REVIEW

# Mechanisms of ligand specificity of the mineralocorticoid receptor

**Peter J Fuller, Yizou Yao, Jun Yang** and **Morag J Young**

Prince Henry's Institute and the Monash University, Department of Medicine, PO Box 5152, Clayton, Victoria 3168, Australia
(Correspondence should be addressed to P J Fuller; Email: peter.fuller@princehenrys.org)

## Abstract

The mineralocorticoid receptor (MR) differs from the other steroid receptors in that it responds to two physiological ligands, aldosterone and cortisol. In epithelial tissues, aldosterone selectivity is determined by the activity of 11β-hydroxysteroid dehydrogenase type 2, while in other tissues, including the heart and regions of the central nervous system, cortisol is the primary ligand for the MR where it may act as an antagonist. Clinical trials have demonstrated the potential of MR antagonists in the treatment of cardiovascular disease, though their use has been limited by concurrent hyperkalaemia. In order to better target the MR, an understanding of the structural determinants of tissue- and ligand-specific MR activation is needed. Interactions of the MR have been identified, which exhibit ligand discrimination and/or specificity. These interactions include those of the ligand-binding domain with ligand, with the N-terminal domain and with putative co-regulatory molecules. Agonist and antagonist binding have been characterised using chimeras between the human MR and the glucocorticoid receptor or the zebra fish MR together with molecular modelling. The interaction between the N-terminus and the C-terminus is aldosterone dependent but is unexpectedly antagonised by cortisol and deoxycorticosterone in the human MR. Nuclear receptor-mediated transactivation is critically dependent on, and modulated by, co-regulatory molecules. Proteins that interact with the MR in the presence of either aldosterone or cortisol, but not both, have been identified. The successful identification of ligand-specific interactions of the MR may provide the basis for the development of novel MR ligands with tissue specificity.
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## Introduction

The mineralocorticoid receptor (MR) is arguably unique amongst the nuclear receptors in that it has two physiological ligands, aldosterone and cortisol (corticosterone in rodents). There is increasing evidence that the consequences of this binding to the MR are not equivalent for these ligands. Previous discussion of specificity at the MR (Fuller *et al*. 2000, Farman & Rafestin-Oblin 2001) has tended to focus on specificity between the MR and the closely related glucocorticoid receptor (GR), both of which can be activated by physiological glucocorticoids. In the intervening decade, there have been a number of studies that have not only highlighted the differences between aldosterone and cortisol, acting at the MR, but also identified mechanisms by which this specificity may be conferred. This review seeks to highlight the molecular mechanisms that may contribute to differential activation of the MR by cortisol and aldosterone (Fig. 1) and explores the structural basis of these differences. This question is much more than an exercise in molecular dissection in that discrimination of ligand-mediated effects is the basis for a number of current and emerging therapies directed at other members of the steroid receptor subfamily of the nuclear receptor superfamily. In the case of the MR, clinical trials have demonstrated the value of MR antagonists in the treatment of cardiac failure, but their use has been limited by concurrent hyperkalaemia. Understanding relevant mechanisms at the MR opens the possibility of novel therapeutic approaches, particularly in the area of cardiovascular disease.

## Pre-receptor mechanisms

The concept that access of ligands to their receptors may be regulated through pre-receptor mechanisms, including both activation and inactivation of the ligand, is well established in endocrinology. In the case of the MR, the work of both Edwards *et al*. (1988) and Funder *et al*. (1988) over 20 years ago clearly established that in epithelial tissues, access of aldosterone and a lack of access of cortisol to the MR were determined by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). These landmark studies resolved a longstanding conundrum in the field that the MR bound


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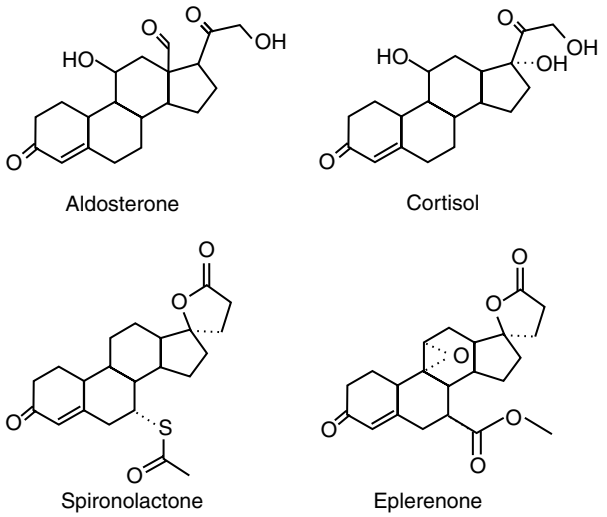


**Figure 1** Structures: chemical structures of the mineralocorticoid receptor agonists, aldosterone and cortisol and the antagonists, spironolactone and eplerenone.

cortisol with an affinity close to that of aldosterone, yet cortisol circulates at concentrations that are orders of magnitude higher than that of aldosterone. Even allowing for issues of plasma binding, the MR would, with normal cortisol levels, be permanently occupied by cortisol except perhaps at the nadir of the diurnal cycle. Subsequent studies have shown the intimate association in sodium-transporting epithelial tissues of 11β-HSD2 with the MR (Odermatt *et al.* 2001). 11β-HSD2 metabolises cortisol to cortisone (or corticosterone to 11-deoxycorticosterone in rodents), cortisone being unable to bind or activate the MR. Both the physiology and the pathophysiology of 11β-HSD2 have been extensively reviewed (see for instance Odermatt & Atanasov (2008)) and will not be further addressed in this review.

## Non-epithelial tissues

In the initial characterisation of receptors for the corticosteroids, the existence of two distinct receptors was clearly identified, and these were originally termed type 1 and type 2 receptors (Feldman *et al.* 1973). Subsequently, the type 1 receptor was identified as being the primary target for the mineralocorticoid hormone aldosterone and hence is now called the MR. To some extent, this nomenclature is unfortunate in the sense that although the role of aldosterone is arguable as implied by the term 'mineralocorticoid', i.e. the regulation of vectorial electrolyte transport, the role of the MR is much broader. The MR is widely distributed in a range of tissues, not only epithelial but also non-epithelial tissues (Fuller & Young 2005, Viengchareun *et al.* 2007). The function of the MR in many of these tissues remains to be determined. In some epithelial tissues such as lung and breast, it is not clear that its role relates specifically to electrolyte transport. In other tissues such as adipose tissue, where the receptor appears to play a fundamental role in adipocyte differentiation (Caprio *et al.* 2007), there is no suggestion that sodium transport is relevant. In the cardiovascular system, the MR is expressed not only in the cardiomyocytes but also in the endothelium and smooth muscle cells of the vasculature. In the vasculature, there is a co-expression of 11β-HSD2 but not in the cardiomyocyte (Fuller & Young 2005).

Similarly, the MR is expressed extensively in the central nervous system (CNS), and indeed, the hippocampus arguably has the highest abundance of MR of any tissue in the body with the possible exception of the distal colon (Fuller & Verity 1990). The function of the MR in the CNS has been the subject of extensive investigation (for a review, see Joëls *et al.* (2007)); it appears to have fundamental roles in diverse behavioural responses including memory and affect. In only a small number of nuclei in the CNS, there is co-expression of 11β-HSD2 and the MR; in these nuclei, there is compelling evidence that the role does relate to sodium balance, in this case, the regulation of salt appetite (Geerling & Loewy 2009).

Our recent studies (Rickard *et al.* 2009) and subsequent studies from other groups (Usher *et al.* 2010) have established a very fundamental role for the MR in the regulation of the cells of the monocyte/macrophage lineage. These cells have previously been well characterised with respect to their response to synthetic glucocorticoids acting via the GR, which predominantly serve to mediate an anti-inflammatory response (Rickard & Young 2009). Hoppmann *et al.* (2010) have recently reported similar findings for inflammatory adipocyte responses. The current evidence would therefore suggest that the activation of the MR in a range of tissues mediates a pro-inflammatory response, and thus, we see in this context an interesting 'yin and yang' with respect to the MR and GR.

Perhaps fundamental to interpreting the significance of this situation are the dose–response curves, with the MR responding to cortisol at much lower concentrations than the GR, a fact that is often overlooked. Indeed, in monocyte/macrophages perhaps more than any other, the postulate that the two receptors may expand the dynamic range of the response to cortisol, with the MR responding in the physiological range associated with a normal diurnal rhythm, and the GR responding to supraphysiological levels associated with stress and disease (Evans & Arriza 1989), would seem to apply. Thus, a parsimonious interpretation is that the MR is involved in the initial activation of the response, and the GR serves to limit the extent of the response, and indeed perhaps turn off the response.

The MR is also expressed in the reproductive tract including the granulosa cells of the ovary (Fru *et al.* 2006). Here, there is evidence that the MR may play a role in the regulation of progesterone synthesis; in this context, it is curious that progesterone, which is itself an MR antagonist, is the parent molecule for the MR antagonists in clinical use, spironolactone and eplerenone.

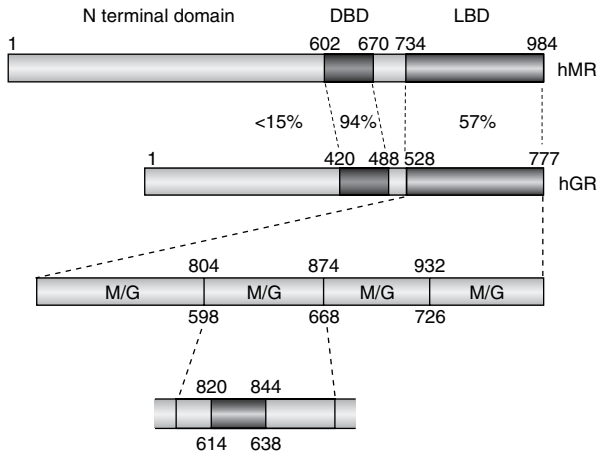


**Figure 2** Schematic representation of the strategies used to create LBD chimeras between the hMR and the hGR. The structures of the hMR and hGR are shown with the N-terminal, DNA-binding (DBD) and ligand-binding (LBD) domains. The amino acid numbers are shown above the receptors, and the homology is also shown as a percentage. The first set of chimeras where the LBD was broken into four regions resulted in 16 chimeras (Rogerson *et al.* 1999), and subsequently targeted chimeras of just the second region hMR (804–874)/hGR (598–668) were created (Rogerson *et al.* 2007). The 25 amino acids that conferred aldosterone-binding hMR (820–844) are shown at the bottom.

## MR: structural considerations

The MR (Arriza *et al.* 1987), as with other members of the nuclear receptor superfamily, consists of three principal domains (Mangelsdorf *et al.* 1995, Gronemeyer *et al.* 2004; Fig. 2). A central cysteine-rich DNA-binding domain (DBD) defines the superfamily. Structural analysis has shown that the cysteine residues are coordinated by two zinc atoms creating the so-called zinc fingers, in reality α-helices, one of which lies in the major groove of DNA where it makes nucleotide-specific contacts, the sequence of which defines the response element (Luisi *et al.* 1991). This region is highly conserved across the superfamily and consists of 66 or 68 amino acids. C-terminal to the DBD is a hinge region that is highly variable between receptors and links the DBD to the ligand-binding domain (LBD). This region is also highly conserved within the superfamily. In the case of the steroid receptors across the androgen receptor (AR), progesterone receptor (PR), GR and MRs, there is a 50–60% amino acid identity. The tertiary structure of the LBD is highly conserved across the superfamily and consists of 12 α-helices orientated in three anti-parallel layers with a ligand-binding pocket in the lower third of the molecule. The C-terminal helix, helix 12, in the presence of ligand forms a specific confirmation, which creates a groove into which co-activator molecules, with a leucine-rich LxxLL motif, bind. This function in the LBD is termed activation function 2 (AF-2) and it is ligand dependent. In the steroid receptors, including the MR (Bledsoe *et al.* 2005, Fagart *et al.* 2005, Li *et al.* 2005), helix 2 is unstructured, and so there are in fact only 11 α-helices, although the numbering observes the general convention for the family. The N-terminal domain of the nuclear receptors is remarkably diverse with respect to both sequence and length. The N-terminal domain of the MR is the longest in the nuclear receptor (NR) superfamily, and it shares little homology with the other NRs, including the other steroid hormone receptors (Arriza *et al.* 1987). The N-terminal domain contributes to receptor-mediated transactivation through AF-1. The N-terminal domain is largely unstructured, and it has been shown to be an exemplar of a class of proteins whose function depends on this lack of structure (Fischer *et al.* 2010).

In effect, the structure of this region is defined by its other interacting protein, and thus, this flexibility provides a degree of promiscuity that enables a diversity of interactions.

## Aldosterone vs cortisol

In classic mineralocorticoid target tissues such as the distal nephron or the distal colon, the response to activation of the MR by aldosterone or cortisol, in the absence of 11β-HSD2 activity, appears equivalent (Fuller & Young 2005). This appears not to be universally true in other tissues. Thus, for instance in the heart, we have presented evidence that corticosterone induces different, albeit overlapping patterns of gene expression (Wilson *et al.* 2009). Sato & Funder (1996) reported that aldosterone-induced hypertrophy in neonatal myocytes was blocked by corticosterone. Similarly, Rossier *et al.* (2008) showed that the MR-mediated chronotropic response in cardiomyocytes was activated by aldosterone but not by corticosterone, except in the presence of an altered redox state. In a transgenic study in which 11β-HSD2 was overexpressed in cardiomyocytes, the authors argued that normal occupancy of the MR by corticosterone was neutral in contrast to the deleterious outcomes that were mediated by the inappropriate activation by aldosterone (Qin *et al.* 2003). In isolated rabbit cardiomyocytes, an antagonistic effect of cortisol on aldosterone activation of PKC signalling has been reported (Mihailidou & Funder 2005). In the CNS, similar ligand discrimination has also been reported: Gomez-Sanchez *et al.* (1990) reported that the effects of i.c.v. infusion of aldosterone were blocked by both MR antagonists and corticosterone.

It has been argued that the differences seen between cortisol/corticosterone and aldosterone at the MR reflect the fact that despite having similar binding affinity constants, kinetic studies show a much faster off-rate for the glucocorticoids than for aldosterone, which in turn mediates a much stronger transcriptional response (Lombes *et al.* 1994, Hellal-Levy *et al.* 2000). That the interaction with the MR may be different for these ligands was demonstrated some years ago by Trapp & Holsboer (1995) who showed that the aldosterone-bound MR was more resistant to proteolysis than either spironolactone- or cortisol-bound MR.

## Binding to the MR LBD: aldosterone vs cortisol

In order to understand the structural basis of the ability of the MR to bind both aldosterone and cortisol whilst the GR effectively binds only cortisol, our group exploited a chimeric approach (Rogerson *et al.* 1999). We and others (Benhamou *et al.* 1992, Keightley *et al.* 1998) had previously used this approach to determine the structural basis of differences in ligand binding across species. Rogerson *et al.* (1999) created 16 MR:GR LBD chimeras with the three break points chosen on the basis of sequence conservation to avoid disturbing secondary structure (Fig. 2). Although the strategy preceded the publication of the crystal structures of the GR (Bledsoe *et al.* 2002) and MR (Bledsoe *et al.* 2005, Fagart *et al.* 2005, Li *et al.* 2005) LBD, the structural integrity was retained in that all the chimeras were able to bind cortisol. When the second of the four segments of the LBD was derived from the MR (amino acids 804–874), aldosterone binding and transactivation was observed irrespective of the source of the first, third and fourth segments. This same region also conferred the ability of the chimeras to bind spironolactone (Rogerson *et al.* 2003) and eplerenone (Rogerson *et al.* 2004). These amino acids were the starting point for a series of smaller MR sequence substitutions in the GR LBD described in a subsequent study (Rogerson *et al.* 2007). Amino acids 820–844 of the human MR were found to be responsible for the binding specificity (Fig. 2) with at least four amino differences, MR vs GR, being responsible. Regions overlapping these 24 amino acids have been identified in other studies using AR:PR (Vivat *et al.* 1997) and GR:PR (Robin-Jagerschmidt *et al.* 2000) chimeras as well as a subsequent study using MR:GR chimeras (Martinez *et al.* 2005). Although all chimeras that bound aldosterone were also transactivated by aldosterone, this was not true for cortisol where binding occurred with all 16 chimeras, but transactivation was only observed when the chimera contained MR sequence at both the second and the fourth regions of the LBD or GR sequence (Rogerson *et al.* 1999, 2007). We interpret this observation as further evidence that the interactions between cortisol and the MR are not equivalent to those for aldosterone and the MR.

## Antagonists of the MR

An extension of the distinction between ligands for the MR is the response to antagonists (Fig. 1). Bledsoe *et al.* (2005) have suggested that antagonism may occur through several different mechanisms. Competitive antagonism occurs where the LBD is largely unaltered but the necessary internal conformational changes fail to occur. Spironolactone is seen as fitting this 'passive' antagonism. Other categorisations include antagonism where an active conformational change occurs that precludes receptor dimerisation and DNA binding, and promotes degradation, and selective antagonists or agonists of transrepression, as has been described for the GR (de Bosscher *et al.* 2010). In addition to spironolactone, the other MR antagonist used in clinical practice is eplerenone, essentially a derivative of spironolactone, which is a derivative of progesterone. Although eplerenone has a lower affinity for the MR than spironolactone, it has greater specificity in that in contrast to spironolactone, it has a very low affinity for the other steroid receptors (Fagart *et al.* 2010). The distinction between antagonist and agonist in this context is subtle in that a single amino acid change at serine 810 in the human MR results in spironolactone and progesterone, both becoming agonist at the MR with significant clinical consequences (early onset hypertension that was catastrophically aggravated in pregnancy) in the kindred in which this mutation was identified (Geller *et al.* 2000, Zhang *et al.* 2005). Subsequently, it was shown that this Ser 810 Leu mutation also confers cortisone responsitivity on the MR (Rafestin-Oblin *et al.* 2003). Similarly, other studies have been able to manipulate the 'direction' of the response with point mutations in the LBD (Hultman *et al.* 2005, Li *et al.* 2005). Many studies have found that spironolactone alone has a weak ability to increase MR-mediated transcription, a response that can be enhanced by treatment with cyclic AMP analogues (Nordeen *et al.* 1995), or in a cell- and promoter-dependent context (Massaad *et al.* 1997).

The small difference between an agonist and an antagonist is further reinforced by the finding that in lower vertebrates, specifically the teleost fish, rainbow trout and zebra fish, the MR shows a similar pattern of response to both physiological mineralocorticoids and glucocorticoids to that observed for the mammalian MR, but in contrast, spironolactone and indeed progesterone show predominant agonist activity (Sturm *et al.* 2005, Pippal *et al.* 2011). The homology within the LBD of the human and zebra fish MR is 81%, confirming that fairly subtle changes of amino acid sequence in the LBD can change the direction of the response. Indeed, the subtlety of the distinctions is such that eplerenone is predominantly an antagonist of aldosterone action at the zebra fish MR, as it is at the human MR (Pippal *et al.* 2011). The calcium channel antagonist, nimodipine, which shows antagonist activity at the human MR (Dietz *et al.* 2008), is also an antagonist at the zebra fish MR (Pippal *et al.* 2011). Dihydropyridine has been used as the basis for the development of non-steroidal MR antagonists (Dietz *et al.* 2008, Fagart *et al.* 2010).

## N/C interaction

Although the various domains of the nuclear receptors are often regarded as modular and somewhat independent, it is clear that specific interactions may occur between domains within the receptor. An interaction between the N-terminal domain and the LBD (N/C interaction) has been extensively characterised for the AR (Langley *et al.* 1995, He *et al.* 1999). Indeed, some patients with the androgen insensitivity syndrome have been shown to have mutations in the AR

that quite specifically impair this interaction without impacting on other functions of the receptor (Thompson *et al.* 2001, Quigley *et al.* 2004). The significance of this interaction in the AR is further reinforced by the use of the interaction as a screen in the development of non-steroidal selective AR modulators (Miner *et al.* 2007). An N/C interaction has also been identified in other nuclear receptors, including the progesterone (Tetel *et al.* 1999) and oestrogen receptors (Métivier *et al.* 2002). This interaction is usually demonstrated using a mammalian-2 hybrid assay (M-2-H), which serves to identify protein–protein interactions (Fig. 3). One protein is linked to the GAL4 DBD and the other to an activation domain, often VP-16. These two components are independently transfected into cells, usually COS-1 cells, with the GAL4 promoter linked to a luciferase reporter gene. If there is a productive interaction between the two proteins of interest, then the activation domain will be brought into relationship with the GAL4 DBD bound to its promoter, and hence transcription will be initiated (Rogerson & Fuller 2003). This assay has been used extensively in the characterisation of the N/C interaction in the AR. Some years ago, we reported an interaction between the MR LBD and the N-terminus (Rogerson & Fuller 2003) using the M-2-H assay under the same conditions as those used to demonstrate an interaction for the AR. Curiously, we were not able to demonstrate an interaction between the N-terminus and the LBD of the GR. The MR N/C interaction was found to be ligand dependent, occurring in the presence of aldosterone. The analysis was, however, confounded to some extent by the strong transactivation activity in the AF-2 region of the MR LBD, which resulted in a significant level of activity in the absence of the N-terminus VP-16 construct. If this activity was specifically eliminated through mutation of the glutamic acid at position 962 to alanine in the human MR (Fuse *et al.* 2000), the interaction was still observed (Rogerson & Fuller 2003). Although this mutation reduces the magnitude of interaction, it does not eliminate the interaction in contrast to what is seen with this mutation for interactions of the AF-2 region with co-regulatory molecules (see below). Moreover, the interaction induced by aldosterone was antagonised by both spironolactone and eplerenone (Rogerson & Fuller 2003); however, somewhat to our surprise, the aldosterone-mediated interaction was also antagonised by cortisol, which exhibited very low induction of the interaction. We were even more surprised to find that although the interaction was observed in the presence of the synthetic mineralocorticoid, α-fludrocortisone, deoxycorticosterone was also a predominant antagonist of the interaction for the human MR (Pippal *et al.* 2009). Using a GST pulldown assay, we provided evidence that the interaction was a direct interaction between the N-terminus and the LBD (Pippal *et al.* 2009). This N/C interaction was observed in a range of cell lines, which is to say there was no evidence that cell-specific factors mediate the interaction. An important caveat with this observation is that this does not preclude the possibility that the consequences of the N/C interaction may exhibit cell specificity. The structural basis of the interaction remains unclear at this stage. The AR has specific motifs in the N-terminal domain, FxxLF and WxxLF, which are known to mediate the interaction with the AF-2 region of the AR LBD (He *et al.* 2000). Such motifs do not exist in the MR N-terminus. Pascual-Le Tallec *et al.* (2003) have identified four sumoylation motifs in the N-terminal domain; however, mutation of the lysine in each of these mutations to an arginine, which will preclude sumoylation, does not compromise the N/C interaction (Pippal *et al.* 2009). A series of truncation and internal deletion mutations of the N-terminus have similarly failed to identify a specific region mediating the interaction, leading us to conclude that more than one region may be involved in mediating the interaction (Pippal *et al.* 2009). A key question with this interaction is whether it occurs within a given receptor molecule, i.e. is an intra-molecular interaction or an inter-molecular interaction between two receptors, perhaps bound, as is often the case, as dimers on the response element that is classically an inverted palindrome. Schaufele *et al.* (2005) have provided compelling evidence for an initial intra-molecular interaction in the unliganded AR but an inter-molecular interaction in the liganded AR when it has migrated to the nucleus. The notion that there might be more than one interacting domain in the N-terminus is consistent with multiple interactions across receptors to promote synergy in the interaction. It is perhaps of relevance that the regions of the MR N-terminal domain that contain the sumoylation motifs were initially identified as synergy motifs (Iñiguez-Lluhi & Pearce 2000). Although it would appear that the AF-2 region makes some contribution to the N/C interaction in that the strength of the interaction is diminished, albeit not eliminated, by mutation in the AF-2 region (Rogerson & Fuller 2003), it would appear that residues elsewhere are also important. The same has been found for the AR where residues in helices 3, 4 and 12 have

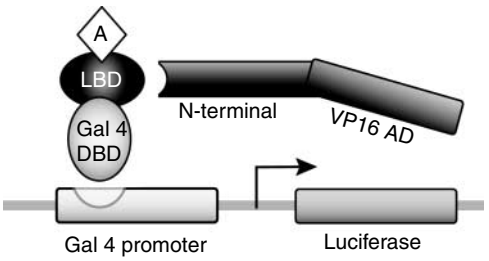


**Figure 3** Schematic representation of the mammalian-2 hybrid assay used to demonstrate the N/C interaction. Cells in culture are transfected with three plasmids. The two expression plasmids encode a chimera of the GAL4 DBD with the MR LBD and a chimera of the MR N-terminal domain with the VP16 activation domain (VP16 AD). The reporter plasmid contains five GAL4 response elements in the promoter that regulates expression of the luciferase gene. Ligand, in this case aldosterone (A), is then added. The yeast-2-hybrid assay used to identify co-regulatory molecules follows a similar principle.

been identified as important in mediating the N/C interaction (He *et al.* 2000, Estébanez-Perpiñá *et al.* 2005). Clearly, much work remains to be done in understanding both the nature and the significance of the N/C interaction observed in the MR. Although one can argue by analogy with the AR that the N/C interaction is functionally significant, this remains to be formally demonstrated. One approach to addressing this question is to ask whether this interaction is conserved across evolution for the MR. Pippal *et al.* (2011) have recently reported that the N/C interaction is also observed for the zebra fish MR. We were able to show that the interaction also occurs across species. Curiously, in contrast to the human MR, both deoxycorticosterone and cortisol were predominant agonists for the interaction, although spironolactone remains an antagonist. Although the significance of this latter observation is not clear, it does suggest that there are very subtle differences in sequence that profoundly alter the direction of the interaction. This again emphasises that subtle, but functionally significant, differences in the confirmation of the MR can be induced by different ligands.

## Post-translational modifications

As with other steroid receptors, the MR is subject to a number of post-translational modifications including phosphorylation, sumoylation, ubiquitination and even acetylation (for reviews see Viengchareun *et al.* (2007) and Odermatt & Atanasov (2008)). Kino *et al.* (2010) have recently identified a role for cyclin-dependent kinase 5-mediated phosphorylation in modulating MR transcriptional activity. Although one can speculate that the different conformations induced by aldosterone and cortisol might alter post-translational modifications, we are unaware of any direct evidence that this can occur.

## Co-activators

The central role of co-regulatory molecules in mediating the genomic response to nuclear receptor activation is now well established, and indeed, over 350 co-regulatory molecules have been identified (Bulynko & O'Malley 2011). In contrast to the other members of the nuclear receptor superfamily, including the steroid receptors, only a small number of co-regulatory molecules have been identified for the MR. These have been reviewed in some detail recently (Viengchareun *et al.* 2007, Yang & Young 2009, Yang & Fuller 2012). In addition to interacting with known well-characterised co-regulators such as the steroid receptor co-regulator 1 (SRC1), SRC2, SRC3 and PGC1α, a number of other MR co-regulators have been identified. Much of the work identifying MR-specific co-regulators has focused on identifying co-regulatory molecules that will discriminate between the MR and the GR. The strategy has primarily, but not exclusively, been to screen using the unique N-terminal domain of the MR to identify receptor-specific interacting proteins (for recent reviews, see Yang & Young (2009) and Yang & Fuller (2012)). Conversely, if co-regulatory molecules are to be ligand discriminant, it arguably makes more sense that they would be interacting with the LBD rather than the N-terminus, although as is described below, this is not entirely true; indeed, the N/C interaction confirms that these domains are not truly independent. The classic interaction of a co-activator molecule with the LBD of the nuclear receptors involves an interaction between the AF-2 region of the LBD formed by helices 3, 4 and 12 interacting with an LxxLL motif in the co-regulatory molecule. The stability of this interaction is determined by a charge clamp between residues in helices 12 and 3 (Savkur & Burris (2004)). In general, co-regulatory molecules have more than one of these motifs (Savkur & Burris 2004, Li *et al.* 2005). Two studies sought to define the pattern of interaction by taking the LxxLL motifs from a series of known co-regulatory molecules and examining their interaction with the MR in a mammalian-2 hybrid assay (Hultman *et al.* 2005) or an alpha screen (Li *et al.* 2005). The first study examined 50 LxxLL motifs from 23 known co-regulators and the second, 38 motifs from 18 known co-regulators. What was remarkable about both of these studies was the relatively small number of these motifs that interacted with the MR. In addition, ligand discrimination was not observed unless mutations were inserted into the MR LBD (Li *et al.* 2005). We have recently addressed this question using a different approach (Yang *et al.* 2011). In this study, phage display was used to screen a library of random 19 amino acid peptides, half of which contained the LxxLL motif at their core. The library was screened with full-length MR, which identified a number of interactions, some of which occurred in the presence of aldosterone but not cortisol. Of particular interest was the finding that one of the random 19-mers was ligand discriminant in that it interacted with the full-length MR in the presence of aldosterone but not cortisol. The physiological significance remains to be demonstrated. Of the published full-length co-regulatory molecules identified, which interact with the MR, only one has, to date, shown ligand specificity. Kitagawa *et al.* (2002) screened HeLa cell extracts using a region of the N-terminus of the rat MR known to have transcriptional activity. They identified a complex containing RNA helicase A (RHA). When they explored the ability of the RHA-containing complex to mediate transactivation with the full-length receptor, transactivation was observed in the presence of aldosterone but not cortisol. It is tempting of course to speculate that this relates to the N/C interaction. The concept that the N-terminal domain is important in defining the response has been reported previously: Jausons-Loffreda *et al.* (1994) demonstrated that the N-terminal region was important if glucocorticoids were to mediate transactivation of the MR, whereas aldosterone was not dependent on this region. We have recently sought to identify ligand-dependent and ligand-discriminant

interacting proteins for the MR using a yeast-2 hybrid screen (PJ Fuller, Y Yao and FM Rogerson 2009, unpublished observations; Fig. 3). Two commercially available yeast-2 hybrid libraries have been screened with the LBD of the MR in the presence of aldosterone or cortisol. A large number of interacting proteins were identified, including SRC1 and PGC1α, which are of course reassuring *de facto*-positive controls. A small number of interacting peptides were isolated from that screen whose interaction with the MR appeared to occur only in the presence of aldosterone but not cortisol, and, in the case of one clone, cortisol but not aldosterone. Some but not all of these fragments contained LxxLL motifs. The characterisation of these clones is the subject of ongoing work in the laboratory. The results of the yeast-2 hybrid assay have been first validated in the mammalian-2 hybrid assay, and those in which the interaction is confirmed in the mammalian system have then been further analysed by the creation of full-length cDNAs for co-expression with the full-length human MR. At least two of these show clear aldosterone- but not cortisol-mediated transactivation in the presence of the full-length receptor, and the preliminary results of these analyses have been reported (Fuller *et al*. 2010). At this stage, the physiological significance of these observations remains to be clearly established. One can conclude from this analysis that, as previously shown by Kitagawa *et al*. (2002), co-regulatory molecules exhibit ligand-specific interactions with the MR under controlled conditions in an experimental system. The physiological studies in which aldosterone and cortisol have been shown not to be equivalent in activating the MR in the absence of 11β-HSD2 might therefore be explained by one of the mechanisms described. This specificity undoubtedly involves interactions with co-regulatory molecules, which can have distinct tissue-specific profiles of expression. In addition, the N/C interaction may play a critical role in some situations.

In addition to the implications for the diversity of physiological responses mediated through activation of the MR, we would suggest that the importance of these studies lies in the clear demonstration that the direction of the response is determined by the choice of ligand. This is consistent with other steroid receptors where ligands that are selective modulators of the receptor can be identified. It is clear that the conformation induced by ligands is not equivalent, and there is considerable plasticity in the system such that subtle differences in the conformation induced in the receptor will result in structural differences at the surface of the receptor, which in turn will determine interactions with other factors and will ultimately lead either to productive transcriptional activation or indeed to repression of transcription.

In this discussion, we have not focused on the alternate mechanisms of action for the MR. Classically, we think of the receptor acting through an interaction with a response element in the regulatory region of the target gene. This classic transcriptional or genomic mechanism of action has been the focus of most studies, particularly for the MR. There is now clear evidence that MR signalling may also be mediated through so-called non-genomic or rapid effects of the MR in the cytoplasm where second messenger system signalling pathways are triggered (Fuller & Young 2005). These mechanisms have been extensively characterised by the work of Grossmann & Gekle (2012) and also by the group of Harvey (Dooley *et al*. 2012). As noted previously, tethering or transrepression is an important genomic, but response element independent, method of signalling for the other steroid receptors. This has not been characterised for the MR, but it would seem likely that, as with the other receptors, this is a component of the response. It is important to recognise that many of the above analyses and studies do not presume the mechanism of action, and thus, observations on the N/C interaction or indeed interacting proteins may be relevant, not in the classic pathway, but rather in the cytoplasmic compartment or indeed as part of tethering of other transcription factors.

The relevance of the possibility of modulation at the MR lies in the limitations of the current therapeutic agents. Although RALES (Pitt 2004), EPHESUS (Pitt 2004), EMPHASIS (Zannad *et al*. 2011) and other clinical trials have shown a clear benefit of the addition of MR antagonist (spironolactone or eplerenone) to standard therapy for cardiac failure and/or ventricular dysfunction in a number of clinical situations, the benefits are limited by the physiology of the antagonism, which is to say by the hyperkalaemia (Juurlink *et al*. 2004), that may result from blocking the MR in epithelial tissues, particularly the distal nephron and presumably the distal colon. The results above therefore provide support for the concept that a ligand for the MR may be identified, which is selective and/or enriched for antagonism in the cardiovascular system but not in the epithelial sodium-transporting tissues. Given the role and distribution of the MR in the CNS, other therapeutic possibilities also remain to be explored.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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