Novel Evolution of the Mineralocorticoid Receptor in Humans compared to Chimpanzees, Gorillas and Orangutans

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Abstract

We identified five distinct full-length human mineralocorticoid receptor (MR) genes containing either 984 amino acids (MR-984) or 988 amino acids (MR-988), which can be distinguished by the presence or absence of Lys, Cys, Ser, Trp (KCSW) in their DNA-binding domain (DBD) and mutations at codons 180 and 241 in their amino terminal domain (NTD). Two human MR-KCSW genes contain either (Val-180, Val-241) or (Ile-180, Val-241) in their NTD and three human MR-984 genes contain either (Ile-180, Ala-241) or (Val-180, Val-241) or (Ile-180, Val-241). Human MR-KCSW with (Ile-180, Ala-241) has not been cloned. In contrast, chimpanzees contain four MRs: two MR-988s with KCSW in their DBD or two MR-984s without KCSW in their DBD. Chimpanzee MRs only contain (Ile180, Val-241) in their NTD. A chimpanzee MR with either (Val-180, Val-241) or (Ile-180, Ala-241) in the NTD has not been cloned. Gorillas and orangutans each contain one MR-988 with KCSW in the DBD and one MR-984 without KCSW, and these MRs only contain (Ile-180, Val-241) in their NTD. A gorilla MR or orangutan MR with either (Val-180, Val-241) or (Ile-180, Ala-241) in the NTD has not been cloned. Together, these data suggest that human MRs with (Val-180, Val-241) or (Ile-180, Ala-241) in the NTD evolved after humans and chimpanzees diverged from their common ancestor. Considering the multiple functions in human development of the MR in kidney, brain, heart, skin, and lungs, as well as MR activity in interaction with the glucocorticoid receptor, we suggest that the evolution of human MRs that are absent in chimpanzees may have been important in the evolution of humans from chimpanzees. Studies are underway to characterize transcriptional activation of the five human MRs by aldosterone, cortisol, and other corticosteroids for comparison with each other to elucidate the roles of these MRs in human physiology.

Key words: Mineralocorticoid Receptor, DNA-binding domain, Evolution, Human MR, Chimpanzee MR, Aldosterone

Running title: Evolution of the DNA-binding domain of the Mineralocorticoid Receptor

Introduction

The mineralocorticoid receptor (MR) is a ligand-activated transcription factor, belonging to the nuclear receptor family, a diverse group of transcription factors that arose in multicellular animals [1–5]. The traditional physiological function of the MR is to maintain electrolyte balance by regulating sodium and potassium transport in epithelial cells in the kidney and colon [6–10]. In addition, the MR has important physiological functions in many other tissues, including brain, heart, skin and lungs [10–16].

The MR and its paralog, the glucocorticoid receptor (GR), descended from an ancestral corticoid receptor (CR) in a cyclostome (jawless fish) that evolved about 550 million years ago at the base of the vertebrate line [17–24]. A descendent of this ancestral steroid receptor, the CR in lamprey (*Petromyzon marinus*), is activated by aldosterone [25,26] and other corticosteroids [25,26]. Lampreys contain two CR isoforms, which differ only in the presence of a four amino acid insert Thr, Arg, Gln, Gly (TRQG) in their DNA-binding domain (DBD) [27] (Figure 1). We found that several corticosteroids had a similar half-maximal response (EC50) for lamprey CR1 and CR2 [26]. However, these corticosteroids had a lower fold-activation of transcription for CR1, which contains the four amino acid insert, than for CR2 suggesting that the deletion of

the four amino acid sequence in CR2 selected for increased transcriptional activation by corticosteroids of CR2 [26,27].

A distinct MR and GR first appear in sharks and other cartilaginous fishes (Chondrichthyes) [19,21,28–31]. The DBD in elephant shark MR and GR lacks the four amino acid sequence found in lamprey CR1 [26] (Figure 1). We inserted this four-residue sequence from lamprey CR1 into the DBD in elephant shark MR and GR and found that in HEK293 cells co-transfected with the TAT3 promoter, the mutant elephant shark MR and GR had lower transcriptional activation by corticosteroids than did their wild-type elephant shark MR and GR counterparts, indicating that the insertion of the four amino acid sequence into the DBD of wild-type elephant shark MR and GR had a similar effect on transcriptional activation as the KCSW insert had in the DBD of lamprey CR1 [27].

We then analyzed the DBD sequence of human MR, which had been cloned, sequenced and characterized by Arriza et al. [32], and found that like elephant shark MR, this human MR (MR1) lacks a four-residue segment in its DBD. This human MR has been widely studied [8,10,12,13,33–35]. Unexpectedly, our BLAST [36] search with the DBD from this human MR found a second, previously described, human MR splice variant with a KCSW insert (MR-KCSW) in its DBD [27,37–39] (Figure 1). A homology model of human MR with KCSW in the DBD constructed by Wickert et al. [39] found no distortion by the four amino acids on adjacent secondary structures of the DBD in human MR.

As described later, further BLAST searches found two full-length human MRs with this insert (MR-KCSW) and three full-length human MRs without this insert. The three human MRs without the KCSW insert contain either (Ile-180, Ala-241) or (Val-180, Val-241) or (Ile-180, Val-241) in their amino terminal domain (NTD) (Figure 2). The two human MR-KCSW splice

variants contain either (Val-180, Val-241) or (Ile-180, Val-241) in their NTD (Figure 2). A human MR-KCSW with (Ile-180, Ala-241) has not been cloned.

Transcriptional activation of any human MR-KCSW by corticosteroids has not been reported. To remedy this deficiency, we have studies underway to characterize transcriptional activation of the five full-length human MRs by a panel of corticosteroids. Here, in this brief report, we describe our evolutionary analysis of human MR from a comparison of five full-length human MRs with four full-length MRs in chimpanzees and two full-length MRs in gorillas, and orangutans. We find that chimpanzees, gorillas, and orangutans lack an MR with either (Ile-180, Ala-241) or (Val-180, Val-241) in their NTD. We propose that MRs with these amino acids in the NTD evolved in humans after divergence of humans from chimpanzees. Due to the multiple functions in human development of the MR alone [11,15,16,40], as well as due to the interaction of the MR with the GR [41–45], we suggest that the evolution of three distinct MRs in humans that are absent in chimpanzees may have been important in the evolution of humans from chimpanzees.

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Human MR1:          CLVCGDEASGCHYGVVTCGSCKVFFKRAVEG----QHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Human MR-KCSW:      CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGKCSWQHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Rat MR:             CLVCGDEASGCHYGVVTCGSCKVFFKRAVEG----QHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Rat MR-KCSW:        CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGKCSWQHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Platypus MR:        CLVCGDEASGCHYGVVTCGSCKVFFKRAVEG----QHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Platypus MR-KCSW:   CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGKCSWQHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Turtle MR:          CLVCGDEASGCHYGVVTCGSCKVFFKRAVEG----QHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Turtle MR-KCSW:     CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGKCSWQHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Xenopus MR:         CLVCGDEASGCHYGVVTCGSCKVFFKRAVEG----QHSYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Xenopus MR-KCSR:    CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGKCSRQHSYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
Lungfish MR:        CLVCGDEASGCHYGVVTCGSCKVFFKRAVEG----QHNYLCAGRNDCIIDKIRRKNCPACRVRKCLQAGM
Zebrafish MR:       CLVCGDEASGCHYGVVTCGSCKVFFKRAVEG----QHNYLCAGRNDCIIDKIRRKNCPACRVRKCLQAGM
Elephant shark MR:CLVCSDEASGCHYGVLTCGSCKVFFKRAVEG----QQNYLCAGRNDCIIDKIRRKNCPACRLRKCLKAGM
Lamprey CR1:        CLICSDEASGCHYGVLTCGSCKVFFKRAVEGTRQGQHNYLCAGRNDCIIDKIRRKNCPACRLRKCIQAGM
Lamprey CR2:        CLICSDEASGCHYGVLTCGSCKVFFKRAVEG----QHNYLCAGRNDCIIDKIRRKNCPACRLRKCIQAGM
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Figure 1. Comparison of the DNA-binding domain on human MR1, human MR-KCSW, rat MR, rat MR-KCSW, Platypus MR, Platypus MR-KCSW, Turtle MR, Turtle MR-

KCSW, Xenopus MR, Xenopus MR-KCSR, lungfish MR, zebrafish MR, elephant shark MR, and lamprey CR1 and CR2.

Figure legend: The DBD of the human MR-KCSW splice variant has an insertion of four amino acids that is absent in human MR1. Otherwise, the rest of the sequences of human MR and human MR-KCSW are identical. Moreover, except for the four amino acid insert in the DBD of human MR, the DBDs of rat MR, platypus MR and turtle MR are identical. The four amino acid splice variant in the DBD of *Xenopus* MR differs from these MRs by one amino acid. Differences between the DBD sequence in human MR and selected vertebrate MRs are shown in red. Protein accession numbers are AAA59571 for human MR, XP_011530277 for human MR-KCSW, NP_037263 for rat MR, XP_038953451 for rat MR-KCSW, XP_007669969 for platypus MR, XP_016083764 for platypus MR-KCSW, XP_043401725 for turtle MR, XP_037753298 for turtle MR-KCSW, NP_001084074 for *Xenopus* MR, XP_018098693 for *Xenopus* MR-KCSR, BCV19931 for lungfish MR, NP_001093873 for zebrafish MR, XP_007902220 for elephant shark MR, XP_032811370 for lamprey CR1, and XP_032811371 for lamprey CR2.

Results and Discussion

Humans have five full-length MRs.

A BLAST [36] search of GenBank retrieved five distinct full-length human MR genes (Figure 2). We retrieved three full-length human MRs without a KCSW insert in the DBD. These three human MRs contain either (Ile-180, Ala-241), (Val-180, Val-241) or (Ile-180, Val-241) in the NTD (Figure 2). We also retrieved two human MR-KCSWs containing either (Val-180, Val-241) or (Ile-180, Val-241) in their NTD. A human MR-KCSW sequence with (Ile-180, Ala-241) has not been deposited in GenBank.

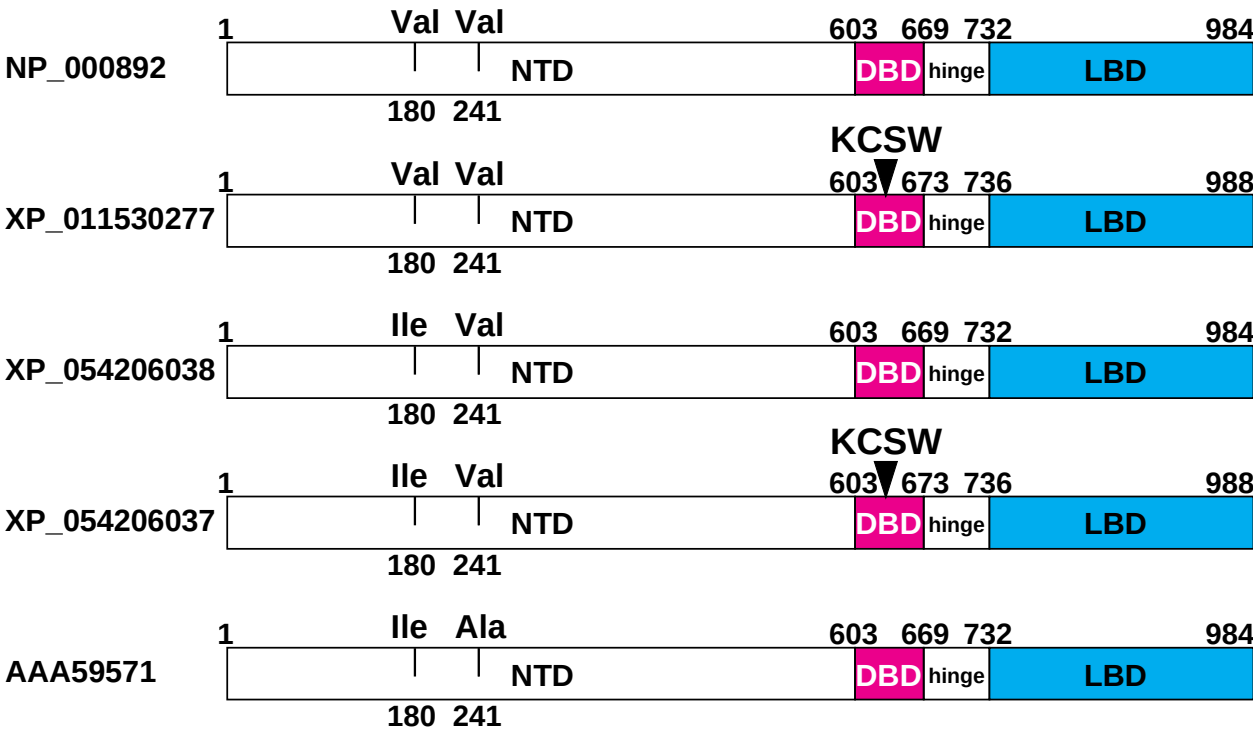

Figure 2. Comparison of five full-length human MRs.

Figure legend: There are three human MRs with 984 amino acids. They contain either valine-180 and valine-241, isoleucine-180 and valine-241 or isoleucine-180 and alanine-241 in the amino-terminal domain (NTD). There are two human MRs with 988 amino acids and KCSW in the DBD and either valine-180 and valine-241 or isoleucine-180 and valine-241 in the NTD. A human MR with KCSW and isoleucine-180 and alanine-241 in the NTD was not found in GenBank.

Chimpanzees have four full-length MRs.

Chimpanzees contain two full-length MRs that have DBDs that are identical to the DBD in human MRs without the KCSW insert and two MRs that have DBDs that are identical to the DBD in human MR-KCSW (Figure 1, Figure 3). All four chimpanzee MRs contain (Ile-180, Val-241) in their NTD, which is similar to the NTD in one human MR (Figure 2). Chimpanzees lack an MR with either (Ile-180, Ala-241) or (Val-180, Val-241) in their NTD. The different

chimpanzee MR sequences contain a Ser-591 to Asn-591 mutation in their NTD (Figure 3). Human MRs contain a serine-591 corresponding to serine-591 in chimpanzee MR.

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MR1:       METKGYHSLPEGLDMERRWGQVSQAVEHSSLGPTERTDENNYMEIVNVSCVSGAIPNNSTQGSSKEKHELLPCLQQDNNRPGILTSDIKT  90
MR2-KCSW:  METKGYHSLPEGLDMERRWGQVSQAVEHSSLGPTERTDENNYMEIVNVSCVSGAIPNNSTQGSSKEKHELLPCLQQDNNRPGILTSDIKT  90
MR3:       METKGYHSLPEGLDMERRWGQVSQAVEHSSLGPTERTDENNYMEIVNVSCVSGAIPNNSTQGSSKEKHELLPCLQQDNNRPGILTSDIKT  90
MR4-KCSW:  METKGYHSLPEGLDMERRWGQVSQAVEHSSLGPTERTDENNYMEIVNVSCVSGAIPNNSTQGSSKEKHELLPCLQQDNNRPGILTSDIKT  90
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MR1:       ELESKELSATVAESMGLYMDSVRDADYSYEQQNQQGSMSPAKIYQNVEQLVKFYKGNGHRPSTLSCVNRPLRSFMSDSGSSVNGGVMRAI 180
MR2-KCSW:  ELESKELSATVAESMGLYMDSVRDADYSYEQQNQQGSMSPAKIYQNVEQLVKFYKGNGHRPSTLSCVNRPLRSFMSDSGSSVNGGVMRAI 180
MR3:       ELESKELSATVAESMGLYMDSVRDADYSYEQQNQQGSMSPAKIYQNVEQLVKFYKGNGHRPSTLSCVNRPLRSFMSDSGSSVNGGVMRAI 180
MR4-KCSW:  ELESKELSATVAESMGLYMDSVRDADYSYEQQNQQGSMSPAKIYQNVEQLVKFYKGNGHRPSTLSCVNRPLRSFMSDSGSSVNGGVMRAI 180
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MR1:       VKSPIMCHEKSPSVCSPLNMTSSVCSPAGINSVSSTTASFGNFPVHSPITQGTPLTCSPNVENRGSRSHSPAHASNVGSPLSSPLSSMKS 270
MR2-KCSW:  VKSPIMCHEKSPSVCSPLNMTSSVCSPAGINSVSSTTASFGNFPVHSPITQGTPLTCSPNVENRGSRSHSPAHASNVGSPLSSPLSSMKS 270
MR3:       VKSPIMCHEKSPSVCSPLNMTSSVCSPAGINSVSSTTASFGNFPVHSPITQGTPLTCSPNVENRGSRSHSPAHASNVGSPLSSPLSSMKS 270
MR4-KCSW:  VKSPIMCHEKSPSVCSPLNMTSSVCSPAGINSVSSTTASFGNFPVHSPITQGTPLTCSPNVENRGSRSHSPAHASNVGSPLSSPLSSMKS 270
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MR1:       SISSPPSHCSVKSPVSSPNNVTLRSSVSSPANINNSRCSVSSPSNTNNRSTLSSPAASTVGSICSPVNNAFSYTASGTSAGSSTLRDVVP 360
MR2-KCSW:  SISSPPSHCSVKSPVSSPNNVTLRSSVSSPANINNSRCSVSSPSNTNNRSTLSSPAASTVGSICSPVNNAFSYTASGTSAGSSTLRDVVP 360
MR3:       SISSPPSHCSVKSPVSSPNNVTLRSSVSSPANINNSRCSVSSPSNTNNRSTLSSPAASTVGSICSPVNNAFSYTASGTSAGSSTLRDVVP 360
MR4-KCSW:  SISSPPSHCSVKSPVSSPNNVTLRSSVSSPANINNSRCSVSSPSNTNNRSTLSSPAASTVGSICSPVNNAFSYTASGTSAGSSTLRDVVP 360
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MR1:       SPDTQEKGAQEVPFPKTEEVESAISNGVTGQLNIVQYIKPEPDGAFSSSCLGGNSKINSDSSFSVPIKQESTKHSCSGTSFKGNPTVNPF 450
MR2-KCSW:  SPDTQEKGAQEVPFPKTEEVESAISNGVTGQLNIVQYIKPEPDGAFSSSCLGGNSKINSDSSFSVPIKQESTKHSCSGTSFKGNPTVNPF 450
MR3:       SPDTQEKGAQEVPFPKTEEVESAISNGVTGQLNIVQYIKPEPDGAFSSSCLGGNSKINSDSSFSVPIKQESTKHSCSGTSFKGNPTVNPF 450
MR4-KCSW:  SPDTQEKGAQEVPFPKTEEVESAISNGVTGQLNIVQYIKPEPDGAFSSSCLGGNSKINSDSSFSVPIKQESTKHSCSGTSFKGNPTVNPF 450
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MR1:       PFMDGSYFSFMDDKDYYSLSGILGPPVPGFDGNCEGSGFPVGIKQEPDDGSYYPEASIPSSAIVGVNSGGQSFHYRIGAQGTISLSRSAR 540
MR2-KCSW:  PFMDGSYFSFMDDKDYYSLSGILGPPVPGFDGNCEGSGFPVGIKQEPDDGSYYPEASIPSSAIVGVNSGGQSFHYRIGAQGTISLSRSAR 540
MR3:       PFMDGSYFSFMDDKDYYSLSGILGPPVPGFDGNCEGSGFPVGIKQEPDDGSYYPEASIPSSAIVGVNSGGQSFHYRIGAQGTISLSRSAR 540
MR4-KCSW:  PFMDGSYFSFMDDKDYYSLSGILGPPVPGFDGNCEGSGFPVGIKQEPDDGSYYPEASIPSSAIVGVNSGGQSFHYRIGAQGTISLSRSAR 540
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MR1:       DQSFQHLSSFPPVNTLVESWKSHGDLSSRRSDGYPVLEYIPENVSSSTLRSVSTGSSRPSKICLVCGDEASGCHYGVVTCGSCKVFFKRA 630
MR2-KCSW:  DQSFQHLSSFPPVNTLVESWKSHGDLSSRRSDGYPVLEYIPENVSSSTLRSVSTGSSRPSKICLVCGDEASGCHYGVVTCGSCKVFFKRA 630
MR3:       DQSFQHLSSFPPVNTLVESWKSHGDLSSRRSDGYPVLEYIPENVSSSTLRNVSTGSSRPSKICLVCGDEASGCHYGVVTCGSCKVFFKRA 630
MR4-KCSW:  DQSFQHLSSFPPVNTLVESWKSHGDLSSRRSDGYPVLEYIPENVSSSTLRNVSTGSSRPSKICLVCGDEASGCHYGVVTCGSCKVFFKRA 630
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MR1:       VEG----QHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGMNLGARKSKKLGKLKGIHEEQPQQQQPPPPPPPPQSPEEGTTYIAPAKE 716
MR2-KCSW:  VEGKCSWQHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGMNLGARKSKKLGKLKGIHEEQPQQQQPPPPPPPPQSPEEGTTYIAPAKE 720
MR3:       VEG----QHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGMNLGARKSKKLGKLKGIHEEQPQQQQPPPPPPPPQSPEEGTTYIAPAKE 716
MR4-KCSW:  VEGKCSWQHNYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGMNLGARKSKKLGKLKGIHEEQPQQQQPPPPPPPPQSPEEGTTYIAPAKE 720
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MR1:       PSVNTALVPQLSTISRALTPSPVMVLENIEPEIVYAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLEDQITLIQYSW 806
MR2-KCSW:  PSVNTALVPQLSTISRALTPSPVMVLENIEPEIVYAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLEDQITLIQYSW 810
MR3:       PSVNTALVPQLSTISRALTPSPVMVLENIEPEIVYAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLEDQITLIQYSW 806
MR4-KCSW:  PSVNTALVPQLSTISRALTPSPVMVLENIEPEIVYAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLEDQITLIQYSW 810
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MR1:       MCLSSFALSWRSYKHTNSQFLYFAPDLVFNEEKMHQSAMYELCQGMHQISLQFVRLQLTFEEYTIMKVLLLLSTIPKDGLKSQAAFEEMR 896
MR2-KCSW:  MCLSSFALSWRSYKHTNSQFLYFAPDLVFNEEKMHQSAMYELCQGMHQISLQFVRLQLTFEEYTIMKVLLLLSTIPKDGLKSQAAFEEMR 900
MR3:       MCLSSFALSWRSYKHTNSQFLYFAPDLVFNEEKMHQSAMYELCQGMHQISLQFVRLQLTFEEYTIMKVLLLLSTIPKDGLKSQAAFEEMR 896
MR4-KCSW:  MCLSSFALSWRSYKHTNSQFLYFAPDLVFNEEKMHQSAMYELCQGMHQISLQFVRLQLTFEEYTIMKVLLLLSTIPKDGLKSQAAFEEMR 900
           ******************************************************************************************

MR1:       TNYIKELRKMVTKCPNNSGQSWQRFYQLTKLLDSMHDLVSDLLEFCFYTFRESHALKVEFPAMLVEIISDQLPKVESGNAKPLYFHRK 984
MR2-KCSW:  TNYIKELRKMVTKCPNNSGQSWQRFYQLTKLLDSMHDLVSDLLEFCFYTFRESHALKVEFPAMLVEIISDQLPKVESGNAKPLYFHRK 988
MR3:       TNYIKELRKMVTKCPNNSGQSWQRFYQLTKLLDSMHDLVSDLLEFCFYTFRESHALKVEFPAMLVEIISDQLPKVESGNAKPLYFHRK 984
MR4-KCSW:  TNYIKELRKMVTKCPNNSGQSWQRFYQLTKLLDSMHDLVSDLLEFCFYTFRESHALKVEFPAMLVEIISDQLPKVESGNAKPLYFHRK 988
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Figure 3. Multiple alignment of four chimpanzee MRs.

Figure legend: The four chimpanzee MRs were aligned with Clustal Omega [46]. MR1 and MR2-KCSW have serine-591 and MR3 and MR4-KCSW have asparagine-591 in the NTD. In contrast to human MRs, chimpanzee MRs only contain an isoleucine at codon 180 and a valine

at codon 241. Chimpanzees also contain an MR with either a serine or an asparagine at codon 591, while human MRs only contain a serine at codon 591.

Gorillas and orangutans have two full-length MRs.

Gorillas and orangutans each contain two full-length MRs: one MR with 984 amino acids and one MR with 988 amino acids. The MRs with 988 amino acids have the KCSW sequence in the DBD. All full-length gorilla MRs and orangutan MRs have isoleucine-180 and valine-241 in the amino-terminal domain (NTD) (Figure 4). A gorilla MR or orangutan MR with either valine-180 and valine-241 or isoleucine-180 and alanine-241 in the NTD was not found in GenBank.

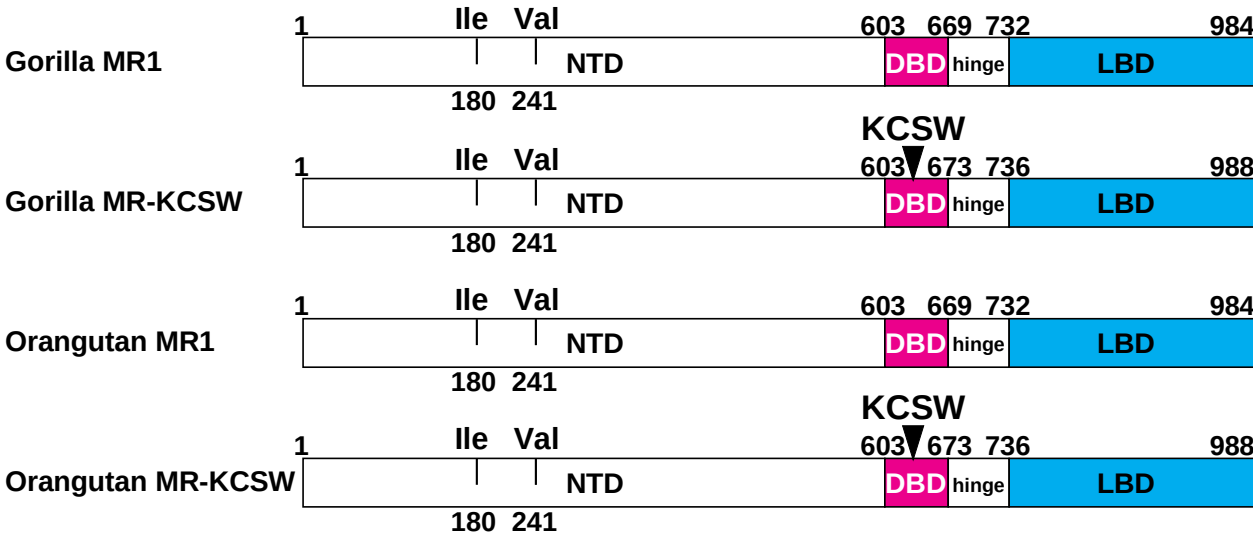

Figure 4. Comparison of gorilla and orangutan MR sequences.

Figure legend: Gorilla MRs and Orangutan MRs conserve an isoleucine-180, valine-241 pair corresponding to isoleucine-180 and valine-241 in the NTD of human MR. A gorilla MR or an orangutan MR with either valine-180 and valine-241 or isoleucine-180 and alanine-241 in the NTD was not found in GenBank. Gorillas and orangutans also only contain an MR with a serine at codon 591. Human MRs only contain a serine at codon 591.

Evolutionary divergence of human and chimpanzee MRs.

Our analysis of human and chimpanzee MRs indicates that a human MR with either (Ile-180, Ala-241) or (Val-180, Val-241) in the NTD evolved after the divergence of humans and chimpanzees from a common ancestor. The physiological consequences of (Ile-180, Ala-241) or (Val-180, Val-241) in the NTD of human MR remain to be elucidated. Moreover, the absence of an asparagine at codon 591 in human MR is intriguing considering the presence of an asparagine at codon 591 in the MR in chimpanzees. As a first step, to determine if there are differences in transcriptional activation of these MRs by corticosteroids, we have cloned these human MR sequences and are screening them for transcriptional activation by aldosterone, cortisol, and other corticosteroids.

Evolution of the MR DBD in basal terrestrial vertebrates

The evolution of an MR with a DBD containing a four amino acid insert in human MR and other terrestrial MRs was surprising to us (Figure 1). We did not expect to find the KCSW insert in human MR at the position homologous to the position of TRQG in the DBD of lamprey CR because an insert at this position was not present in the DBD of elephant sharks and lungfish MRs (Figure 1). Moreover, the rest of the DBD sequence is highly conserved in terrestrial vertebrates (Figure 1) suggesting that the evolution of a four amino acid sequence in the DBD has an important function in terrestrial vertebrates. The evolution of the KCSR sequence into the DBD *Xenopus* MR (Figure 1) places the re-emergence of this motif close to the origin of terrestrial vertebrates. Evolution of aldosterone synthesis in lungfish is thought to be important in the evolution of terrestrial vertebrates [4,6,21,47–49]. The evolution of KCSR in Xenopus

MR in the homologous position to the DBD in human MR suggests that insertion of KCSR in Xenopus MR also may have been important early in the evolution of terrestrial vertebrates. Moreover, turtles contain an MR with KCSW in the homologous position in DBD (Figure 1) of human MR DBD, indicating that KCSW also has an ancient origin in MRs in terrestrial vertebrates. Indeed, the conservation of KCSW in the MR DBD in turtles and humans suggests an important function for KCSW in terrestrial vertebrates.

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Contributions.

Yoshinao Katsu: Investigation, Conceptualization, Supervision, Formal Analysis, Writing – original draft, Writing – review & editing.

Jiawen Zhang: Data curation, Investigation, Methodology.

Michael E. Baker: Conceptualization; Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

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