboratories, Roumainville, France) were added to a final concentration of 10^{-6}M in serum-free medium. To monitor redistribution of GGMNL1- to the cytoplasm following hormone withdrawal, cells were pretreated with aldosterone (10^{-6}M) for 1h, and withdrawal was initiated by three washes of the cells with PBS and two washes in serum-free medium supplemented with bovine serum albumin (BSA, Sigma, Oakville, ON) to 5%. For direct visualization of the fluorescent proteins (fusion protein containing the Enhanced Green Fluorescent Protein, EGFP), the cells were fixed for 30 min at 4°C with 3% paraformaldehyde pH 7.4 in PBS and washed for 10 min with 0.1M glycine pH 8.0. The glass coverslips were then mounted on a micro slide (VWR, Mississauga, ON) and sealed with nail polish.

For indirect immunofluorescence after hormone induction, the cells were fixed and washed as for direct visualization of fluorescent proteins. The cells were then permeabilized with 0.5% Triton-X in PBS for 30min, blocked with 5% Normal Goat Serum (Gibco BRL, Burlington, ON) in PBS for 1h and the protein of interest was probed for the BuGR tag by incubation with the FiGR antibody (dilution 1:400 v/v) overnight at 4°C. The next morning, the cells were washed 3 times with PBS for 2 min. Cells were then incubated with the secondary antibody, Rhodamine Red™-X-conjugated AffiniPure Donkey Anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) (dilution 1:150 v/v) in PBS for 45 min at room temperature and the cells were washed again 3 times with PBS for 2 min each before the glass coverslips were mounted.

Slides were examined for subcellular localization of the protein of interest on a Nikon TE 300 microscope using filter cubes for rhodamine and FITC. Digital images were captured using a cooled CCD camera (Hamamatsu Orca ER) and Simple PCI imaging software. For each condition or time point, at least 200 stained cells were counted in duplicate. Each experiment was repeated two or three times over a period of several weeks. The localization of the protein of interest in each cell was classified into one of five categories (N, N>C, N=C, C>N and C). Briefly, cells were classified as N when the fluorescent signal was visible only in the nucleus and not detectable in the cytoplasm. For cells characterized as N>C, the signal was predominantly in the nucleus with a slightly detectable signal in the cytoplasm. When the fluorescence was equally

distributed in the nucleus and in the cytoplasm, the cells were classified as N=C, while cells with a signal predominantly in the cytoplasm were characterized as C>N. Finally, cells with no fluorescence in the nucleus were scored as C. The categories N and N>C were fused and also C and C>N were fused so that the graphs were not saturated. Three categories of classification were determined: N + N>C, N=C and C + C>N. Counting was performed by double-blind encryption. On graphs, the error bars indicate the standard errors of the means. The validity of this scoring system for monitoring the nucleocytoplasmic trafficking of steroid receptors has been previously demonstrated by Ylikomi *et al.* (1992) and Sackey *et al.* (1996).

Transient transfection analysis of reporter gene activation

i) Cytoplasmic extract preparation

Cos7 cells were transfected with 50ng of the receptor of interest, 100ng RSV-βgal and 100ng MMTV-237Luc per 60mm dish, as described above. After 24h, transcription was induced with either 10^{-6}M aldosterone or cortisol as appropriate. Cytoplasmic extracts were prepared using 400µl of Reporter Lysis Buffer (Promega, Madison, WI, USA) per 60mm dish according to the manufacturer's instructions.

ii) Analysis of gene expression by transient transfection

Cytoplasmic extract (20µl) of Cos7 cells transiently transfected with MMTV-237Luc reporter construct, RSV-βgal construct and the vector containing the protein of interest was used to measure the relative luminescence unit (RLU) by the Analytical Luminescence Laboratory Monolight 2010, using Luciferase assay substrate (Promega,

Madison, WI, USA). For data analysis, the RLU were normalized for transfection efficiency by the β-galactosidase activity. To measure the activity of β-galactosidase, 100µl cytoplasmic extract was used. Z buffer (100µl) (60mM Na_2HPO_4, 40mM $NaH_2PO_4 \cdot H_2O$, 10mMKCl, 1mM $Mg_2SO_4 \cdot 7H_2O$, 50mM 2-β-mercaptoethanol, pH 7.0) was added to the cytoplasmic extract. To initiate the enzymatic reaction, 40µl of O-Nirophenyl β-d-galactopyranoside (ONPG, Sigma, Oakville, ON) (4mg/ml in 100mM phosphate buffer (0.1M KH_2PO_4 pH 7.2, 0.15mM NaCl)) was added and the tubes were incubated at 30°C. Following the appearance of yellow colour, 100µl of sodium bicarbonate (Na_2CO_3) was added to stop the reaction. The activity of samples was measured by determination of absorption at OD_{420} (A_{420}) and the following formula was used to determine β-galactosidase activity in units/ml: A_{420}/(0.0045 X reaction time (min) X volume of extract (ml)).

Stability assay

Cos7 cells were transfected with 500ng of the cDNA expression plasmid of the receptor of interest per 60mm dish, as described above. Cells were treated with 1µM steroid (cortisol or aldosterone) and/or MG132 (10^{-6}M) (N-CBZ-Leu-Leu-Leu-AL, Sigma, Oakville, ON) for 20-24h as indicated. Proteins were then harvested by WCE and analyzed by Western blotting as described.

Results

Part I: Potential NL2 in MR LBD

Sequence alignment of GR and MR allowed the identification of three clusters of basic amino acids in the hinge region of MR, which aligned with the three clusters of the hinge region of GR. Preliminary results in our laboratory had shown that mutations of the three lysines of MR, at position 677, 678 and 681, to asparagines (MRNL1-) strongly reduced steroid-dependent translocation to the nucleus, but had only a small effect on the distribution of the naïve receptor, which is equally distributed in the nucleus and the cytoplasm (Claudia Bayer, unpublished data). After steroid treatment, MRNL1- shifted from the cytoplasm to a partially nuclear distribution, a behaviour similar to that of GRNL1-, which had been demonstrated to be due to the NL2 located in the LBD of GR (Savory *et al.*, 1999).

To determine whether MR also contains an NL2-like activity, I employed two approaches: indirect immunofluorescence and direct visualization of fluorescent protein. These techniques were employed depending on whether EGFP was present on the MR construct or not. Immunofluorescence had previously been shown to be a reliable and powerful means to examine nucleocytoplasmic trafficking of nuclear receptors (Ylikomi *et al.*, 1992; Sackey *et al.*, 1996).

To express MR, Cos 7 cells were transiently transfected with mammalian expression plasmids, followed by culture in serum-free media to synchronize cells in G_0. Cells were forced to exit the cell cycle by serum withdrawal in order to have cells in which there

was minimal new synthesis or degradation of MR. We wished to study cells in G_0 because the majority of cells responsive to corticoids are primarily in G_0 and G_1 phase of the cell cycle, with the important exception of the immune system. It has also been shown that subcellular localization of receptors is affected by the stage of the cell cycle (Hsu *et al.*, 1992). After synchronization to G_0/G_1, cells were hormone-treated and localization of the receptor was visualized by direct fluorescence (if the receptor was fused to the fluorescent protein EGFP) or by indirect immunofluorescence of the receptor protein by using a fluorescent-conjugated antibody, if the receptor had not been fused to a fluorescent protein. Localization of receptors in cells was scored into 3 categories (N +N>C, N=C and C + C>N) (Fig. 4).

MR LBD moves to the nucleus in an agonist-dependent manner

To investigate whether the C-terminal portion of MR possessed an NLS, preliminary localization studies were done to compare the localization of MR, MRNL1- and of a construct comprising the LBD of MR (from amino acids 690 to 981) cloned in frame with the Enhanced Green Fluorescent Protein (EGFP). When transiently transfected into Cos7 cells, it had been shown previously by other members of the laboratory (C. Bayer, unpublished data) that mutation of the NL1 of MR did not appear to affect the expression or stability of MRNL1- compared to wild-type MR, since they are expressed at similar levels (Fig. 5A). The fusion protein $EGFPMR_{690C}$ was also appropriately expressed (Fig. 5B), as the molecular weight corresponded approximately to the theoretical molecular weight of 61kDa.

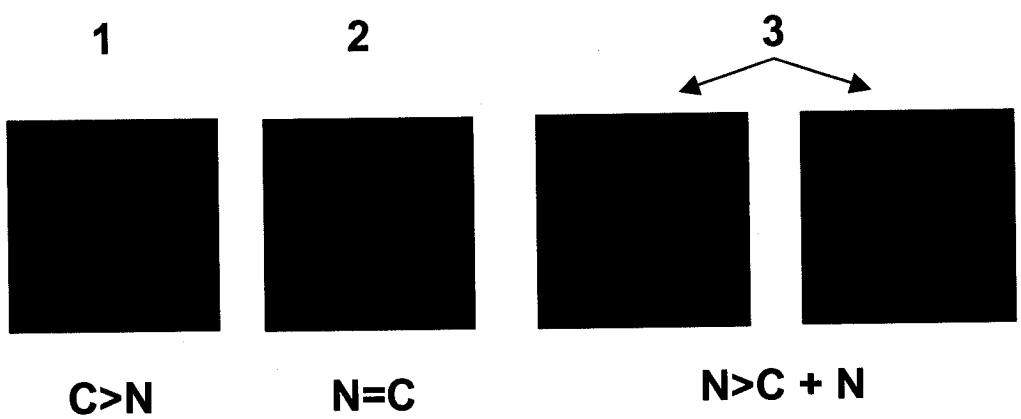
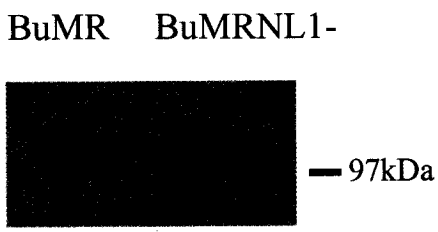

1
2
3
C>N
N=C
N>C + N

Fig. 4
Classification of subcellular localization of MR constructs.
Cells with the fluorescent signal predominantly in the cytoplasm were characterized as having a protein localization of C + C>N (1). When the fluorescent signal was evenly distributed throughout the cell, they were classified as N=C (2). For cells characterized as N + N>C (3), immunofluorescence was predominantly in the nucleus with a slightly detectable signal in the cytoplasm or completely in the nucleus.

A)



B)

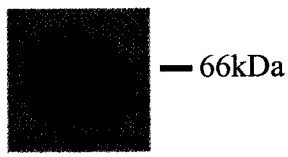


Fig. 5
Expression levels of BuMR, BuMRNL1- and $EGFPMR_{690C}$.
A) Cos7 cells were transiently transfected with 500ng of pTL_2BuMR or $pTL_2BuMRNL1$- B) Cos7 cells were transiently transfected with 2.5μg of $pEGFPMR_{690C}$. Whole cell extract (100 μg) was run on a denaturing SDS-PAGE gel and transferred onto a PVDF membrane and probed with FiGR antibody (1:400) to detect BuMR and BuMRNL1- and with mouse α-GFP (1:1000) to detect $EGFPMR_{690C}$.

To determine if the LBD of MR had a second nuclear localization signal located in its LBD, we compared the subcellular localization of $EGFPMR_{690C}$ to the subcellular localization of MR and MRNL1- after treatment with hormone. As published previously, naïve MR is equally distributed between the nucleus and the cytoplasm (Nishi *et al.*, 2001, Fejes-Thot *et al.*, 1998 and Odermatt *et al.*, 2001). Upon agonist binding, the receptor translocates virtually completely to the nucleus (Nishi *et al.*, 2001, Fejes-Thot *et al.*, 1998 and Odermatt *et al.*, 2001). Mutation of the NL1 of MR reduced agonist-dependent translocation to the nucleus (Fig. 6), such that only 74% of the cells had a mostly nuclear fluorescent signal, as opposed to 97% with wild type MR. The LBD of MR prior to hormone treatment was distributed between the nucleus and the cytoplasm (about 80%) identical to the full-length wild type MR. However, in the other 20% of cells the LBD was mainly cytoplasmic in localization instead of mainly nuclear as had been the case with wild type MR. After aldosterone treatment for 1h, $EGFPMR_{690C}$ localized in the nucleus in 30% of the cells. Antagonist (spironolactone) treatment caused translocation to the nucleus of MR wild type to almost the same extent as agonist, but did not cause the translocation of MRNL1- or $EFGPMR_{690C}$. These results suggest that the LBD of MR contains a sequence that triggers nuclear translocation. However, the MR NL2 is less efficient than the NL1, and unlike NL1 appears to be agonist-specific.

Addition of EGFP to the MR LBD increased the size of the protein to slightly over 61kDa, but a Western blot showed that the fusion protein was smaller than 60kDa. The original N=C distribution of the $EGFPMR_{690C}$ was of some concern as the size of the protein was close to the cut-off for passive diffusion. To be certain that the size of the

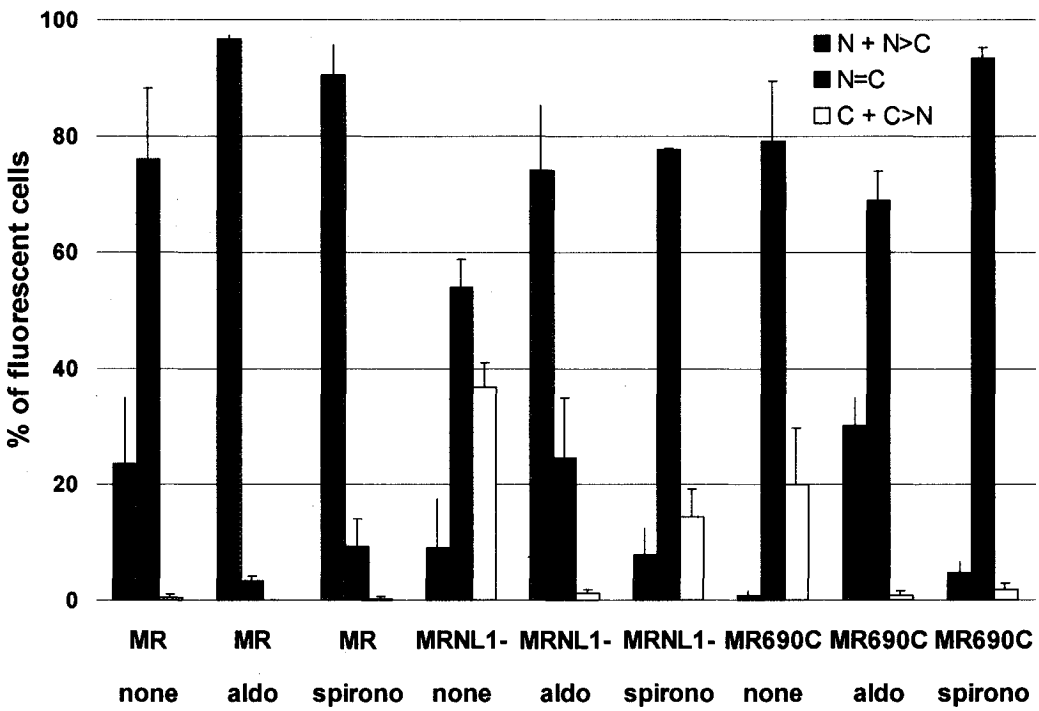

100
80
60
40
20
0
% of fluorescent cells
N + N>C
N=C
C + C>N
MR none
MR aldo
MR spirono
MRNL1- none
MRNL1- aldo
MRNL1- spirono
MR690C none
MR690C aldo
MR690C spirono

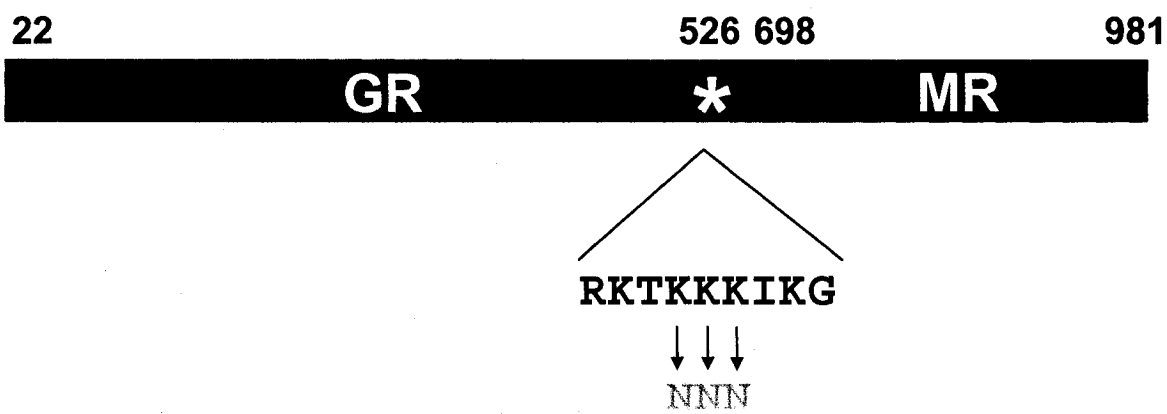
Fig. 6
Immunofluorescence analysis of subcellular localization of BuMR, BuMRNL1- and $EGFPMR_{690C}$.
Cos7 cells prepared for immunofluorescence either before treatment or following treatment with aldosterone (1μM) or spironolactone (1μM) for 1h. Localization of the MR constructs was visualized as follows: $EGFPMR_{690C}$ by direct fluorescence after fixation and BuMR and BuMRNL1- by indirect immunofluorescence using FiGR antibody (1:400) followed by a Rhodamine Red™-X-conjugated AffiniPure Donkey Anti-mouse secondary antibody (1:150). Over 200 cells were counted in duplicate for each condition from three different experiments. The localization of each cell was categorized as completely or mostly nuclear (blue bars), equally distributed (red bars) or localized predominantly or exclusively in the cytoplasm (yellow bars). The error bars indicate the standard errors of the means of three experiments.

protein would not allow passive diffusion into the nucleus, I created a chimeric construct, called GGMNL1-, containing the N-terminal portion of GR with its DBD (amino acids 22 to 526) including the mutated NL1 (^{513}KKK^{515} to ^{513}NNN^{515}) fused to the LBD of MR (amino acids 698 to 981) (Fig. 7A). With this construct, the size of the chimeric protein is similar to GR full-length (i.e. theoretical molecular weight of 90kDa). As mentioned earlier, replacement of the three lysines of the core NLS of GR abrogates the nuclear localization of GR after hormone treatment. A difference in nuclear localization of GGMNL1- compared to GRNL1- should be attributable to the LBD of MR. The chimeric protein GGMNL1- was properly expressed (Fig. 7B), as the molecular weight corresponded approximately to the theoretical molecular weight of 90kDa.

GGMNL1- is transcriptionally active

Before doing any subcellular localization studies of GGMNL1-, I verified its ability to function as a transcription regulator. Therefore, I measured the transcriptional activation of the reporter gene MMTV-237Luc by the chimeric GGMNL1-. The mouse mammary tumor virus long terminal repeat sequence contains a GRE that is responsive to both MR and GR. As controls, GR and GRNL1- were included in the experiment to determine the effect of the disruption of the nuclear localization signal on transcription, and MR, to have a control that activates transcription upon aldosterone binding. It has been published that restricting nuclear occupancy of GR is an effective way to control the level of its transcriptional activation (Savory *et al.*, 1999). In that study, expression of a cotransfected chloramphenicol acetyltransferase reporter gene whose transcription was dependent on the promoter-proximal steroid-regulatory region of MMTV was three-

A)



B)

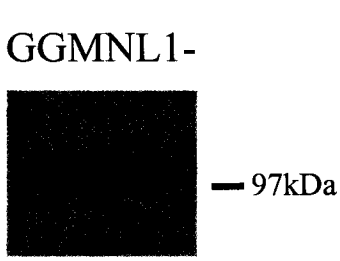


Fig. 7
The chimeric protein GGMNL1-.
A) Schematic representation of the chimeric protein GGMNL1-.
The MR LBD (amino acid 698 to 981) was cloned in frame to the amino-terminal portion of GR with the DBD and part of the hinge domain (amino acid 22 to 526). The NLS of GR (^{513}KKK^{515}) had been mutated to ^{513}NNN^{515}.
B) Western blot indicating the expression level of GGMNL1-. Cos7 cells were transfected with 500ng of pTL_2GGMNL1- using Lipofectamine. Whole cell extract (50 μg) was run on a denaturing SDS-PAGE gel and transferred onto a PVDF membrane and probed with FiGR antibody (1:400).

to fourfold lower when GRNL1-, as opposed to wild-type GR, was transfected with the reporter gene. I was able to reproduce these results using a luciferase reporter gene (Fig. 8). When GGMNL1- was transiently cotransfected with the luciferase reporter gene, the activation of transcription was also three- to four-fold lower than activation of transcription by the wild type GGM, which NLS was not mutated. The decrease in activation of transcription by GGMNL1- can be explained by the reduction in nuclear occupancy compared to GGM. The stronger activation of transcription by wild type GGM may be due to synergy between the activation function AF-1 of GR and the activation function AF-2 from MR. These results suggest that GGMNL1- activates transcription, even if its nuclear localization is restricted by mutation of the NLS.

Spironolactone is an MR antagonist

Spironolactone has been used during the last four decades in the treatment of the sodium-retaining state and as an antihypertensive agent (Corvol *et al.*, 1981). Certain antagonists bind to the receptor with an affinity identical to that of aldosterone and potentially induce a receptor conformation that is transcriptionally silent (Lombes *et al.*, 1993 and Couette *et al.*, 1996). To verify that spironolactone could displace aldosterone and block transcription, we included spironolactone treatment in the last experiment (Fig. 8). As a control, the antagonist RU486 was used with GR and GRNL1-. The results showed clearly that spironolactone treatment of wild type MR, MRNL1- and GGMNL1- does not result in activation of transcription of the reporter gene MMTVLuc and that ten times higher concentration of spironolactone can displace aldosterone and inhibit transcriptional activation.

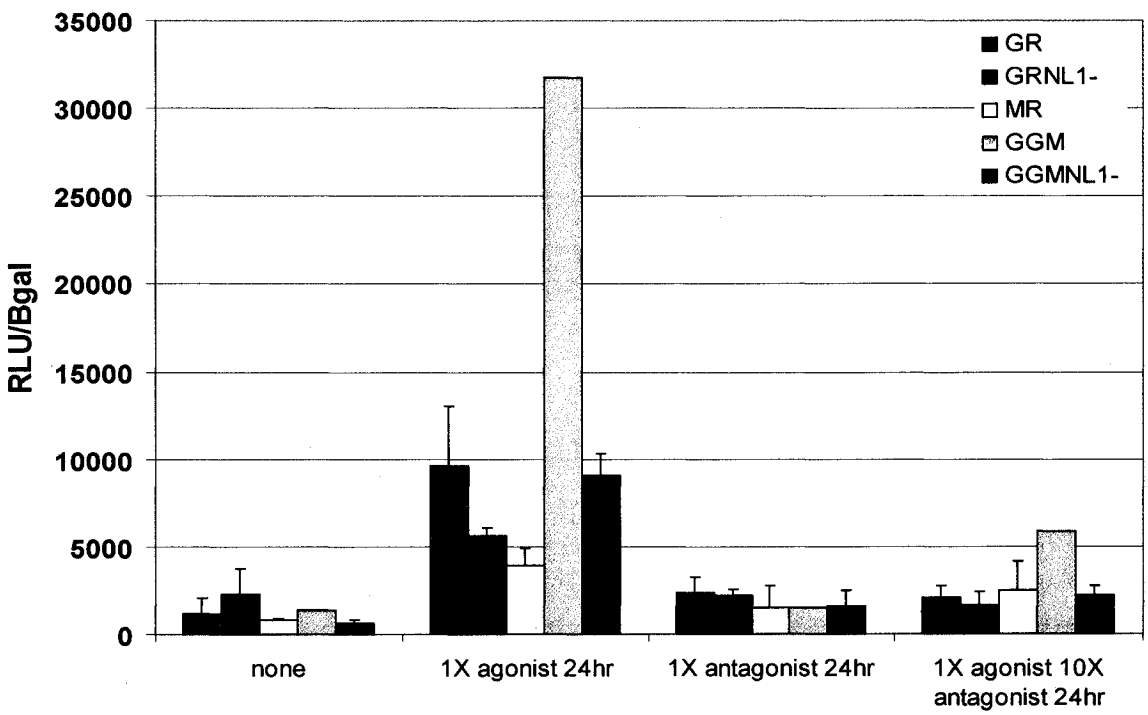

35000
30000
25000
20000
15000
10000
5000
0
RLU/Bgal
GR
GRNL1-
MR
GGM
GGMNL1-
none
1X agonist 24hr
1X antagonist 24hr
1X agonist 10X antagonist 24hr

Fig. 8
Transcriptional activation of the reporter gene MMTV-237Luc by GR, GRNL1-, MR, GGM and GMMNL1- is agonist-dependent.
Cos7 cells were transiently transfected with 50ng of receptor (GR, darker blue, GRNL1-, red, MR, yellow, GGM, lighter blue or GGMNL1-, green), 100ng of RSV-βgal and 100ng of MMTV-237Luc per 60mm dish. Twenty-four hours following transfection, transcription was induced with 10^{-6}M agonist (cortisol for GR and GRNL1- or aldosterone for MR, GGM and GGMNL1-), antagonist (RU486 for GR and GRNL1- or spironolactone for MR, GGM and GGMNL1-) or with 10^{-6}M agonist in combination with 10^{-5}M of the appropriate antagonist. Cytoplasmic extract was prepared using 400µl of Reporter Lysis Buffer (Promega, Madison, WI, USA) per 60mm dish and luciferase activity of 20µl of cytoplasmic extract was measure by the Analytical Luminescence Laboratory Monolight 2010. For data analysis, the RLU were normalized for transfections efficiency by the β-galactosidase activity.

MR LBD has an agonist-dependent NL2 function

To study the kinetics of nuclear import of GGMNL1-, a time course analysis of subcellular localization after aldosterone treatment was done. GGMNL1- was predominantly evenly distributed between the nucleus and the cytoplasm (1.3% ± 0.6% N + N>C, 61.1% ± 6.8% N=C, 37.6% ± 7.2% C + C>N) prior to aldosterone treatment (Fig. 9). Upon aldosterone treatment, there was a slow (in comparison to wild type GR, which is completely nuclear after 10 minutes) and incomplete transfer of GGMNL1- to the nucleus (~40% N + N>C). In comparison to GRNL1- though, the transfer is much faster since GRNL1- took 6 hours to equilibrate in the nucleus (Savory *et al.*, 1999). Nuclear transfer was noticeable after 10 minutes and equilibrium had been attained in one hour. However, the localization remained predominantly N=C. Treatment of GGMNL1- with the antagonist spironolactone did not significantly trigger nuclear localization of the receptor (Fig. 10). Even after six hours, only ~15% of C + C>N moved to N=C. Together these results suggest the presence of an agonist-dependent NL2 in the LBD of MR.

GGMNL1- does not redistribute to the cytoplasm after hormone withdrawal

It has been shown that GRNL1- redistributes rapidly to the cytoplasm following hormone withdrawal (Savory *et al.*, 1999). Savory *et al.* showed that GRNL1- completely returns to the cytoplasm after only 2 hours, compared to GR, which remains mostly nuclear (80% of the cells) even 24 hours after withdrawal of cortisol. The conclusion of those previous experiments was that the extended maintenance of GR in the nucleus subsequent to hormone withdrawal required an intact NL1. As opposed to GRNL1-, GGMNL1- remained in a more nuclear localization up to 6h after removal of

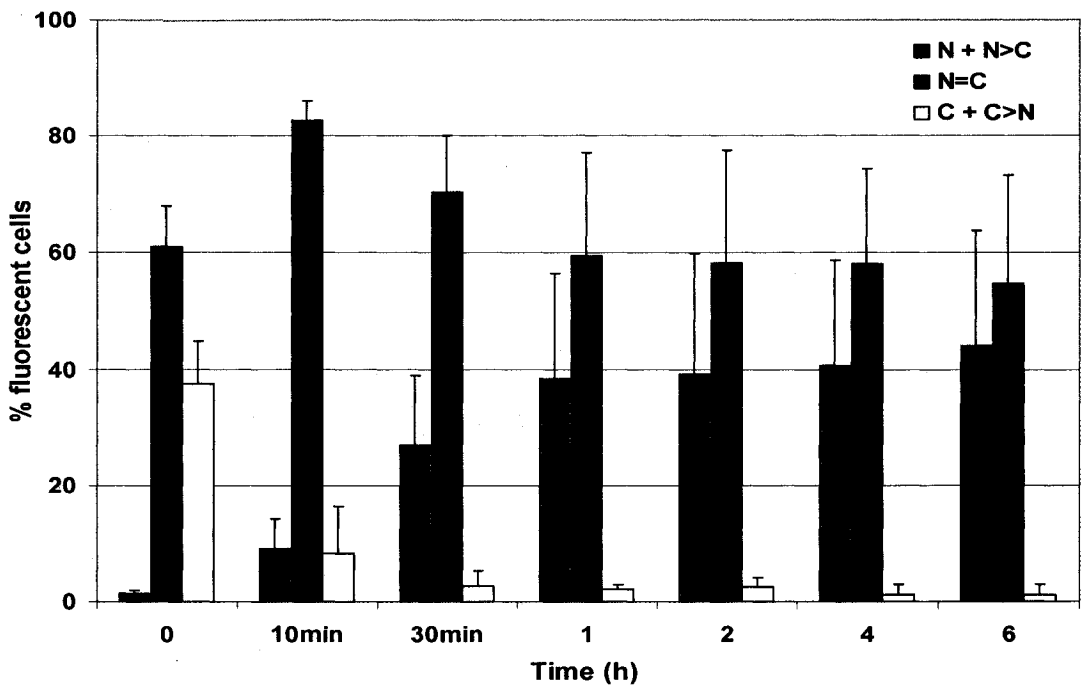

100
80
60
40
20
0
% fluorescent cells
N + N>C
N=C
C + C>N
0
10min
30min
1
2
4
6
Time (h)

Fig. 9
MR LBD has an NL2 function.
Cos7 cells prepared for immunofluorescence were treated with 1μM aldosterone for various lengths of time. Localization of GGMNL1- was visualized using FiGR antibody (1:400) followed by a Rhodamine Red™-X-conjugated AffiniPure Donkey Anti-mouse secondary antibody (1:150 v/v). Over 200 cells were counted for each condition, in duplicate from three different experiments. The localization of each cell was categorized as completely or mostly nuclear (blue bars), equally distributed (red bars) or localized predominantly or exclusively in the cytoplasm (yellow bars). The error bars indicate the standard errors of the means.

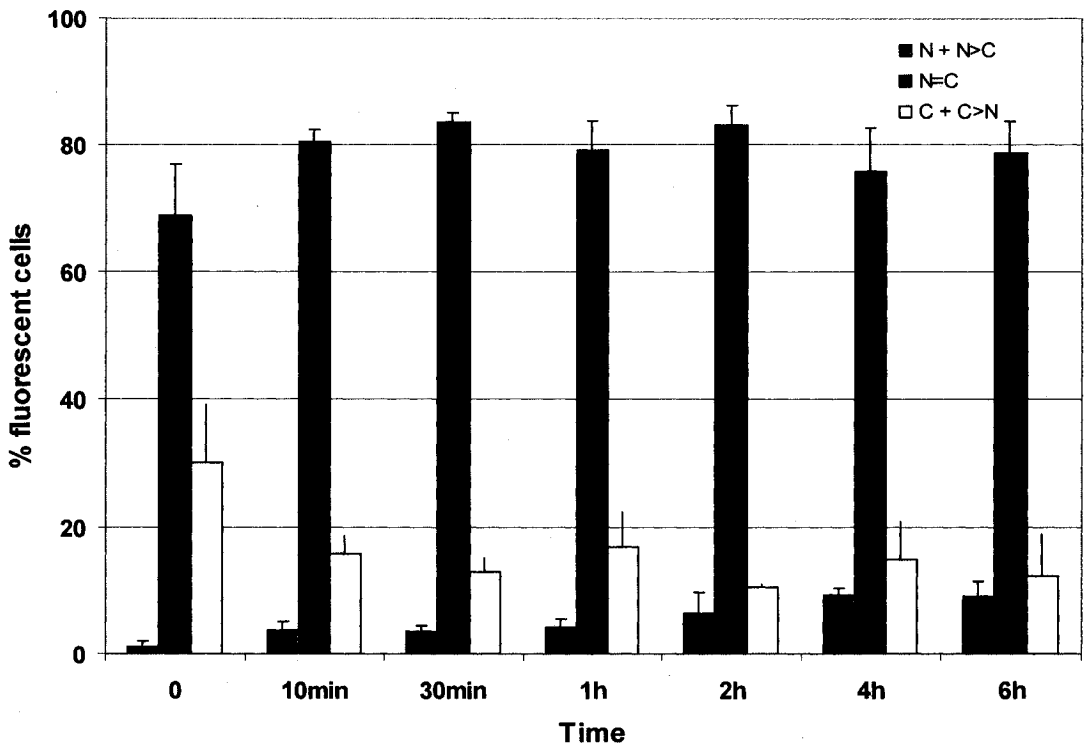

100
80
60
40
20
0
% fluorescent cells
N + N>C
N=C
C + C>N
0
10min
30min
1h
2h
4h
6h
Time

Fig. 10
The MR NL2 is agonist-dependent.
Cos7 cells prepared for immunofluorescence were treated with 1μM spironolactone for increasing lengths of time. Localization of GGMNL1- was visualized using FiGR antibody (1:400) followed by a Rhodamine Red™-X-conjugated AffiniPure Donkey Anti-mouse secondary antibody (1:150 v/v). Over 200 cells were counted for each condition, in duplicate from three different experiments. The localization of each cell was categorized as completely or mostly nuclear (blue bars), equally distributed (red bars) or localized predominantly or exclusively in the cytoplasm (yellow bars). The error bars indicate the standard errors of the means.

aldosterone from the culture media (Fig. 11). Over time, the % of cells with GGMNL1- located mainly in the nucleus increased slightly (~10% more cells as N + N>C at 6 hours after withdrawal compared to after a 1-hour treatment with aldosterone only) consistent with data from our laboratory concerning the localization of MRNL1- after hormone withdrawal. Upon hormone withdrawal, MRNL1- translocated continuously to a more nuclear localization even 24 hours after removal of the hormone (data not shown). These results suggest that the MR LBD contains a sequence that prolongs nuclear subcellular localization of GGMNL1- in contrast to the LBD of GR.

Part II: Stability of MR

MR constructs are less stable than wild-type GR

In our laboratory, it had been observed that, independent of the amount of MR cDNA transiently transfected in Cos7 cells, the expression level of MR was always markedly lower than for GR. It was hypothesised that the lower level of MR could be due to a more rapid degradation of MR than GR. To explore this hypothesis, we determined the expression levels of GR and MR in the absence or presence of the proteasome inhibitor (MG132) before and after treatment with ligand.

In our experiments, for each construct, an equal amount of DNA was transiently transfected into Cos7 cells. The quality of the DNA was verified before transfection, to be certain that the effect on the expression level was not due to differences in quality of the DNA transfected. All DNAs had the same ratio of supercoiled to nicked DNA (Fig. 12).

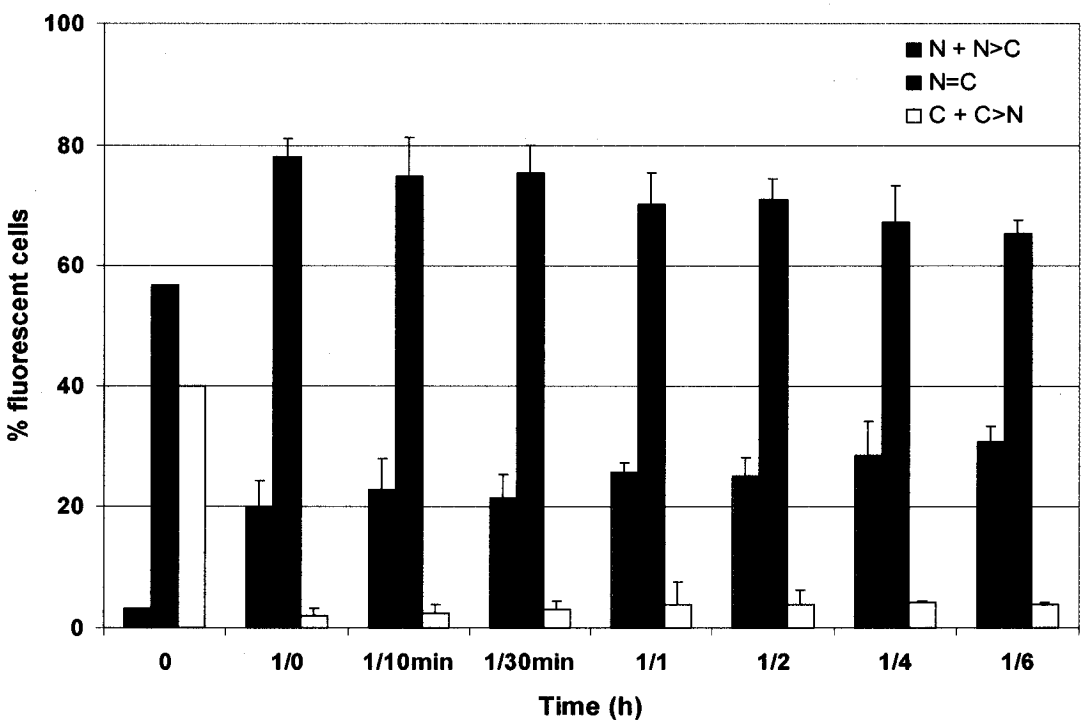

100
80
60
40
20
0
% fluorescent cells
N + N>C
N=C
C + C>N
0
1/0
1/10min
1/30min
1/1
1/2
1/4
1/6
Time (h)

Fig. 11
GGMNL1- does not redistribute to the cytoplasm after hormone withdrawal. Cos7 cells prepared for immunofluorescence were pretreated with 1μM aldosterone for 1h. Hormone withdrawal was initiated by three washes with PBS and two washes followed by incubation in serum-free medium supplemented with bovine serum albumin to 5%. Specific localization was visualized using FiGR antibody (1:400 v/v) followed by a Rhodamine Red™-X-conjugated AffiniPure Donkey Anti-mouse secondary antibody (1:150 v/v). Over 200 cells were counted for each condition, in duplicate from three different experiments. The localization of each cell was categorized as completely or mostly nuclear (blue bars), equally distributed (red bars) or localized predominantly or exclusively in the cytoplasm (yellow bars). The error bars indicate the standard errors of the means.1/2 represents 1hour treatment with aldosterone/ 2hours withdrawal of aldosterone.

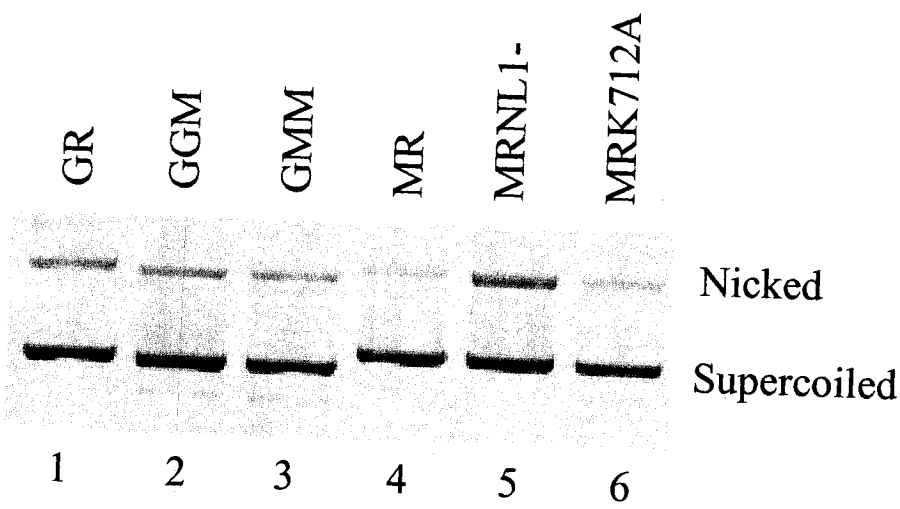

GR
GGM
GMM
MR
MRNL1-
MRK712A
Nicked
Supercoiled
1
2
3
4
5
6

Fig. 12
DNA quality for transient transfection
Ethidium bromide stained 0.8% agarose gel loaded with 100ng of uncut DNA. In lane 1: pTL_2GR, lane 2, pTL_2GGM, lane 3, pTL_2GMM, lane 4, pTL_2MR, lane 5, pTL_2MRNL1- and lane 6, $pTL_2MRK712A$.

It has been shown that mouse GR undergoes ligand-dependent down-regulation via the ubiquitin-proteasome pathway in Cos1 cells (Wallace and Cidlowski, 2001). In my experiments using rat GR in Cos7 cells, we reproduced the cortisol-dependent down-regulation of the receptor. However, the proteasome inhibitor MG132 could not block the ligand-dependent degradation as had been reported previously (Wallace and Cidlowski, 2001) (Fig. 13).

The expression of MR was markedly lower than GR, even though the expression was driven by the same SV40 promoter, which is not regulatable by steroids. That expression system is different than the endogenous GR gene that is repressed by Dex. Upon aldosterone treatment, MR levels decreased. Furthermore, a higher molecular weight form appeared with nearly complete loss of the wild type form (see Fig. 15 for darker exposure). Addition of the proteasome inhibitor induced the accumulation of MR to higher level although still not to the level of GR. In the presence of aldosterone and MG132, a small increase in the level of expression compared to that observed in the absence of MG132 was visible, with again, the appearance of two forms of the receptor.

Mutation of the NL1 of MR changed the pattern of expression of the receptor. There was no change in the expression level of MRNL1- compared to MR but the expression level of MRNL1-, when treated with proteasome inhibitor, was lower and there was no appearance of the higher molecular form of the receptor in presence of ligand.

These results suggest that down-regulation of MR is ligand-dependent. Furthermore, as no higher molecular weight form was observed after induction of GR with cortisol, and

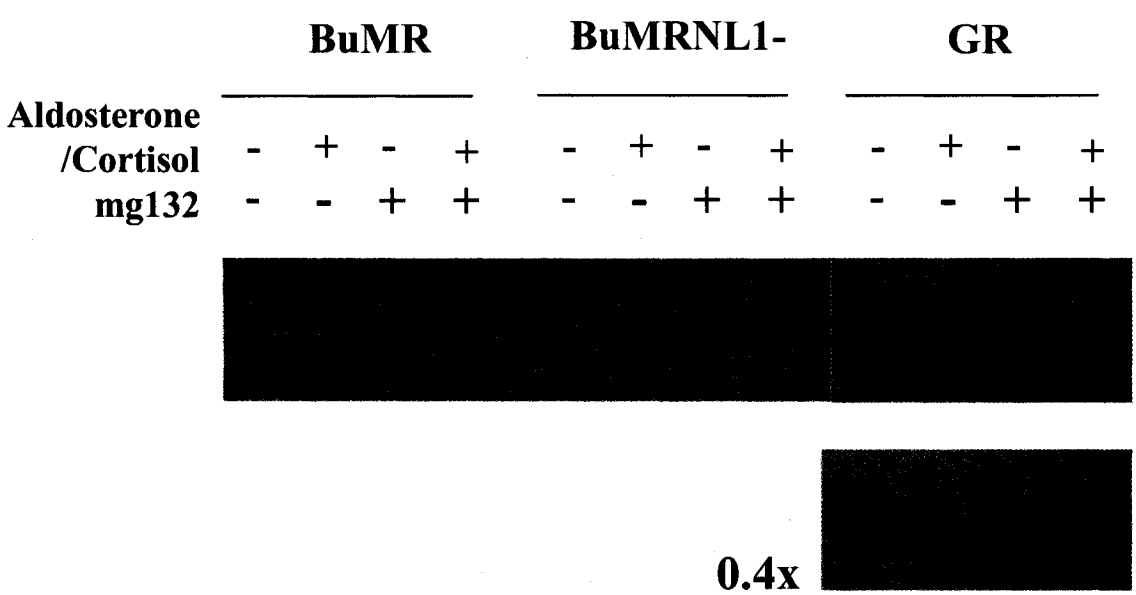
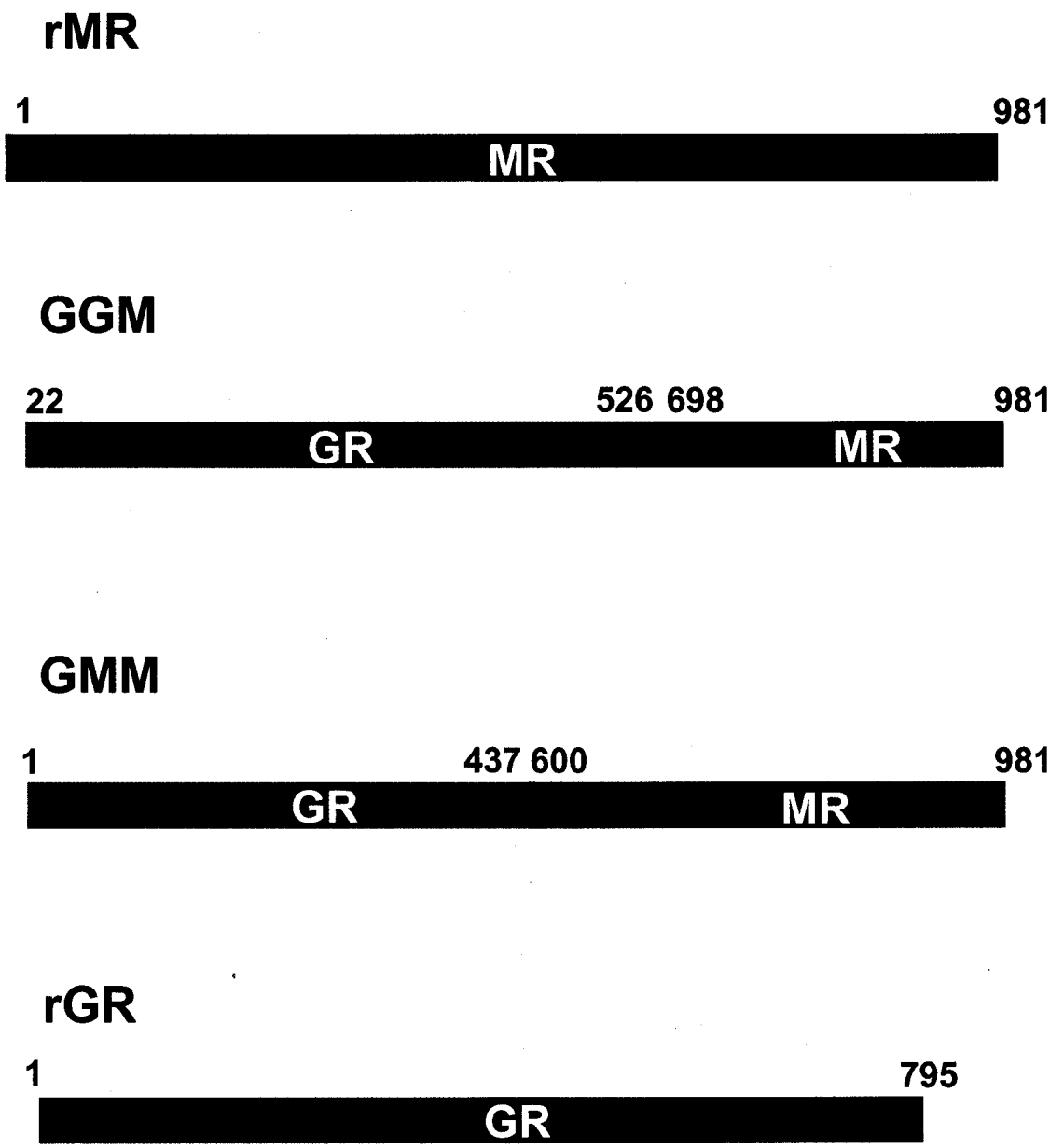

BuMR
BuMRNL1-
GR
Aldosterone
/Cortisol
mg132
- + - + - + - + - + - +
- - + + - - + + - - + +
0.4x

Fig. 13
MR constructs are less stable than wild-type GR.
Cos7 cells were transiently transfected with 500ng of pTL_2BuMR, $pTL_2BuMRNL1$- or pTL_2GR using Lipofectamine. 50 μg of whole cell extract was run on a denaturing SDS-PAGE gel and transferred onto a PVDF membrane and probed with FiGR antibody (1:400). 0.4X represents the fraction of time exposure (40%) compared to the exposure above.

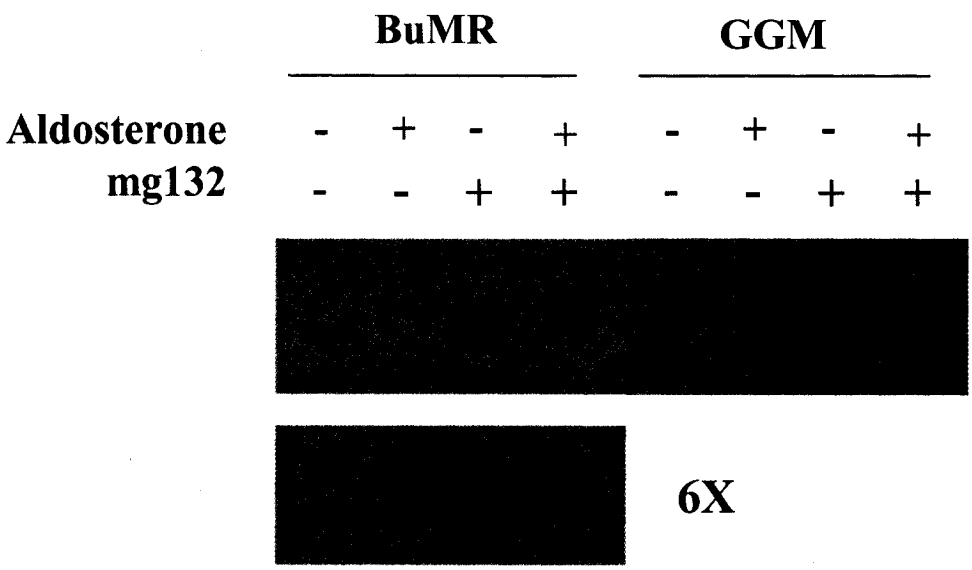

rMR
1
981
MR
GGM
22
526 698
981
GR
MR
GMM
1
437 600
981
GR
MR
rGR
1
795
GR

that MG132 did not stabilize GR, it suggests that MRNL1- is regulated in a different manner than GR. These results also suggest that the presence of the wild type MR NL1 is necessary for the receptor to undergo the degradation that results in a different molecular form of MR.

The N-terminal portion of MR is responsible for the higher molecular form of MR and the decrease in stability of the receptor

To define the region of MR responsible for the aldosterone-dependent change in molecular weight of MR, other chimeric receptors were made, by swapping different domains of MR for GR. GGM was constructed by the fusion of the N-terminal domain and DBD of GR to the LBD of MR. GMM contains the N-terminal of GR fused to the DBD and LBD of MR (Fig. 14). The two chimeras, GGM and GMM, had similar stability patterns as full-length GR (Fig. 15). Ligand-dependent degradation was detectable upon treatment with agonist but the proteasome inhibitor produced a slight accumulation of receptor. Neither the presence of the LBD of MR alone nor the presence of the DBD with the LBD of MR provoked the shift in molecular weight of the receptor. The only construct that produced the higher molecular form of the receptor in the presence of aldosterone is the full-length MR. The presence of a lower molecular weight form with GGM and GMM has always been seen in all the conditions and is probably a degradation form of the receptor due to our method of protein extraction. From these results, we can deduce that the N-terminal portion of MR is responsible for the higher molecular form of the receptor in presence of aldosterone.

Fig. 14
Schematic representation of MR, GGM, GMM and GR.
GGM contains the N-terminal domain and DBD of GR to the LBD of MR.
GMM contains the N-terminal of GR, and the DBD and LBD of MR


BuMR
GGM
Aldosterone
mg132
- + - + - + - +
- - + + - - + +
6X

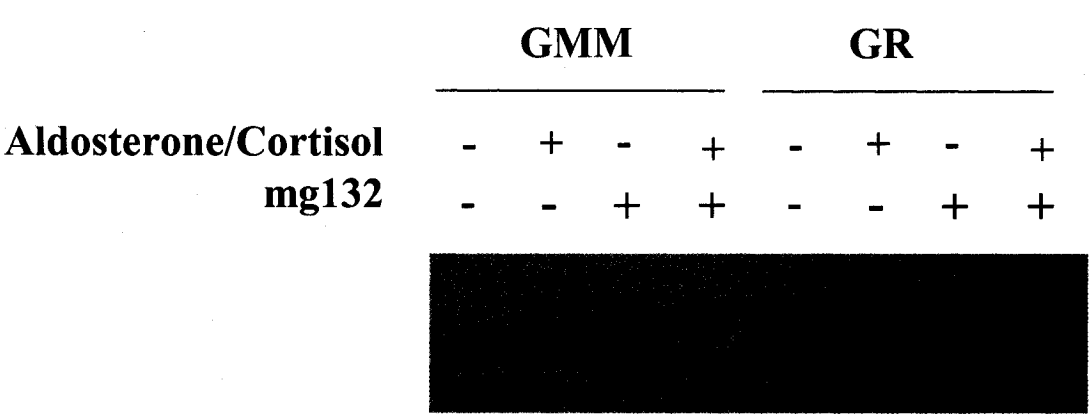

GMM
GR
Aldosterone/Cortisol
mg132
- + - + - + - +
- - + + - - + +

Fig. 15
The N-terminal portion of MR seems to be responsible for the higher molecular form of MR and the decrease in stability of the transiently transfected protein.
Cos7 cells were transfected with 500ng of pTL_2BuMR, pTL_2GGM, pTL_2GMM or pTL_2GR using Lipofectamine. 50 μg of whole cell extract was run on a denaturing SDS-PAGE gel and transferred onto a PVDF membrane and probed with FiGR antibody (1:400). 6X represents how many times longer was the exposure time compared to the exposure above.

Identification of a PEST signal in MR

The PESTfind program allowed identification of one potential PEST motif from amino acids 686 to 711 in rMR, as opposed to two signals in GR, both located in the N-terminal of the protein (Fig. 16). According to the program, this signal has a score of +15.41. On a scale from –50 to +50, a value above +5 has to be taken into consideration as a potential PEST signal (Rechsteiner and Rogers, 1996). Wallace and Cidlowski (2001) have showed that mutagenesis of the lysine residue following the PEST signal from amino acids 407 to 426 in mouse GR, abrogated the ligand-dependent down-regulation of the receptor suggesting that this lysine serves as an acceptor ubiquitin site that plays a important role in GR degradation through the proteasome-dependent pathway. Since MR had a lysine directly after its PEST signal also, at position 712, we considered this residue as a potential site for anchoring ubiquitin. The lysine was mutated to an alanine to observe the consequences on degradation of the receptor.

It has been published that mutagenesis of the lysine following the PEST signal in GR inhibits its ligand-dependent down-regulation and that transcriptional activation by the lysine mutant was enhanced in the presence of MG132 (Wallace and Cidlowski, 2001). Experiments on the expression level of the mutant MR (MRK712A) in the presence or absence of the proteasome inhibitor (MG132) demonstrated that there was no difference in expression level of this mutant MR compared to the wild type MR (Fig. 17). In an experiment measuring the activation of transcription of a reporter gene by MRK712A, there was no difference in transcriptional activation in the absence of MG132. (Fig. 18). The study could not be done in the presence of MG132 since after only a 20-hour treatment, the cells began to die. Therefore, it was not possible to compare the

rMR PEST signal

^{680}GKLKGLHEEQPQQPPPPPPQSPEEGTTYIAPTKEP^{714}

rGR PEST signals

^{157}PENPKSSTSATGCATPTEKEFP^{178}

^{415}SPGMRPDVSSPPSSSSAATGPPPKLCL^{441}

Fig. 16
Sequence of the PEST signals in rMR and rGR.
The PEST signal is identified in red letters and the flanking sequences are in black.

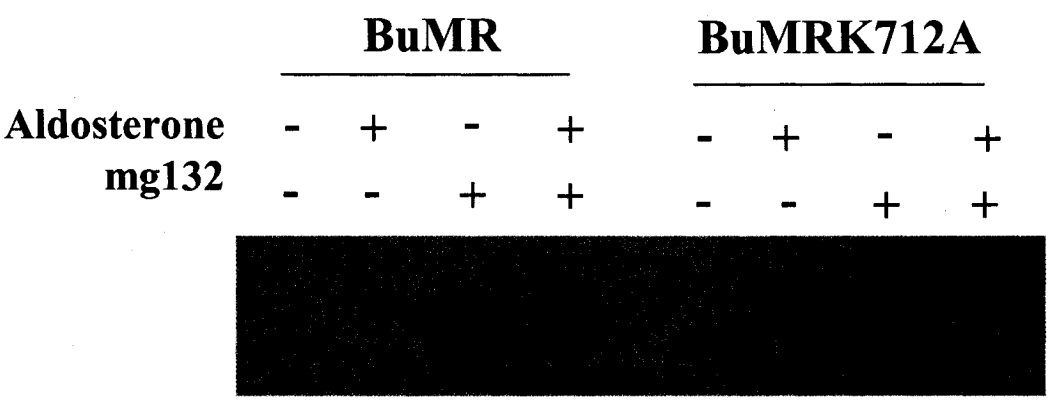

BuMR
BuMRK712A
Aldosterone - + - + - + - +
mg132 - - + + - - + +

Fig. 17
Mutation of the lysine next to the predicted PEST signal (K712A) in rMR does not affect the stability of the receptor.
Cos7 cells were transiently transfected with 500ng of pTL_2BuMR or $pTL_2MRK712A$ using Lipofectamine. 50 μg of whole cell extract was run on a denaturing SDS-PAGE gel and transferred onto a PVDF membrane and probed with FiGR antibody (1:400).

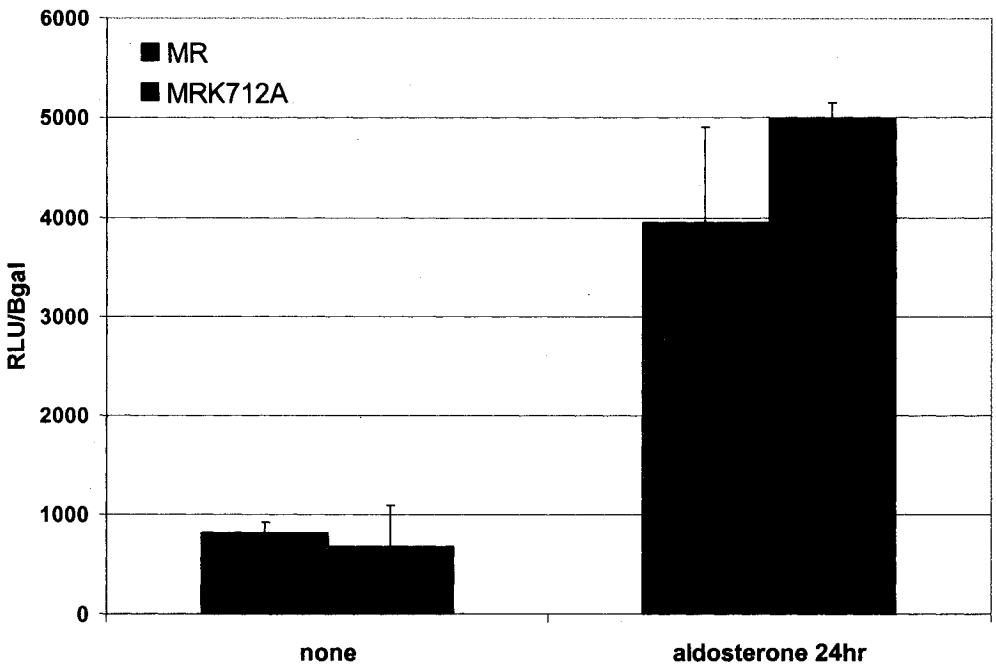

6000
5000
4000
3000
2000
1000
0
RLU/Bgal
MR
MRK712A
none
aldosterone 24hr

Fig. 18
Transcriptional activation of the reporter gene MMTV-237Luc by MRK712A. Cos7 cells were transiently transfected with 50ng of receptor (pTL_2MR in blue and $pTL_2MRK712A$ in red), 100ng of RSV-βgal and 100ng of MMTV-237Luc per 60mm dish.Twenty-four hours after transfection, transcription was induced with 10^{-6}M aldosterone. Cytoplasmic extract was prepared using 400µl of Reporter Lysis Buffer (Promega, Madison, WI, USA) per 60mm dish and luciferase activity of 20µl of cytoplasmic extract was measure by the Analytical Luminescence Laboratory Monolight 2010. For data analysis, the RLU were normalized for transfection efficiency by the β-galactosidase activity.

transcriptional activation of the cells treated with cells not treated with proteasome inhibitor. To conclude the experiments with MRK712A, the subcellular localization of the mutant was studied to confirm that other aspects of MR, such as its trafficking, were not affected by the mutation of the lysine residue. MRK712A behaved the same as MR wild type in subcellular localization (Fig. 19). Prior to aldosterone treatment, MRK712A was equally distributed between the nucleus and the cytoplasm and moved to the nucleus after treatment with either aldosterone or spironolactone. From these studies of MRK712A, we conclude that the lysine at position 712 in rat MR does not serve as acceptor ubiquitin site that plays an important role in MR degradation.

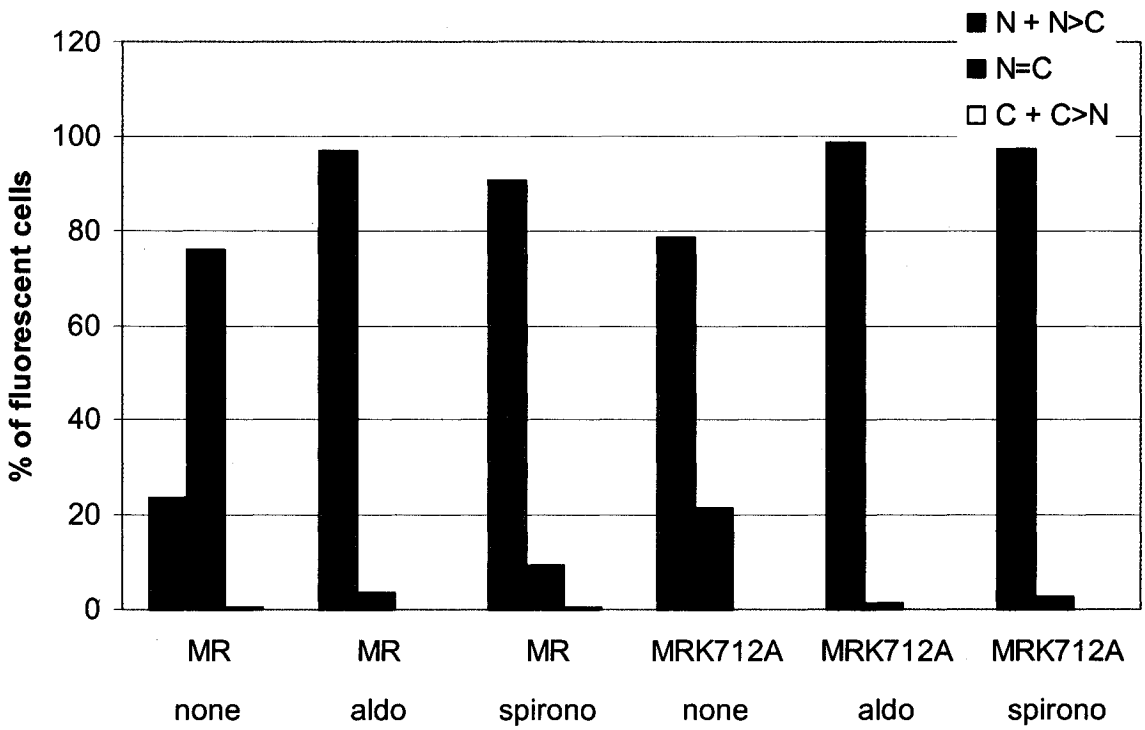

N + N>C
N=C
C + C>N
% of fluorescent cells
120
100
80
60
40
20
0
MR none
MR aldo
MR spirono
MRK712A none
MRK712A aldo
MRK712A spirono

Fig. 19
Mutagenesis of lysine 712 of MR does not affect its subcellular localization. Cos7 cells prepared for immunofluorescence were pretreated with 1μM aldosterone or spironolactone for 1 hour. Specific localization was visualized using FiGR antibody (1:400) followed by a Rhodamine Red™-X-conjugated AffiniPure Donkey Anti-mouse secondary antibody (1:150). Over 200 cells were counted for each condition, repeated in duplicate in two different experiments. The localization of each cell was categorized as completely or mostly nuclear (blue bars), equally distributed (red bars) or localized predominantly or exclusively in the cytoplasm (yellow bars). The error bars indicate the standard errors of the means.

Discussion

Nucleocytoplasmic trafficking of MR

In our study on nucleocytoplasmic trafficking of MR, we identified and characterized an agonist-specific NL2 function in the LBD of MR. We also observed that the LBD sequence contains either a nuclear retention signal compared to the LBD of GR or no export signal, as the LBD of MR stays nuclear even after 24h after hormone withdrawal.

Preliminary results obtained in our laboratory showed that by contrast to nuclear transfer mediated by the NL1 of MR, the nuclear transfer of MR mediated by the NL2 appeared to be hormone-dependent as MRNL1- stayed mostly N=C after treatment with spironolactone. The MR LBD, presumably containing the NL2 also moved to the nucleus only in presence of aldosterone and not in the presence of spironolactone.

The expression level of $EGFPMR_{690C}$ could not be compared to the expression level of MR and MRNL1- since different antibodies were used to detect the proteins. To compare the expression levels, we could have added a BuGR epitope to $EGFPMR_{690C}$. Another better possibility could have been to use an antibody that recognizes the C-terminal part of MR.

It is recognized that naïve GR is located in the cytoplasm of a cell, but it has been shown that overexpression of mouse GR in Chinese hamster ovary cells causes the receptor to localize to the nucleus with its chaperone proteins (Sanchez *et al.*, 1990). This observation raised the possibility that the subcellular localization of MR, which is equally distributed in the cytoplasm and the nucleus, could be due to an overexpression

of the receptor. However, other laboratories have published the same subcellular localization as we have found for MR, using less DNA with different transient transfection methods in different cell lines (Nishi *et al.*, 2001, Fejes-Thot *et al.*, 1998 and Odermatt *et al.*, 2001). Therefore, the nuclear localization of MR does not seem to be due to overexpression of the receptor.

Creation of the chimeric protein GGMNL1- allowed us to have a protein of similar size to GR and MR. The drop of 70% in transcriptional activation by GGMNL1- as opposed to GGM wild type was very similar to the drop in transcriptional activation between GR and GRNL1- (Savory *et al.*, 1999). That was an indication that activation can be lower due to a decrease in nuclear occupancy. Measurement of subcellular localization after treatment of GGMNL1-, at different times, with aldosterone and spironolactone clearly showed that the kinetics of agonist-dependent nuclear import of GGMNL1- mediated by the NL2 of MR was different then the kinetics of nuclear import of wild type MR when mediated by the NL1. Upon agonist binding, wild type MR translocates virtually completely to the nucleus within 30 minutes (Nishi *et al.*, 2001, Fejes-Thot *et al.*, 1998 and Odermatt *et al.*, 2001). MRNL1- and GGMNL1- translocate to the nucleus only partially and attain equilibrium after 1h.

The LBD of MR contains binding domains for the chaperone proteins (Rafestin-Oblin *et al.*, 1989, Couette *et al.*, 1998) and a ligand-dependent activation function (AF-2). As it has been shown, MR binds agonists or antagonists while still in a complex with the Hsps (Couette *et al.* 1996), suggesting that the conformational changes of the LBD precede the release of the Hsps. Upon aldosterone treatment, GGMNL1- moved slowly to the

nucleus, in comparison to wild type GR, which is completely nuclear after 10 minutes. The transfer of GGMNL1- was also incomplete with only ~40% of the cells having a signal classified as N + N>C. Nuclear transfer was measurable after 10 minutes and equilibrium was attained after 1 hour. A possibility is that the chaperone proteins mask the NL2, even after the ligand binding, and that the displacement of the Hsps occurs more slowly than does exposure of the NL1. These results suggest a significant difference in mechanism of transport, although for now, it is impossible to say exactly what are the differences.

It has been published that antagonists (i.e. progesterone) bind to MR with an affinity identical to that of aldosterone and induce a receptor conformation that is transcriptionally silent (Lombes *et al.*, 1993). Spironolactone theoretically could inhibit the action of aldosterone through MR by at least three different mechanisms. In the first one, the antagonist could prevent the receptor from translocating to the nucleus. In the second situation, translocation could occur but the receptor-antagonist complex would have low affinity for the chromatin. In the last mechanism, the complex could translocate to the nucleus and bind chromatin with the same affinity but fail to interact with coactivators to trigger transcription. Our transcription activation experiments confirm that the receptor-antagonist complex does not activate transcription without providing other details on the possible mechanism of inhibition of transcription. Data obtained by Marver and colleagues (1974) and Claire and colleagues (1979) were in favour of the mechanism in which the trafficking of the receptor was blocked. Our subcellular localization experiments, on the other hand, gave indications that trafficking of the receptor is partially affected by antagonist binding. NL2-mediated trafficking of

MR was blocked by use of spironolactone. MRNL1-, EFGPMR$_{690C}$ and GGMNL1- almost did not move into the nucleus upon spironolactone binding. MR full-length, on the other hand, upon exposure to antagonist, moved into the nucleus but not to the same extent as with agonist. Cells displaying nuclear localization of MR after treatment with spironolactone could be subcategorized: 41% of the cells had a completely nuclear pattern of localization and 49% had a N>C pattern, as opposed to 85% completely nuclear for MR treated with aldosterone. This phenomenon might be explained by the fact that the antagonists, not having the same structure as agonists, do not contact properly important amino acids of the receptor for MR to generate a conformational change such that helix 12 is stabilized and in its active position. The conformational changes, not being the same as when receptor contacts agonist, might not unmask the NLS properly to expose the signal to trigger subcellular trafficking of the receptor.

GRNL1- has been shown to redistribute rapidly to the cytoplasm following hormone withdrawal compared to wild type GR (Savory *et al.*, 1999). The conclusion drawn from these previous experiments was that the extended maintenance of GR in the nucleus subsequent to hormone withdrawal required an intact NL1. As opposed to GRNL1-, MRNL1- remained nuclear after hormone withdrawal, and at increasing times after withdrawal, the number of cells with nuclear localization increased (data not shown), suggesting that the NL1 of MR is not required for extended maintenance of the receptor in the nucleus. In the case of GGMNL1-, after 1 hour, 30% of the cells were nuclear and stayed nuclear up to 6 hours after removal of aldosterone. These results suggest that the LBD of MR, in contrast to the LBD of GR, contains a sequence that prolongs nuclear subcellular localization of GGMNL1- or lacks an export signal. It is interesting to see

drastic differences in nuclear import kinetics between receptors with a high degree of similarity in sequence. Studies on the reassociation of MR with Hsps in the nucleus should be done to compare results with the data published on GR, which reassociates with the chaperone proteins even before being exported from the nucleus. It would also be interesting to know whether the NL2-mediated import of MR is done with the participation of importin-α or if it is achieved by another pathway, as is the case for GR (Savory *et al.*, 1999), consistent with a lack of noticeable basic motif in the LBD.

Stability of MR

In the second part of my work, we identified that MR is degraded by the proteasome, that addition of steroid induces further degradation and that the N-terminal portion of MR is responsible for the appearance of higher molecular weight forms of the receptor after aldosterone treatment. We also identified a PEST-like signal starting at position 686 to 711 and determined that the lysine residue at position 712 is not an acceptor ubiquitin site, therefore, it does not play a role in the degradation of MR.

In our laboratory, it was noticed that expression level of MR was always lower than the expression level of GR after transient transfection into Cos7 cells. Since it has been demonstrated that GR undergoes proteasomal degradation upon ligand binding (Deroo *et al.*, 2002), probably to diminish cellular responsiveness to ligand (Wallace *et al.*, 2001), it was hypothesised that the lower level of MR could also be due to the degradation of the receptor.

Our experiments have shown that MR is degraded when it is in presence of aldosterone, independently of the presence of proteasome inhibitor, concomitantly, a higher molecular weight form of the receptor appears. The steroid stimulation does not lead to the appearance of higher molecular weight forms. These results suggest that down-regulation of MR is ligand-dependent. Furthermore, the appearance of a higher molecular weight form of MR suggests that MR is regulated in a different manner than GR.

Mutation of the NL1 of MR blocked the appearance of the higher molecular weight form of the receptor after ligand treatment suggesting that the presence of the wild type NL1 is necessary for the receptor to undergo what I propose to be a post-translational modification decreasing mobility on SDS-PAGE. A possible explanation could be that MRNL1- is not entering the nucleus to the same extent as wild type MR and not activating transcription. Also, by not entering the nucleus, it might not be accessible to the nuclear factors that do the modification.

To identify the domain of MR responsible for the appearance of the higher molecular weight form, chimeras of GR and MR were created. The two chimeras, GGM and GMM, although they were slightly less stable than GR full-length, had a similar stability pattern as the degradation patter of rGR in Cos7 cells: a ligand-dependent degradation after agonist treatment. They also had a minor accumulation of receptor upon proteasome inhibitor treatment, a feature different from the pattern of GR. As opposed to Wallace's results using mouse GR in Cos1 cells, in my experiments, MG132 treatment could not block the ligand-dependent degradation of rGR in Cos7 cells. The reason for

this could be the difference in cells used for the experiment or the differences in sequence between mouse GR and rGR.

The only protein that produced the higher molecular form of the receptor in the presence of aldosterone was full-length MR. From these results, we can conclude that the N-terminal portion of MR is, at least in part, responsible for the higher molecular weight forms of the receptor in the presence of aldosterone. This domain is the least conserved of the domains of steroid receptors, with less than 15% homology to the N-terminal domain of GR and could well explain the difference in proteolysis pattern between the two receptors. Experiments with a construct containing the N-terminal of MR and the DBD and LBD of GR (MGG) would confirm this hypothesis.

The last aspect of my research was an investigation of a PEST signal in the sequence of MR. The motif was identified between amino acids 686 and 711 of rMR. It has been shown that the lysine following the PEST signal, in mouse GR, was a potential site for anchoring ubiquitin to mediate proteasome-dependent degradation of the receptor (Wallace and Cidlowski, 2001). Mutation of the lysine residue following the PEST signal in rMR (MRK712A) did not seem to have the same result as mutation of the lysine residue following the PEST signal in GR. The degradation pattern of mutated MR remained identical to the one of wild type MR. MRK712A was still degraded in presence of aldosterone, suggesting that this residue is not critical to the ligand-dependent regulation of MR. Other results confirmed that conclusion: the mutated receptor activated transcription to the same level as wild type MR and the subcellular localization was not affected by the mutation as it was comparable to the localization of

MR in response to aldosterone and spironolactone. The difference in cell line used to do the experiments could explain the difference between Cidlowski's results and mine. Cos7 cells might lack the PEST-dependent E3 so that the lysine after the PEST motif is not targeted by ubiquitin.

The PEST signal, being close to the NL1 of MR constituted of clusters of basic amino acids (i.e. lysines), is surrounded by many other lysines residues. It has not been shown that a specific location for the lysine residue, around the PEST signal, is a more likely acceptor site for ubiquitination of a protein. It has been shown that a lysine following the PEST signal in the GR sequence had a role for the ubiquitin-mediated proteolysis, but nothing has been reported for the role of lysines elsewhere around the PEST motif. The difference in proteolysis pattern and transcriptional activation of MRNL1- can be explained by the limited access to the nucleus, but the fact that these lysines could play a role in the ubiquitin-dependent degradation pathway of MR has to be considered.

In summary, I have contributed to two aspects of the mechanism of action of MR about which relatively little was known. First, I identified an NL2 function in the LBD of MR, which is agonist-specific. I also observed that the LBD sequence contains either a nuclear retention signal or no export signal, as the LBD of MR stays nuclear even after 24h after hormone withdrawal. Second, I identified that MR is degraded by the proteasome upon agonist binding and that the N-terminal portion of MR is responsible for the appearance of higher molecular weight forms of the receptor after aldosterone treatment. Finally, I identified a PEST signal starting at position 686 to 711 and determined that the lysine residue at position 712 is not an acceptor ubiquitin site.

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Appendix 1

Primers:

MR690 forward	5' - CGG GGT ACC CCA CAG CAG CCC CCA CCA C - 3'
MR981 3' XmaI:	5' - TCC CCC CGG GTC ACT TTC TGT GAA AGT AAA G - 3'
MR700 SmaI:	5' - CGT CCC GGG AGT CCA GAA GAG GGG ACC - 3'
MR 981-3':	5' - CGG GAT CCT CAC TTT CTG TGA AAG TAA AG - 3'
GR22 KpnI forward:	5' - GCG GTA CCA TGG GGA GGG GGA GCG TAA TG - 3'
GR526 EcoRV rev:	5' - GTG ATA TCG ACT CCT GCA GTG GCT TGC TG - 3'
MRK712A upper:	5' - CAT ACA TCG CTC CGA CCG CGG AGC CAT CGG TGA AC - 3'
MRK712A lower:	5' - GTT CAC CGA TGG CTC CGC GGT CGG AGC GAT GTA TG - 3'

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- Function of CstF in coactivation of genic transcription.
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Ottawa
Recipient of NSERC Undergraduate Student Research Award in Industry

• Production of novel *Trichoderma reesei* strains.

F- Academic laboratory, January 1998-April 1998
Dr D. Liao's laboratory
Microbiology and infectiology department
Centre Universitaire de Santé de l'Estrie
University of Sherbrooke
Recipient of Institutionnal Award

• Expression and purification of the tumor suppressor p53 as a GST-fusion protein.

Extra-academic skills:

Basic computer skills:	-Word, Excel, Power Point, Internet
Biology-related computer skills:	-Literature search on PubMed Medline -Knowledge of standard DNA sequence analysis programs (GCG from University of Ottawa, Swiss-Prot and ExPASy) and public DNA and protein sequence databases (NCBI)