Evolution of the Mineralocorticoid Receptor: Sequence, Structure and Function

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Abstract. The mineralocorticoid receptor (MR) is descended from a corticoid receptor (CR), which has descendants in lamprey and hagfish, cyclostomes (jawless fish), a taxon that evolved at the base of the vertebrate line. A distinct MR and GR first appear in cartilaginous fishes (Chondrichthyes), such as sharks, skates, rays and chimaeras. Skate MR has a strong response to corticosteroids that are mineralocorticoids and glucocorticoids in humans. The half-maximal responses (EC50s) for skate MR for the mineralocorticoids aldosterone and 11-deoxycorticosterone are 0.07 nM and 0.03 nM, respectively. EC50s for the glucocorticoids cortisol and corticosterone are 1 nM and 0.09 nM, respectively. The physiological mineralocorticoid in ray-finned fish, which do not synthesize aldosterone, is not fully understood because several 3-ketosteroids, including cortisol, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone and progesterone are transcriptional activators of fish MR. Divergence of the MR and GR in terrestrial vertebrates, which synthesize aldosterone, led to increased selectivity of the MR for aldosterone, coupled with a diminished response to cortisol and corticosterone. Here, we combine sequence analysis of the CR and vertebrate MRs and GRs, analysis of crystal structures of human MR and GR and data on transcriptional activation by 3-ketosteroids of wild-type and mutant MRs and GRs to investigate the evolution of selectivity for
3-ketosteroids by the MR in terrestrial vertebrates and ray-finned fish, as well as the basis for binding of some glucocorticoids by human MR and other vertebrate MRs.

**Introduction**

In this special issue of JOE, we celebrate the thirtieth anniversary of cloning of the mineralocorticoid receptor (MR) in the Evans laboratory at the Salk Institute (Arriza, et al. 1987). This was an impressive achievement. Indeed, it was not an easy task, as the MR was the last cloned receptor from the adrenal and sex steroid receptor family, which also includes the glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR) and estrogen receptor (ER) (Baker, et al. 2015; Evans 1988; Markov, et al. 2009; Baker, 2011 #31). The MR and other steroid receptors belong to the nuclear receptor family, a diverse group of transcription factors that arose in multicellular animals, which have key roles in the physiology of humans and other vertebrates (Baker, et al. 2013; Bridgham, et al. 2010; Huang, et al. 2010; Markov et al. 2009).

The availability of recombinant human MR facilitated the cloning of MRs from a wide variety of vertebrates and an analysis of the evolution of the MR. These MR sequences and those of the GR confirmed the original observation of kinship between the MR and GR (Arriza et al. 1987; Baker, et al. 2007; Bridgham, et al. 2006; Evans 1988). The MR and GR are descended from the corticoid receptor (CR) (Baker et al. 2007; Baker et al. 2015; Bridgham et al. 2006; Thornton 2001). Descendants of the ancestral CR are found in lampreys and hagfish, which are cyclostomes (jawless fish), a taxon that evolved at the base of the vertebrate line (Figure 1) (Osorio and Retaux 2008; Sauka-Spengler and Bronner-Fraser 2008). A distinct MR first appears in cartilaginous fishes (Chondrichthyes), such as sharks, skates, rays and chimaeras (Baker et al. 2015; Bridgham et al. 2006; Carroll, et al. 2008).

Studies with recombinant human MR yielded some unexpected findings. First, Arriza et al. (Arriza et al. 1987) showed that aldosterone (Aldo), cortisol (F), 11-deoxycortisol (S), corticosterone (B), 11-deoxycorticosterone (DOC) and progesterone (Prog) (Figure 2) had similar equilibrium binding constants (Kds) for human MR. This indicated that the steroid binding site on the human MR was not selective for physiological mineralocorticoids (Aldo, DOC) over glucocorticoids (F, B, S) and Prog.
Figure 1. MR, GR, PR, AR and ER in vertebrates. The ER, CR and PR are found in lampreys and hagfish, jawless fishes (cyclostomes) that evolved at the base of the vertebrate line. A separate MR and GR are found in skates, rays, sharks and chimaeras, cartilaginous fishes that are basal jawed vertebrates. The AR first appears in cartilaginous fishes. Land vertebrates are descended from lobe-finned fishes [coelacanths and lungfishes].
Figure 2. Structures of Mineralocorticoids and Glucocorticoids. Aldosterone [Aldo] and 11-deoxycorticosterone [DOC] are the main physiological mineralocorticoids in vertebrates. Cortisol [F] and corticosterone [B] are the main physiological glucocorticoids in vertebrates. 11-deoxycortisol [S] has activity as mineralocorticoid and glucocorticoid in lamprey CR (Baker 2011). Progesterone [Prog] is an antagonist for human MR (Rupprecht et al. 1993, Geller, 2000 #148) and an agonist for fish MRs (Pippal et al. 2011; Sturm et al. 2005; Sugimoto et al. 2016).

The similar affinity of F, B and Aldo for the MR raised the question of how Aldo could occupy the MR in the presence of F and B, which have from 100 to 1000 fold higher concentrations in human and rodent serum, respectively, than that of Aldo. Human MR should be occupied by F and rodent MR by B. Selective binding of Aldo to the MR in the presence of either F or B arises from a novel enzymatic mechanism involving expression of 11β-hydroxysteroid dehydrogenase-type 2 (11βHSD2) in epithelia containing the MR. This enzyme metabolizes the 11β-OH of F and B to a ketone, yielding cortisone and 11-dehydrocorticosterone, respectively, two inactive steroids (Baker 2010; Chapman, et al. 2013; Draper and Stewart 2005;
Edwards, et al. 1988; Funder, et al. 1988; Odermatt and Kratschmar 2012). Aldo is inert to 11βHSD2 and can occupy the kidney and colon MRs in the presence of 11βHSD2. However DOC, S and Prog lack an 11β-OH, which allows these steroids to compete with Aldo for the MR.


Important in understanding MR evolution is the pathway for the synthesis of corticosteroids that are ligands for the MR (Figure 3). Progressive modification of Prog yields steroids with substituents that are ligands for either the MR and GR or both. The position of each steroid in this pathway appears to coincide with the evolution of steroids in vertebrates as physiological activators of the CR, MR and GR. For example, Aldo, which is at the end of one synthetic pathway, is not present in either lamprey or hagfish serum (Bridgham et al. 2006). Potential activators of the CR are S, DOC and Prog, which have been found in Atlantic sea lamprey serum (Close, et al. 2010; Roberts, et al. 2014; Wang, et al. 2016). These steroids are at the beginning of the pathway. S has mineralocorticoid activity in lamprey (Close et al. 2010); the roles of DOC, which is a mineralocorticoid in mammals (Hawkins, et al. 2012; Lam, et al. 2006) and of Prog are not known.
Figure 3 Pathway for synthesis of Aldosterone from Progesterone and Cortisol from 17α-OH-Progesterone. Progesterone is hydroxylated at C21 to form 11-deoxycorticosterone, which is hydroxylated at C11 to form corticosterone. Hydroxylated of corticosterone at C18 followed by oxidation of the C18 hydroxyl forms aldosterone. In a second pathway, progesterone is hydroxylated at C17 and then hydroxylated at C21 to form 11-deoxycortisol, which is hydroxylated at C11 to form cortisol (Baker 2011; Rossier et al. 2015).
**Figure 4. Comparison of Domains on MR, GR, PR, AR and CR.** The A/B domain to E domains are schematically represented with the numbers of amino acid residues and the percentage of amino acid identity between the domains compared to human MR. For example, the entire human MR sequence is 82% identical to that of chicken MR, while domain E (LBD) on human MR is 93% identical to that of chicken MR. Accessions are in Supplement Table 1.

Chondrichthyes contain B and a novel derivative 1α-OH-B, which has not been found in other vertebrates. F and B, but not Aldo, are found in ray-finned fish (Jiang, et al. 1998; Sakamoto, et al. 2011). Aldo first appears in tetrapods (Joss, et al. 1994; Rossier, et al. 2015), which also contain F and B.

Interestingly, Aldo has low EC50s for the MR in vertebrates that do not synthesize Aldo. The EC50 of Aldo for hagfish CR is 0.4 nM (Bridgham et al. 2006). The EC50s of Aldo, DOC

<table>
<thead>
<tr>
<th>Domain</th>
<th>Amino Acid Residues</th>
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<tr>
<td>A/B</td>
<td>603 669 732 984</td>
</tr>
<tr>
<td>C</td>
<td>604 670 729 981</td>
</tr>
<tr>
<td>D</td>
<td>600 666 727 979</td>
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<tr>
<td>E</td>
<td>601 667 718 970</td>
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<tr>
<td>F</td>
<td>585 651 704 956</td>
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<tr>
<td>G</td>
<td>237 303 367 619</td>
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<tr>
<td>H</td>
<td>421 487 526 777</td>
</tr>
<tr>
<td>I</td>
<td>21 94 29 56</td>
</tr>
<tr>
<td>J</td>
<td>567 633 681 933</td>
</tr>
<tr>
<td>K</td>
<td>20 79 30 51</td>
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**Accessions are in Supplement Table 1.**
and B for skate MR are 0.07 nM, 0.03 nM and 0.09 nM, respectively (Carroll et al. 2008).

11βHSD2 first appears in Chondrichthyes (Baker et al. 2015; Rossier et al. 2015), in which
11βHSD2 may regulate access of B to the MR. Although Aldo is not found in ray-finned fish,
Aldo is a strong transcriptional activator of fish MR (Greenwood, et al. 2003; Pippal, et al. 2011;
mineralocorticoid in ray-finned fish is not fully understood because several 3-ketosteroids,
including F, DOC, B, S and Prog are transcriptional activators of the MR (Greenwood et al.
2003; Pippal et al. 2011; Stolte et al. 2008; Sturm et al. 2005; Sugimoto et al. 2016), and one or
more of these steroids could be a physiological mineralocorticoid.

Thus, during the evolution of the MR in cartilaginous fishes, ray-finned fishes and
terrestrial vertebrates, there have been changes in MR specificity for corticosteroids, as well as
the MR’s physiological function (Hawkins et al. 2012; Jaisser and Farman 2016; Martinerie, et al.
To gain a deeper understanding of the evolution of the MR, we have taken advantage of the
sequencing, in the last five years, of a cornucopia of genomes from vertebrates at key
evolutionary transitions, including lamprey, elephant shark and coelacanth, to investigate regions
of conservation and divergence among and between MRs and GRs. We use these sequence
analyses, the crystal structures human MR (Bledsoe, et al. 2005; Edman, et al. 2015; Fagart, et al.
2005; Li, et al. 2005) and GR (Bledsoe, et al. 2002; He, et al. 2014) and functional studies of
human MR mutants (Bledsoe et al. 2005; Fagart, et al. 1998; Geller et al. 2000; Jimenez-Canino,
et al. 2016; Li et al. 2005; Shibata, et al. 2013,Mani, 2016 #272) to investigate the evolution of
selectivity for Aldo and other corticosteroids by the MR in terrestrial vertebrates (Baker et al.
2013) and ray-finned fish (Arterbery, et al. 2011; Bury and Sturm 2007; Prunet, et al. 2006;
Sugimoto et al. 2016), as well as the basis for binding of some glucocorticoids by human MR
and other vertebrate MRs (Li et al. 2005; Mani et al. 2016). Together these data provide
molecular and structural fingerprints for investigating the evolution of selectivity for 3-
ketosteroids by the MR in vertebrates.

The MR is a multi-domain transcription factor

Like other steroid receptors, the MR is composed of several functional domains (Figure
4). The MR contains an A/B domain at the N-terminus (NTD), a DNA-binding domain (DBD)
(C domain) near the center, a short hinge domain (D domain) and a steroid-binding domain (LBD) (E domain) at the C-terminus (Arriza et al. 1988; Huang et al. 2010; Huyet, et al. 2012; Pascual-Le Tallec and Lombes; Yang and Young 2009). The A/B domain contains an activation function 1 [AF1] and the E domain contains an AF2 domain (Faresse 2014; Huyet et al. 2012; Li et al. 2005; Pascual-Le Tallec and Lombes 2005). Each domain in MR is important for transcriptional responses (Faresse 2014; Fuller, et al. 2012).

As shown in Figure 4, the DBD is highly conserved in vertebrate MRs, while the NTD and hinge domains are poorly conserved. The LBD has intermediate sequence conservation, which makes it useful for phylogenetic analysis of the MR (Baker et al. 2015; Bridgham, et al. 2008; Rossier et al. 2015). In addition, the sequence of the LBD in vertebrate MRs is of interest because mutations in the LBD have been correlated with changes in transcriptional activation by Aldo and other steroids (Bledsoe et al. 2005; Bridgham et al. 2006; Fagart et al. 2005; Fagart et al. 1998; Funder 2013; Geller et al. 2000; Li et al. 2005; Shibata et al. 2013, Mani, 2016 #272). Thus, a multiple alignment of the LBD of key vertebrate MRs and GRs can be used for a phylogenetic analysis of the MR, as well as to identify sites that could be important in functional divergence of vertebrate MRs from each other and from the GR.

**Evolution of vertebrate MR steroid-binding domain: Divergence from its GR paralog**

Figure 5 shows a multiple alignment of the steroid-binding domain on various MRs, GRs, CRs, PRs and AR from vertebrates at key evolutionary transitions. Figure 5 also shows the α-helices on the MR as determined by X-ray crystallography (Bledsoe et al. 2005; Edman et al. 2015; Fagart et al. 2005; Li et al. 2005) and amino acids that have been found to be important in either steroid binding or transcriptional activation of the MR or CR (Bledsoe et al. 2005; Bridgham et al. 2006; Edman et al. 2015; Fagart et al. 1998; Geller et al. 2000; Hultman, et al. 2005; Jimenez-Canino et al. 2016; Li et al. 2005; Mani et al. 2016; Shibata et al. 2013) or the GR (Bledsoe et al. 2002; Edman et al. 2015; He et al. 2014; Mani et al. 2016). A striking feature, discussed in more detail below, is the strong conservation of amino acids among the CR, MR, GR, PR and AR, including lamprey PR and CR. Indeed, some amino acids are conserved in vertebrate MRs, GRs and CRs and even in lamprey and hagfish PRs.

We used this multiple alignment to construct an up-dated phylogeny of the steroid-binding domain on the MR and other 3-ketosteroid receptors (Figure 6). This phylogeny
indicates that the CR and PR evolved from an ancestral 3-ketosteroid receptor through gene duplication and divergence (Baker et al. 2015; Rossier et al. 2015; Thornton 2001); that the MR and GR evolved from an ancestral CR; that the MR is closer than the GR is to the CR, and that the CR ancestor of the MR and GR appears to be lost in Pacific and Atlantic lamprey. Also, the presence of the PR and the absence of an AR in cyclostomes indicates that the AR evolved from a duplication of an ancestral PR.
Figure 5 Alignment of the steroid binding domain on vertebrate MRs, CRs, GRs, PRs and AR. The steroid binding domains were collected with BLAST searches of GenBank. Clustal W was used to construct the multiple alignment (Larkin, et al. 2007). The crystal structure of human MR (PDB: 2A3I) {Li, 2005 #10} was used to locate α-helices. Amino acids that contact Aldo are shown above human MR. The highly conserved Glu-962 is part of AF2, which contacts co-activator proteins. The functions of Ser-949 and His-950, remain to be elucidated.

Figure 6 Phylogenetic analysis of vertebrate MRs, GRs, CRs, PRs and ARs. Steroid binding domains were collected with BLAST searches of GenBank. Then Clustal W (Larkin et al. 2007) was used to construct the multiple alignment, and phylogenetic trees were constructed with Maximum likelihood (ML) analysis conducted using JTT+G+I model (Guindon and Gascuel 2003). Statistical confidence for each branch in the tree was evaluated with 1,000 bootstrap runs. MEGA5 program was used for these analysis (Tamura, et al. 2011). A gene duplication of an ancestral 3-ketosteroid receptor in cyclostomes produced the ancestors of the CR and PR in modern lampreys and hagfishes. Lamprey and hagfish CR and lamprey PR cluster in one branch. The MR and GR are in another branch. The CR ancestor of the MR and GR appears to be lost in lamprey and hagfish. In Gnathostomes, a gene duplication produced the AR and PR.
Figure 7. Contacts between the MR and Aldo and two water molecules. Contacts between Aldo with human MR (PDB: 2AA2) (Bledsoe et al. 2005) are shown. Two water molecules mediate contacts between Ser-810 and the A ring of Aldo.

Evolution of contacts between the MR and A and B rings on Aldo and other 3-ketosteroids

Stabilizing interactions between α-helix 3 and α-helix 5 with each other and with the A and B rings on corticosteroids are important in transcriptional activation of the MR (Baker et al. 2013; Bledsoe et al. 2005; Fuller et al. 2012; Geller et al. 2000; Huyet et al. 2012; Li et al. 2005), as well as other 3-ketosteroid receptors. Consistent with the common ancestry of the MR, GR, PR, AR and structural similarities of the A and B rings in their canonical ligands, some key amino acids in the MR are conserved in the GR, PR, AR and CR (Baker et al. 2013; Li et al. 2005; Mani et al. 2016). However, other amino acids are not conserved, providing specificity for mineralocorticoids (Baker et al. 2007; Baker et al. 2013; Bledsoe et al. 2005; Huyet et al. 2012), glucocorticoids (Bledsoe et al. 2002; He et al. 2014), progestins (Williams and Sigler 1998) and androgens (Sack, et al. 2001) in their cognate receptors.

For example, in human MR, Gln-776 (helix 3) and Arg-817 (helix 5) are conserved in corresponding positions in vertebrate MR, GR, PR, AR and CR (Figure 5, Figure 7). Also conserved in human MR, lamprey CR, as well as the GR, PR and AR are contacts between the side chain on Phe-829 (human MR) with the A ring on corticosteroids and between the backbone oxygen on Phe-829 with Nε and Nη2 on Arg-817 (Figures 5 and 7).
Ser-810 (helix 5) in human MR: Evolution in the common ancestor of ray-finned and lobe-finned fish

Ser-810 in human MR also is important in binding of the A ring of steroids. The crystal structure of human MR with Aldo reveals that Ser-810 stabilizes the A ring on Aldo through a hydrogen bond network with two water molecules (Bledsoe et al. 2005). In one hydrogen bond network, a water molecule contacts $\text{O}_{\gamma}$ on Ser-810 and the C3-ketone on Aldo; in another network, a water molecule contacts the backbone oxygen on Ser-810, $\text{N}_{\eta2}$ on Arg-817 and the C3-ketone on Aldo (Bledsoe et al. 2005) (Figure 7).

A serine corresponding to Ser-810 in human MR first appears in ray-finned fish and lobe-finned fish (Figure 5) (Baker et al. 2007; Baker et al. 2013; Baker, et al. 2011). In contrast, chondrichthian MRs and cyclostome CRs contain a methionine corresponding to Ser-810. Moreover, the GR, PR, and AR also have a methionine at this position (Figure 5) (Baker et al. 2007; Baker et al. 2013; Baker et al. 2011). Thus, this water-mediated hydrogen bond between $\text{O}_{\gamma}$ on Ser-810 and C3-ketone on Aldo, which emerged in the MR in common ancestor of ray-finned fish and tetrapods, is unique among 3-ketosteroid receptors. The evolution of this serine in the MR affects binding to 3-ketosteroids because, as noted by Bledsoe et al. (Bledsoe et al. 2005), methionine at this position cannot participate in a water-mediated hydrogen bond with the C3-ketone on corticosteroids, indicating that there was a change in the mechanism for stabilization of the C3-ketone in the MR in ray-finned fish and tetrapods.

Moreover, as discussed below, it appears that replacement of methionine with serine was important the loss transcriptional activation of the MR by Prog (Geller et al. 2000) and cortisone (Rafestin-Oblin, et al. 2003).

Evolution of the contact between Ser810 (Helix 5)-Ala773 (Helix 3) in human MR: Role in the divergence of the MR and GR

Important evidence for a physiological role of Ser-810 in human MR comes from a report in 2000 by Geller et al. (Geller et al. 2000), who identified a Ser810Leu mutation in the MR, which was activated by Prog (EC50 of ~ 1nM). Prog activation of the MR is unexpected because Prog is an antagonist for wild-type human MR (Geller et al. 2000; Rafestin-Oblin et al. 2003; Rupprecht et al. 1993; Sugimoto et al. 2016). The mineralocorticoid activity of Prog for
Leu-810 MR explained high blood pressure in pregnant woman with this mutant MR. In addition, cortisone, which binds poorly to human MR, is an agonist for the Leu-810 MR (Rafestin-Oblin et al. 2003) and could cause hypertension in people with this mutant MR. Moreover, spironolactone, an MR antagonist, activated the Ser810Leu MR in COS-7 cells. Thus, the evolution of an ancestral Ser-810 in the MR in ray-finned fish and tetrapods has an important physiological consequence in preventing activation of the MR by Prog and cortisone.

A 3D model of Leu810-MR found a van der Waals contact between Leu-810 and Ala-773 in the mutant MR, which stabilized the contact between helix 3 and helix 5 (Geller et al. 2000). Transcriptional analyses of MRs with mutations at 810 and 773 supported stabilization of the helix 3-helix 5 contact in the agonist activity of Prog. Subsequent crystal structures of Leu810MR found a stabilizing interaction between helix 3 and helix 5 (Bledsoe et al. 2005; Fagart et al. 2005). This contact between Ala-773 and Ser-810 is not found in crystal structure of wild-type human MR (Bledsoe et al. 2005; Li et al. 2005).

As mentioned previously, Ser-810 evolved in the MR in ray-finned fish and tetrapods. Lamprey and hagfish CR have cysteine (Cys-227) and methionine (Met-264) corresponding to Ala-773 and Ser-810, respectively. A 3D model of lamprey CR found a van der Waals contact between Cys-227 and Met-264. In skate and elephant shark MR, this cysteine is replaced with the alanine that is conserved MR descendants. Based on mutagenesis studies of Geller et al. (Geller et al. 2000), we predict that Ala-191 and Met-238 in skate MR and Ala-745 and Met-782 in elephant shark MR (corresponding to human MR Ser-810), will have van der Waals contacts and, thus, Prog will be an agonist for skate and elephant shark MRs.

The evolution of this helix 3-helix 5 contact in the GR affects its response to 3-ketosteroids and the divergence of the GR and MR. Gly-106 in skate GR and Gly-227 in elephant shark GR correspond to Ala-191 in skate MR and Ala-745 in elephant shark MR. The GR in tetrapods and ray-finned fish conserves a corresponding glycine (helix 3) and methionine (helix 5). The human GR crystal structure (Bledsoe et al. 2002; Zhang, et al. 2005) reveals, as expected, that Gly-567 (helix 3), which lacks a side chain, does not contact Met-604 (helix 5). Interestingly, replacement of Gly-567 with Ala-567 decreases the response to F, B and DEX by at least 10-fold (Zhang et al. 2005). In skate and elephant shark MR, the corresponding site contains an alanine suggesting that the emergence in cartilaginous fish GRs of a glycine
corresponding to Gly-567 in human GR was important in evolution of specificity for glucocorticoids.

**Evolution of contacts between the MR and C and D rings on 3-ketosteroids.**

Crystal structures of the MR reveal that differences in contacts between the MR and hydroxyl groups on the C and D rings of 3-ketosteroids (Figure 2) influence their transcriptional activity for the various MRs, as well as for the GR and other steroid receptors (Bledsoe et al. 2005; Bledsoe et al. 2002; Huang et al. 2010; Huyet et al. 2012).

Vertebrate MRs, CRs and chondrichthian GR and PR conserve many amino acids in human MR (Figure 5) that contact the C and D rings on Aldo (Figure 7), DOC and B. These include Asn-770 (helix 3), Met-852 (helix 7), Phe-941 (helix 11), Cys-942 (helix 11) and Thr-945 (helix 11) (Figure 5). Tetrapod and ray-finned fish GRs also conserve amino acids corresponding to Asn-770, Met-852, Cys-942 and Thr-945, but not Phe-941 in human MR. Interestingly lamprey PR conserves amino acids corresponding to Asn-770, Met-852, Phe-941, Cys-942 and Thr-945 in human MR.

**Ser-843 (helix 6) in human MR: Role in divergence from the GR**

Analysis of the crystal structure of the human MR with B (Li et al. 2005) and human GR with DEX (Bledsoe et al. 2002) identified a pocket containing helices 6 and 7 that was present in the GR and not in the MR. This pocket on the GR could accommodate a 17α-hydroxyl group on F and DEX and glucocorticoids with other 17α substituents. Two amino acid differences between human MR and GR (Ser-843 and Leu-848 in human MR, Pro-637 and Gln-642 in human GR) (Figure 5) were identified as important in this conformational change. Indeed, when human GR and MR are superimposed, Ser-843 in the MR is displaced by over 5 Å from Pro-637 in the GR and Leu-848 is 4.5 Å from C16 on B (Baker et al. 2013; Rossier et al. 2015) (Figure 8), which could be important in different responses between MR and GR to F and DEX (Li et al. 2005). In human GR, Gln-642 has a hydrogen bond with the 17α-hydroxyl on DEX (Figure 8) (Bledsoe et al. 2002; He et al. 2014). In the MR, the hydrophobic side chain on Leu-848 was proposed to clash with the 17α-hydroxyl on F and DEX. In contrast, B, Aldo and DOC, which lack a 17α-hydroxyl, would not clash with Leu-848, explaining the stronger response of the MR to these steroids. However, a crystal structure of the MR with DEX (Edman et al. 2015) did not
find a steric clash between Leu-848 on the MR with the 17α-hydroxyl on Dex suggesting that
other sites on the MR and GR are important in their transcriptional response to F and other
corticosteroids with a 17α-hydroxyl group.

Figure 8. Comparison of Ser-843/Leu-848 on human MR with Pro-637/Gln-642 on human
GR. Human GR complexed with dexamethasone (PDB: 1M2Z) (Bledsoe et al. 2002) and
human MR complexed with dexamethasone (PDB: 4UDA) (Edman et al. 2015) were
superimposed. In human MR, Cδ1 on Leu-848 is 6.2 Å and 3.8 Å, respectively, from 17α-OH
and 16α-CH3 on DEX. In human GR, Oε1 is 3.0 Å and 3.6 Å, respectively, from 17α-OH and
16α-CH3 on DEX. Ser-843 and Pro-637 are displaced by over 5 Å.

Supporting this hypothesis are the low EC50s of F for fish MR, which conserve a serine
and leucine corresponding to Ser-843 and Leu-848 in human MR. The EC50 of F is 0.02 nM for
cichlid (Greenwood et al. 2003), 1.1 nM for trout (Sturm et al. 2005), 2.4 nM for carp (Stolte et
al. 2008) and 0.22 nM for zebrafish (Pippal et al. 2011).

Nevertheless, the Ser-Pro mutation in helix 6 in the MR likely has some biochemical
effect that is important in divergence of the GRs in ray-finned fish and terrestrial vertebrates
from the GR and MR in cartilaginous fish. Indeed, mutagenesis of amino acids in an ancestral
CR (AncCR) corresponding to Ser-843 and Leu-848 was incorporated into a novel model to
investigate the evolution specificity for steroids with a 17α-hydroxyls such as F for the GR
(Bridgham et al. 2006). First, AncCR was transfected into cells and exposed to Aldo or F. The AncCR had a strong response to Aldo and a weak response to F. Then Ser-106 and Leu-111 on AncCR, corresponding to Ser-843 and Leu-848, were mutated to Pro and Gln, as found in ray-finned fish and terrestrial vertebrate GRs. The AncCR-Gln111 mutant had low activity for Aldo, F and DOC, while AncCR-Pro106 was activated by Aldo, DOC and F. The subsequent double AncCR-Pro106/Gln111 mutant had an increased response to F and low response to Aldo, indicating that the GR evolved from AncCR through a step wise mutation of Ser-106 to Pro followed by Leu-111 to Gln. However, studies with human MR (Li et al. 2005; Mani et al. 2016) find that Leu843Gln human MR mutant has a favorable response to F, unlike that of the AncCR, leaving unresolved the pathway for the formation of Pro and Gln in the GR. Future studies with mutations at the corresponding serine and leucine residues in GRs and MRs in cartilaginous fish should provide more direct data on the pathway for the evolution of specificity for corticosteroids in the GR and MR.

**Phosphorylation of Ser-843 inactivates human MR**

An important discovery of another physiological role of Ser-843, also relevant for the Ser to Pro mutation in the GR ancestor of lobe-finned and ray-finned fish, comes from a report by Shibata et al. (Shibata et al. 2013) showing that under normal conditions Ser-843 in human MR is phosphorylated in intercalated cells in the kidney distal tubule and this phosphorylated MR is inactive. De-phosphorylation of Ser-843 by a phosphatase induced by angiotensin II activates the MR, such that binding of Aldo leads to sodium chloride absorption and potassium secretion. Interestingly, high potassium levels increase phosphorylation of Ser-843 (Funder 2013; Jimenez-Canino et al. 2016; Shibata et al. 2013). Phosphorylated Ser-843 human MR has only been found in intercalated cells in the kidney distal tubule; other cells do not contain phosphorylated MR.

A serine corresponding to Ser-843 is found in lamprey and hagfish CR, cartilaginous fish MRs and GRs and lamprey PR. This serine also is conserved in descendent MRs, PRs and ARs. If skate MR Ser-261 and GR Ser-176 and elephant shark MR Ser-815 and GR Ser-297, are phosphorylated in vivo, then the evolution of a corresponding proline in the GR lobe-finned and ray-finned fishes would provide a mechanism for specificity for regulation of transcriptional activation of the MR through a kinase/phosphatase that would not affect the GR.
At this time, it is not known if this serine is phosphorylated in lamprey PR or other PRs and ARs, or if phosphorylation alters the response to steroids.

**Unanswered questions.**

Dobzhansky’s aphorism “Nothing in Biology Makes Sense Except in the Light of Evolution” (Dobzhansky.T 1973) is our lodestar for investigating the evolution of the MR as well as other steroid receptors and steroidogenic enzymes. In this spirit we discuss other properties of the MR that merit further investigation to shed light on the evolution of the MR.

**Transcriptional activation of fish MR by Prog, a possible mineralocorticoid**

The absence of Aldo in fish has led to speculation that F and DOC may be a physiological mineralocorticoid for fish (Arterbery et al. 2011; Baker 2003; Baker et al. 2007; Bury and Sturm 2007; McCormick, et al. 2008; Prunet et al. 2006; Sakamoto et al. 2011; Sakamoto et al. 2016; Sturm et al. 2005; Takahashi and Sakamoto 2013). Interestingly Prog is a transcriptional activator of ray-finned fish, which also respond to 19-norProg and spironolactone (Pippal et al. 2011; Sturm et al. 2005; Sugimoto et al. 2016). This response is unexpected because Ser-810 in human MR is crucial for the absence of transcriptional activation by Prog, spironolactone and 19-norProg (Baker et al. 2013; Bledsoe et al. 2005; Fagart et al. 2005; Geller et al. 2000), and fish MR contain a serine corresponding to Ser-810 in human MR. The basis for this novel response to Prog, spironolactone and 19-norProg is not known. Nevertheless, Prog may be a physiological mineralocorticoid in fish. Like DOC, Prog lacks an 11β-hydroxyl and thus is inert to 11β-HSD2 (Chapman et al. 2013; Odermatt and Kratschmar 2012).

**Function of Ser-849 in human MR and its deletion in tetrapod and ray-finned fish GRs**

Human MR contains Ser-949 in the loop connecting helix 11 and helix 12. A corresponding serine is found in other MRs, shark GR, the CR, lamprey and human PR and human AR, but not in the GR in tetrapods and ray-finned fish [Figure 5] (Baker et al. 2007; Baker et al. 2013). The physiological consequences of this serine in human MR and its deletion in the GR are not known. This difference between the GR and MR appears to alter the conformation of helix 12, which contains AF2, in human GR and MR [Figure 9]. Differences in the conformation of AF2 may be important in selective binding of co-activators to MR and GR

**Figure 9. Superposition of helix 12 and the preceding loop in human MR and human GR.**

Human GR has a deletion corresponding to Ser-949 in human MR, which is in the loop connecting helix 11 and helix 12 in human MR. In human GR, this deletion displaces Oδ2 on Glu-755 in helix 12 on human GR from Oδ2 on Glu-962 in human MR by 3.5 Å. In the loop preceding helix 12, Nζ on Lys-743 on human GR is 3 Å from Ne2 on His-950 on human MR. Glu-755 and Glu-962 in the AF2 domain are highly conserved and are part of charge clamp 1 between co-activators and the GR and MR (Kattoula and Baker 2014; Li et al. 2005).

**His-950 in human MR: role in the evolution in old world monkeys**

A histidine corresponding to His-950 evolved in the MR in old world monkeys (Baker et al. 2007), which separated from new world monkeys about 40 million years ago. The MR in new world monkeys, other primates, birds, amphibians, coelacanths and ray-finned fish contains glutamine at this position [Figure 5] (Baker et al. 2013). The functional basis for mutation of a highly conserved glutamine to a histidine, amino acids with different structures, is not known.
Nevertheless, the differences between glutamine and histidine suggest that this is not a neutral mutation.

**Transcriptional activation by MR-GR heterodimers**

Mammalian MR and GR regulate gene transcription as homodimers (Liu, et al. 1995; Mifsud and Reul 2016). However, reflecting the kinship of the MR and GR, there is evidence that they form functional heterodimers with different properties than their homodimers (Bradbury, et al. 1994; Liu et al. 1995; Ou, et al. 2001; Trapp, et al. 1994). In human hippocampus, stress increases cortisol to levels that occupy the MR and GR. Cortisol activated MR-GR heterodimers bind to glucocorticoid response elements, regulating glucocorticoid target genes (Mifsud and Reul 2016). Recently, heterodimers between trout MR and GR were studied in detail, and the MR in the presence of either cortisol or DOC was found to be a dominant negative repressor of trout GR (Kiilerich, et al. 2015). Thus, the actions of the MR and GR in cells that co-express both receptors is complex and can be influenced by steroids that bind both receptors or are selective for each receptor.

Conservation of functional MR-GR heterodimers over 400 million years suggests that MR-GR heterodimers confer some selective advantage(s) in vertebrates. One possible activity of MR-GR heterodimers in fish comes from cortisol activation of the GR in fish, regulating electrolyte balance (Cruz, et al. 2013; Kumai, et al. 2012). Mineralocorticoid activity of the GR is surprising and raises the question: what role, if any, does the MR have in osmoregulation in fish? One possibility is that fish MR influences osmoregulation through formation of MR-GR heterodimers. In any event, conservation of heterodimer formation between the MR and its GR kin during the evolution of tetrapods and ray-finned fish and suggests new avenues of research to elucidate physiological responses to corticosteroids by the MR and GR.

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**Author contributions**

M.E.B and J.K. conceived of and wrote the paper.

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