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The Role of N-terminal Domain in Regulation of Mineralocorticoid Receptor Function

By
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Abstract

The N-terminal domain of the mineralocorticoid receptor (MR) exhibits no significant sequence homology to the highly related glucocorticoid receptor. I report here that amino acids 450-602 of the N-terminus play a role in three aspects of MR degradation: (1) degradation in the absence of aldosterone mediated by the 26S proteasome, (2) degradation in the presence of aldosterone mediated by the 26S proteasome, and (3) degradation in the presence of aldosterone not mediated by the 26S proteasome. Concurrently I determined that aldosterone treatment following transient expression of MR in Cos-7 cells induced the appearance of higher molecular weight forms of the receptor. I determined that two regions within the N-terminus of MR were required for the appearance of the aldosterone-stimulated shift in molecular weight. Furthermore, the type of agonist, nuclear occupancy and the cell type employed influenced the shift in molecular weight of MR.

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Abbreviations

11βHSD2	11β hydroxysteroid dehydrogenase 2
AF	activation function
DBD	DNA binding domain
DMEM	Dulbecco's Modified Eagle Medium
ENaC	epithelial sodium channel
ER	estrogen receptor
FBS	fetal bovine serum
GFP	green fluorescent protein
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HRE	hormone response element
LBD	ligand binding domain
MMTV	mouse mammary tumor virus long terminal repeat sequence
MR	mineralocorticoid receptor
NLS	nuclear localization signal
NR	nuclear receptor
ONPG	O-Nitrophenyl β-D-galactopyranoside
PBS	phosphate-buffered saline
PR	progesterone receptor
RLU	relative light unit
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFBS	charcoal-stripped fetal bovine serum

SHR	steroid hormone receptor
SUMO	small ubiquitin-like modifier
SV40	simian virus 40
WCE	whole cell extract

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Introduction

One mechanism of maintaining homeostasis in the human body is through secretion of hormones by the endocrine system. The classical steroid hormones, including the corticosteroids, retinoids, thyroid hormones and vitamin D3, were identified in the early 20th century based on their association with human diseases and their ability to affect homeostasis, embryonic development, metamorphosis and differentiation. The corticosteroids are classified as either glucocorticoids or mineralocorticoids, based on their ability to control glycogen metabolism and the stress response or the metabolism of minerals, sodium and posstassium, respectively (1,2). With the exception of the retinoids, the steroid hormones are synthesized from cholesterol within endocrine glands and circulate throughout the body (3). Due to their lipophilic nature they are able to pass through the lipid bilayer of the cell membrane and bind to intracellular receptors, unlike the hydrophilic peptide hormones and growth factors, which require cell surface receptors (3).

The cloning of the human glucocorticoid receptor in 1985 (4) and the human estrogen receptor a year later (5) began a decade of characterization of the intracellular receptors which bind the steroid hormones, and the identification of the superfamily to which these and other structurally related receptors belong, the nuclear receptor superfamily. The nuclear receptor superfamily mediates diverse physiological processes such as development, reproduction and metabolism through their actions as ligand-inducible transcription factors. The nuclear receptor superfamily has been subdivided into three classes of receptors: type I receptors (the steroid hormone receptors) dimerize and translocate to the nucleus where they bind DNA half-sites organized as inverted

repeats, type II receptors (the thyroid/retinoid/vitamin D receptor family) heterodimerize with RXR and characteristically bind to direct repeats, and type III receptors or "orphan nuclear receptors" are those receptors which resemble other nuclear receptors in their domain structure but for whom ligands have not been identified (as reviewed in (6)).

Characterization of the Mineralocorticoid Receptor

The mineralocorticoid receptor (MR) is a member of the steroid hormone receptor (SHR) family which includes the glucocorticoid receptor (GR), the progesterone receptor (PR), the androgen receptor (AR) and the estrogen receptor (ER). MR was the second last receptor of the SHR family to be cloned with the cloning of human MR in 1987 (7) followed by rat MR in 1989 (8). Both human and rat MR were cloned based on their high degree of structural similarity to GR through low-stringency hybridization to GR cDNA.

MR was identified as the intracellular receptor that binds aldosterone, the primary physiological mineralocorticoid, with high affinity and activates transcription (7). However, it was also demonstrated *in vitro* that MR exhibits similar binding affinity for the physiological glucocorticoid, cortisol (or corticosterone in rats and mice) (7) . In addition, MR was shown to activate transcription at a glucocorticoid-responsive promoter. *In vivo* the circulating levels of cortisol are several orders of magnitude higher than aldosterone, thus the ability of MR to bind and mediate effects in response to glucocorticoids is functionally significant.

The Actions of 11βHSD2 in Aldosterone-target Tissues

To ensure steroid-specific activation of MR in mineralocorticoid target tissues, such as the sweat and salivary glands, the distal tubules of the kidney and the distal colon, an enzyme is expressed that converts the physiologically active cortisol into the inactive metabolite, cortisone (9-12). This conversion is performed by 11β hydroxysteroid dehydrogenase 2 (11βHSD2) (13). In the absence of 11βHSD2, MR would be saturated by cortisol due to its prevalence and similar binding affinity in comparison to aldosterone. 11βHSD2 is NAD+-requiring, has a nanomolar affinity for glucocorticoids and is essentially unidirectional (14). Unlike cortisol, aldosterone is protected from enzymatic attack by 11βHSD2 due to its unique chemistry whereby the aldehyde group at C18 cyclizes with the hydroxyl group at C11 in solution to form a hemiacetal group that is not susceptible to attack (13). The actions of 11βHSD2 in converting cortisol to cortisone and thereby preventing it from acting as a mineralocorticoid are crucial as it has been determined that the hypertension associated with the syndrome of 'apparent mineralocorticoid excess' is caused by inactivating mutations in 11βHSD2 (15).

Physiological Role of MR

The phenotype of MR knock-out mice reveals the obligatory role of MR in mediating the actions of aldosterone, as mice show the features of aldosterone deficiency with a severe salt-wasting state, water and weight loss leading to death by day 8 (16). These mice display a strongly activated renin-angiotensin system which controls

aldosterone secretion leading to the high levels of aldosterone also observed in the MR knock-out mice.

The principal effects of aldosterone are on maintenance of normal sodium and potassium concentrations and extracellular volume (3). In epithelial tissues such as the kidney collecting duct and distal colon, MR is protected from competition by cortisol through the actions of 11βHSD2, as discussed previously. Therefore, in these tissues aldosterone promotes sodium reabsorption across epithelial Na+ channels (ENaC) through an MR-dependent induction of serum and glucocorticoid-regulated kinase (sgk) which subsequently increases ENaC activity (17-19). If the actions of 11βHSD2 are blocked or deficient, cortisol can occupy MR and act as an agonist, mimicking the aldosterone effect on ion transport (20).

In non-epithelial tissues such as the hippocampus and the heart, MR is not protected by 11βHSD2 and therefore both cortisol and aldosterone have equal access to MR (20). Interestingly, in contrast to epithelial tissues where cortisol acts as an agonist of MR function, in non-epithelial tissues cortisol can act as an antagonist of MR. This is illustrated in the actions of MR in the rat CNS where aldosterone results in an increase in blood pressure, whereas, corticosterone acts through MR to antagonize the of effects of aldosterone (21).

Structure of MR

MR, like other members of the SHR family as well as members of the nuclear receptor superfamily, is characterized by a centrally located DNA binding domain

(DBD), flanked by a C-terminal ligand binding domain (LBD) (Fig. 1). MR exhibits 94% sequence homology to GR within the DBD and 57% homology within the LBD (7); in contrast, the N-terminal domain exhibits less than 15% sequence homology with GR and other SHRs (2). The highly conserved structure of SHRs is reflected in their analogous receptor function, whereby the inactive receptor associated with heat-shock proteins binds hormone, dissociates from its heat-shock protein complex, homodimerizes, translocates to the nucleus and interacts with specific hormone response elements (HREs) in order to regulate target gene transcription.

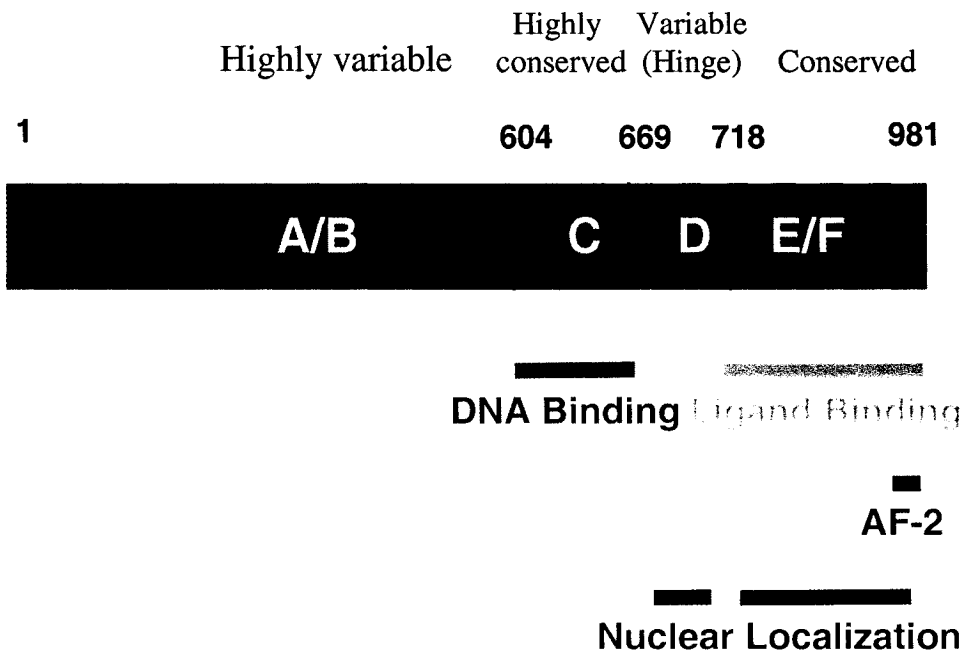
Extensive crystallographic studies of other members of the nuclear hormone receptor superfamily have revealed that the tertiary structure of the DBD and LBD is highly conserved amongst family members (22-31). Although no direct structural data has been published for MR, it is assumed that MR adopts a tertiary structure similar to other family members given the high degree of sequence homology and biochemical characterization of MR performed to date (7,32-43).

The N-terminus

The N-terminus (or A/B domain) of nuclear receptors remains largely uncharacterized as compared to the extensive studies that have been pursued concerning the other receptor domains. The lack of knowledge is in part due to the fact that the initial characterization of nuclear receptors focused on their shared properties as members of a superfamily of transcription factors, and therefore due to the unconserved nature of the N-terminus it was not a primary focus. Despite the unconserved nature of the N-terminus, a transactivation domain termed activation function (AF-1) has been identified in all SHRs (33,36,44-47).

Figure 1. Schematic representation of rat MR and its functional domains.

A schematic representation of rat MR with boxes that highlight the major domains. Amino acid positions for boundaries of major domains, are indicated by numbers above the diagram. Some of the functions within each domain are summarized below the diagram. The N-terminal domain (A/B), possesses an AF-1 activity; however, the exact region has not been delimited. The C-terminus consists of the DNA binding domain (C), the hinge region (D) and the ligand binding domain (E/F). Nuclear localization signals, NL1 and NL2, are located in the hinge region and ligand binding domain, respectively.


Highly variable
Highly conserved
Variable (Hinge)
Conserved
1
604
669
718
981
A/B
C
D
E/F
DNA Binding
Ligand Binding
AF-2
Nuclear Localization

It has been postulated that the N-terminus of nuclear receptors achieves its folded, functional state after the receptor has bound its ligand and interacted with cofactors (48). In addition, interaction between the N- and C-terminus in a ligand-dependent manner has been demonstrated for MR (49), PR (50) and AR (51). In the case of MR, aldosterone stimulated the strongest interaction between the N- and C-terminus; whereas, cortisol stimulated only a weak interaction and spironolactone (an MR antagonist) inhibited interaction of the N- and C-terminus in a mammalian-2-hybrid assay (49).

The DNA Binding Domain

The DNA binding domain (or C domain) contains the amino acids that interact directly with the specific DNA bases composing hormone response elements during receptor binding to DNA. The DBD is rich in cysteine, lysine and arginine residues, including 9 cysteine residues that are conserved within the nuclear receptor superfamily (2). Eight of these conserved cysteines, grouped into two groups of four, form the α-helical zinc fingers that each co-ordinate a single zinc atom and interact directly with DNA (52). A short amino acid sequence within the second zinc finger, termed the D box, has been shown to play a role in receptor dimerization (53).

The zinc fingers of the DBD mediate the binding of nuclear receptors to palindromic DNA sequences termed hormone response elements (HREs). The SHRs MR, GR, AR and PR bind as ligand induced homodimers to an inverted hexameric palindrome separated by a non-conserved three base pair spacer termed the glucocorticoid response element (GRE): AGAACA NNN TGTTCT (54). No unique mineralocorticoid response element (MRE) has been defined to date. Unlike the other members of the SHR family, ER binds as a ligand induced homodimer to a different half

site sequence, AGGTCA, identical to the half-site used by the nonsteroid nuclear receptors such as TR, RAR and VDR (52).

The Hinge Region

The hinge region (or D domain) links the DBD to the LBD and is largely unconserved amongst nuclear receptors. Interestingly, the hinge region of MR contains 24 additional residues not found in the hinge of GR (7). Within these 24 amino acids resides a stretch of 4 glutamines followed by 8 prolines, the significance of which is unknown; however structure-breaking prolines are thought to provide flexibility within the hinge region of SHRs (53). The best characterized functional region of the hinge is the nuclear localization sequence (NL1) which has been identified in SHRs such as GR (55), AR (56) and recently in MR by our lab (C. Bayer, unpublished data).

The Ligand Binding Domain

As suggested by the name, the first function of the ligand binding domain (or E/F domain) is ligand binding. MR has been shown to bind agonists (aldosterone and cortisol) or antagonists (spironolactone and progesterone) (40,57). Mutational studies have demonstrated that the majority of the sequence of the LBD must be intact otherwise ligand binding is compromised. In contrast, the other functions associated with the LBD, such as heat-shock protein association, dimerization, nuclear localization and transactivation require only small stretches of amino acid sequence within the LBD (53). Ligand-dependent transactivation is accomplished in part by the C-terminal helix of the LBD which contains a ligand-dependent activation function (AF-2) (23).

Crystallographic analysis of nuclear receptor LBDs has revealed that the LBD is composed of twelve α-helices and one β sheet organized into an antiparallel α-helical structure enclosing the ligand binding pocket (23,24,27,28,30,31). Upon agonist binding a conformational change occurs that results in the repositioning of the C-terminal (AF-2) helix over the ligand binding pocket. The repositioning of the AF-2 helix stabilizes the receptor in an active conformation and enables specific interaction with coactivator molecules, such as the p160 family (58). In contrast, upon antagonist binding the AF-2 helix adopts an alternative conformation and in this position some nuclear receptors recruit corepressor molecules such nuclear corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone (SMRT) (59,60).

For nuclear receptors such as RAR and TR, the shape and volume of the ligand binding pocket matches that of the ligand thereby maximizing hydrophobic contacts (30,31). This contributes to the stability and selectivity of the ligand binding pocket for its ligand. In contrast, for SHRs the selectivity for the structurally similar ligands is driven more by case specific interactions than by many hydrophobic interactions due to the large volume of the ligand binding pocket in comparison to the ligand (58).

The exact determinants of steroid binding within the ligand binding pocket of MR remain undefined. Given the high sequence homology within the LBD of MR and GR, it is not surprising that they overlap in their ability to bind cortisol. However, only MR binds aldosterone with high affinity. In an effort to determine the precise region(s) of the MR LBD that confers the ability to bind aldosterone with high affinity, Rogerson *et al.* (38) employed MR/GR chimeric fusion proteins. They determined that amino acids 804-874 of the MR LBD were required for high affinity aldosterone binding specificity. In

addition, it was demonstrated that although they were not critical, amino acids 932-984 also contributed to the high affinity binding of aldosterone.

The recent crystallization of the GR LBD (23) provides clues as to the reason for the specificity of the MR ligand binding pocket for aldosterone and cortisol. This crystal structure of the GR LBD revealed that helices 6 and 7 align differently than within the LBD of ER, AR and PR and produce a unique side pocket. Glucorticoids have larger substituents at the C17α position than progesterone or the sex steroids. Therefore, the authors speculate that the side pocket may account for the specificity of GR for glucocorticoids. Furthermore, given that the residues that form the side pocket within the GR LBD are conserved in the MR LBD and that aldosterone possesses a similar substituent at C17α, they propose that MR may have a similar pocket that accounts for its specificity for aldosterone and cortisol (23).

Localization and Trafficking of SHRs

The subcellular localization of the SHRs prior to ligand binding (ie. the naïve receptor) differs from receptor to receptor. While GR is exclusively cytoplasmic (55), AR is distributed between the nucleus and the cytoplasm (61) and ER and PR are constitutively nuclear (62,63). Reports on the localization of naïve MR have varied. Some groups report that naïve MR is mainly cytoplasmic (64,65) while others report that naïve MR is distributed between the nucleus and cytoplasm (66,67).

In contrast with other nuclear receptors, all unliganded SHRs, localized to the nucleus or cytoplasm, are associated with a large, multiprotein complex of chaperones, including the heat-shock proteins Hsp90 and Hsp70, and the immunophilin Hsp56.

Similar to GR, the role of Hsp90 in MR function is two-fold: to keep the receptor in an inactive state and to maintain the LBD in a structural conformation that promotes high affinity ligand binding (68). *In vivo*, the absence of specific interactions with Hsp90 results in significantly impaired hormone induction by GR (69).

After ligand binding and chaperone dissociation, the SHRs that were located in the cytoplasm translocate to the nucleus in order to regulate transcription. This nuclear import is mediated by a nuclear localization signal (NLS) which may be hidden when the receptor is unliganded and bound by Hsps. An NLS, characteristically rich in basic amino acids such as lysine and arginine, has been identified in all members of the SHR family (61,70,71). Most recently, our lab identified two NLSs in MR, NL1 (C. Bayer, unpublished data) and NL2 (Y. Rouleau, unpublished data), similar in function to the two NLSs described in GR (55,72). NL1, located in the hinge region of both GR and MR, is constitutively active when separated from the inhibiting function of the LBD and mutation of key residues results in significantly reduced steroid-dependent translocation to the nucleus (72)(C. Bayer, unpublished data). NL2, located in the LBD of GR and MR, mediates a slower, agonist-specific transfer to the nucleus (72)(Y. Rouleau, unpublished data).

Transcriptional Regulation by Nuclear Receptors

Initially it was thought that nuclear receptors activate transcription by stabilizing the preinitiation complex through direct interactions with general transcription factors (73,74). However, the identification of a large number of factors that interacted with nuclear receptors in a ligand-dependent or –independent manner and potentiated their

ability to activate transcription, led to the current belief that nuclear receptors require these coactivator proteins in order to achieve maximal transactivational activity. These coactivators are often found in large, multiprotein complexes that act both by local chromatin remodeling enabling the transcriptional machinery to access the DNA and direct modification and recruitment of basal transcription factors and RNA polymerase II (75).

Coregulatory proteins, including coactivators as well as corepressors, interact with the receptors via the two major activation functions (AF-1 and AF-2) identified within nuclear receptors (45). AF-1, located in the N-terminal domain, is considered to be ligand-independent; however, the conformation adopted by the unliganded full-length receptor inhibits AF-1 activity (53). AF-2, located in the C-terminal helix of the LBD, is ligand-dependent as it is able to interact with coactivator molecules following the conformational change associated with agonist binding. Several coactivators which interact with the AF-2 of SHRs such as GR, ER and PR have been well-characterized and include the p160 family members, SRC-1 (76), GRIP-1/TIF2 (77,78) and p/CIP/SRC-3 (79-81) as well as coactivators that possess strong histone and factor acetyltransferase activity such as p300/CBP (82) and p/CAF (83).

The AF-2 of nuclear receptors, including MR, is highly conserved; in contrast, there is no highly conserved region within the N-terminal domain that represents the AF-1 of all nuclear receptors. The reports to date characterizing AF-1 within MR have been conflicting. In one report MR AF-1 was delimited to amino acids 328-382 within the MR N-terminus by fusion of this region to a GR mutant lacking its AF-1 and examining transcriptional activation (33). In contrast, another group characterized MR AF-1 in the

context of a receptor possessing only the N-terminus and DBD (ie. in the absence of the AF-2 of MR) and determined that MR AF-1 is composed of two regions within the N-terminus: amino acids 1-169 (termed AF-1a) and 451-603 (termed AF-1b) (36). This group also reported that SRC-1, p300 and TIF2 acted as coactivators for the AF-2 of MR; whereas the AF-1a activity was potentiated only by p300 and AF-1b activity was potentiated by p300 and TIF2. In a subsequent paper the group demonstrated that RNA helicase A (RHA) and CBP interact with the AF-1a region of and cooperatively potentiate transcription in response to aldosterone, but not cortisol (35).

The Ubiquitin-Proteasome Pathway

In order to maintain intracellular homeostasis cells must balance the levels of protein synthesis and degradation. The turnover of unnecessary proteins provides the cells with amino acids for synthesis of other proteins as well as a mechanism to eliminate abnormal or damaged proteins (84). The two main pathways responsible for protein degradation in eukaryotic cells are the lysosomal pathway and the ubiquitin-proteasome pathway. The lysosomal pathway is considered to be non-specific and mainly involved in the degradation of cell-surface proteins and extracellular proteins taken up by endocytosis (85). In contrast, the ubiquitin-proteasome pathway is highly specific and is responsible for the degradation of many abnormal proteins as well as many normal intracellular short-lived proteins (86,87).

Two discrete steps are involved in degradation of proteins by the ubiquitin-proteasome pathway: (1) tagging of the substrate by covalent attachment of multiple ubiquitin moieties and (2) degradation of the tagged protein by the 26S proteasome. The

conjugation of ubiquitin, a highly conserved 76-residue polypeptide, requires the sequential actions of three enzymes: an activating enzyme (E1), a conjugating enzyme (E2) and a ligase enzyme (E3) which links the C-terminus of ubiquitin to the epsilon-amino group of a substrate lysine residue (88). Ubiquitin moieties are then successively added to the previously conjugated Lys residue to form a polyubiquitin chain. The polyubiquitination is catalyzed by E3s, or by a subfamily of E3s termed E4s (89). While eukaryotic organisms encode a single or at most a few E1s and substantially more E2s (over 20 in mammals), it is thought that there are hundreds of E3s that mediate the highly efficient and specific reaction of ubiquitination (90). E3 ubiquitin ligases recognize structural motifs, known as ubiquitination signals, within substrates and are therefore the central determinants of specificity in ubiquitination (88). The PEST motif, characterized by a region rich in the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T), is a common structural motif recognized by E3s that triggers ubiquitination of the protein at the lysine present or in close proximity to the PEST motif (91).

Proteins tagged with polyubiquitin chains are recognized by the 26S proteasome and degraded through an ATP-dependent process into small peptides as well as into intact ubiquitin moieties that are re-used. The 26S proteasome is found in both the nucleus and cytoplasm of eukaryotic cells (92) and is composed of a 20S proteolytic core particle and a 19S regulatory cap. The regulatory particle binds polyubiquitin chains, unfolds the substrate protein through ATP hydrolysis and translocates the substrate to the proteolytic core (93). The 20S proteolytic core particle is composed of four stacked rings creating an internal chamber containing the proteases which mediate degradation through chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolyzing activities (94).

Degradation of SHRs

The ubiquitin-proteasome pathway regulates a broad range of eukaryotic cell functions such as cell-cycle progression, signal transduction and transcriptional regulation. Although the degradation of transcription factors through the ubiquitin-proteasome pathway requires the expenditure of cellular ATP, it is crucial in the efficient and appropriate regulation of transcription as well as maintenance of cellular homeostasis. Indeed, the ability of transcription factors to activate transcription appears, at least sometimes, to be directly correlated with the cell's desire to selectively degrade them through the ubiquitin-proteasome pathway, as the activation domain overlaps with the degradation signals in short lived transcription factors such as Myc, p53, E2F-1, Jun, Fos and HIF-1α (95).

Some members of the nuclear receptor family have been shown to be degraded through the ubiquitin-proteasome pathway in a ligand-dependent manner. It is believed that this degradation is an important component of the feedback response that prevents overstimulation by the hormones (96). Ligand-dependent downregulation by the 26S proteasome has been observed for the SHRs GR, PR, ER and AR (97-100).

The protease activity of the proteasome can be inhibited by treatment with the proteasome inhibitors MG132 or lactacystin (also referred to as β-lactone) (101). Interestingly, the effect on SHR function following treatment with MG132 or lactacystin was not the same for all receptors. Treatment with proteasome inhibitors suppressed transcriptional activation by ER, PR and AR (100,102,103). In contrast, treatment with MG132 or lactacystin significantly enhanced GR-mediated transcriptional activation (99,104). In addition, Wallace and Cidlowski identified a PEST degradation motif within

GR and demonstrated that mutation of Lys-426 within the PEST element abolished ligand-dependent down-regulation of over-expressed GR by the 26S proteasome as well as enhanced GR-mediated transcriptional activation (99).

Post-translational Modification of SHRs

In addition to ubiquitination, a modification that targets several SHRs for degradation by the 26S proteasome (99,105,106), SHRs are subject to many other forms of post-translational modification that affect receptor function. Both AR and ER are acetylated by the coactivator p300 and this acetylation affects receptor-mediated transactivation {Wang, 2001 #129;Fu, 2000 #127}. Another modification that SHRs, as well as other transcription factors, undergo is sumoylation. Sumoylation involves the conjugation of a small ubiquitin-like modifier (SUMO) to a target protein and has been shown to affect in subcellular localization, transcriptional activity and protein stability. Both AR and GR have been demonstrated to be sumoylated in their N-termini; furthermore, the sumoylation motifs that are sumoylated in AR and GR exist in the N-termini of PR and MR as well (109-111). Sumoylation of AR appeared to decrease testosterone-induced transactivation (109); whereas, overexpression of SUMO-1 potentiated GR-mediated transactivation as well as induced GR degradation (110).

Phosphorylation of SHRs

Steroid hormone receptors are subject to extensive phosphorylation which has been implicated in DNA binding, transcriptional activation and receptor stability. SHRs are basally phosphorylated in the absence of ligand when bound by the heat-shock

protein complexes and are hyperphosphorylated upon ligand binding (112). Most of the phosphorylated residues are serines in the N-terminal domain and the phosphorylation is mediated by proline-directed kinases such as the cyclin-dependent kinases or MAP kinases (113). Although it has been demonstrated that MR undergoes phosphorylation (114), the characterization of phosphorylated residues in MR has lagged behind that of all other SHRs.

Studies on GR and PR have revealed a crucial role for phosphorylation in regulating receptor degradation by the 26S proteasome. Mutation of 7 or 8 of the phosphorylation sites in mouse GR significantly extended GR half-life and rendered the mutated receptor resistant to ligand-induced down-regulation (115), which was subsequently shown to be mediated by the 26S proteasome (99). Lange *et al.* (98) demonstrated that ligand binding induces PR phosphorylation by MAP kinases at Ser-294, which then targets the receptor for degradation by the 26S proteasome. In subsequent papers, the group demonstrated that phosphorylation of Ser-294 is also involved in transcriptional hyperactivity and nucleocytoplasmic shuttling (102,116).

Objectives

Previous research in the lab examining protein levels of MR and GR in *Xenopus laevis* oocytes indicated that MR is present at lower levels than GR (M. Liao, unpublished data). Furthermore, it was demonstrated that exchange of the N-terminus of GR for the N-terminus of MR, creating the chimeric fusion protein GMM (indicating a fusion protein with the N-terminus of GR fused to the DBD and LBD of MR), resulted in increased protein levels compared to wildtype MR. In addition, it was observed by

Western blotting that, independent of the amount of MR or GR cDNA transiently transfected into Cos-7 cells, MR protein levels were consistently lower than GR protein levels. To date all SHRs except MR have been shown to undergo degradation by the 26S proteasome. Therefore, we hypothesized that this difference in MR versus GR protein levels is due to greater degradation of MR by the 26S proteasome. The first objective of my project was to determine whether the increased degradation of MR is a property conferred by the highly variable N-terminus and to delimit the region(s) within MR that are required for degradation by the 26S proteasome.

Previous research in the lab revealed that aldosterone treatment following transient expression of MR in Cos-7 cells induced the appearance of higher molecular weight forms of the receptor (Y. Rouleau, unpublished data). Therefore, the second objective of my project was to delimit the region(s) of MR required for this shift in molecular weight as well as examine the conditions which are required to induce the higher molecular weight forms of MR.

Materials and Methods

Plasmids

All MR and GR constructs examined in this study are of rat origin and are expressed by the pTL2 vector (a derivative of pSG5 with an expanded multiple cloning site) (117) under the control of an SV40 promoter. In order to simultaneously detect MR and GR constructs using the same antibody, the BuGR epitope tag (amino acids 408-422 of rat GR) was fused to the N-terminus of all MR constructs in the context of the pTL2 vector backbone (pTL2Bu). The nomenclature employed for rat MR N-terminal deletion constructs is that the amino acid at which the construct begins is listed followed by "C" indicating that the construct includes all the following residues of the full-length receptor up to and including the C-terminal residue (amino acid 981). For example, MR 300C begins at amino acid 300 and ends at the C terminus (therefore lacking the first 300 amino acids).

pTL2Bu MR 604C was created by PCR amplification (using the PTC-200 Peltier Thermocycler, MJ Research, Reno, CA) of amino acids 604 to 981 of wildtype rat MR, using pTL2 MR as a template. The primers employed were: KpnI BuGR MR 604 Fwd (see Appendix 1 for primer sequences) and BamHI MR 981 Rev. All primers were purchased from Invitrogen (Burlington, ON). The PCR product was purified by agarose gel electrophoresis and extracted using the Gel Extraction kit (QiaGEN, Mississauga, ON). The PCR product and the vector backbone pTL2 were then digested with KpnI and BamHI, purified by agarose gel electrophoresis and ligated. All enzymes used for cloning were purchased from New England Biolabs (Mississauga, ON). pTL2Bu MR

151C was created using the same cloning strategy and same vector backbone (pTL2). The primers employed for PCR amplification of rat MR 151-981 were KpnI BuGR MR 151 Fwd and SmaI MR 981 Rev. pTL2Bu MR 151C was constructed such that the entire MR insert could be cut out of the vector backbone and the vector re-ligated including the BuGR epitope yielding pTL2Bu which was employed in subsequent subcloning of MR.

pTL2Bu MR 51C was created by PCR amplification of amino acids 51 to 981 of rat MR. The primers employed were: NheI MR 51 Fwd and Not1 MR 981 Rev. The PCR product and the vector backbone pTL2Bu were then digested with NheI and NotI and ligated. A similar strategy was used to subclone pTL2Bu MR 101C. Amino acids 101-981 of rat MR were PCR amplified using primers NheI MR 101 Fwd and Not1 MR 981 Rev.

pTL2Bu MR Δ100-150 was created through a two-step PCR amplification strategy. Firstly, amino acids 1-100 of rat MR were amplified using primers NheI MR 5' Fwd and EcoRV MR 100 Rev. MR 1-100 was then ligated into pTL2Bu and used as the vector for the next step. Secondly, amino acids 150-981 of rat MR were PCR amplified using primers EcoRV MR 151 Fwd and NotI MR 981 Rev. MR 151-981 was then ligated into pTL2Bu MR1-100 to create pTL2Bu MR Δ100-150. pTL2Bu MR Δ450-500 and pTL2Bu MR Δ450-550 were created using the same two-step PCR strategy. The primers used in the first PCR reaction were the same for both constructs: NheI MR 5' Fwd and EcoRV MR 450 Rev. For the second PCR amplification, the forward primer used was EcoRv MR 500 Fwd (for MR Δ450-500) or EcoRV MR 550 (for MR Δ450-550) along with NotI MR 981 Rev as the reverse primer.

Several plasmids were made by other members of the lab for the purposes of this thesis. pTL2Bu MR 300C was created by PCR amplification of amino acids 300-981 of rat MR. pTL2Bu MR 450C was created by PCR amplification of amino acids 450-981. pTL2Bu MR Δ450-602 was constructed by a two-step PCR amplification strategy. First, amino acids 1-450 were amplified and ligated into pTL2Bu. Then, amino acids 602-981 were amplified and ligated into pTL2Bu MR1-450. pTL2Bu MR Δ550-602 and pTL2Bu MR Δ590-602 were created using the same two-step PCR amplification strategy.

Several of the plasmids employed were made previously. pTL2Bu MR contains full-length wildtype rat MR inserted into pTL2 with a BuGR tag on the N-terminus. The pTL2Bu MR NL1- construct was created in order to abolish the MR NL1 nuclear localization signal (NLS) in the context of the full-length receptor. The construct was designed with the lysines within the NL1 mutated to asparagines (^{677}KKLGK^{681} to ^{677}NNLGN^{681}). pTL2 GR contains full-length wildtype GR (therefore including the BuGR epitope) inserted into pTL2. pMMTV-237 Luc contains the mouse mammary tumor virus (MMTV) long terminal repeat sequence (-237 to +105) followed by the luciferase reporter gene. pRSV β-Gal contains the β-galactosidase gene under the control of the Rous sarcoma virus (RSV) promoter. pEGFP-C1 was purchased from Clonetech (Mississauga, ON).

Cell Culture and Transient Transfection

Cos-7 cells (ATCC CRL-1651), CV-1 cells (ATCC CCL-70), HeLa cells (ATCC CCL-2), 293 cells (ATCC CRL-1573) and 293T cells (ATCC CRL-11268) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (for a complete list of the sources of chemicals see Appendix 2) supplemented with 10% fetal bovine serum (FBS)

at 37°C with 5% CO_2. Transient transfection of Cos-7 and CV-1 cells with cDNA expression plasmids was performed using Lipofectamine according to manufacturer's instructions. Briefly, cells were incubated for 16 h with 500 ng DNA and 10 μL Lipofectamine per 60 mm dish in OptiMem. After 16 h the transfection was stopped by adding DMEM containing 20% charcoal-stripped fetal bovine serum (SFBS) to reach a final concentration of 10% SFBS. HeLa cells were transiently transfected with 500 ng DNA using ExGen as per manufacturer's directions. The ratio of ExGen:DNA used was 8 μL ExGen per μg DNA. DNA and ExGen were diluted in 150 mM NaCl in separate tubes. ExGen-NaCl solution was added to tube containing DNA in NaCl. The transfection mixture was incubated for 10 min at room temperature then serum-free DMEM was added to tube and the mixture was dropped onto cells in serum-free DMEM (total volume per 60 mm dish= 300 μL). The transfection reaction proceeded for 3 h and was stopped by the addition of 20% SFBS in DMEM to a final concentration of 10% SFBS. 293 and 293T cells were transiently transfected using FuGene according to manufacturer's directions. The ratio of FuGene:DNA used was 3:1 (3 μL FuGene per μg DNA). FuGene and serum-free DMEM were combined in one tube and DNA and serum-free DMEM were combined in another tube. The DNA-DMEM tube contents were transferred to the FuGene-DMEM tube, mixed and incubated at room temperature for 30 min. The transfection mix was dropped onto cells in DMEM + 10% SFBS. After 16 h the media on the plates was removed by aspiration and fresh DMEM + 10% SFBS was added.

For all three transient transfection techniques, cells were allowed to recover after stopping the transfection for at least 6 h in DMEM + 10% SFBS. For degradation assays,

the cells were treated with or without the proteasome inhibitors MG132 or lactacystin (at 1 μM), and with or without hormone (1 μM aldosterone for constructs with the MR LBD or 1 μM cortisol for constructs with the GR LBD). Generally the treatment length was 20 h, unless otherwise noted, at which time whole cell extracts were prepared for Western blotting. For the transcription assay, Cos-7 cells transiently expressing MR constructs were treated with or without 1 μM aldosterone for 20 h and harvested for transcription assay.

Preparation of Whole Cell Extracts

Following 48 h of transient expression of the desired DNA construct for the degradation assay, cell lines were harvested for Western blotting. Plates (60 mm) were washed twice with phosphate-buffered saline (PBS), harvested using a rubber policeman and centrifuged at 5000 g (Heraeus Instruments Biofuge Pico, Germany) for 2 min. Cell pellets were then resuspended in 100 μL whole cell extract buffer (WCE) (for solution formulations please see Appendix 3) and allowed to swell on ice for 10 min. Extracts were sonicated for 15 s using a Branson Sonifier 450 on constant duty cycle (output control=1). Following sonication extracts were centrifuged at 13 000 rpm for 10 min at 4°C to remove cellular debris and the protein concentration of the supernatant was quantified using the Bradford Assay.

Protein Concentration Determination

The Bradford Assay was used to determine the protein concentration in whole cell extracts. A small volume of extract (usually 2 μL) was added to 800 μL of water followed by addition of 200 μL of Bio-Rad Protein Assay Dye Reagent. The solution

was mixed and allowed to develop for 5 min at room temperature. The absorbance of each sample was measured at 595 nm on a spectrophotometer (Biochrom Ultrospec 3000). The amount of protein present in the extract was determined by comparison to a bovine serum albumin standard curve.

Western Blotting

Protein extract (50 μg) with the appropriate amount of 6x SDS loading dye was loaded onto a denaturing gel and separated by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) using the Bio-Rad Mini-Protean III Cell System. Typically an 8% polyacrylamide gel was used. Proteins were transferred to Immunoblot PVDF membranes for 1 h at 100V then blocked for 1 h in 5% skim milk in PBS-T (PBS with 0.1% Tween-20 w/v) at room temperature. The membrane was incubated with primary antibody in PBS-T for 16 h at 4°C. The membrane was then washed four times, 10 min each in PBS-T at room temperature and incubated with secondary antibody for 1 h at room temperature. Unbound secondary was removed by 10 min washes in PBS-T. The antibody-bound protein was detected using Western Lightning Chemiluminescence Reagent (Enhanced Luminol) followed by exposure to film.

Antibodies

(i) Primary Antibodies: MR constructs tagged with the BuGR epitope or wildtype rat GR were detected using the mouse FiGR antibody (raised against BuGR epitope: rat GR amino acids 408-422) at a dilution of 1/400 (v/v). Green fluorescent protein was detected with the GFP primary antibody (JL-8) at a dilution of 1/1000 (v/v).

(ii) Secondary Antibody: The primary antibodies FiGR and GFP were recognized by the sheep anti-mouse secondary antibody conjugated to horseradish peroxidase.

Densitometry

Densitometry of Western blots was employed in order to quantify the relative protein levels of the various constructs in the different conditions. Densitometry was performed using the ImageQuant software. After correcting for background, the absolute values were either converted to a percentage as compared to untreated MR or expressed as the fold difference compared to untreated for each construct set.

Transcription Assay

Cos-7 cells were transiently transfected with 300 ng of either pTL2Bu MR or pTL2Bu MR 150C, 100 ng of RSV β-Gal and 200 ng of MMTV-237 Luc per 60 mm dish, as described above. 20 h prior to harvesting, cells were treated with or without 1μM aldosterone. After 20 h cells were washed twice with PBS and cytoplasmic extracts were prepared using 400 μL of Reporter Lysis Buffer per 60 mm dish, according to the manufacturer's directions. In order to determine the extent of reporter gene activation in each condition, the luciferase activity in relative light units (RLU) was measured in each 20 μL extract aliquot combined with the Luciferase Assay Substrate using the Analytical Luminescence Laboratory Monolight 2010 Luminometer.

β-Galactosidase Assay

In order to control for transfection efficiency within all the conditions, the RLU were normalized using the β-Gal activity of each extract. To quantify β-Gal activity, 50

μL of extract was added to 150 μL of Z buffer, followed by the addition of 40 μL of ONPG. The solution was mixed and incubated at 30°C until a faint yellow colour developed. The incubation time of each reaction was recorded and the reaction was stopped by the addition of 100 μL 1M Na_2CO_3 and the absorbance at 420 nm was measured using a spectrophotometer. The formula used to determine β-Galactosidase activity is as follows:

β-Gal units/mL= A420/(0.0045 x incubation time (min) x volume of extract(mL))

Small-scale Plasmid Preps by Alkaline Lysis

Clones were grown in 5 mL selective LB liquid media overnight at 37°C. The bacterial culture was pelleted and resuspended in 100 μL of GTE (see Appendix 3 for solution formulations). Lysis solution (200 μL) was added and the suspension was mixed and briefly stored on ice. Potassium acetate solution (150 μL) was added, vortexed and incubated on ice for 5 min. The suspension was centrifuged at 12 000 g for 5 min at 4°C and the supernatant was transferred to a new tube and extracted with an equal volume of phenol:chloroform. DNA was precipitated with 2 volumes 100% ethanol for 2 min at room temperature. The solution was centrifuged 12 000 g for 5 min at 4°C and the supernatant removed. The pellet was then washed with 70% ethanol, centrifuged as above, dried and resuspended in 50 μL TE containing RNAase (20 μg/mL).

Large-scale Plasmid Preps by Alkaline lysis and Cesium-chloride Gradient

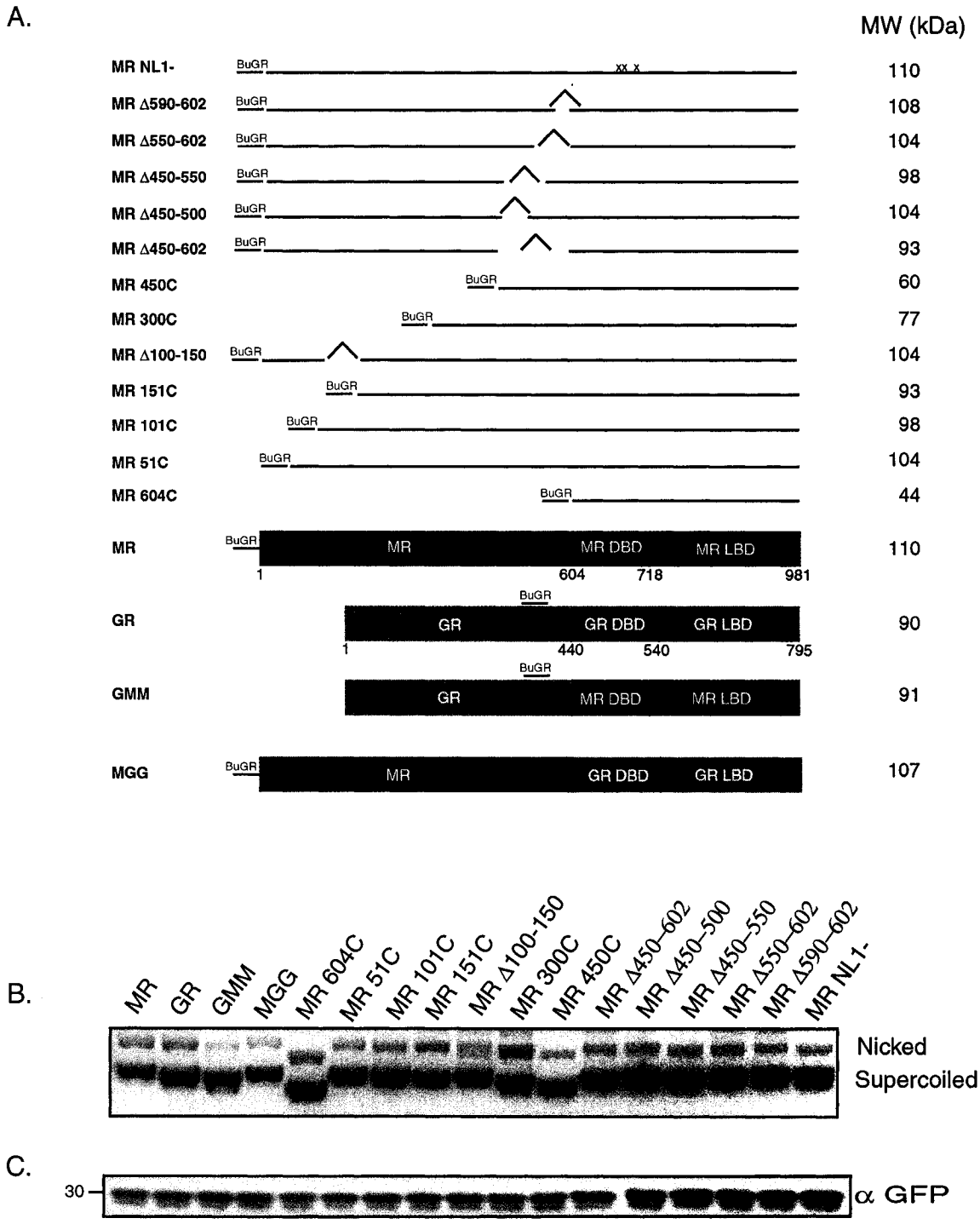
A 1 L bacterial culture was pelleted by centrifugation at 4500 g for 10 min at 4°C, resuspended in GTE and a small amount of lysozyme was added to the suspension. After 10 min incubation on ice, 20 mL of lysis solution was added and the suspension was incubated on ice for 10 min. Potassium acetate solution (15 mL) was added, the suspension was mixed and incubated on ice for 10 min. The suspension was centrifuged at 15 000 g for 10 min at 4°C to pellet bacterial debris and the supernatant was filtered through gauze. Isopropanol (20 mL) was added and the solution was incubated at room temperature for 15 min followed by centrifugation 4000 g for 10 min at 4°C. The supernatant was discarded and the pellet was dried at room temperature followed by resuspension in 8 mL TE. 5 M LiCl (8 mL) was added and incubated on ice for 15 min. The suspension was centrifuged 4000 g for 5 min at 4°C and the supernatant was filtered through gauze. 100% ethanol (18 mL) was added and incubated for 1 h at -20°C to precipitate DNA. The suspension was centrifuged 4000 g for 15 min at 4°C and the DNA pellet was resuspended in 2.8 mL TE, 3.08g CsCl and 30 µL EtBR (2 mg/mL). The solution was transferred to a polypropylene ultracentrifuge tube, the tube was sealed and centrifuged 95 000 rpm overnight at 22°C in a Beckman TLN 100 fixed angle rotor. The following day the supercoiled DNA band was extracted from the tube by syringe using an 18 gauge needle. The supercoiled DNA was transferred to another polypropylene ultracentrifuge tube, filled to the top with CsCl solution (R.I. = 1.397- 1.399), sealed and centrifuged 95 000 rpm for 6 h at 22°C in a Beckman TLN 100 fixed angle rotor. The supercoiled DNA was removed as above and ethidium bromide was extracted using

water/salt saturated isopropanol. When the ethidium bromide was no longer detectable in either phase, the aqueous phase was transferred to dialysis tubing and dialyzed for 1 h at room temperature in 2 L TE. The TE buffer was then changed and dialysis was allowed to proceed overnight at 4°C. The DNA quality and quantity was determined by measuring the absorbance at 260 and 280 nm and by running it on an agarose gel to assess supercoiling.

Results

In order to examine the role of the N-terminus of MR in receptor degradation and in the shift of molecular weight following aldosterone treatment, MR/GR chimeric fusion proteins and a series of MR constructs were expressed by transient transfection in Cos-7 cells (Fig. 2A). All expression plasmids were constructed from cDNA and expressed from the pTL2 backbone under the control of the SV40 promoter. This enabled the direct comparison of the relative protein levels of the MR or GR constructs in transient transfection experiments if comparable DNA quality and equal transfection efficiency had been achieved. In order to ensure that the DNA quality of all expression plasmids was comparable, equal amounts of the uncut MR or GR expression plasmids were resolved on an agarose gel. As illustrated in Figure 2B, all of the MR or GR constructs employed were of comparable quality with the ratio of supercoiled to nicked DNA in each plasmid preparation being approximately equal. In order to demonstrate that transfection efficiency was constant when different constructs were transiently transfected into different plates of Cos-7 cells, each MR or GR DNA preparation illustrated in Figure 2B was co-transfected with a control plasmid expressing enhanced green fluorescent protein (GFP) and the relative protein levels of GFP were visualized by Western blotting (Fig. 2C). The relative protein levels of GFP were approximately constant in the whole cell extract from each plate of Cos-7 cells despite co-transfection with MR or GR expression plasmids. This result suggests that the difference in relative protein levels observed in subsequent experiments was not due to differences in transfection efficiency.

Figure 2. DNA quality and transfection efficiency controls for expression plasmids.
A. A schematic representation of a series of rat MR constructs and rat MR/GR chimeric constructs with amino acid numbering for GR and MR and including the location of DBD and LBD domains. The theoretical molecular weight of each construct is listed on the right. The location of the BuGR epitope which is detected by the FiGR primary antibody to be used in subsequent Western blotting is labelled on each construct.
B. 500 ng of uncut plasmid DNA for each construct was resolved by agarose gel electrophoresis on a 1% gel. Bands were visualized under UV light on an ethidium bromide-stained gel. Supercoiled and nicked plasmid species are indicated.
C. Cos-7 cells were transiently co-transfected with 500 ng of the indicated MR or GR construct and pEGFP-C1, in order to control for transfection efficiency. Cells were allowed 48 hours for transient expression and harvested for Western blotting. Whole cell extract (50 μg) for each condition was resolved on a 12% gel by SDS-PAGE, transferred to PVDF and probed with the GFP primary antibody (1:1000).

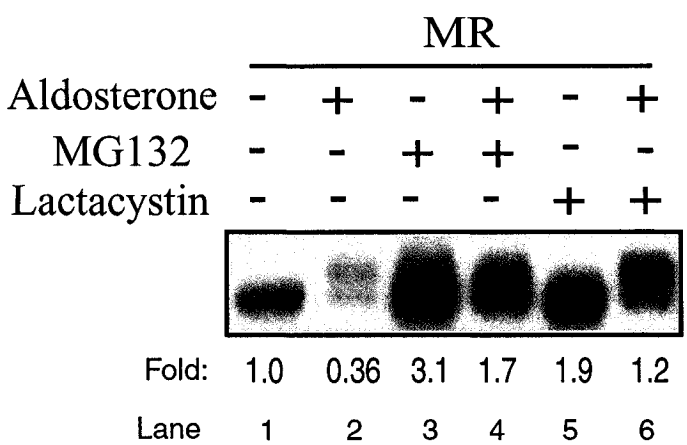

A.
MW (kDa)
MR NL1-
MR Δ590-602
MR Δ550-602
MR Δ450-550
MR Δ450-500
MR Δ450-602
MR 450C
MR 300C
MR Δ100-150
MR 151C
MR 101C
MR 51C
MR 604C
BuGR
xx x
110
108
104
98
104
93
60
77
104
93
98
104
44
MR
MR DBD
MR LBD
1
604
718
981
GR
GR DBD
GR LBD
440
540
795
GMM
MGG
90
91
107
B.
MR
GR
GMM
MGG
MR 604C
MR 51C
MR 101C
MR 151C
MR Δ100-150
MR 300C
MR 450C
MR Δ450–602
MR Δ450–500
MR Δ450–550
MR Δ550–602
MR Δ590–602
MR NL1-
Nicked
Supercoiled
C.
30
α GFP

Part I: MR Degradation

In order to examine the role of the N-terminus of MR in receptor degradation, I transiently transfected MR constructs or MR/GR chimeric constructs into my model cell line, Cos-7 cells, treated the cells for 20 hours in the presence and absence of proteasome inhibitors and hormone, and harvested the cells for analysis by Western blotting. Densitometry of the Western blots was performed to quantify the relative levels of protein present in each condition.

In the initial experiment, Cos-7 cells transiently expressing MR were treated with one of two proteasome inhibitors, MG132 or lactacystin, in the presence and absence of hormone (Fig. 3). Densitometry results were calculated as the fold difference compared to the MR protein level in untreated cells (lane 1). The relative levels of MR present in the aldosterone treated cells (lane 2) was only 0.36 fold of that present in the untreated cells, indicating that hormone treatment accelerated MR degradation. Treatment with aldosterone, in the presence or absence of proteasome inhibitors, also resulted in the appearance of higher molecular weight forms of MR (lanes 2, 4 and 6). This shift in molecular weight was not observed upon treatment with proteasome inhibitor alone and is the subject of further analysis which will be discussed later. Treatment with either MG132 or lactacystin in the absence of hormone resulted in stabilization of the receptor (3.1 fold by MG132 versus 1.9 fold by lactacystin). This observation indicated that MR is, like other steroid hormone receptors, degraded by the 26S proteasome and this degradation can be blocked by proteasome inhibitors. Neither proteasome inhibitor was able to completely stabilize the liganded receptor as treatment with both aldosterone and

Figure 3. Effect of aldosterone, MG132 and lactacystin on MR degradation.
Cos-7 cells were transiently transfected with 500 ng of wildtype MR. For 20 hours prior to harvesting cells were treated with or without 1 μM aldosterone, 1 μM MG132 or 1 μM lactacystin, as indicated. Whole cell extract (50 μg) for each condition was resolved on a 10% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blot shown is representative of three independent experiments. Densitometry of the entire region shown for each lane was calculated and is expressed below the blot as the fold difference compared to untreated MR.


MR
Aldosterone - + - + - +
MG132 - - + + - -
Lactacystin - - - - + +
Fold: 1.0 0.36 3.1 1.7 1.9 1.2
Lane 1 2 3 4 5 6

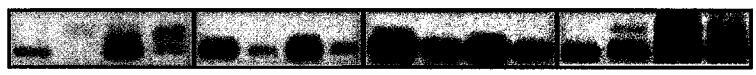
either proteasome inhibitor (MG132 or lactacystin, lanes 4 and 6, respectively) resulted in lower levels of MR as compared to MR treated with inhibitor in the absence of hormone.

The effect on inhibition of MR degradation by the 26S proteasome was similar for both MG132 and lactacystin, and given that MG132 has been more commonly used in examining SHR degradation by the 26S proteasome, only MG132 was employed in subsequent experiments. On the basis of these results, three aspects of MR degradation were examined: (1) degradation in the absence of hormone mediated by the 26S proteasome (ie. MG132-sensitive), (2) degradation in the presence of hormone mediated by the 26S proteasome (ie. MG132-sensitive, aldosterone-sensitive), (3) degradation in the presence of hormone not mediated by the 26S proteasome (ie. MG132-insensitive, aldosterone-sensitive).

The N-terminus of MR affects receptor degradation

In order to examine the role of the N-terminus of MR in the three aspects of receptor degradation, MR/GR chimeric fusion proteins were created, as depicted in Figure 2A. GMM possesses the GR N-terminus fused to the MR DBD and LBD, and therefore was treated with aldosterone during hormone treatment as aldosterone is the natural ligand of the MR LBD. In contrast, MGG possesses the MR N-terminus fused to the GR DBD and LBD, and therefore was hormone treated with cortisol, the natural ligand of GR. Densitometry of the Western blot of MR, GMM, GR and MGG (Fig. 4) was performed and is expressed both as a percentage of untreated MR and as the fold difference compared to the untreated protein level for each construct set. From Figure 4 it can be seen that substitution of the GR N-terminus for the MR N-terminus resulted in

Figure 4. Exchange of the MR or GR N-terminus affects receptor degradation. Cos-7 cells were transiently transfected with 500ng of MR, GMM, GR or MGG. For 20 hours prior to harvesting cells were treated with or without 1μM steroid (aldosterone for MR and GMM, cortisol for GR and MGG) or 1μM MG132, as indicated. Whole cell extract (50 μg) for each condition was resolved on a 8% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blots shown are representative of three independent experiments. The lower blot represents a darker exposure of the four MR lanes depicted above it. Densitometry of the entire region shown for each lane was calculated and is expressed below the blot both as a percentage of untreated MR and as the fold difference compared to untreated for each construct set.



	MR				GMM				GR				MGG			
Hormone	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
MG132	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+

132 kDa
116 kDa

Lane	1	2	3	4												
Absolute value expressed as a % of untreated MR (lane 1)	100	26	413	183	585	88	622	136	1774	1047	1375	778	545	642	2144	1276
Fold difference (ie. treated/untreated) for each construct set	1.0	0.26	4.1	1.8	1.0	0.88	1.1	0.23	1.0	0.59	0.78	0.44	1.0	1.2	3.9	2.3

increased protein levels in the untreated condition (ie. GMM is 585% of MR). Conversely, substitution of the MR N-terminus for the GR N-terminus decreased the protein levels in the untreated condition (ie. 545% for MGG versus 1774% for GR). This suggests that the N-terminus of MR conferred a degree of protein instability to the receptor which was not present in the GR N-terminus. This protein instability appeared, at least in part, to be due to degradation by the 26S proteasome given that treatment with MG132 significantly increased both MR and MGG protein levels: 4.1 fold and 3.9 fold, respectively. In contrast, neither GR nor GMM were significantly stabilized by treatment with MG132: a 1.1 fold increase for GMM and a 0.78 fold decrease for GR. These results indicate that sensitivity to treatment with MG132 resulting in increased MR accumulation resides within the N-terminus of MR.

The MR/GR chimeras also reveal information concerning the aldosterone-stimulated higher molecular weight forms of MR. The shift in MR mobility was dependent upon the presence of the N-terminus of MR because GMM was not modified following treatment with aldosterone; in contrast, higher molecular weight forms of MGG appeared upon treatment with cortisol confirming that the N-terminus of MR is required (Fig. 4). It should be noted that the shift in mobility of MGG was present not only following aldosterone treatment but also when the cells were treated with MG132 alone; in constrast, for MR the shift in mobility was only observed following aldosterone treatment.

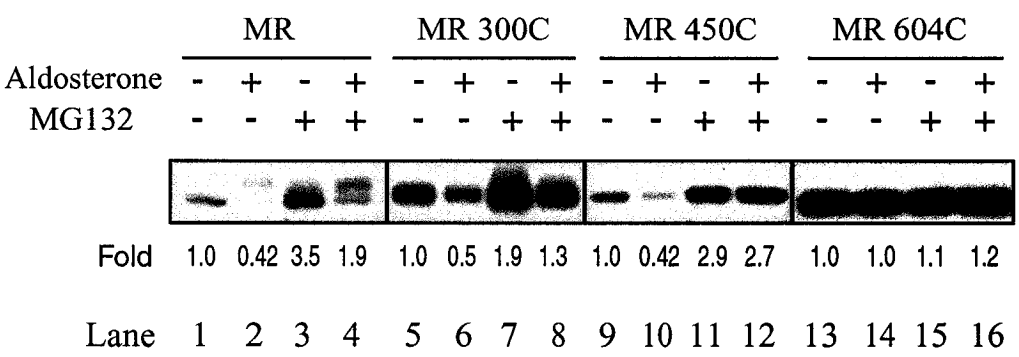
Amino acids 450-602 are involved in MR degradation

In order to begin to characterize the region of the N-terminus of MR that conferred sensitivity to MG132, N-terminal deletion constructs were created. Figure 5 reveals that an MR construct lacking the N-terminus, MR 604C, was essentially insensitive to both MG132 stabilization and aldosterone-stimulated degradation (a fold difference of no more than 1.2 fold was observed for the four conditions) and was significantly more stable than wildtype MR in the absence of hormone. However, extension of the MR construct by 150 amino acids towards the N-terminus (ie. MR 450C) restored both aldosterone sensitive degradation (0.42 fold decrease, lane 10) and MG132-mediated receptor stabilization (2.9 fold increase, lane 11). Intriguingly, however, MG132 stabilized MR 450C to essentially the same degree in both the absence and presence of aldosterone (2.9 fold versus 2.7 fold, lanes 11 and 12), in contrast to wildtype MR where MG132 was only partially effective in the presence of aldosterone.

A further N-terminal extension, MR 300C, resulted in a receptor that was more stable than both wildtype MR and MR 450C in the untreated condition (Fig. 5, lane 5). Furthermore, MR 300C was stabilized by MG132 in the absence of hormone (1.9 fold, lane 7) and began to exhibit MG132-insensitive, aldosterone-stimulated degradation as evidenced by the fact that the fold stabilization in the presence of MG132 decreased when co-treated with aldosterone (1.3 fold, lane 8). Interestingly, none of the three constructs examined in Figure 5 exhibited the higher molecular weight forms of MR upon aldosterone treatment suggesting that the first 300 amino acids of MR are required.

These results suggest that amino acids 450-602 played a role in MG132 sensitivity; however, they also indicate that the method of N-terminal truncations may not

Figure 5. Amino acids 450-602 play a role in conferring sensitivity to aldosterone-stimulated degradation and MG132-mediated stabilization.
Cos-7 cells were transiently transfected with 500 ng of MR or the MR N-terminal deletion constructs, as illustrated. For 20 hours prior to harvesting cells were treated with or without 1 μM aldosterone or 1 μM MG132, as indicated. Whole cell extract (50 μg) for each condition was resolved on a 8% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blot shown is representative of three independent experiments. Densitometry of the entire region shown for each lane was calculated and is expressed as the fold difference compared to untreated for each construct set.


MR
MR 300C
MR 450C
MR 604C
Aldosterone - + - + - + - + - + - + - + - +
MG132 - - + + - - + + - - + + - - + +
Fold 1.0 0.42 3.5 1.9 1.0 0.5 1.9 1.3 1.0 0.42 2.9 2.7 1.0 1.0 1.1 1.2
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

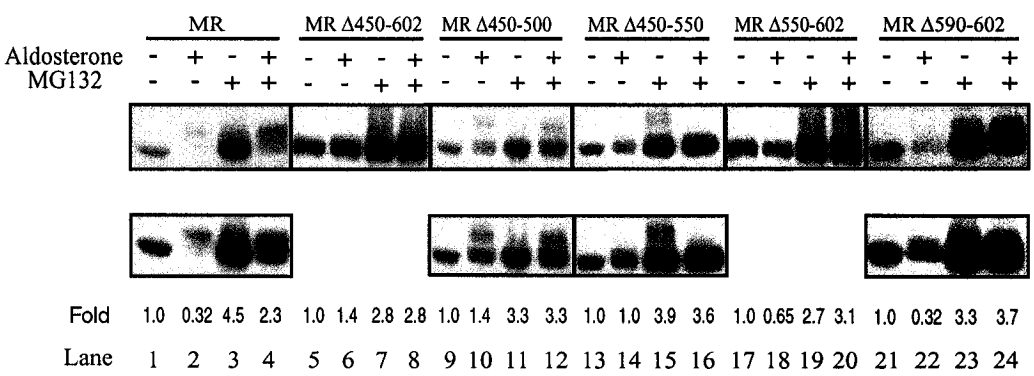
be an appropriate method of determining which regions of the N-terminus of MR are required for the three modes of degradation. Some potential limitations of this method will be presented in the discussion. The progressive N-terminal truncations did not behave as was expected if discrete regions were required for the distinct modes of degradation. Instead, the results suggest that receptor stability and sensitivity to aldosterone and MG132 was influenced by the overall structure of the N-terminus of MR.

Examination of the role of amino acids 450-602 in MR degradation

In order to determine the contribution of amino acids 450-602 to MR sensitivity to aldosterone-stimulated degradation and MG132 stabilization, MR constructs with deletions within this region were created, as depicted in Fig. 2A. Firstly, from Figure 6 it can be seen that deletion of amino acids 450-602 eliminated aldosterone-stimulated degradation (lanes 6 and 8). Somewhat unexpectedly, however, stabilization by MG132 was retained (2.8 fold, lane 7). In addition, all the constructs examined with smaller deletion constructs within amino acids 450-602 retained MG132 sensitivity (MR Δ450-500, lane 11; MR Δ450-550, lane 15; MR Δ550-602, lane 19; and MR Δ590-602, lane 23). This result indicates that while amino acids 450-602 play a role in conferring MG132 sensitivity to MR, other amino acids of the N-terminus also contribute to MG132 sensitivity. On the other hand, deletion of amino acids 450-602 revealed that those residues were required in order to confer aldosterone-stimulated degradation to the receptor.

As revealed by densitometry, neither MR Δ450-500 nor MR Δ450-550 were degraded in response to aldosterone (Fig. 6, lanes 10 and 14, respectively). However,

Figure 6. Examination of regions within amino acids 450-602 that were required for degradation and aldostosterone-stimulated shift in MR mobility.
Cos-7 cells were transiently transfected with 500 ng of MR or MR deletion mutants. For 20 hours prior to harvesting cells were treated with or without 1 μM aldosterone or 1 μM MG132, as indicated. Whole cell extract (50 μg) for each condition was resolved on a 8% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blots shown are representative of three independent experiments. The lower blots represent a darker exposure of the four lanes depicted above them. Densitometry of the entire region shown for each lane was calculated and is expressed as the fold difference compared to untreated for each construct set.


MR
MR Δ450-602
MR Δ450-500
MR Δ450-550
MR Δ550-602
MR Δ590-602
Aldosterone - + - + - + - + - + - + - + - + - + - + - + - +
MG132 - - + + - - + + - - + + - - + + - - + + - - + +
Fold 1.0 0.32 4.5 2.3 1.0 1.4 2.8 2.8 1.0 1.4 3.3 3.3 1.0 1.0 3.9 3.6 1.0 0.65 2.7 3.1 1.0 0.32 3.3 3.7
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

MR Δ550-602 was partially sensitive (0.65 fold decrease in response to aldosterone, lane 18) and MR Δ590-602 was as sensitive as wildtype MR (0.32 fold decrease in response to aldosterone, lane 22). This suggests that the region of MR required for aldosterone-stimulated, MG132-sensitive degradation lies between amino acids 450 and 590.

Interestingly, while MR Δ590-602 was degraded in response to aldosterone, like wildtype MR, it was also completely stabilized by MG132 even in the presence of aldosterone (Fig. 6, 3.3 fold versus 3.7 fold for MG132 versus MG132 + aldosterone, respectively), unlike wildtype MR. This result suggests that amino acids 590-602 were required for aldosterone-stimulated, MG132-insensitive degradation.

In summary, internal deletion constructs within amino acids 450-602 of the N-terminus of MR revealed two regions that appeared to mediate the two aspects of aldosterone-stimulated degradation. Amino acids 450-590 were required for degradation in the presence of aldosterone mediated by the 26S proteasome (ie. MG132-sensitive). Amino acids 590-602 were required for degradation in the presence of aldosterone not mediated by the 26S proteasome (ie. MG132-insensitive). In contrast, identifying the region of the N-terminus of MR that mediated degradation by the 26S proteasome (ie. MG132-sensitive) in the absence of aldosterone was more difficult. While it appeared that amino acids 450-602 were involved in conferring MG132 sensitivity to MR, smaller, internal deletions between amino acids 450 and 602 did not resolve the exact determinants of MG132 sensitivity. Furthermore, the results indicated that other regions of the N-terminus of MR also contributed to MG132 sensitivity. The complicated nature of MG132 sensitivity suggests that it was influenced by the overall structure of the N-terminus of MR.

Part II: MR shift in mobility

The appearance of aldosterone-stimulated higher molecular weight forms of MR (hereafter termed MR shift in mobility) was examined using the same experimental procedure as outlined for MR degradation. MR constructs were transiently transfected into either my model cell line, Cos-7 cells, or other cell lines to test their capacity to induce the shift in MR mobility. The cells were treated for 20 hours (unless otherwise specified) in the presence and absence of MG132 and/or aldosterone, and harvested for Western blotting. As previously mentioned, from Figure 4 it can be seen that the N-terminus of MR was required for the shift in mobility. Following 20 hours aldosterone treatment, the majority of MR species detected had an apparent molecular weight of approximately 132 kDa (Fig. 4, lane 2), which was 16 kDa larger than the apparent molecular weight of untreated MR (116 kDa, lane 1). Therefore, MR species exhibiting this consistent shift of approximately 16 kDa upon aldosterone treatment will be referred to as "completely" shifted. In darker exposures of MR constructs treated with aldosterone or upon treatment with MG132 + aldosterone, intermediate shifted isoforms can be observed as a smearing between MR that had not shifted and completely shifted MR (eg. Figure 4, lane 4).

Residues within amino acids 450-602 play a role in MR shift in mobility

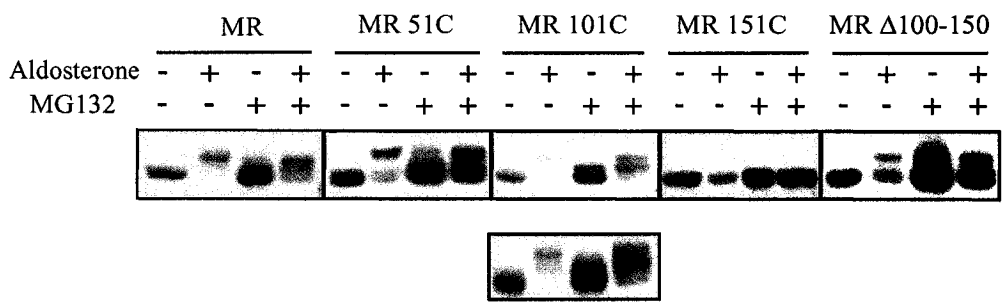
As mentioned, Figure 5 illustrates that the first 300 amino acids of MR were required for the shift in mobility. From Figure 6 it can be seen that another region of MR was also required for aldosterone-stimulated shift in mobility. Whereas MR Δ450-500 was shifted following aldosterone treatment (lane 10), neither MR Δ450-602, MR Δ450-550, nor MR Δ550-602 were shifted upon aldosterone treatment (lanes 6, 14, and 18,

respectively). For MR Δ450-602, MR Δ450-550 and MR Δ550-602, treatment with MG132 resulted in some smearing above the prominent band, however the lack of higher molecular weight forms of MR following aldosterone treatment alone suggests that this smearing is distinct from the aldosterone-stimulated shift in MR. Furthermore, deletion of amino acids 590-602 significantly, if not completely, abolished aldosterone-stimulated shift in MR mobility (lane 22). These results not only suggest that amino acids 500-602 are required for the shift in MR mobility but also that amino acids 590-602 play a significant role in the aldosterone-stimulated shift in mobility.

Amino acids 1-150 are also required for MR shift in mobility

N-terminal deletion constructs of the first 150 amino acids of MR were created in order to determine the contribution of those residues to the aldosterone-stimulated shift in mobility (Fig. 7). Deletion of the first 50 amino acids of the receptor (MR 51C) appeared to induce an intermediate phenotype of mobility shift, as for MR 51C there appeared to be an increased proportion of receptor species that were not shifted following aldosterone treatment compared to wildtype receptor (Fig. 7, lane 6 versus lane 2). Deletion of the first 100 amino acids (MR 101C) yielded a receptor that was shifted in an aldosterone-dependent manner approximately the same as wildtype MR: the majority of MR 101C species visualized in the aldosterone treated lane were shifted (lane 10) and there were intermediate isoforms apparent in the MG132 + aldosterone condition (lane 12). Deletion of the first 150 amino acids (MR 151C) completely abolished the aldosterone-stimulated shift in mobility of the receptor (lanes 14 and 16) indicating that those residues were required for the aldosterone-stimulated shift in mobility. An MR construct lacking

Figure 7. Requirement of the first 150 amino acids of MR for the aldosterone-stimulated shift in mobility.
Cos-7 cells were transiently transfected with 500 ng of MR or MR N-terminal deletion mutants. For 20 hours prior to harvesting cells were treated with or without 1 μM aldosterone or 1 μM MG132, as indicated. Whole cell extract (50 μg) for each condition was resolved on a 8% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blots shown are representative of three independent experiments. The lower blot represents a darker exposure of the four lanes depicted above it.


MR
MR 51C
MR 101C
MR 151C
MR Δ100-150
Aldosterone - + - + - + - + - + - + - + - + - + - + - +
MG132 - - + + - - + + - - + + - - + + - - + +
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

residues 100-150 (MR Δ100-150) displayed an intermediate phenotype of mobility shift similar to MR 51C; however, MR Δ100-150 was also shifted upon treatment with MG132 alone, unlike either wildtype MR or the other N-terminal deletion constructs.

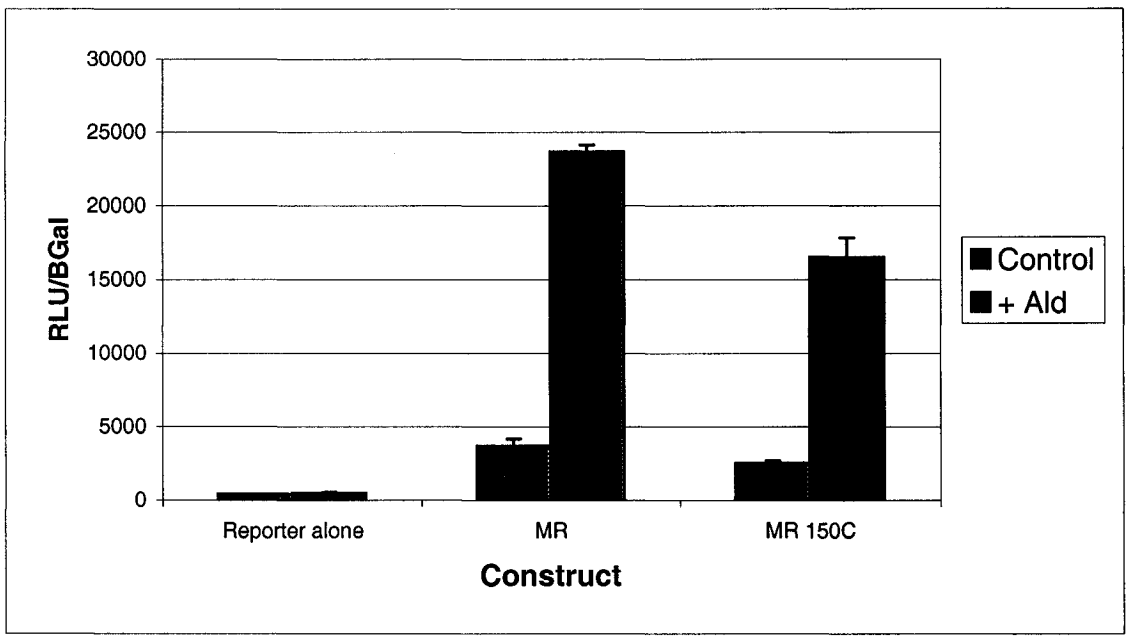
Since deletion of the first 150 amino acids of MR abolished the aldosterone-dependent shift in mobility, I examined whether the lack of shift in mobility of MR 150C affected the ability of MR to activate transcription. MR and MR 150C were transiently transfected into Cos-7 cells along with the reporter MMTV-237 Luc (the mouse mammary tumour virus long terminal repeat promoter from -237 to 105 fused to a luciferase reporter gene). As illustrated in Figure 8, upon aldosterone treatment MR 150C displayed decreased transcriptional activation (as measured by relative light units standardized by β-galactosidase activity) compared to wildtype MR (panel A), and when expressed as fold induction by aldosterone, transfection of wildtype MR activated transcription 49 fold versus 34 fold activation when MR 150C was transfected. Therefore, MR 150C displayed decreased transactivational activity compared to wildtype MR on the MMTV promoter.

Time course of MR shift in mobility in response to 0-24 hours aldosterone treatment

In order to characterize the time course of the aldosterone-stimulated shift in MR mobility, Cos-7 cells transiently transfected with MR were treated with or without aldosterone and MG132 for either 4, 8 or 24 hours. From Figure 9 it can be seen that the aldosterone-stimulated shift in MR mobility was visible at the 4 hour time point and while it appeared that there were intermediate shifted species of MR, a large proportion of MR appeared to be completely shifted. By the 24 hour timepoint it was difficult to detect MR upon treatment with aldosterone alone; in contrast, while there appeared to be

Figure 8. MR 150C exhibited decreased transactivation activity compared to wildtype MR.
Cos-7 cells were transiently co-transfected with 200 ng pMMTV-237 Luc, 100 ng pRSV β-Gal and 300 ng MR or MR 150C. For 20 hours prior to harvesting cells were treated with or without 1 μM aldosterone. Cytoplasmic extracts were prepared followed by measurement of luciferase activity and β-Galactosidase activity. (A) The transcriptional activation in each condition is expressed as relative light units standardized by the β-Galactosidase activity and depicted by bar graph. (B) The fold induction by aldosterone versus reporter alone was calculated for each construct and depicted by bar graph.

A.


30000
25000
20000
15000
10000
5000
0
RLU/BGal
Control
+ Ald
Reporter alone
MR
MR 150C
Construct

B.

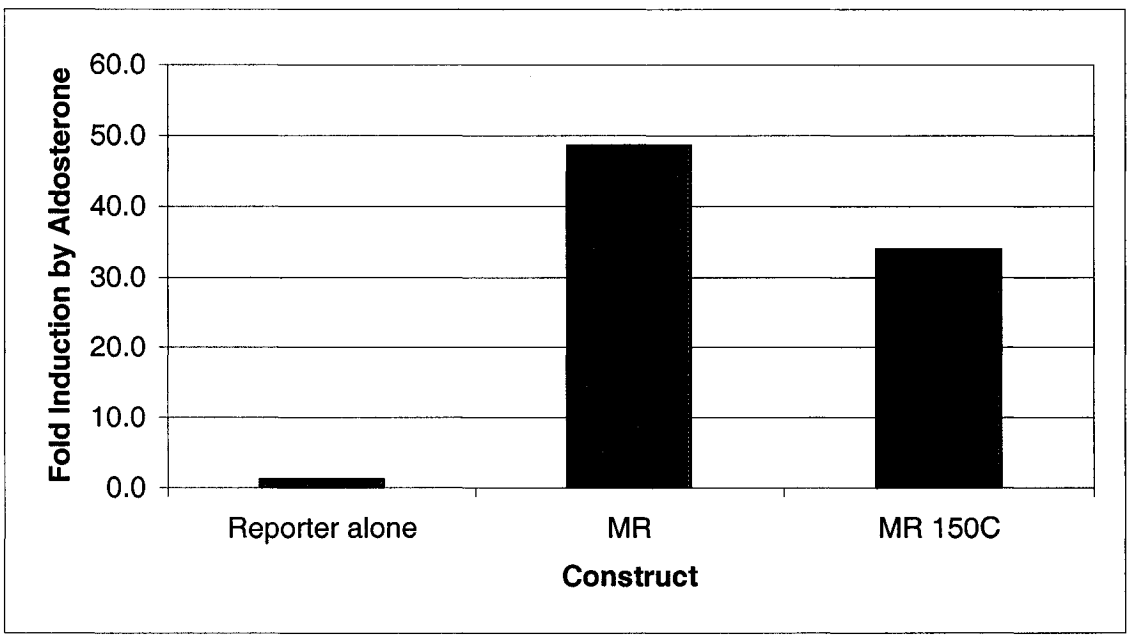
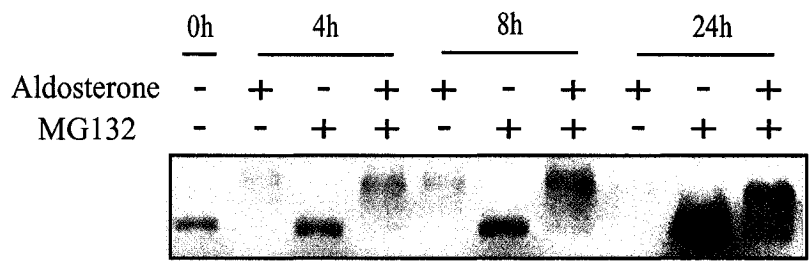

60.0
50.0
40.0
30.0
20.0
10.0
0.0
Fold Induction by Aldosterone
Reporter alone
MR
MR 150C
Construct

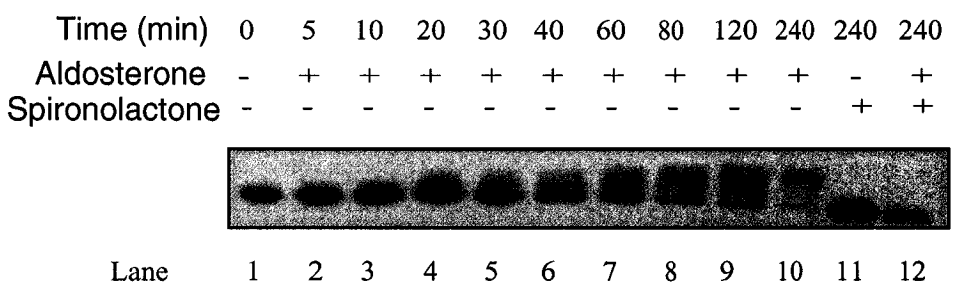
Figure 9. Time course of aldosterone and MG132 treatment.
Cos-7 cells were transiently transfected with 500 ng of wildtype MR. For 4, 8 or 24 hours prior to harvesting cells were treated with or without 1 μM aldosterone or 1 μM MG132, as indicated. Whole cell extract (50 μg) for each condition was resolved on a 6% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blot shown is representative of three independent experiments.


0h
4h
8h
24h
Aldosterone - + - + + - + + - +
MG132 - - + + - + + - + +

a slight stabilization of shifted MR in the MG132 + aldosterone condition at the 8 hour time point, the stabilizing effects of MG132 treatment did not significantly increase MR protein levels until the 24 hour timepoint, in both the presence and absence of aldosterone.

A shorter time course of aldosterone treatment was performed in order to determine the length of treatment required to induce the shift in MR mobility. In addition, the effect of treatment with an MR antagonist, 1 μM spironolactone, and a condition in which Cos-7 cells were treated with both 1 μM aldosterone and 100 μM spironolactone were included in the experiment. Figure 10 illustrates that after 20 minutes aldosterone exposure, intermediate forms of shifted MR species were detected. By 40 minutes aldosterone treatment many intermediate shifted forms were detected as visualized by the smearing above MR that was not shifted. Between 40 and 120 minutes aldosterone treatment (lanes 6-9) the proportion of shifted MR increased and at 240 minutes the majority of MR species detected were completely shifted (lane 10). In contrast, treatment with spironolactone for 240 minutes did not result in a shift in MR mobility (lane 11); furthermore, upon treatment with 100 fold spironolactone compared to aldosterone for 240 minutes (lane 12), the aldosterone-induced degradation of MR was observed but the shift in MR mobility was not observed indicating that spironolactone was able to effectively compete with aldosterone and inhibited the shift in MR mobility.

Figure 10. Time course of agonist-dependent MR shift in mobility.
Cos-7 cells were transiently transfected with 500 ng of MR. For increasing lengths of time between 0 and 240 minutes cells were treated with either 1 μM aldosterone (lanes 2-10), 1 μM spironolactone (lane 11) or 1 μM aldosterone and 100 μM sprironolactone (lane 12). Whole cell extract (50 μg) for each condition was resolved on a 6% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blot shown is representative of three independent experiments.


Time (min) 0 5 10 20 30 40 60 80 120 240 240 240
Aldosterone - + + + + + + + + + - +
Spironolactone - - - - - - - - - - + +
Lane 1 2 3 4 5 6 7 8 9 10 11 12

Cortisol, but not dexamethasone, stimulated the shift in MR mobility

Given that spironolactone did not induce a shift in MR mobility, the effects of the steroid hormones cortisol, the natural ligand for GR, and dexamethasone, a synthetic analog of cortisol, on the shift in MR mobility were examined. Both cortisol and dexamethasone have been shown to bind MR with approximately equal affinity as aldosterone (7). It can be seen in Figure 11A that whereas aldosterone induced a complete shift in MR mobility after 20 hours treatment, treatment with cortisol for the same length of time resulted in many intermediate shifted forms of MR. It appeared that after 20 hours cortisol treatment the majority of MR species were not shifted which is more similar to a shorter aldosterone treatment (ie. 40-60 minutes, depicted in Fig. 10, lanes 6 and 7). In sharp contrast, treatment with dexamethasone did not result in a mobility shift of MR. Figure 11B illustrates that none of the three steroid hormones induced a shift in mobility of GR; however, all three stimulated degradation of GR.

Decreased nuclear occupancy decreased the proportion of MR that exhibited the shift in mobility

In order to determine whether nuclear occupancy of MR was important to the aldosterone-stimulated shift in MR mobility, an MR construct lacking the nuclear localization signal NL1 (MR NL1-) was employed. MR NL1- has been shown to have significantly reduced steroid-dependent nuclear translocation as compared to wildtype MR (C. Bayer, unpublished data). Figure 12 illustrates that the proportion of MR NL1- that was shifted upon aldosterone treatment is decreased compared to wildtype MR. The majority of wildtype MR detected was completely shifted following aldosterone

Figure 11. Aldosterone and cortisol, but not dexamethasone, stimulated the shift in MR mobility.
Cos-7 cells were transiently transfected with 500 ng of MR or GR. For 20 hours prior to harvesting cells were treated without (NT) or with 1 μM aldosterone, 1 μM cortisol, or 1 μM dexamethasone, as indicated. Whole cell extract (50 μg) for each condition was resolved on a 8% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blots shown are representative of three independent experiments.

A.

B.

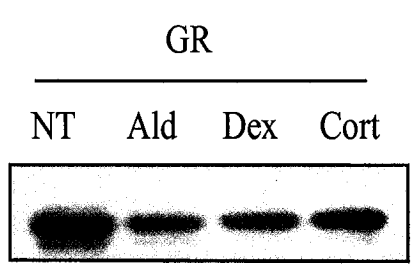
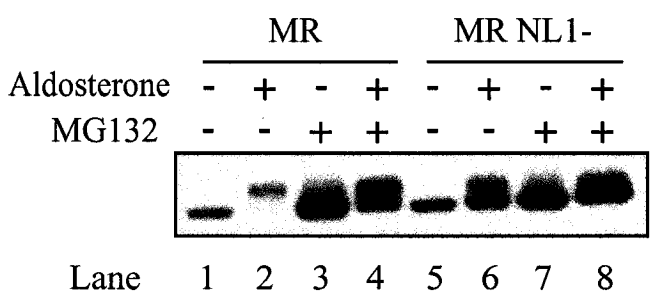


Figure 12. The proportion of MR NL1- that exhibits the aldosterone-stimulated shift in mobility is decreased compared to wildtype MR.
Cos-7 cells were transiently transfected with 500 ng of MR or MR NL1-. For 20 hours prior to harvesting cells were treated with or without 1 μM aldosterone or 1 μM MG132, as indicated. Whole cell extract (50 μg) for each condition was resolved on an 8% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blot shown is representative of three independent experiments.


MR
MR NL1-
Aldosterone - + - + - + - +
MG132 - - + + - - + +
Lane 1 2 3 4 5 6 7 8

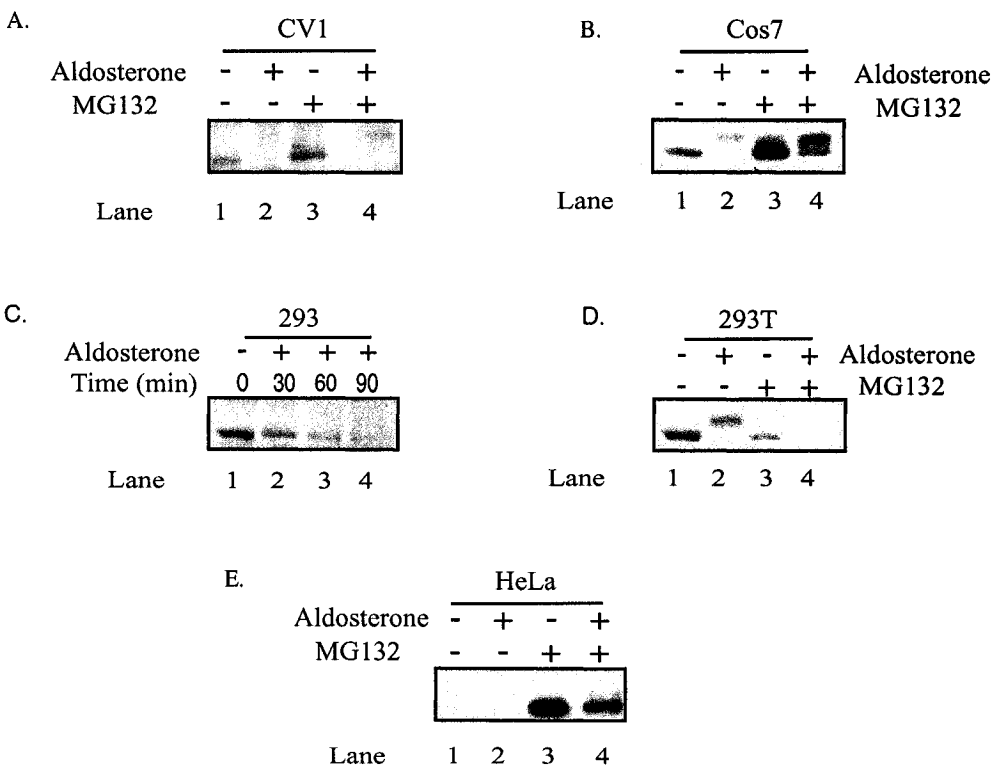
treatment (lane 2); in contrast, a larger proportion of MR NL1- was not shifted while some MR NL1- species were partially shifted (lane 6). The same trend appeared following treatment with MG132 + aldosterone: the majority of MR detected was completely shifted with some MR species only partially shifted, whereas the majority of MR NL1- was not shifted with some MR NL1- species partially shifted. This result suggests that the degree of nuclear occupancy affected the proportion of MR that exhibited the aldosterone-stimulated shift in mobility.

The shift in MR mobility was dependent upon cell line employed

In order to determine whether the aldosterone-stimulated shift in MR mobility was observable in kidney cell lines other than Cos-7 cells, MR was transiently transfected into other cell lines and the mobility of MR was assessed by Western blotting. CV-1 cells, the parent cell line of Cos-7 cells which, unlike Cos-7 cells, are not transformed with the SV40 large T antigen were examined (Fig. 13A). As illustrated in Figure 13A, it was difficult to detect MR following aldosterone treatment in CV-1 cells and although there may be a hint of a shift in mobility visible in the MG132 + aldosterone condition (panel A, lane 4), a significant shift in MR mobility, such as that observed in Cos-7 cells (panel B, lanes 2 and 4), was not detected in CV-1 cells. Interestingly, treatment with MG132 did not increase MR protein levels in CV-1 cells to the same extent as observed in Cos-7 cells.

In order to investigate the status of the aldosterone-stimulated shift in MR mobility in other kidney cell lines, the human embryonic kidney cell lines, 293 and 293T cells were employed. Similar to Cos-7 cells, 293T cells are the SV40 large T antigen-

Figure 13. Examination of the aldosterone-stimulated shift in MR mobility in various cell lines.
CV1 (A), Cos-7 (B), 293 (C), 293T (D) and HeLa (E) cells were transiently transfected with 500 ng MR. For 20 hours (panels A, B, D and E) prior to harvesting cells were treated with or without 1 μM aldosterone or 1 μM MG132, as indicated. 293 cells (panel C) were treated with 1 μM aldosterone for 30, 60 and 90 min, as indicated, and harvested for Western blotting. Whole cell extract (50 μg) for each condition was resolved on an 8% gel by SDS-PAGE, transferred to PVDF and probed with the FiGR primary antibody (1:400). The blots shown are representative of three independent experiments.


A.
CV1
Aldosterone - + - +
MG132 - - + +
Lane 1 2 3 4
B.
Cos7
- + - + Aldosterone
- - + + MG132
Lane 1 2 3 4
C.
293
Aldosterone - + + +
Time (min) 0 30 60 90
Lane 1 2 3 4
D.
293T
- + - + Aldosterone
- - + + MG132
Lane 1 2 3 4
E.
HeLa
Aldosterone - + - +
MG132 - - + +
Lane 1 2 3 4

transformed version of 293 cells. Perhaps due to cell toxicity, MG132 did not stabilize MR protein levels in either 293 (data not shown) or 293T cells (Fig. 13D); in fact, as illustrated in Figure 13D, MG132 reduced our ability to detect MR. In the case of 293T cells, MR was detectable following 20 hours aldosterone treatment (panel D, lane 2); however, 293 cells do not transfect as well as 293T cells and therefore MR was not detectable following 20 hours aldosterone treatment (data not shown). For this reason, a short time course of aldosterone treatment from 30-90 minutes was employed in 293 cells in order to detect MR before it was completely degraded (Fig. 13C). As described previously, the shift in MR mobility occurred within 30 minutes of aldosterone treatment in Cos-7 cells (Fig. 10) and therefore we expect that if the mobility of MR were shifted in 293 cells, we would detect the shift in mobility following the short aldosterone treatments employed. As illustrated in Figure 13, while MR was completely shifted in 293T cells following 20 hours aldosterone treatment (panel D, lane 2), no shift in MR mobility was detected following aldosterone treatment in 293 cells (panel C, lanes 2-4). These results are similar to those obtained from CV-1 and Cos-7 cells in that the parent cell lines (CV-1 and 293) which are not transformed with the SV40 large T antigen, did not exhibit a shift in MR mobility following aldosterone treatment. The aldosterone-stimulated mobility shift of MR was assessed in the cervical carcinoma cell line, HeLa (Fig. 13E), which are also not transformed with the SV40 large T antigen. Although MR could not be detected in the absence of MG132, it was clear that upon treatment with MG132 + aldosterone (panel E, lane 4) no shift in MR mobility occurred. The combination of results from the 5 cell lines examined suggests that the SV40 large T antigen may play a role in the aldosterone-stimulated shift in MR mobility.

Discussion

The characterization of MR has lagged behind that of all other members of the steroid hormone receptor family. This thesis addressed a key regulatory mechanism for transcription factors such as MR, their turnover. Degradation of SHRs enables the cell to prevent overstimulation by hormones as well as to maintain a primed state allowing a quick, efficient transcriptional response to hormonal stimuli. Ligand-dependent degradation by the 26S proteasome has been demonstrated for GR, PR, ER and AR (97-100). The results presented here indicate that MR is susceptible to ligand-dependent degradation by the 26S proteasome. In addition, two other aspects of degradation were identified: ligand-independent degradation by the 26S proteasome and ligand-dependent degradation not mediated by the 26S proteasome.

The comparison of MR and GR proteins levels following treatment with MG132 in the absence of hormone revealed that, unlike MR, GR protein levels were not stabilized by MG132. This result concurs with the previous study by Wallace and Cidlowski where they observed that GR was not stabilized by MG132 in the absence of hormone (99). However, my results differ from the previous results in that I observed that GR, like MR, was susceptible to degradation in the presence of hormone that was not blocked by treatment with MG132. In contrast, Wallace and Cidlowski reported that treatment with MG132 completely stabilized GR in the presence of hormone. This difference could be due to the fact that they pretreated cells with MG132 for 1 hour followed by 12 hours hormone treatment, whereas I treated cells with MG132 and hormone simultaneously for 20 hours. Wallace and Cidlowski also identified a PEST motif within the N-terminus of GR and observed that ligand-dependent degradation of

GR was abolished upon mutation of the lysine residue within the PEST motif (99). A PEST motif in the hinge region of MR was identified in our lab. However, an MR construct with the lysine residue within the PEST motif mutated exhibited the same pattern of degradation as wildtype MR, indicating that the mutated lysine is not involved in ligand-dependent downregulation of MR (Y. Rouleau, unpublished data).

I established that the N-terminus of MR appeared to be involved in all three modes of degradation; however, delimiting the precise regions involved in the three modes of degradation proved to be difficult using our deletion construct approach. I hypothesize that the MR N-terminal deletion constructs perturbed the secondary and tertiary structure of MR to such an extent that normal receptor function was affected. Therefore, interpretation of the significance of the results using the MR N-terminal deletion constructs was difficult. In an attempt to preserve more of the overall structure of the MR N-terminus we created smaller, internal deletions within amino acids 450 and 602. Examination of the protein levels of these MR constructs revealed two regions that appeared to confer sensitivity to aldosterone-stimulated degradation. Amino acids 450 to 590 were required for degradation in the presence of aldosterone mediated by the 26S proteasome (ie. MG132-sensitive). Amino acids 590-602 were required for degradation in the presence of aldosterone not mediated by the 26S proteasome (ie. MG132-insensitive). In contrast, while it appeared that amino acids 450 to 602 were involved in aldosterone-independent degradation by the 26S proteasome (ie. MG132 sensitive), smaller, internal deletions between amino acids 450 and 602 did not resolve the exact determinants of MG132 sensitivity in the absence of hormone. The complicated nature

of MG132 sensitivity in the absence of hormone suggests that it was influenced by the overall structure of the N-terminus of MR.

A possible explanation for the unexpected behaviour of the MR deletion constructs is that the deletion interfered with proper folding of the receptor. All the MR constructs examined, with the exception of MR 604C, were stabilized by MG132 and therefore it was difficult to resolve the region of the N-terminus required for MG132 sensitivity. Given that improperly folded proteins are degraded by the 26S proteasome (86), if the deletions affected proper folding then treatment with MG132 would stabilize those receptor species regardless of whether the region that conferred sensitivity to MG132 in the wildtype receptor was present in the deletion construct.

In addition to examining the aspects of MR of degradation, it was observed that aldosterone treatment following transient expression of MR in Cos-7 cells induced the appearance of higher molecular weight forms of the receptor. Following 20 hours aldosterone treatment the majority of MR species detected by Western blotting appeared to be approximately 16 kDa larger than untreated MR. In addition, treatment with both aldosterone and MG132 resulted in intermediate shifted forms of MR. I hypothesize that these aldosterone-stimulated higher molecular weight forms of MR were as a result of post-translational modification(s) of the receptor.

As outlined in the introduction, steroid hormone receptors undergo a variety of post-translational modifications. Ubiquitination was a likely candidate modification of MR given that I demonstrated that MR is degraded through the ubiquitin-proteasome pathway. In addition, ubiquination of other SHRs, such as GR, AR and ER, has been demonstrated (99,105,106). However, ubiquitination of GR, AR and ER resulted in the

formation of the characteristic laddering pattern of higher molecular weight species up to at least 180 kDa, which is not consistent with the pattern observed for MR. Furthermore, mutation of the lysine residue within the PEST site identified in MR did not abolish the appearance of the aldosterone-stimulated shift in MR mobility (Y. Rouleau, unpublished data), suggesting that the shift is not a result of ubiquitination at that lysine.

Acetylation is another possible modification, as AR and ER have been shown to be acetylated by the coactivator p300. However, in the AR and ER studies, acetylation could not be directly visualized as a mobility shift on an SDS-PAGE gel. In contrast, the higher molecular weight forms of MR observed were easily detected on a Western blot suggesting that they are not the result of an acetylation event.

Sumoylation of MR is another candidate modification, as AR and GR have been shown to be sumoylated (109-111); in addition, four sumoylation motifs are present in the N-terminus of MR. However, visualization of sumoylation of AR and GR by Western blotting required co-transfection with a SUMO-1 expressing vector and treatment with a de-sumoylation inhibitor during harvesting of cell extracts. Neither of these techniques was required in order to visualize the shift in MR mobility. Furthermore, each sumoylation event of AR or GR resulted in an incremental increase in molecular weight and was visualized as a distinct band (109,111). While the highest molecular weight form of MR detected was approximately 16 kDa larger, which could be consistent with one sumoylation event, the intermediate isoforms detected following MG132 and aldosterone treatment would not be consistent with sumoylation.

Phosphorylation is the most likely candidate modification given that SHRs are phosphoproteins that are hyperphosphorylated following hormone treatment (112).

Furthermore, PR exhibits a mobility shift on an SDS-PAGE gel associated with ligand-induced phosphorylation (98). It was determined that deletion of the region between amino acids 590 and 602 of the MR N-terminus significantly reduced, if not abolished, the appearance of the aldosterone-stimulated higher molecular weight forms. Interestingly, this twelve amino acid stretch contains five serines and one threonine, which could serve as sites of phosphorylation. Preliminary results in our lab suggested that a MR construct with serines 594, 597, 598 and threonine 595 mutated to alanines exhibited the aldosterone-stimulated higher molecular weight forms similar to wildtype MR, indicating that phosphorylation of those residues was not involved in the MR shift in mobility. However, these results were inconclusive because mutation of those serine and threonine residues appeared to destabilize the MR Ser/Thr-Ala construct to such an extent that it was difficult to detect by Western blotting (A. Edgecombe, unpublished data).

The other region of MR that was demonstrated to be required for the aldosterone-stimulated mobility shift was the first 150 amino acids of the N-terminus. If the shift in mobility was the result of a modification of MR, it is possible that the modifier is a cofactor that binds MR, similar to the mechanism by which p300 binds and acetylates AR and ER {Fu, 2000 #127;Wang, 2001 #129}. This phenomenon could explain why two distinct regions of the MR N-terminus were required for the shift in mobility. Perhaps amino acids 1 to 150 acted as a binding pocket for the cofactor which modified MR downstream between amino acids 590 and 602. This hypothesis would be consistent with the observation that amino acids 1 to 150 were required for the shift in mobility; however smaller deletions within that region did not abolish the shift, but rather altered the proportion of shifted MR. The smaller deletions may have inhibited binding of the

cofactor to different extents, but not completely abrogated cofactor binding. Interestingly, amino acids 1 to 169 of MR have been shown to recruit a multiprotein complex containing RNA helicase A (RHA) and CBP in an aldosterone-dependent manner (35). Therefore, modification of MR by RHA, CBP or one of the other proteins in the multiprotein complex recruited to this region of MR is a distinct possibility.

Deletion of the first 150 amino acids of MR moderately decreased receptor transactivation on the MMTV promoter. This decreased transactivation could be a result of the lack of aldosterone-stimulated shift in MR mobility; however, as discussed in the introduction, that region has been reported by one group (36), in constrast with another group (33), to possess an activation function (AF-1a). The other region of the MR N-terminus required for modification, amino acids 590 to 602, was also reported by the first group to be a part of an activation function (AF-1b). Therefore, the effect of the shift in MR mobility on receptor transactivation could not be separated from putative activation functions. If future characterization of the shift in MR mobility reveals a specific amino acid that is required, then site-directed mutagenesis could be employed in order to generate an MR construct with which to gauge the importance of the shift in MR mobility on receptor-mediated transactivation.

The aldosterone time course experiment revealed that the hypothesized modification of MR is a progressive event which was first detected after 20 minutes of aldosterone treatment. Increasing the length of aldosterone treatment up to 2 hours resulted in the detection of higher and higher molecular weight forms of MR. The pattern observed following 4 to 24 hours aldosterone treatment suggests that modified MR may be more stable than unmodified MR, since the majority of MR detected at these time

points was the highest molecular weight species, approximately 16 kDa larger than untreated MR. The observation that MR NL1-, a construct with decreased steroid-induced nuclear occupancy, exhibited a decreased proportion of shifted MR species compared to wildtype MR, suggests that the modification of MR is a nuclear event perhaps mediated by a cofactor localized in the nucleus.

The ability of the MR antagonist, spironolactone, to induce the shift in MR mobility was examined. It has been demonstrated that, upon binding spironolactone, MR translocates to the nucleus and interacts with hormone response elements; however it does not activate transcription efficiently because spironolactone induces a transcriptionally silent conformation (40,118). Therefore, the observation that treatment with spironolactone did not induce the appearance of the higher molecular weight forms suggests that the MR shift in mobility was independent of DNA binding and indicates that receptor conformation may have an impact on the shift.

I demonstrated that treatment with the glucorticoid, cortisol, but not dexamethasone, a synthetic analog of cortisol, resulted in the appearance of the shifted forms of MR; furthermore, treatment with cortisol resulted in a smaller proportion of MR species at the higher molecular weights than treatment with aldosterone. Whereas cortisol and aldosterone are metabolized, dexamethasone is not, and this may have been involved in the inability of dexamethasone to stimulate the MR shift in mobility. However, it is more likely that, similar to spironolactone, each agonist induced a different conformation of MR which affected the formation of the higher molecular weight forms.

Receptor conformation can influence the ability of the receptor to recruit cofactors as well as the type of cofactors recruited. Interestingly, two recent studies

suggest that the MR agonists, aldosterone and cortisol, differentially control receptor function. Firstly, the interaction between the N- and C-terminus of MR in a mammalian two-hybrid assay was observed to be induced strongly by aldosterone, weakly by cortisol and inhibited by the antagonist, spironolactone (49). Intriguingly, these observations closely parallel the results observed concerning the appearance of the higher molecular weight forms of MR. Secondly, aldosterone, but not cortisol, was observed to stimulate MR recruitment of RHA and CBP to native MR target gene promoters through the AF-1a region of MR (35). Both of these studies suggest that MR agonists and antagonists differentially regulate MR function and therefore might differentially stimulate the shift in MR mobility.

Altered receptor conformation may also explain the unexpected observation that MR Δ100-150 exhibited the higher molecular weight forms following treatment with MG132 in the absence of aldosterone. For all other MR constructs that exhibited the shift in MR mobility, treatment with aldosterone was a requirement. It may be that removal of amino acids 100 to 150 removed the requirement for aldosterone such that the higher molecular weight forms were visualized following treatment with MG132. MGG, a chimeric receptor construct consisting of the MR N-terminus fused to the GR DBD and LBD, exhibited a similar pattern whereby the higher molecular weight forms were detected following treatment with the hormone cortisol but also upon treatment with MG132 in the absence of hormone. This result indicates that the shift in MR mobility is a property of the N-terminus of MR which can be transferred to the MR/GR chimeric receptor, MGG. The observation that treatment with cortisol is not an absolute requirement for detection of the higher molecular weight forms of MGG leads to the

hypothesis that, similar to MR Δ100-150, MGG adopts a conformation which relieves the hormone-dependent property of the shift in mobility.

The last aspect of the MR shift in mobility to be examined was the dependence on the cell line employed. In the parent cell line, CV-1, it was difficult to detect MR. However, in CV-1 cells no significant aldosterone-stimulated shift in MR mobility was observed, in contrast to the SV40 large T antigen-transformed daughter cell line, Cos-7. Similarly, in the parent cell line, 293, no aldosterone-stimulated shift in MR mobility was observed, in contrast to the SV40 large T antigen-transformed daughter cell line, 293T. In addition, no aldosterone-stimulated shift in MR mobility was detected in the cervical carcinoma HeLa cell line, which is not transformed with the SV40 large T antigen. While the absence of the aldosterone-stimulated higher molecular weight forms of MR in CV-1, 293 and HeLa may be due to lower MR expression levels as compared to Cos-7 and 293T cells, it is also possible that the presence of the SV40 large T antigen was required for or enhanced the MR shift in mobility. Future experiments in which TAg and MR are co-transfected into CV-1 and 293 cells followed by aldosterone treatment and assessment of the MR shift in mobility would determine the significance of TAg in stimulating the higher molecular weight forms.

The SV40 large T antigen (TAg) is a powerful oncoprotein that transforms a variety of cell types through inactivation of the tumor suppressors, p53 and Rb (119). Interestingly, TAg has also been shown to bind p300/CBP. Although TAg has been demonstrated to inhibit the transcriptional activity of p300/CBP on certain promoters (120), the molecular mechanism of inhibition is unknown. Given that p300/CBP is a coactivator for SHRs, it is possible that a TAg-p300/CBP interaction in Cos-7 and 293T

cells altered the composition of the cofactor complexes recruited to aldosterone-bound MR and stimulated the modification of MR. Therefore, the aldosterone-stimulated higher molecular weight forms of MR could be a cell culture artifact. However, a cell transformed with TAg could also mimic a pathophysiological state such a carcinoma. Examination of MR in renal carcinoma cell lines, such as 786-0, following aldosterone treatment could reveal similar higher molecular weight forms of the receptor.

Conclusion

The results presented here examined three aspects of MR degradation: (1) ligand-dependent degradation by the 26S proteasome, (2) ligand-independent degradation by the 26S proteasome and (3) ligand-dependent degradation not mediated by the 26S proteasome. It was demonstrated that amino acids 450 to 602 within the N-terminus of MR were involved in all three modes of degradation. In addition, aldosterone-stimulated higher molecular weight forms of MR were identified and two regions within the N-terminus of MR, specifically residues 1 to 150 and 590 to 602, were shown to be required for the shift in mobility. Furthermore, ligand-dependence, length of ligand treatment, nuclear occupancy and cell type specificity were examined in their contribution to the shift in MR mobility.

Examination of the degradation of MR is crucial to understanding receptor function. Whereas GR and other SHRs are degraded in a ligand-dependent manner, it was demonstrated that MR is also susceptible to ligand-independent degradation by the 26S proteasome. In addition, MR, but not GR, exhibited agonist-stimulated higher molecular weight forms. These higher molecular weight forms of MR may be a means of conferring receptor specificity in tissues where MR and GR are co-expressed and interact

with the same hormone response elements. Furthermore, the observation that aldosterone and cortisol differentially stimulated the shift in MR mobility may be of significance in non-epithelial tissues such as the heart and brain, where MR is not protected from saturation by cortisol by the actions of 11βHSD2. In conclusion, research into the mechanisms mediating differential regulation of MR is of fundamental importance in order to understand the final transcriptional response and the physiological roles of MR.

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Appendices

Appendix 1: Primers

KpnI BuGR MR 604 Fwd:

5' GGG GTA CCA TGT CAG TGT TTT CTA ATG GGT ACT CAA GCC CTG GGA TGA GAC CAG ATG TAA GCT GTT TGG TGT GTG GAG ATG AGG C 3'

BamHI MR 981 Rev:

5' CGG GAT CCT CAC TTT CTG TGA AAG TAA AG 3'

KpnI BuGR MR 151 Fwd:

5' TTG GTA CCA TGT CAG TGT TTT CTA ATG GGT ACT CAA GCC CTG GAA TGA GAC CAG ATG TAA GCG CTA GCG ATA TCT GCG GCC GCT CCT CCA CAC TGA GTG C 3'

SmaI MR 981 Rev:

5' GTA CCC GGG TCA CTT TCT GTG AAA GTA AAG GGG 3'

NotI MR 981 Rev:

5' TAT GCG GCC GCT CAC TTT CTG TGA AAG TAA AGG GG 3'

NheI MR 51 Fwd:

5' CTG GCT AGC GTT TCC GGT GCT ATT CCG 3'

NheI MR 101 Fwd:

5' CTG GCT AGC GTA GCT GAG TCC ATG GG 3'

NheI MR 5' Fwd:

5' CTG GCT AGC ATG GAA ACC AAA GGC TAC 3'

EcoRV MR 100 Rev:

5' CTG GAT ATC CGT GGC TGA AAG TTC C 3'

EcoRV MR 151 Fwd:

5' CGT GAT ATC TCC TCC ACA CTG AGT G 3'

EcoRV MR 450 Rev:

5' CTG GAT ATC TGG GTT GAC CGT GGG GTT C 3'

EcoRV MR 500 Fwd:

5' CTG GAT ATC GGG AGC TAT TAC CCT G 3'

EcoRV MR 550 Fwd:

5' CTG GAT ATC TTC CCA CCT GTC AAT AC 3'

Appendix 2: Sources of Chemicals, Reagents and Other Materials

Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Burlington, ON)

Fetal Bovine Serum (FBS lot#100201, HyClone, Logan, UT)

Lipofectamine (Invitrogen, Burlington, ON)

Charcoal-stripped Fetal Bovine Serum (SFBS lot#100201, HyClone, Logan, UT)

ExGen (Fermentas, Burlington, ON)

FuGene (Roche, Laval, QC)

MG132 (Sigma, Mississauga, ON)

Lactacystin (Sigma, Mississauga, ON)

Aldosterone (Sigma, Mississauga, ON)

Cortisol (Sigma, Mississauga, ON)

Bio-Rad Protein Assay Dye Reagent (BioRad, Mississauga, ON)

Immunoblot PVDF membranes (BioRad, Mississauga, ON)

Sheep anti-mouse secondary (Amersham Biosciences, Baie d'Urfe, QC)

Western Lightining Chemiluminescence Reagent (Enhanced Luminol) (PerkinElmer Life Sciences Inc., Boston, MA, USA)

JL-8 GFP primary antibody (Clontech, Mississauga, ON)

Reporter lysis buffer (Promega, Madison, WI, USA)

Appendix 3: Solution Formulations

WCE: 150mM NaCl, 1mM EDTA, 50mM HEPES pH 7.4, 10% glycerol, 0.5% Nonidet P40 (NP-40), 20mM molybdate, 1mM dithiothreitol and 1X protease inhibitor cocktail (Roche, Laval, QC)

Z buffer: 60mM Na_2PO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM Mg_2SO_4, 50mM β-Mercaptoethanol

ONPG: 4mg/mL ONPG in 100mM phosphate buffer (0.1M KH_2 pH 7.2, 0.15M NaCl)

GTE: 50mM glucose, 10mM EDTA, 25mM Tris pH 8.0

Lysis solution: 0.2M NaOH, 1% SDS

Potassium acetate solution: 60mL 5M KOAc, 11.5 mL glacial acetic acid, 28.5mL ddH_20

Katy Swan

Curriculum Vitae

Education

2001-present: 2nd year Biochemistry Master's student at the University of Ottawa
Recipient of Ontario Graduate Scholarship (2001-2003), University of Ottawa Excellence Scholarship (2001-2003) and Strategic Areas of Development Award (2001-2002)

1997-2001: Queen's University, Honours Biochemistry Graduate
Average in Fourth Year: 87.5%, Dean's Scholar
Recipient of the Eric Horsey May Scholarship (1997-98), the Ottawa Ladies' College Scholarship (2000-01) and the Society of Chemical Industry Student Merit Award (2001)

1992-1997: Glebe Collegiate Institute. OAC Average: 93%.

Related Work Experience

2001-present **Graduate Studies in Biochemistry**
The focus of my Master's thesis was to identify degradation signal(s) within a steroid hormone receptor, the mineralocorticoid receptor. Through the course of my Master's I gained valuable experience in self-directed research as well as the ability to critically analyze scientific publications. I became very familiar with techniques such as mammalian cell tissue culture, SDS-PAGE, Western blotting, transcriptional assays, (co)immunoprecipitation, GST pull-downs, cloning techniques (PCR, restriction enzyme digests, ligations, agarose gel electrophoresis, manual DNA sequencing, etc), as well as many other basic laboratory techniques.

2000-2001 **4th year Biochemistry Honours Student at Queen's University**
My first term was spent examining the ability of calpain, a Ca^{+2}-dependent cysteine protease, to bind phospholipid vesicles, as measured by light scattering. My second term project examined the role of the putative Stat3 response elements in the hepatocyte growth factor (HGF) promoter region. I gained valuable experience in generating site-directed mutants and examining their role in transcription using luciferase assays.

Summer 2000 **Summer Student- Ottawa Health Research Institute**
My project involved screening a cDNA library to identify proteins which interact with the glucocorticoid receptor DNA binding domain. Techniques I was exposed to included Far Westerns and radio-labelled probe preparation.

Summer 1998 **Summer Student- Agriculture and Agri-Food Canada**
1996 –1997 **Co-op Placement: Lab Technician - Agriculture and Agri-Food Canada**
Skills acquired: plant tissue culture, agrobacterium-mediated transformations, plant/greehouse maintenance

Other Work Experience

Summer 1999 Data Administrator- Natural Sciences and Engineering Research Council
Summer 1997 Park and Pool Supervisor- City of Ottawa
Summer 1995-96 Park and Pool Programmer- City of Ottawa
Winter 1995- 1997 Ski Instructor

Volunteer Work

Let's Talk Science: Graduate students teaching highschool and elementary students (University of Ottawa)
Volunteer for Pediatric Ward (Child Life Program) at Kingston General Hospital
Exec Member of Kids 4 Kids- Queen's committee to raise money for the Child Life program at KGH
Key Club, a part of the Kiwanis organization in high schools

Katy Swan

Awards and Scholarhips

Post-secondary school

- Ontario Graduate Scholarship, 2001-2003
- University of Ottawa Excellence Scholarship, 2001-2003
- Strategic Areas of Development Award, 2001
- Society of Chemical Industry Student Merit Award: Awarded to the graduating student with the highest average in 4th Biochemistry, 2001
- Ottawa Ladies' College Scholarship: Queen's University, 2000
- Eric Horsey May Scholarship: Queen's University, 1997

Secondary School

- OAC Biology Academic Excellence Award: Awarded to highest ranking student in each OAC course, 1997
- Henry Birks & Sons Medal: Awarded to student who has contributed the most to the life and spirit of the graduating class, 1997
- Fellowship Award, 1997: Recognizes the Glebe student who has done social action work on behalf of the community within and beyond the school for humanitarian values
- Glebe Top Ten Students Award, 1994, 1996 & 1997
- Ottawa Board of Education Special Scholastic Attainment Silver Medal (over 90% average), 1994, 1996 & 1997
- Glebe Honours Society, 1993-1997
- Ontario Scholar

Other

- City of Ottawa Best Safety and Supervision Park (Brantwood Park), 1997
- City of Ottawa Best Water Play Instructor (Ottawa South-East), 1996
- Top I.I.T. (Instructor In Training) Award, Snowhawks Ski School

Other Qualifications

- National Lifeguard Service, 1995
- Canadian Ski Instructor's Alliance Level I
- Red Cross First Aid & CPR
- Outdoor Education Course

Other Achievements

- Queen's University Orientation Week:
  Co-ordinator (1999), Frosh leader (1998)
- Treasurer of the Grad Committee (1997)
- Head of the Fundraising Committee for the Glebe Kiwanis Club (1997)
- Sports
  Soccer (competitive)
  Ultimate Frisbee (university level)
  Downhill skiing
  Volleyball, Touch Football (recreational)
- Piano
  Royal Conservatory Grade 6

References

Available upon request.