30 YEARS OF THE MINERALOCORTICOID RECEPTOR

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 chimeras. 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. Further divergence of the MR and GR in terrestrial vertebrates, which synthesize aldosterone, led to emergence of aldosterone as a selective ligand for the MR. 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.

Key Words

- ▶ corticosteroid receptor evolution
- ▶ mineralocorticoid receptor evolution
- ▶ glucocorticoid receptor evolution
- ▶ lamprey
- ▶ cartilaginous fishes

Journal of Endocrinology
(2017) **234**, T1–T16

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) (Evans 1988, Markov *et al.* 2009, Baker *et al.* 2015). 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 (Markov *et al.* 2009, Bridgham *et al.* 2010, Huang *et al.* 2010, Baker *et al.* 2013). A 3-ketosteroid receptor (SR) ancestor of the MR, GR, PR and AR first

Figure 1

MR, GR, PR, AR and ER in vertebrates. The ER, CR and PR are found in lampreys and hagfish, which are jawless fishes (cyclostomes) that evolved at the base of the vertebrate line. A separate MR and GR are found in cartilaginous fish (Chondrichthyes), which are divided into the subclass Elasmobranchii containing sharks, skates and rays and the subclass Holocephali (chimeras) containing elephant sharks and which together are basal jawed vertebrates. These basal jawed vertebrates, which contain cartilage instead of bone, are forerunners of bony fishes, which are divided into the subclass Actinopterygii (ray-finned fish) containing, for example, sturgeon, zebrafish and salmon, and the subclass Sarcopterygii (lobe-finned fish) containing coelacanths and lungfish, which are forerunners of tetrapods (land vertebrates). The AR first appears in cartilaginous fishes.

appears in amphioxus, a close ancestor of vertebrates (Bridgham *et al.* 2008, Paris *et al.* 2008, Markov *et al.* 2009, Katsu *et al.* 2010, Baker *et al.* 2015).

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, Evans 1988, Bridgham *et al.* 2006, Baker *et al.* 2007, Kassahn *et al.* 2011). The MR and GR are descended from the corticoid receptor (CR) (Thornton 2001, Bridgham *et al.* 2006, Baker *et al.* 2007, Kassahn *et al.* 2011, Baker *et al.* 2015). 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 (Fig. 1) (Osorio & Retaux 2008, Sauka-Spengler & Bronner-Fraser 2008). A distinct MR first appears in cartilaginous fishes (Chondrichthyes), which consist of two subclasses Elasmobranchii containing sharks, skates and rays, and Holocephali containing chimeras, such as the elephant shark (Bridgham *et al.* 2006, Carroll *et al.* 2008, Baker *et al.* 2015).

Studies with recombinant human MR yielded some unexpected findings. (Arriza *et al.* 1987) Aldosterone (Aldo), cortisol (F), 11-deoxycortisol (S), corticosterone (B) and 11-deoxycorticosterone (DOC) (Fig. 2) had similar equilibrium binding constants (Kds) for human MR (Arriza *et al.* 1987, Rupprecht *et al.* 1993b). Similar findings were reported for rat MR (Krozowski & Funder 1983, Myles & Funder 1994) and guinea pig MR (Myles & Funder 1994). Progesterone (Prog) also has high affinity for human MR (Arriza *et al.* 1987, Rupprecht *et al.* 1993b, Sugimoto *et al.* 2016), rat MR (Myles & Funder 1996) and guinea pig MR (Myles & Funder 1996). 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.

The similar affinity of F, B and Aldo for the MR raised the question of how Aldo could occupy human MR in the presence of F and mouse and rat MR in the presence of B, which have from 100 to 1000 fold higher concentrations in serum than that of Aldo. Human MR should be occupied by F and rat and mouse MR by B. Selective activation by Aldo of the MR in the presence of either F or B arises from a novel enzymatic mechanism involving expression of 11 β -hydroxysteroid dehydrogenase-type 2 (11 β HSD2) in epithelia containing the MR. This enzyme metabolizes the 11 β -OH of F and B to a ketone, yielding cortisone and 11-dehydrocorticosterone, respectively, two inactive steroids (Edwards *et al.* 1988, Funder *et al.* 1988, Draper & Stewart 2005, Baker 2010, Odermatt & Kratschmar 2012, Chapman *et al.* 2013). Aldo is inert to 11 β HSD2 and can occupy the kidney and colon MRs in the presence of 11 β HSD2, activating the MR. However, DOC, S and Prog lack an 11 β -OH, which allows these steroids to compete with Aldo for the MR.

Specificity of human MR for Aldo compared to other corticosteroids also comes from Aldo's strong transcriptional activation of the MR. Indeed, a follow-up paper (Arriza *et al.* 1988) showed that the half-maximal response (EC50) of Aldo for human MR was over 100X lower than that of F. Subsequent reports extended the stronger response of the human MR to Aldo compared to other corticosteroids (Rupprecht *et al.* 1993b, Lombes *et al.* 1994, Hellal-Levy *et al.* 1999, Mani *et al.* 2016, Sugimoto *et al.* 2016). The basis for the stronger response to Aldo is not understood. Among the possible contributors to the stronger transcriptional activation of the MR by Aldo

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.* 1993b, Geller 2000) and an agonist for fish MRs (Sturm *et al.* 2005, Pippal *et al.* 2011, Sugimoto *et al.* 2016).

than by other corticosteroids are differences in cell types, which have been shown to alter the response of the MR to Aldo and F (Lim-Tio *et al.* 1997). The longer off-time Aldo from the MR has been proposed to be important in Aldo activation of human MR (Hellal-Levy *et al.* 1999). Lastly, the stronger transcriptional activity of Aldo compared to F and other corticosteroids may be due to Aldo's stronger stabilization of an interaction between the N-terminal A/B domain and the ligand-binding domain (Rogerson & Fuller 2003, Pippal *et al.* 2009, 2011). In this regard, allosteric interactions between the A/B domain and the ligand-binding domain also are important in differences among corticosteroids in transcriptional activation of the GR (Pearce & Yamamoto 1993, Rupprecht *et al.* 1993a, Oka *et al.* 2015, Katsu *et al.* 2016). Interestingly, Prog is an antagonist for human MR (Kagawa 1958, Wambach & Higgins 1978, Funder & Adam 1981, Rupprecht *et al.* 1993b, Geller *et al.* 2000, Sugimoto *et al.* 2016).

Important in understanding MR evolution is the pathway for the synthesis of corticosteroids that are ligands for the MR (Fig. 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 (Lam *et al.* 2006, Hawkins *et al.* 2012), and of Prog are not known.

Chondrichthyes contain B and a novel derivative 1 α -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 lungfish (Joss *et al.* 1994, Rossier *et al.* 2015), which also secrete F and B. Lungfish are the closest extant forerunners of tetrapods (Woolston 2013).

Figure 3

Pathway for the synthesis of aldosterone from progesterone and cortisol from 17 α -OH-Progesterone. Progesterone is hydroxylated at C21 to form 11-deoxycorticosterone, which is hydroxylated at C11 to form corticosterone. Hydroxylated 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).

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 and B for skate MR are 0.07 nM, 0.03 nM and 0.09 nM, respectively (Carroll *et al.* 2008). 11 β HSD2 first appears in Chondrichthyes (Baker *et al.* 2015, Rossier *et al.* 2015), in which 11 β HSD2 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, Sturm *et al.* 2005, Stolte *et al.* 2008, Pippal *et al.* 2011, Sugimoto *et al.* 2016). Indeed, the physiological 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, Sturm *et al.* 2005, Stolte *et al.* 2008, Pippal *et al.* 2011, 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 the EC₅₀ of the MR for 3-ketosteroids (Sturm *et al.* 2005, Carroll *et al.* 2008, Pippal *et al.* 2011, Sugimoto *et al.* 2016), as well as the MR's physiological function (Vize & Smith 2004, Hawkins *et al.* 2012, Martinerie *et al.* 2013, Rossier *et al.* 2015, Jaisser & Farman 2016, Mifsud & Reul 2016, Sakamoto *et al.* 2016). It needs to be noted that transcriptional activation by corticosteroids of fish MRs is studied in mammalian cells, which may provide different responses than in assays using homologous fish cells. 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, a jawless fish, elephant shark, a basal jawed fish and coelacanth, which, along with lungfish, belongs to the lobe-finned fish clade, forerunners of terrestrial vertebrates, 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,

Fagart *et al.* 2005, Li *et al.* 2005, Edman *et al.* 2015) and GR (Bledsoe *et al.* 2002, He *et al.* 2014) and functional studies of human MR mutants (Fagart *et al.* 1998, Geller *et al.* 2000, Bledsoe *et al.* 2005, Li *et al.* 2005, Shibata *et al.* 2013, Jimenez-Canino *et al.* 2016, Mani 2016) 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 (Prunet *et al.* 2006, Bury & Sturm 2007, Arterbery *et al.* 2011, 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 (Fig. 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, Pascual-Le Tallec & Lombes 2005, Yang & Young 2009, Huang *et al.* 2010, Huyet *et al.* 2012). The A/B domain contains an activation function 1 [AF1], and the E domain contains an AF2 domain (Li *et al.* 2005, Pascual-Le Tallec & Lombes 2005, Huyet *et al.* 2012, Faresse 2014). Each domain in MR is important for transcriptional responses (Faresse 2014, Fuller *et al.* 2012).

As shown in Fig. 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 (Bridgham *et al.* 2008, Baker *et al.* 2015, 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 (Fagart *et al.* 1998, Geller *et al.* 2000, Bledsoe *et al.* 2005, Fagart *et al.* 2005, Li *et al.* 2005, Bridgham *et al.* 2006, Funder 2013, Shibata *et al.* 2013, Mani 2016). 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

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 [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article).

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 W2 was used to construct the multiple alignment (Larkin *et al.* 2007). The crystal structure of human MR (PDB: 2A3I) (Li *et al.* 2005) 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 coactivator proteins. The functions of Ser-949 and His-950 remain to be elucidated.

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, Fagart *et al.* 2005, Li *et al.* 2005, Edman *et al.* 2015) and amino acids that have been found to be important in either steroid binding or transcriptional activation of the MR or CR (Fagart *et al.* 1998, Geller *et al.* 2000, Bledsoe *et al.* 2005, Hultman *et al.* 2005, Li *et al.* 2005, Bridgham *et al.* 2006,

Shibata *et al.* 2013, Edman *et al.* 2015, Jimenez-Canino *et al.* 2016, Mani *et al.* 2016) or the GR (Bledsoe *et al.* 2002, He *et al.* 2014, Edman *et al.* 2015, 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 across vertebrate MRs, GRs and CRs and even in lamprey and hagfish PRs.

We used this multiple alignment to construct an updated phylogeny of the steroid-binding domain on the MR and other 3-ketosteroid receptors (Fig. 6). This phylogeny indicates that the CR and PR evolved from an ancestral 3-ketosteroid receptor through gene duplication and divergence (Thornton 2001, Baker *et al.* 2015, Rossier *et al.* 2015); 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

Figure 6

Phylogenetic analysis of vertebrate MRs, GRs, CRs, PRs and ARs. Steroid-binding domains were collected with BLAST searches of GenBank. Then Clustal W2 ([Larkin et al. 2007](#)) was used to construct the multiple alignment. The phylogenetic tree was constructed using Maximum Likelihood based on the JTT matrix-based model ([Jones et al. 1992](#)). Statistical confidence for each branch in the tree was evaluated with 1,000 bootstrap runs ([Tamura et al. 2011](#)). The tree indicates that the CR/PR ancestor in an ancestral cyclostome duplicated to form genes that evolved into the CR and PR in modern lampreys and hagfishes. Subsequently, the ancestral CR duplicated, with the surviving CR found in the branch containing descendants in lamprey and hagfish that cluster with lamprey and hagfish PR. The MR and GR are descendants of the other CR that appears to be lost in lamprey and hagfish ([Baker et al. 2015](#), [Rossier et al. 2015](#)). In Gnathostomes, a gene duplication produced the AR and PR.

be lost in Pacific and Atlantic lamprey (Baker *et al.* 2015, Rossier *et al.* 2015). Also, the presence of the PR in lamprey and hagfish and the absence of an AR in lamprey indicate that the AR evolved from a duplication of an ancestral PR.

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 (Geller *et al.* 2000, Bledsoe *et al.* 2005, Li *et al.* 2005, Fuller *et al.* 2012, Huyet *et al.* 2012, Baker *et al.* 2013), 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 (Li *et al.* 2005, Baker *et al.* 2013, Mani *et al.* 2016). However, other amino acids are not conserved, providing specificity for mineralocorticoids (Bledsoe *et al.* 2005, Baker *et al.* 2007, Huyet *et al.* 2012, Baker *et al.* 2013), glucocorticoids (Bledsoe *et al.* 2002, He *et al.* 2014), progestins (Williams & 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 (Figs 5 and 7) (Baker *et al.* 2007, 2013). 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 N ϵ and N η 2 on Arg-817 (Figs 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 O γ on Ser-810 and the C3-ketone on Aldo; in another network, a water molecule contacts the backbone oxygen on Ser-810, N η 2 on Arg-817 and the C3-ketone on Aldo (Bledsoe *et al.* 2005) (Fig. 7).

A serine corresponding to Ser-810 in human MR first appears in ray-finned fish and lobe-finned fish (Fig. 5) (Baker *et al.* 2007, Baker *et al.* 2011, Baker *et al.* 2013). In contrast, skate and elephant shark MRs and lamprey and hagfish CRs contain a methionine corresponding to Ser-810. Moreover, the GR, PR and AR also have a methionine at this position (Fig. 5) (Baker *et al.* 2007, 2011, 2013). Thus, this water-mediated hydrogen bond between O γ on Ser-810 and C3-ketone on Aldo, which emerged in the MR in cartilaginous fish (skates and elephant shark), is unique among 3-ketosteroid receptors. The evolution of this serine in the MR affects binding to 3-ketosteroids because, as noted by Bledsoe and coworkers (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 in the loss transcriptional activation of the MR by Prog (Geller *et al.* 2000) and cortisone (Rafestins-Oblin *et al.* 2003).

Evolution of the contact between Ser810 (Helix 5) and 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 and coworkers (Geller *et al.* 2000), who identified a Ser810Leu mutation in the MR, which was activated by Prog (EC₅₀ of ~ 1 nM). Prog activation of the MR is unexpected because Prog is an antagonist for wild-type human MR (Kagawa 1958, Wambach & Higgins 1978, Funder & Adam 1981, Rupprecht *et al.* 1993b, Geller *et al.* 2000, Rafestins-Oblin *et al.* 2003, 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 (Rafestins-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.

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.

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 the 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 ([Baker *et al.* 2011](#)). In skate and elephant shark MR, this cysteine is replaced with the alanine that is conserved in MR descendants. Based on mutagenesis studies of Geller and coworkers ([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 (Fig. 2) influence their transcriptional activity for the various MRs, as well as for the GR and other steroid receptors (Bledsoe *et al.* 2002, Bledsoe *et al.* 2005, Huang *et al.* 2010, Huyet *et al.* 2012).

Vertebrate MRs, CRs and chondrichthyan GR and PR conserve many amino acids in human MR (Fig. 5) that contact the C and D rings on Aldo (Fig. 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) (Fig. 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.

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, C δ 1 on Leu-848 is 6.2 Å and 3.8 Å, respectively, from 17 α -OH and 16 α -CH₃ on DEX. In human GR, O ϵ 1 is 3.0 Å and 3.6 Å, respectively, from 17 α -OH and 16 α -CH₃ on DEX. Ser-843 and Pro-637 are displaced by over 5 Å.

Ser-843 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 17 α -hydroxyl group on F and DEX and glucocorticoids with other 17 α 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) (Fig. 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) (Fig. 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 17 α -hydroxyl on DEX (Fig. 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 17 α -hydroxyl on F and DEX. In contrast, B, Aldo and DOC, which lack a 17 α -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 and the 17 α -hydroxyl on DEX due to plasticity in helix6-helix7 in the MR, which allows for an open conformation that can accommodate DEX. A molecular mechanics (Monte Carlo simulations) and crystallographic analysis of the MR, GR, PR, ER and AR revealed that such plasticity is present in MR, GR, PR

and ER, but not in the AR. Interestingly, the MR undergoes a larger rearrangement of this region than the GR, which is in a more open configuration (Edman *et al.* 2015).

Interestingly, F has low EC₅₀s for fish MRs, 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 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 17 α -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 stepwise 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 and coworkers (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. However, because intercalated cells lack 11 β -HSD2, activation of the intercalated cell MR by de-phosphorylation is followed by F or B acting as an MR agonist (Funder 2013, Shibata *et al.* 2013). Interestingly, high potassium levels increase phosphorylation of Ser-843 (Funder 2013, Shibata *et al.* 2013, Jimenez-Canino *et al.* 2016). Phosphorylated Ser-843 human MR has only been found in intercalated cells in the kidney distal tubule; other tissues such as brain, aorta, heart and colon do not contain phosphorylated Ser-843. Phosphorylation of Ser-843 in the MR in breast and ovary has not been investigated (Funder 2013, Shibata *et al.* 2013).

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 descendant 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 the regulation of transcriptional activation of the MR through a kinase/phosphatase mechanism 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 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 (Baker 2003, Sturm *et al.* 2005, Prunet *et al.* 2006, Baker *et al.* 2007, Bury & Sturm 2007, McCormick *et al.* 2008, Arterbery *et al.* 2011, Sakamoto *et al.* 2011, Takahashi & Sakamoto 2013, Sakamoto *et al.* 2016). Interestingly, Prog is a transcriptional activator of ray-finned fish, which also respond to 19-norProg and spironolactone (Sturm *et al.* 2005, Pippal *et al.* 2011, 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 (Geller *et al.* 2000, Bledsoe *et al.* 2005, Fagart *et al.* 2005, Baker *et al.* 2013), and fish MR contains 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 11 β -hydroxyl and thus is not metabolized by 11 β -HSD2 (Odermatt & Kratschmar 2012, Chapman *et al.* 2013). However, serum protein(s) may sequester Prog, reducing its access to fish MRs, as has been found for the effect of corticosteroid binding globulin on access of DOC, B and F to rat MR (Krozowski & Funder 1983).

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 (Fig. 5) (Baker *et al.* 2007, Baker *et al.* 2013). The physiological consequences of this serine in human MR

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 Nε2 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 coactivators and the GR and MR (Li *et al.* 2005, Kattoula & Baker 2014).

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 (Fig. 9). Differences in the conformation of AF2 may be important in selective binding of coactivators to MR and GR (Hultman *et al.* 2005, Li *et al.* 2005, Hu & Funder 2006, Baker *et al.* 2007, Yang & Young 2009, Fuller *et al.* 2012, Baker *et al.* 2013).

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 (Fig. 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 & Reul 2016).

However, reflecting the kinship of the MR and GR, there is evidence that they form functional heterodimers with different properties from their homodimers (Bradbury *et al.* 1994, Trapp *et al.* 1994, Liu *et al.* 1995, Ou *et al.* 2001). 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 & 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 coexpress both receptors are 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 (Kumai *et al.* 2012, Cruz *et al.* 2013). 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.

Conclusion

The cloning of the MR (Arriza *et al.* 1987) coincides with the beginning of a renaissance in evolutionary biology due to the explosive increase in the number sequences of receptors, transcription factors and enzymes deposited in GenBank. This cornucopia of sequences required new software to extract and manage the information in the sequences for application to control diseases, which facilitated analyses that provided insights into the evolution of steroid receptors and other nuclear receptors (Evans 1988, Laudet *et al.* 1992, Baker 1997). The insight that the MR and GR were kin has been important for applying information about each receptor to understanding mineralocorticoid and glucocorticoid responses in normal physiology and in treatment of endocrine-dependent diseases. With the current explosive increase in genomic sequences, in which thousands of human genomes can be studied for mutations, we can expect new insights into the molecular basis for diseases dependent on the MR, GR and their ligands (Markov *et al.* 2009, Hawkins *et al.* 2012, Nelson *et al.* 2013, de Kloet 2014, Gomez-Sanchez & Gomez-Sanchez 2014, Baker *et al.* 2015, Fuller 2015, Rossier *et al.* 2015, de Kloet *et al.* 2016, Jaisser & Farman 2016).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0661>.

Declaration of interest

M E B and Y K have no conflict of interest.

Funding

Y K was supported in part by Grants-in-Aid for Scientific Research [26440159] from the Ministry of Education, Culture, Sports, Science and Technology of Japan. M E B was supported by Research fund #3096.

Author contribution statement

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

Acknowledgement

We gratefully acknowledge valuable discussions with Dr Romain Studer regarding the phylogenetic analysis of the MR and other steroid receptors. We thank Drs Karl Edman and Victor Guallar for informative discussion on Monte Carlo simulations of the MR and other steroid receptors.

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Received in final form 13 April 2017

Accepted 3 May 2017

Accepted Preprint Published Online 3 May 2017