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.

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

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-deoxycorticoster (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 F, 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 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 cortisol 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 11βHSD2 and can occupy the kidney and colon MRs in the presence of 11βHSD2, activating the MR. However, DOC, S and Prog lack an 11β-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...
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 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 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).
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). 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, 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,

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 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 -helices.
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). 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
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 Ne and Nq2 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 Oγ on Ser-810 and the C3-ketone on Aldo; in another network, a water molecule contacts the backbone oxygen on Ser-810, Nq2 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 Oγ 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 (Rafestin-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, Rafestin-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 (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 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.
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 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) (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 17α-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 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 and the 17α-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 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 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 11β-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 11β-hydroxyl and thus is not metabolized by 11β-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
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 (Küllerich 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 (Evens 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, Jaiiser & Farman 2016).

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