30 YEARS OF THE MINERALOCORTICOID RECEPTOR

Coregulators as mediators of mineralocorticoid receptor signalling diversity

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Abstract

The cloning of the mineralocorticoid receptor (MR) 30 years ago was the start of a new era of research into the regulatory processes of MR signalling at target genes in the distal nephron, and subsequently in many other tissues. Nuclear receptor (NR) signalling is modified by interactions with coregulatory proteins that serve to enhance or inhibit the gene transcriptional responses. Over 400 coregulatory proteins have been described for the NR super family, many with functional roles in signalling, cellular function, physiology and pathophysiology. Relatively few coregulators have however been described for the MR although recent studies have demonstrated both ligand and/or tissue selectivity for MR-coregulator interactions. A full understanding of the cell, ligand and promoter-specific requirements for MR-coregulator signalling is an essential first step towards the design of small molecular inhibitors of these protein-protein interactions. Tissue-selective steroidal or non-steroidal modulators of the MR are also a desired therapeutic goal. Selectivity, as for other steroid hormone receptors, will probably depend on differential expression and recruitment of coregulatory proteins.

Introduction

The cloning of the mineralocorticoid receptor (MR) by Jeff Arriza working in the laboratory of Ron Evans (Arriza et al. 1987) marked a critical inflexion point for research on aldosterone action. It followed the cloning of the other steroid hormone receptors (SHRs) and indeed other members of the nuclear receptor (NR) superfamily of ligand-dependent transcription factors. The determination of the amino acid sequences of the SHR enabled the characterization of shared structural domains, and the cloned MR was used in ‘cis-trans’ assays to provide functional characterization of the receptor including its ability to bind both aldosterone and cortisol (Evans & Arriza 1989). These transcription factors enhanced gene expression in ‘trans’, which is to say they interacted with response elements in the chromatin, which were not contiguous with the promoter being at some distance from the transcripational start site. As a consequence, it became clear that a mechanism was required for the receptor to interact with the transcriptional machinery assembled on the promoter and thus, by analogy with
other transcription factors, there must exist coregulators that bridge the activation function in the NR to the promoter complex.

The search for SHR coactivators was on, and in 1995, the O’Malley group (Onate et al. 1995) cloned the first coactivator for SHR, steroid receptor coactivator-1 (SRC-1). Other groups identified co-repressors which modulated NR (Chen & Evans 1995, Horlein et al. 1995) and other coactivators including SRC-2 and SRC-3, both of which have multiple aliases reflecting each laboratory’s desire to place their imprimatur on the molecule (or more charitably, reflecting the disparate contexts in which each laboratory identified these molecules). SRC-1, SRC-2 and SRC-3 comprise the extensively characterized p160 family of coactivators. Coactivators both transduce and integrate signalling well beyond merely being coactivators of steroid receptors (York & O’Malley 2010). The coregulators also serve as docking platforms for further coregulator binding to form larger transcriptional complexes (McInerney et al. 1998). These complexes are recruited to the target gene by the activated, DNA-bound, receptors to enable many of the actions needed for gene expression. These include chromatin remodelling, histone modification, initiation of transcription, elongation of RNA chains, RNA splicing and termination of transcriptional responses (Auboeuf et al. 2007, O’Malley 2007). Corepressors, conversely, reverse many of these processes; for instance, while coactivators promote acetylation of histones, coregulators inhibit gene expression by inducing histone deacetylase activity. There are now over 400 published molecules designated as coregulators (O’Malley 2016) which have been recognized to play a central role in modulating gene expression mediated by NR, and are thought to impart tissue and ligand specificity to receptor activity (Lonard & O’Malley 2006).

**Structural determinants in the MR**

The cloning of the MR confirmed that, as with other members of the NR superfamily, the MR has three major functional domains: an N-terminal domain (NTD), a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Arriza et al. 1987).

The MR-NTD is the longest among all the receptors (602 amino acids) and is the least conserved domain with respect to both sequence and structure across the steroid receptors (Lavery & McEwan 2005). The NTD is considered to be intrinsically disordered in the absence of other binding proteins, thereby providing structural flexibility for protein-protein interactions (McEwan et al. 2007); its structure is therefore effectively determined by its interacting partner. Various studies have identified at least three regions in the NTD, termed activation functions (AFs), which are thought to recruit coactivators: AF1a (amino acids 1–169), middle domain (MD, amino acids 247–385) and AF1b (amino acids 451–602) (Govindan & Warrier 1998, Fuse et al. 2000, Pascual-Le Tallec et al. 2003, Fischer et al. 2010). However, AF-1b has been reported to adopt a stable secondary structure and can interact with protein targets in the absence of induced folding (Fischer et al. 2010). A central inhibitory region (amino acids 163–437) has also been described (Pascual-Le Tallec et al. 2003) although it may reflect the ability of MD to recruit corepressors (Fischer et al. 2010). The fact that the regions identified may vary between studies may reflect the intrinsic structural plasticity and therefore context dependence of the NTD. The NTD also interacts with the LBD in an N-C interaction to stabilize receptor conformation (Rogerson & Fuller 2003).

The DBD (66 amino acids) is highly conserved. It is characterized by two ‘zinc fingers’ each containing a zinc ion tetrahedrally coordinating four cysteine residues. It determines the specificity of DNA recognition and binding, as well as having a role in receptor homo- and hetero-dimerization (Pippal & Fuller 2008). It is linked to the LBD by a hinge region (61 amino acids) that also plays a role in receptor homo-dimerization. The structure of the DBD has been solved using both nuclear resonance spectroscopy and crystallography for many of the NRs including recently, the MR (Hudson et al. 2014).

The LBD (251 amino acids), the crystal structure of which was solved independently by 3 groups in 2005 (Bledsoe et al. 2005, Fagart et al. 2005, Li et al. 2005), is a complex structure organized in eleven α-helices (labelled by convention 1 to 12; helix 2 is unstructured in the SHR) and four β-strands forming three anti-parallel layers (Bledsoe et al. 2005, Fagart et al. 2005, Li et al. 2005). It contains a ligand-dependent activation function 2 (AF-2) made up of helices 3, 4, 5 and 12. The LBD interacts with chaperone proteins in the absence of ligand and undergoes conformational change upon ligand binding such that helix 12 forms a stable interaction that creates a hydrophobic cleft on the surface of the LBD (Huyet et al. 2007) which serves as a docking platform (AF-2) for transcriptional coregulators (Fig. 2). Many coactivators bind to the AF-2 region via a preserved ‘NR-box’ that contains one or more LxxLL (where L is leucine and x is any amino acid) motifs (Heery et al. 1997, Darimont et al. 1998).
Coregulators for the MR

Although over 400 coregulatory molecules have been identified (O’Malley 2016), only a relatively small number have been characterized for the MR (reviewed in Yang & Fuller 2012). Of the relatively ‘generic’ coactivators, SRC-1, SRC-2 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) interact strongly with the AF-2 region of the MR via their LxxLL motifs. p300/CREB-binding protein (CBP) is able to potentiate MR-induced transcription via both AF-1 and AF-2. Although a direct interaction with the AF-1 region was not initially observed (Fuse et al. 2000) for SRC-2, SRC-3 and CBP, they were found to bind directly to the MR-NTD after stabilization of the NTD structure with the natural osmolyte trimethyl N-oxide (TMAO) (Fischer et al. 2010).
Zennaro and coworkers (Zennaro 2001) addressed this issue using a mutant MR that is unable to form AF-2. They examined three previously described AF-2 interacting steroid receptor coactivators, SRC-1, RIP140 (140-kDa receptor-interacting protein) and TIF1α (TRIM24: tripartite motif containing 24), which were known to interact in an agonist-dependent fashion with the MR (Hellal-Levy et al. 2000). Zennaro and coworkers (Zennaro et al. 2001) identified an interaction of the three coactivators with the mutant MR lacking an AF-2 region. This finding suggests that these coactivator-MR interactions likely involve more than just the interaction of the LxxLL motifs in the coactivator with AF-2 in the MR-LBD; evidence from other systems points to the involvement of the NTD (Zennaro et al. 2001).

Unlike NR, which are structurally conserved, coregulators are structurally and functionally diverse, and are often recruited in a ligand- and cell type-specific manner.

Table 1 Summary of coregulators that interact with the MR (modified from Yang & Fuller 2012).

<table>
<thead>
<tr>
<th>Coregulator</th>
<th>Sites of interaction with the MR</th>
<th>Known functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coactivators</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRC-1</td>
<td>AF-2; AF-1 by SRC-1α isoform</td>
<td>Recruits histone acetylation complex to initiate transcription; weak intrinsic histone acetyltransferase activity.</td>
<td>(Hultman et al. 2005, Meijer et al. 2006, Wang et al. 2004, Zennaro et al. 2001)</td>
</tr>
<tr>
<td>SRC-2, TIF2, GRIP1, p300/CBP</td>
<td>AF-1, AF-2</td>
<td>Enhances transactivation.</td>
<td>(Fuse et al. 2000, Hong et al. 1997, Wang et al. 2004)</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>AF-2</td>
<td>Recruits histone acetyltransferase activity; recruits RNA polymerase II to target gene promoter</td>
<td>(Fuse et al. 2000)</td>
</tr>
<tr>
<td>ELL</td>
<td>AF-1b</td>
<td>RNA polymerase II elongation factor; prevents premature arrest and transient pausing of polymerase II</td>
<td>(Pascual-Le Tallec et al. 2005)</td>
</tr>
<tr>
<td>FLASH</td>
<td>AF-1</td>
<td>Regulates cell apoptosis</td>
<td>(Obradovic et al. 2004)</td>
</tr>
<tr>
<td>FAF-1</td>
<td>AF-1</td>
<td>Regulates cell apoptosis</td>
<td>(Obradovic et al. 2004)</td>
</tr>
<tr>
<td>Ubc9</td>
<td>NTD</td>
<td>SUMO E2-conjugating enzyme; forms coactivation complex with SRC-1</td>
<td>(Yokota et al. 2007)</td>
</tr>
<tr>
<td>TIF1α</td>
<td>NTD</td>
<td>Transcriptional coactivator / corepressor</td>
<td>(Zennaro et al. 2001)</td>
</tr>
<tr>
<td>RIP140</td>
<td>NTD</td>
<td>NR coactivator</td>
<td>(Zennaro et al. 2001)</td>
</tr>
<tr>
<td>Tesmin</td>
<td>LBD</td>
<td>Ligand-specific coactivator</td>
<td>(Obradovic et al. 2004)</td>
</tr>
<tr>
<td>EEFL1A</td>
<td>LBD</td>
<td>Growth and proliferation</td>
<td>(Yang et al. 2014)</td>
</tr>
<tr>
<td>XRCC6</td>
<td>LBD</td>
<td>DNA repair</td>
<td>(Yang et al. 2014)</td>
</tr>
<tr>
<td>Corepressors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMRT</td>
<td>LBD</td>
<td>Recruits histone deacetylase</td>
<td>(Wang et al. 2004)</td>
</tr>
<tr>
<td>NCoR</td>
<td>LBD</td>
<td>Recruits histone deacetylase</td>
<td>(Wang et al. 2004)</td>
</tr>
<tr>
<td>DAXX</td>
<td>NTD</td>
<td>Regulates cell apoptosis; represses MR transactivation in some cell lines</td>
<td>(Obradovic et al. 2004)</td>
</tr>
<tr>
<td>PIAS1</td>
<td>NTD</td>
<td>SUMO E3 ligase; exact mechanism of repression unclear</td>
<td>(Pascual-Le Tallec et al. 2003)</td>
</tr>
<tr>
<td>NF-YC</td>
<td>AF-1</td>
<td>Inhibits aldosterone-induced MR N-C interaction</td>
<td>(Murai-Takeda et al. 2010)</td>
</tr>
<tr>
<td>Gemin 4</td>
<td>LBD</td>
<td>Nuclear organelle-associated transcription elongation factor</td>
<td>(Yang et al. 2015)</td>
</tr>
<tr>
<td>SSRP1</td>
<td>LBD</td>
<td></td>
<td>(Yang et al. 2014)</td>
</tr>
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AF (activation function), CBP (cAMP-response element-binding protein-binding protein), DAXX (DNA-binding domain), ELL (eleven-nineteen lysine-rich leukaemia), FLASH (fas-associated death domain-like IL-1α-converting enzyme -associated huge), FAF-1 (fas-associated factor 1), GRIP1 (glucocorticoid receptor-interacting protein 1), LBD (ligand-binding domain), MR (Mineralocorticoid receptor), NCoR (nuclear receptor corepressor), NTD (N-terminal domain), PGC1 (peroxisome proliferators-activated receptor gamma coactivator 1), PIAS (protein inhibitor of activated signal transducer and activator of transcription), RHA (RNA helicase A), RIP140 (receptor-interacting protein 140), SMRT (silencing mediator of retinoid and thyroid hormone receptor), SRC-1(steroid receptor coactivator 1), SUMO-1 (small ubiquitin-related modifier-1), TIF1α (transcriptional intermediary factor 1α), TIF2 (transcriptional intermediary factor 2), Ubc9 (Ubiquitin-like protein SUMO-1 conjugating enzyme), Gem (nuclear organelle)-associated protein 4, eukaryotic elongation factor 1A1 (EEF1A1), structure-specific recognition protein 1 (SSRP1), X-ray repair cross-complementing protein 6 (XRCC6).
manner as demonstrated for the estrogen receptor (ER), androgen receptor (AR), peroxisome proliferators-activated receptor γ (PPAR γ) and a range of other receptors (Kodera et al. 2000, Kraichely et al. 2000, Bramlett et al. 2001, Klok et al. 2007, McKenna 2011).

The crystal structure of the ligand-bound LBD associated with a LxxLL motif containing coactivator-derived peptide was solved for the ER (Brzozowski et al. 1997, Shiau et al. 1998) and subsequently for many other NRs. Obtaining crystal structures for full-length receptors has been more elusive, but structural information has recently been published for a series of ligand-activated NR heterodimers, bound to DNA and coactivator peptides (Khorasanizadeh & Rastiejad 2016). In these structures, the NTD is not however visualized, consistent with its unstructured state (Chandra et al. 2008). Yi and coworkers (Yi et al. 2015) have used cryoelectron microscopy to visualize the structure of an ER-coactivator complex bound to DNA. This shows that each ER molecule in the homodimer recruits one SRC-3 via AF-2 and that these two SRC-3 bind to different regions of one p300 protein. Using this technology, they were able to identify AF-1 and provide evidence that it has a role in SRC-3 recruitment as previously predicted, but until now not formally demonstrated.

PGC-1α is a tissue-specific coactivator involved in metabolic regulation and energy homeostasis (Knutti et al. 2000). The physiological significance of PGC-1α as a putative coactivator in MR signalling is unclear, especially in the kidney where it is not co-expressed with the MR (Lombes et al. 1990, Portilla et al. 2002). However, the high level of expression of PGC-1α in brown fat may be relevant given increasing evidence of a key role of the MR in adipocyte differentiation (Armani et al. 2014).

Hultman and coworkers (Hultman et al. 2005) used a mammalian two-hybrid (M2-H) system to identify further MR-LBD interacting coregulators using 50 coregulator peptides derived from 23 known NR coactivators and corepressors. Each peptide contained the LxxLL motif (coactivators) or the CoRNR box motif found in corepressors. In a similar approach, Li and coworkers (Li et al. 2005) screened 38 peptides in an alpha screen for competitive binding to ligand-bound MR-LBD against an established LxxLL motif. Neither study identified additional MR binding coregulator proteins beyond those already described. Both studies also sought ligand specificity for the interactions but found no differences between aldosterone and cortisol, the two physiological MR ligands.

Corepressors and the MR

The corepressors nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) interact with the MR-LBD via their C-terminal corepressor-NR interaction (CoRNR) boxes containing a core I/L-xx-I/V-I motif to attenuate aldosterone-dependent MR-mediated transactivation by inducing histone deacetylase activity (Wang et al. 2004). These corepressors were originally identified through their role in transcriptional repression of NRs in the absence of ligand or when bound to synthetic antagonists (Chen & Evans 1995, Horlein et al. 1995). However, NCoR is recruited by the MR upon aldosterone binding and not upon binding of spironolactone, the steroidal MR antagonist, or finerenone, a newly synthesized non-steroidal MR antagonist (Fagart et al. 2010). SMRT and NCoR serve as key corepressors for many different NRs and transcription factors. The amino acids in the core I/L-xx-I/V-I motif as well as the flanking sequences are critical determinants of receptor preference (Hu & Lazar et al. 1999). Both corepressors are subjected to extensive mRNA splicing events which produce ‘custom tailored’ variants with unique CoRNR boxes, resulting in different affinities for the various NRs (Goodson et al. 2005). The splice variant that interacts with the MR is not known.

MR-interacting proteins

A series of putative coregulatory molecules that interact with the MR (Table 1) are described in detail below. These have been identified using a range of approaches, which fundamentally identify a protein-protein interaction. Most have used yeast 2-hybrid (Y2-H) screens in which either the full-length MR or more often a fragment of the MR is expressed in yeast as ‘the bait’ with a cDNA library as ‘the prey’. The bait is fused to the GAL4 DBD, and the prey is fused to the GAL4 activation domain. A productive interaction between bait and prey brings these two elements of the GAL4 transcription factor together and allows transcriptional activation. The yeast contains a GAL4 responsive construct containing the GAL4 response elements and a reporter gene LacZ whose product, β-galactosidase, changes a chromogenic substrate (X-gal) to a blue colour enabling selection of the yeast clone of interest. The system has limitations in that yeast although similar are not identical to mammalian cells so that ligand specificity may not be robust. It is also not 100% efficient so that the entire ‘interactome’ is not captured, and it can
have a high false positive rate as only 1 in 6 of the clones in the cDNA library will be in frame and in the correct orientation. The M-2-H assay follows the same principles but assesses the interaction in mammalian cells, and it is not used for screening but ideal for more detailed analysis of the interaction. We have also used phage display, a technique in which the bait is fixed to a solid surface and the library is incorporated into a cell surface protein of the phage. The phage are then washed across a plate containing the bait; the adherent phage are eluted and the relevant fragment from the library is retrieved. This is a powerful technique particularly for identifying surface interactions; an obvious limitation is that the interaction is in vitro rather than intracellular. Initially, M13 phage were used, which contained short oligonucleotide fragments harbouring a known core sequence, e.g. LxxLL, or random sequences. However, all peptides that were isolated contained the LxxLL motif. Subsequent studies have used T7 phage, which express cDNA fragments derived from tissues or cells (Yang et al. 2014).

**MR-specific coregulators**

In view of the high structural conservation in the LBD and the finding that AF-2-interacting coactivators such as SRC-1 appear to interact with most if not all of the steroid receptors, various investigators focused on identifying putative MR-specific coregulators by looking for NTD interactions. The MR-NTD, which shares less than 15% identity with other NRs, has been used as bait in pull-down and two-hybrid studies (Viengchareun et al. 2007).

This approach identified the elongation factor ELL (eleven-nineteen lysine-rich leukaemia) as an MR-specific interacting partner, which enhances RNA polymerase II activity (Shilatifard et al. 2003, Pascual-Le Tallec et al. 2005). ELL interacts exclusively with the AF-1b region of the MR to potentiate MR-mediated transactivation while conversely repressing GR-mediated transactivation. ELL is co-expressed with the MR in the cortical collecting duct cells of the human kidney and is upregulated by aldosterone (Pascual-Le Tallec et al. 2005). The ability of ELL to differentially regulate the MR and GR may thus be particularly relevant in those tissues that coexpress the two receptors.

Yokota and coworkers (Yokota et al. 2007) found that ubiquitin-like protein SUMO-1 conjugating enzyme (Ubc9) potentiates aldosterone-dependent MR transactivation. Ubc9 is involved in the sumoylation process whereby SUMO-1 (small ubiquitin-related modifier-1) is covalently attached to its protein target (Seeler & Dejean 2003). While sumoylation of NRs has generally been shown to have a repressive effect on gene expression (Bulynko & O’Malley 2011), Ubc9 interacts with the MR-NTD to potentiate aldosterone-dependent MR transactivation, independent of its sumoylation activity (Yokota et al. 2007). No interaction was observed between Ubc9 and the MR-LBD. Interestingly, SRC-1 is recruited simultaneously and can synergistically potentiate MR-mediated transcription with Ubc9. This observation suggests that Ubc9 mediates its effect in part by binding SRC-1 and recruiting other coactivators, which is supported by the co-localization of MR, Ubc9 and SRC-1 in mouse kidney collecting duct cell nuclei (Yokota et al. 2007).

Another sumoylation enzyme, protein inhibitor of activated signal transducer and activator of transcription 1 (PIAS1), interacts with the MR-NTD but as a corepressor (Pascual-Le Tallec et al. 2003). PIAS1 inhibits aldosterone-dependent MR-mediated transactivation. The repressive effect of PIAS1 is partly mediated by promoter-dependent sumoylation of the MR; however, PIAS1 contains two overlapping CoNR boxes, which do not mediate the repression. PIAS1 also contains three LxxLL motifs and has been shown to enhance transactivation mediated by the AR and GR (Tan et al. 2000). PIAS3, like PIAS1, also interacts with the MR-NTD and represses MR-induced transactivation in a neural cell line, but does not affect the transcriptional activity of GR (Tirard et al. 2004).

The overlap between protein-modifying enzymatic activity and coactivation has also been reported for the histone deacetylases (HDACs). Lee and coworkers (Lee et al. 2015) have reported that the class II HDAC, HDAC4 mediates an interaction between HDAC3 and the MR, which coactivates aldosterone-induced, MR-mediated transactivation. Obradovic and coworkers (Obradovic et al. 2004) sought to address the role of coregulators in conferring MR vs GR specificity in neurons where both receptors are responding to the same ligand, cortisol. They also used a Y-2-H screen with the NTD as bait with a human brain cDNA library. Although only a small number of interacting peptides was identified which did not overlap with previous studies, they did identify three proteins, DAXX (death-associated protein), FLASH (FLICE-associated huge) and FAF-1 (Fas-associated factor 1), previously characterized for their role in Fas receptor-mediated apoptosis. In a transactivation assay using a hippocampal cell line, DAXX corepressed both MR- and GR-mediated, ligand (aldosterone and dexamethasone, respectively)-dependent transactivation, whereas FLASH coactivated both MR and GR. FAF-1 (Fas-associated
factor 1) weakly coactivated MR but not GR-mediated transactivation. The results were however different when these assays were repeated in a neuroblastoma cell line, highlighting the importance of cellular context.

Nuclear transcription factor Y subunit gamma (NF-YC) was identified in a Y-2-H screen using full-length MR as bait (Murai-Takeda et al. 2010) as a specific corepressor of the MR. NF-YC, a subunit of the heterotrimeric transcription factor NF-Y, recognizes a CCAAT box motif found in the RNA polymerase II initiation site in many eukaryotic promoters and activates transcription (Nakshatri et al. 1996). It interacts with the MR-NTD and represses MR transactivation in a dose- and agonist concentration-dependent manner. NF-YC is selective for the MR with no effect on GR, AR or PR-mediated transactivation. Endogenous NF-YC is recruited to the MR response element of the human ENaC gene promoter a short time after MR and SRC-1 recruitment, suggesting that it may limit the transactivation induced by coactivators.

These studies therefore provide compelling evidence that for a number of these coregulatory molecules, there are important differences in their interactions between the MR and the GR. In tissues such as the hippocampus or in macrophages where both receptors are abundant and co-expressed, respond to the same ligand (cortisol or corticosterone) and potentially interact with the same response elements in the same genes, coregulators may be important determinants of the distinct, receptor-specific integrated cellular responses to ligand. Changes in coregulator profiles and/or coregulator activation status may integrate other signalling pathways that interact with corticosteroid signalling (Fuller & Young 2016).

Ligand- or cell-specific MR coactivators

The MR was originally identified as the type 1, corticosteroid receptor (Feldman et al. 1973) reflecting the sometimes overlooked fact that both the MR and the GR bind cortisol (corticosterone in rodents) although only the MR binds aldosterone (Rogerson et al. 1999). Prior to the cloning of the MR, there remained uncertainty as to whether there was a renal, ‘aldosterone-prefering’ MR and a ‘hippocampal cortisol-prefering’ MR. An earlier study by the late Zig Krozowski working with John Funder (Krozowski & Funder 1983) had provided compelling evidence that the ‘two’ MR without cellular factors were the same, a conclusion that was unambiguously confirmed by the cloning of the MR, which enabled the demonstration in vitro that the cloned MR bound both aldosterone and cortisol equivalently (Arizza et al. 1987). Subsequently, in 1988, the distinction ‘mineralocorticoid’ vs ‘type 1 corticosteroid’ was found to be conferred at a tissue level by the enzyme 11β hydroxysteroid dehydrogenase type 2 (HSD2) (Edwards et al. 1988, Funder et al. 1988).

In epithelial tissues, the vasculature and discrete subpopulations of hypothalamic neurones (Geerling & Loewy 2009) where HSD2 is co-expressed with the MR, HSD2 confers aldosterone specificity by virtue of its ability to convert cortisol to its inactive metabolite cortisone (or corticosterone to 11-dehydrocorticosterone) (Odermatt & Kratschmar 2012). The MR is however expressed in a diverse range of non-epithelial tissues where it plays a fundamental role in cellular function and pathology; in many of these tissues, there is little or no HSD2, so the MR is undoubtedly acting as a receptor for cortisol (Fuller & Young 2005). In epithelial cells and in vascular smooth muscle cells, when HSD2 is blocked, both aldosterone and cortisol act as MR agonists (Young et al. 2003, Wilson et al. 2009). In non-epithelial cells such as neurons and cardiomyocytes, glucocorticoids can antagonize the effects of aldosterone (Gomez-Sanchez et al. 1990, Sato & Funder 1996, Mihailidou 2006). These observations, together with evidence of differences between the interaction of aldosterone and cortisol with the MR in ligand binding (Rogerson et al. 1999, Rogerson et al. 2007) and the N-C interaction (Rogerson & Fuller 2003, Pippal et al. 2009) supports the concept of tissue- and ligand-specific interactions. It follows that ligand-discriminant activation of the MR may be mediated by differential coregulator interactions, as is well described for the selective estrogen receptor modulators (SERM), tamoxifen and raloxifene (Shang & Brown 2002).

Rogerson and coworkers (Rogerson et al. 2014) described the first ligand-specific coactivator for the MR. This study was also based on a Y-2-H screen, but in this, they used the MR-LBD as bait (Rogerson et al. 2014). Several of the interactions represented known coactivators, including SRC-1 and PGC-1a. The majority of interactions identified were seen with both aldosterone and cortisol, but Y-2-H clones that appeared to be ligand discriminant were identified and one has been fully characterized. This Y-2-H clone encoded a fragment of tesmin or metallothionein-like 5 (MLT5), which is a 508 amino acid protein with a cysteine-rich region that binds zinc. This clone showed aldosterone specificity in the Y-2-H assay; an interaction was also observed in the presence of cortisol in a M-2-H assay, but the M-2-H interaction was 12-fold more active in the presence of aldosterone than cortisol. When full-length tesmin was analysed...
in a transactivation assay, it was found to coactivate aldosterone-induced MR-mediated transactivation but not cortisol-induced MR-mediated transactivation; the interaction was antagonized by spironolactone. Tesmin and MR were co-immunoprecipitated in the presence of aldosterone but not cortisol arguing for a direct interaction; inactivation of the AF-2 region in the LBD inhibited the interaction, again consistent with a direct LxxLL-mediated interaction. Tesmin contains two LxxLL motifs; mutation of the two LxxLL motifs eliminated the interactions in the co-immunoprecipitation, M-2-H and transactivation assays. The actual structural determinants of the coactivation per se remain to be determined although clearly the LxxLL motifs are essential for the primary interaction. The structural basis of the ligand discrimination, aldosterone vs cortisol, also remains to be determined.

In parallel studies using recombinant full-length MR (Clyne et al. 2009) with phage display, Yang and coworkers (Yang et al. 2011) identified novel, LxxLL motif-constrained, 19-mer peptides that interact with the MR in a ligand-dependent manner. A unique consensus binding motif, MPxLxxLL, was demonstrated in peptides that displayed robust interactions with the MR in M-2-H assays (Yang et al. 2011). Sequence alignment of the consensus motif within a protein database identified Gem (nuclear organelle)-associated protein 4 (GEMIN4) as a potential molecular partner of the MR (Yang et al. 2015). When cotransfected with the MR, Gemin4 repressed agonist-induced, MR-mediated transactivation in a cell-specific manner. This repressive effect was observed in a human embryonic kidney (HEK293) cell line, but not in a rat cardiomyocyte (H9c2) cell line, again highlighting the importance of cellular context for coregulator function (Yang et al. 2015). Gemin4 also displayed gene specificity in its corepressive activity whereby its co-expression significantly decreased the expression of four well-characterized, endogenous MR target genes (Yang et al. 2015).

In a study using T7-phage cDNA libraries derived from either human heart or kidney RNA and full-length human MR as bait, Yang and coworkers (Yang et al. 2014) identified several additional putative coregulators that differ between cell type, ligand and promoter in their coregulation of the MR response. These include eukaryotic elongation factor 1A1 (EEF1A1), structure-specific recognition protein 1 (SSRP1) and X-ray repair cross-complementing protein 6 (XRCC6). XRCC6 selectively enhanced cortisol-induced MR transactivation of three promoter constructs in HEK293 cells, while EEF1A1 was uniquely transcriptionally active in H9c2 cells. SSRP1 displayed distinct cell-specific effects as it repressed MR-mediated transactivation in the HEK293 cells but selectively upregulated aldosterone-induced MR activity in the H9c2 cells. Gene expression studies of three MR target genes, GILZ, SGK1 and CNKSR3, also revealed cell- and gene-specific differences. Overexpression of EEF1A1 enhanced transcription of all three MR target genes in H9c2 cells, but its effect in HEK293 cells was limited to CNKSR3 expression. XRCC6 increased GILZ and CNKSR but not SGK1 expression in HEK293 cells, and had no effect in H9c2 cells. Possible mechanisms for these specific effects are postulated below. The biologic function of each coactivator in the setting of MR activation remains to be elucidated although each has known functions in addition to receptor coactivation: EEF1A1 plays an important role in growth, cellular proliferation, signal transduction and nuclear export (Becker et al. 2013); SSRP1 acts as a transcription elongation factor; and XRCC6 plays a role in specific pathways for DNA repair (Boulton & Jackson 1998, Winkler & Lugner 2011).

**Gene specificity**

The nature of the coactivator complex assembled by an activated DNA-bound NR complex in a given cell type will vary with the response element and its context. Gene-specific effects of coregulators for NRs are well described (O’Malley 2003, Won Jeong et al. 2012). This may reflect changes in receptor conformation induced by distinct DNA response elements, which may in turn alter the binding of coactivators (Wood et al. 2001, Klinge et al. 2004). Pascual-Le Tallec and coworkers (Pascual-Le Tallec et al. 2003) reported promoter dependence for MR corepression by PIAS1, and Yang and coworkers (Yang et al. 2014, 2015) reported gene-specific effects for GEMIN4, XRCC6, EEF1A1 and SSRP1. These differences may be mediated by other transcription factors including so-called pioneer/licensing factors such as FOXAI which have been extensively characterized for steroid receptors particularly the AR (Pihlajamaa et al. 2015). Large-scale gene expression profiling and coactivator recruitment assays for novel progesterone receptor modulators have also found that regulation is highly gene specific despite a common coactivator recruitment profile for certain compounds (Berrodin et al. 2009). This level of detail has not yet been achieved for MR biology, and indeed cistromic information remains limited for the MR. A recent study from Le Billan and coworkers
(Le Billan 2015) did not identify an association of mineralocorticoid response elements (MRE) with binding motifs for other transcription factors, although these were found in putative binding sites that lacked MRE, a finding of uncertain significance.

**Tissue specificity**

As noted above, the cellular repertoire of coregulatory molecules will determine the cellular response to activation of the MR. Similarly, the impact of a given coregulatory molecule may be modified by this cellular milieu as, for instance, in the above studies of Obradovic and coworkers (Obradovic et al. 2004) and Yang et al. (2014, 2015) which highlight a clear context dependence for the transactivation assay. Conversely, tesmin shows equivalent ligand-specific coactivation of the MR in two disparate cell lines. Some of the studies discussed have been confined to one cell line so that no conclusions can be drawn. Ultimately it will be critical to understand the role of coregulatory molecules in vivo, particularly for the MR where appropriate cellular model systems with endogenous MR expression are generally lacking (Fuller & Young 2014).

**Conclusions**

The expansion of knowledge in the fields of aldosterone action and MR biology has been exponential in the 30 years since the cloning of the MR. Early models of steroid hormone action conceptualized a rigid, linear process in which once ligand bound, a transformation occurred in the receptor (often likened to a key opening a lock) enabling the receptor to move into the nucleus, interact with DNA and switch genes on or off. It is now clear that the mechanisms are much more complex and nuanced. The ligand-receptor interaction is not rigid but rather a dynamic interaction, with the receptor exhibiting considerable plasticity in that the receptor conformation is determined by the ligand. The resulting signal transduction is determined by interactions with an extensive, cell-specific repertoire of coregulatory molecules discussed in this review. There are thus ligand-, gene-, and cell-specific MR responses determined by coactivator interactions. As with many things, the broad concepts articulated 30 years remain robust but the ‘devil is (very clearly) in the detail’. It is clear, albeit ‘after the fact’ that receptor coactivator interactions determine whether an ER ligand is agonist (estradiol), a SERM (tamoxifen) or an antagonist (fulvestrant). The MR is an important therapeutic target in cardiovascular disease, but the current MR antagonists are limited by their lack of tissue specificity resulting in hyperkalaemia (Juurlink et al. 2004) due to concurrent renal MR blockade. Antagonists that block the MR in cardiovascular tissues, but not in the distal nephron, would seem highly desirable, but have yet to emerge. As the role of the MR in an expanding range of tissues becomes more fully elucidated, it is tempting to speculate that new MR ligands may emerge, selective MR modulators, which exploit the ligand- and tissue-specific MR coactivators to provide tissue specificity, not just in cardiovascular disease but across a range of conditions.

**Declaration of interest**

The authors declare that they have no conflict of interest.

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Thematic Review

Hypertension


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