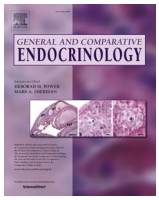
General and Comparative Endocrinology 328 (2022) 114105

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Research paper

A basal actinopterygian melanocortin receptor: Molecular and functional characterization of an Mc2r ortholog from the Senegal bichir (*Polypterus senegalus*)

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ARTICLE INFO

Keywords:
Endocrinology
Evolution
Stress
Mc2r
Mrap1
Chondrostean fish

ABSTRACT

In bony vertebrates, melanocortin 2 receptor (Mc2r) specifically binds adrenocorticotropic hormone (ACTH) and is responsible for mediating anterior pituitary signaling that stimulates corticosteroid production in the adrenal gland/interrenal cells. In bony fishes Mc2r requires the chaperoning of an accessory protein (Mrap1) to traffic to the membrane surface and bind ACTH. Here, we evaluated the structure and pharmacological properties of Mc2r from the Senegal bichir (*Polypterus senegalus*), which represents the most basal bony fish from which an Mc2r has been pharmacologically studied to date. In our experiments, cDNA constructs of the Mc2r from the Senegal bichir (sbMc2r) and various vertebrate Mrap1s were heterologously co-expressed in Chinese hamster ovary (CHO) cells, stimulated by ACTH or melanocyte-stimulating hormone (α-MSH) ligands, and assessed using a luciferase reporter gene assay. When expressed without an Mrap1, sbMc2r was not activated by ACTH. When co-expressed with Mrap1 from either chicken (*Gallus gallus*) or bowfin (*Amia calva*), sbMc2r could be activated in a dose-dependent manner by ACTH, but not α-MSH. Co-expression of sbMrap2 with sbMc2r resulted in no detectable activation of the receptor. Collectively, these results demonstrate that sbMc2r has pharmacological properties similar to those of Mc2rs of later-evolved bony fishes, such as Mrap1 dependence and ACTH selectivity, indicating that these qualities of Mc2r function are ancestral to all bony fish Mc2rs.

1. Introduction

Melanocortin receptors are members of the large rhodopsin family of seven-pass transmembrane G protein-coupled receptors (Cone, 2006; Ramachandrappa et al., 2013). Among bony vertebrates, there are five known melanocortin receptors (Mc1r, Mc2r, Mc3r, Mc4r, and Mc5r) which exhibit varying degrees of selectivity to the proopiomelanocortin (POMC)-derived peptides adrenocorticotropin hormone (ACTH) and melanocyte-stimulating hormone (α-MSH) (Nakanishi et al., 1979). Mammalian Mc1r, Mc3r, Mc4r, and Mc5r can all be activated by both ACTH and α-MSH ligands (Cone, 2006). However, Mc2r can only be activated by ACTH and is designated as the "ACTH receptor" (Mountjoy et al., 1992).

As a receptor for ACTH, Mc2r is important in mediating hypothalamic–pituitary-adrenal/interrenal (HPA/HPI) signaling, wherein the adrenal gland of later-evolved tetrapods is homologous to the interrenal tissue associated with the kidney of amphibians and fishes. In the HPI axis of fishes, corticotropin releasing factor (CRF) is secreted from the hypothalamus to the pituitary, causing the release of ACTH into the vascular system (Wendelaar Bonga, 1997). At the interrenal cells, ACTH binds to Mc2r to initiate steroid biosynthesis, resulting in the production and release of corticosteroids (Dores and Garcia, 2015). Comparative pharmacological investigations on Mc2r have described many functional qualities that appear to have been derived throughout vertebrate evolution (Dores and Chapa, 2021). Of the bony vertebrates (class Osteichthyes) studied to date, Mc2r exclusively binds ACTH and requires cooperative interaction with a melanocortin receptor accessory protein (Mrap1) for membrane trafficking and activation by ACTH (Dores and Chapa, 2021). However, these qualities of bony vertebrate Mc2r function appear to be derived (Dores and Chapa, 2021).

In the group of jawed cartilaginous fishes (class Chondrichthyes), comprised of the elasmobranchs (i.e., sharks, skates, rays) and holocephalans (i.e., chimaeras) (Nelson et al., 2016), the reliance of Mc2r on Mrap1 chaperoning and modulation is less apparent or altogether absent. The Mc2r ortholog of the stingray (*Dasyatis akajei*) (srMc2r) can be activated by both ACTH and α-MSH at non-physiological

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https://doi.org/10.1016/j.ygcen.2022.114105
Received 11 July 2022; Received in revised form 9 August 2022; Accepted 11 August 2022
Available online 13 August 2022

concentrations. Although co-expression with Mrap1 is not required for srMc2r activation, it does greatly increase the sensitivity of srMc2r to stimulation by ACTH (Dores et al., 2018; Takahashi et al., 2016). Similar observations were made for the Mc2r ortholog of the whale shark (*Rhincodon typus*) (wsMc2r), for which it was explained that Mrap1 had no effect on wsMc2r activation but enhanced trafficking of wsMc2r to the plasma membrane (Hoglin et al., 2022). Together, these studies in elasmobranchs suggest that the lack of selectivity for ACTH and the increased sensitivity of Mc2r for ACTH by Mrap1 chaperoning are shared features of Mc2rs in elasmobranchs. Similar studies have been performed in the elephant shark (*Callorhincus milli*), a representative of the sister group to the elasmobranchs, the holocephalans. Like in the elasmobranchs, the Mc2r ortholog of the elephant shark (esMc2r) can be activated by both ACTH and α-MSH, but the sensitivity of esMc2r to ACTH was not appreciably affected by co-expression with Mrap1 (Barney et al., 2019).

Conspicuously absent from previous comparative studies on Mc2r orthologs were representatives from the subclass Chondrostei (*e.g.* bichir, ropefish, paddlefish, sturgeon) (Nelson et al., 2016). In the present study, we sought to evaluate the pharmacological properties of the Mc2r from the Senegal bichir (*Polypterus senegalus*) (sbMc2r). Bichirs are members of the family Polypteridae (order Polypteriformes), which represent the most basal order of ray-finned fishes (class Actinopterygii) (Carroll, 1988). As there have been no functional studies on Mc2r from any of the lobe-finned fishes (the basal representatives of the sister class of bony vertebrates, Sarcopterygii), sbMc2r represents an Mc2r that may be more closely related to the Mc2rs of cartilaginous fishes than any other Mc2r studied to date (Betancur et al., 2017). Indeed, functional studies of Mc2rs from the most basal bony vertebrates (including bichir and lobe-finned fishes) may facilitate an enhanced understanding of how the functional qualities of the bony vertebrate Mc2r arose. Our primary hypothesis was that sbMc2r would exhibit functional qualities characteristic of the Mc2rs of more derived actinopterygians, including ACTH selectivity and Mrap1 dependence. However, we have made the observation that a sequence for an *mrap1* gene is apparently absent from the recently published *P. senegalus* genome assembly (Bi et al., 2021). This observation has raised the possibility that sbMc2r may have properties more similar to the cartilaginous fish Mc2r, which lacks Mrap1-dependance and is capable of being activated by either ACTH or α-MSH (Hoglin et al., 2022; Reinick et al., 2012a). Hence, this study tested an alternative hypothesis that sbMc2r may be an Mrap1-independent Mc2r ortholog that is capable of being activated by either ACTH or α-MSH.

2. Materials and methods

2.1. Sequence discovery and analyses

The genome assembly of *P. senegalus* is available from the National Center for Biotechnology Information (NCBI) GenBank (assembly ASM1683550v1; Bi et al., 2021). Using the BLAST (Basic Local Alignment Search Tool) from NCBI, we surveyed the *P. senegalus* assembly by querying with nucleotide sequences of *mc2r*, *mrap1*, and *mrap2* orthologs from several species of basal bony fishes for which sequences for these genes are available, including spotted gar (*Lepisosteus oculatus*) and bowfin (*Amia calva*). Amino acid sequences for sbMc2r and sbMrap2 were deduced using the Translate tool from ExPASy (https://www.expasy.org). Hypothetical membrane topology of sbMc2r was predicted using the TMHMM tool from the DTU Bioinformatics Server (https://www.bioinformatics.dtu.dk). Multiple sequence alignment and phylogenetic analyses were carried out using a selection of gnathostome Mc2r and Mrap amino acid sequences available from NCBI GenBank. Sequence alignments were performed using the Clustal Omega multiple sequence alignment tool available from the European Bioinformatics Institute (https://www.ebi.ac.uk/tools/msa/), which was also used to obtain percent sequence identities. Multiple sequence alignments were arranged using BioEdit software (Hall, 1999), with modifications to ensure the alignment of functional motifs, following previously described methods (Dores et al., 1996). Phylogenetic analyses using the maximum parsimony method (1,000 bootstrap replicates) were implemented using MEGA10 software (Kumar et al., 2008).

Accession numbers for the amino acid sequences used in our analyses were: elephant shark Mc2r (FAA00704), whale shark Mc2r (XP_020380838), stingray Mc2r (BAU98231), gar Mc2r (XP_006636159), rainbow trout Mc2r (ABV23494), carp Mc2r (CAE53845), zebrafish Mc2r (AAO24743), lungfish Mc2r (XP_043923917), frog Mc2r (XP_002936118), chicken Mc2r (AGR42637), mouse Mc2r (NP_001288301), human Mc2r (NP_001278840), whale shark Mrap1 (XP_020375601), whale shark Mrap2 (XP_020377388), chicken Mrap1 (XR_001470382), chicken Mrap2 (XP_046770071), mouse Mrap1 (NP_084120), and mouse Mrap2 (NP_001346884). Sequences for bowfin (bf) Mc2r, Mrap1, and Mrap2 were obtained as a gift from the authors of the recently published bowfin genome (Thompson et al., 2021).

2.2. Cell Culture, Transfection, and reporter gene assay

Using previously described methods (Hoglin et al., 2022; Liang et al., 2011; Reinick et al., 2012b), sbMc2r activation by melanocortin ligands was analyzed by transfecting cDNA constructs of the bichir *mc2r* and various vertebrate *mrap1s* into Chinese hamster ovary (CHO) cells, then using a *luciferase* reporter gene assay to indirectly measure cAMP production resulting from receptor activation by melanocortin ligands. CHO cells were selected for this project because this cell line does not endogenously express Mc2r or Mrap1 proteins (Noon et al., 2002; Reinick et al., 2012b; Sebag and Hinkle, 2007).

Transfection was performed using cDNA constructs that were commercially obtained as inserts on a pcDNA3.1 + expression vector (GenScript; Piscataway, NJ). Plasmid vectors (2 μg per 1×10^5 cells) were transiently transfected into CHO cells (ATCC; Manassas, VA) using a Solution T kit for the Amaxa Nucleofector 2b system (Lonza; Portsmouth, NH). This system is quoted by the manufacturer as delivering efficiencies of ~ 80 % (https://bioscience.lonza.com), and has been empirically shown to produce such efficiencies (Hannes et al., 2010; Maasho et al., 2004). Our lab has used this system to successfully transfect CHO cells for many years (Liang et al., 2011; Reinick et al., 2012a, 2012b), and we confirm the success of our transfections using positive controls for each functional assay we run. Although the particular arrangement of *mc2r* and *mrap* constructs that were co-transfected varied by experiment, all transfections included the co-transfection of a cAMP reporter construct (*luciferase* gene promoted by a cAMP responsive element (CRE-Luciferase); transfected at 2.5 μg per 1×10^5 cells) (Chepurny and Holz, 2007), totaling a maximum of 3 simultaneous transfections for any experiment. After transfection, CHO cells were seeded in triplicate wells in opaque 96-well cell culture plates (Cat. No. 3912; Corning Life Sciences; Manassas, VA) at a density of 3×10^5 cells cm^{-2} and incubated at 37 °C with 5 % CO_2 for 2 d in a DMEM/F12 media (Cat. No. 11320–033; Gibco, UK) with 10 % fetal calf serum and 1 % penicillin–streptomycin. After 48 h in incubation post-transfection, the culture media was removed, and transfected cells were stimulated with either human ACTH(1–24) (hereafter referred to as ACTH) or α-MSH (New England Peptide, Gardiner, MA) diluted to concentrations ranging from 10^{-6}–10^{-12} M in serum-free DMEM/F12 media, then placed back into incubation for an additional 4 h. After stimulation, media was removed and replaced with a luciferase substrate (BrightGLO; Promega; Madison, WI). The luminescence generated after 5 min was measured spectrophotometrically by a BioTek Synergy HT microplate reader using Gen5 software (Agilent Technologies; Santa Clara, CA). Luminescence readings (in relative light units) were corrected by subtracting the average luminescence values of an unstimulated (0 M ligand) control for each unique transfection.

2.3. Calculations and statistics

The dose–response curve of receptor activation was analyzed using non-linear regression (three-parameter polynomial; log([ligand]) vs luminescence). From the fitted curves, values for half-maximal effective concentration (EC_{50}) and maximal response (V_{max}) were obtained. Values for EC_{50} and V_{max} were compared using the extra-sum-of-squares F test ($\alpha = 0.05$). All statistics and figure preparation were performed using Prism 9 software (GraphPad Inc., La Jolla, CA). All data are presented as mean ± standard error ($n = 3$).

3. Results

3.1. Sequence analyses

In our survey of the *P. senegalus* genome, we were able to identify a sequence for *mc2r* (XM_039738597). However, no *mrap1* ortholog was detected in the *P. senegalus* genome, although we could identify an accessory protein sequence that NCBI BLAST analysis identified as an *mrap2* ortholog (XM_039747869). The deduced amino acid sequence of sbMc2r aligned with a selection of other vertebrate Mc2r sequences, exhibiting high sequence similarity in the 7 transmembrane domains that are characteristic of Mc2r proteins (Fig. 1). In our analysis of vertebrate Mc2r orthologs, the highest sequence similarities were within the mammals (mouse and human; 89 % sequence similarity) and the cyprinids (carp and zebrafish; 83 %), whereas the lowest sequence similarities were between the stingray and the cyprinids (39–41 %) (Fig. 2A). As expected, the sequence similarity of sbMc2r to other vertebrate Mc2rs fell within these two extremes. The sbMc2r ortholog had highest similarity to the Mc2r in gar (65 %), and sbMc2r had a 55 % sequence similarity to human Mc2r and a 46 % sequence similarity to srMc2r (Fig. 2A). In a molecular phylogenetic comparison, Mc2r sequences of sarcopterygians, actinopterygians, and chondrichthyes all formed respective monophyletic clades, with the sbMc2r occupying the basal position among the Mc2rs of actinopterygians (Fig. 2B).

The amino acid sequence for sbMrap2 was aligned with a selection of gnathostome Mrap1 and Mrap2 sequences (Fig. 3A). All gnathostome Mrap sequences had high sequence similarity in the reverse topology motif in the *N*-terminal domain and the trafficking motif in the transmembrane domain, but only the bony vertebrate Mrap1 sequences contained the putative activation motif consisting of a δ-D-Y-δ residue sequence (Dores and Chapa, 2021) (Fig. 3A). The sbMrap2 sequence was missing this putative activation motif (Fig. 3A). In a phylogenetic analysis, the Mrap1 and Mrap2 sequences formed distinct monophyletic clades, with the sbMrap2 sequence being represented as most closely related to the bowfin Mrap2 (Fig. 3B).

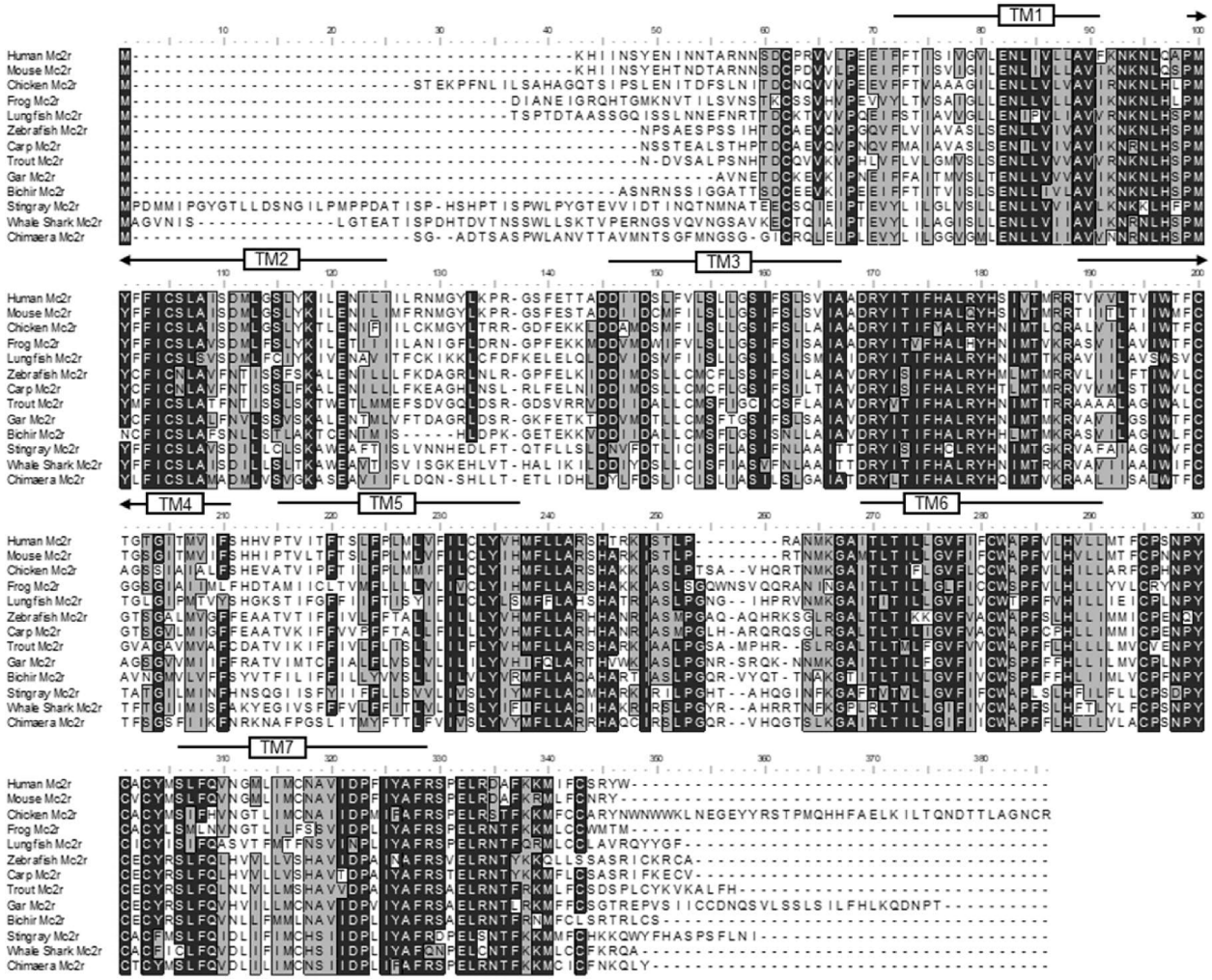


Fig. 1. Multiple alignment of Mc2r amino acids sequences from a selection of jawed vertebrates, including bichir Mc2r. Shading indicates identical (black) and molecularly similar (grey) residues. Horizontal bars indicate the 7 transmembrane regions; arrows indicate continuation of the indicated regions. See Materials and Methods for details of analyses and sequence accession numbers.

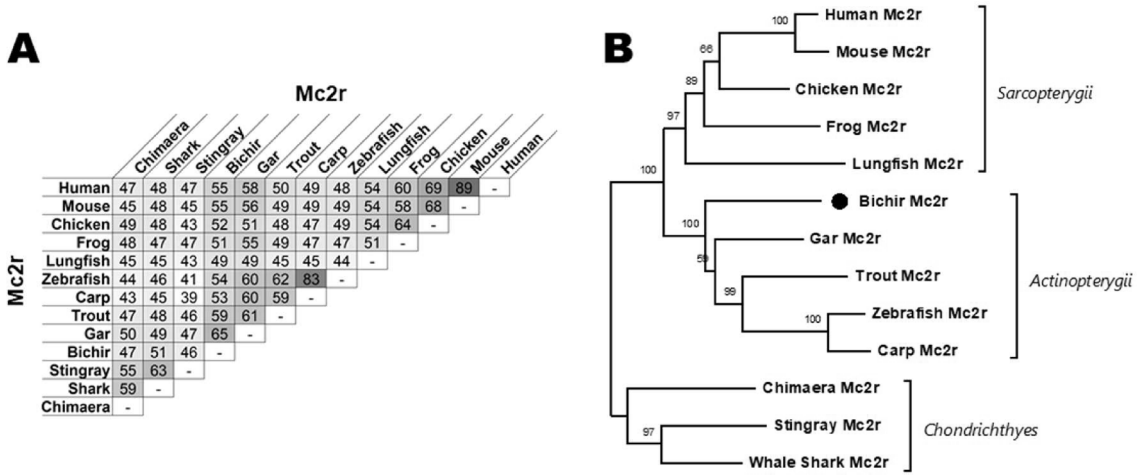


Fig. 2. Sequence similarities and phylogenetic relationships of Mc2r amino acid sequences from a selection of jawed vertebrates. (A) Amino acid sequence similarities of gnathostome Mc2rs are presented as percent identity (shading intensity reflects sequence similarity). (B) Molecular phylogeny of gnathostome Mc2rs, where the clade of Chondrichthyes was rooted as an outgroup. Bichir Mc2r is highlighted by black dot. See Materials and Methods for details of analyses and sequence accession numbers.

3.2. Receptor activation studies

To test the hypothesis that sbMc2r was dependent on chaperoning by an Mrap, we performed receptor activation studies on sbMc2r expressed either alone or with the co-expression of various vertebrate Mrap1s or the sbMrap2. When expressed alone (without co-expression of an Mrap), sbMc2r was not activated by any concentration of ACTH ligand (Fig. 4A). The positive control for this experiment was bfMc2r co-expressed with bfMrap1, which responded in a robust manner to stimulation by ACTH (Fig. 4A).

Given these results, we tested the hypothesis that sbMc2r does indeed require co-expression with an Mrap1 ortholog for functional activation. This experiment was performed using various vertebrate Mrap1 orthologs, including bfMrap1 and chMrap1 orthologs, which contain the δ-D-Y-δ activation motif, and wsMrap1, which lacks the δ-D-Y-δ activation motif. Co-expression of sbMc2r with various vertebrate Mrap1s resulted in activation of sbMc2r by ACTH, but this affect was dependent on the particular Mrap1 being co-expressed (Fig. 4B). When sbMc2r was co-expressed with wsMrap1, no activation of sbMc2r by ACTH was observed. When sbMc2r was co-expressed with either bfMrap1 or chMrap1, a characteristic activation of sbMc2r by ACTH was observed that fit a classic dose–response curve (bfMrap1: $R^2 = 0.91$; chMrap1: $R^2 = 0.90$) (Fig. 4A). The EC_{50} values of sbMc2r modulated by bfMrap1 and chMrap1 were as follows: bfMrap1: $EC_{50} = 9.0 \times 10^{-10}$ M (95 % CI: 4.3×10^{-10} to 1.8×10^{-9} M); chMrap1: $EC_{50} = 1.0 \times 10^{-8}$ (95 % CI: 3.9×10^{-9} to 2.3×10^{-8} M). These EC_{50} values were determined to be significantly different from each other ($F_{1,38} = 13.7$; $P < 0.001$). Although the maximal luciferase activity (V_{max}) was consistently marginally higher when sbMc2r was co-expressed with bfMrap1 compared with chMrap1, the V_{max} for each was not significantly different ($F_{1,38} = 1.78$; $P = 0.190$).

The results of experiments presented in Fig. 4A-B demonstrated the requirement of Mrap co-expression for sbMc2r function. Due to our ability to detect an *mrap2* ortholog, but not an *mrap1* ortholog, in the *P. senegalus* genome, we evaluated whether sbMrap2 could facilitate activation of sbMc2r (Fig. 4C). In this experiment, although sbMc2r co-expressed with bfMrap1 (the positive control) was activated following stimulation with ACTH, sbMc2r co-expressed with sbMrap2 did not respond to stimulation with ACTH (Fig. 4C).

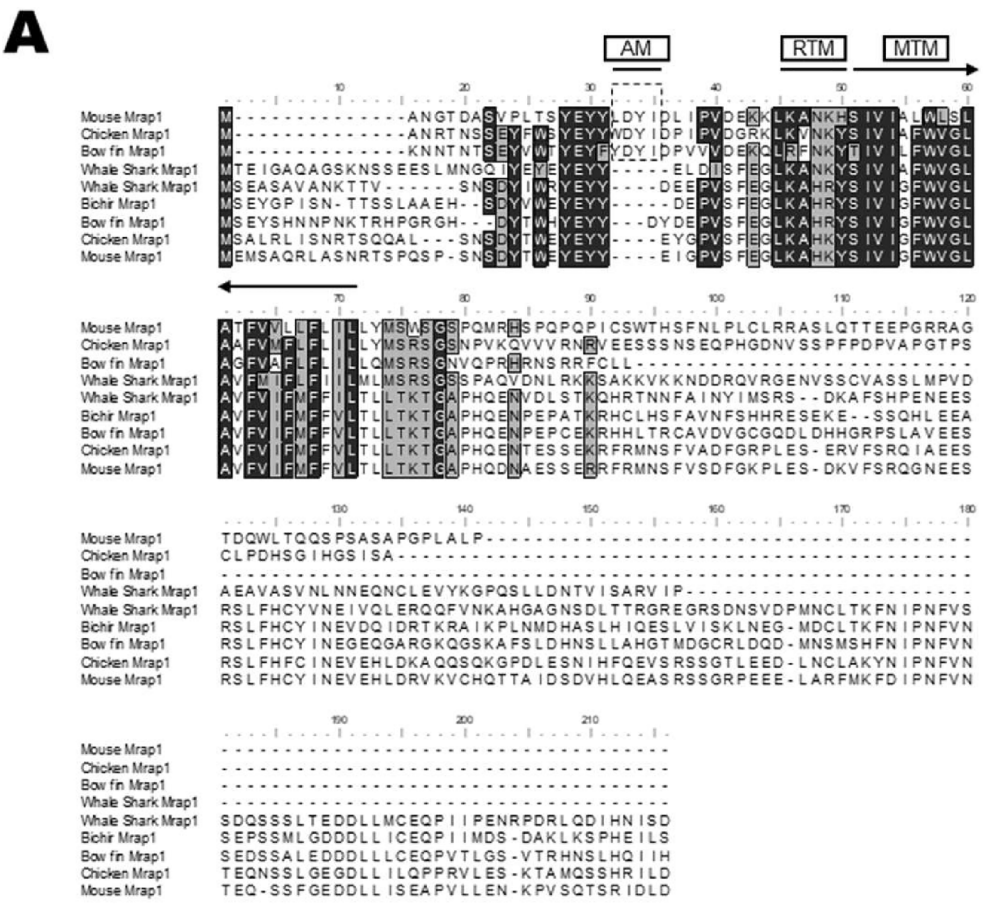
Finally, previous studies have shown that neopterygian and tetrapod Mc2r orthologs are exclusively selective for activation by ACTH, whereas cartilaginous fish Mc2r orthologs could be activated by either ACTH or α-MSH with varying degrees of efficacy (Dores and Chapa, 2021). To evaluative the ligand selectivity of sbMc2r, the receptor was co-expressed with bfMrap1 and stimulated with either ACTH or α-MSH ligands (Fig. 5). As in our previous experiments, sbMc2r co-expressed with bfMrap1 characteristically responded to stimulation by ACTH (the positive control) (Fig. 5). However, no activation of sbMc2r co-expressed with bfMrap1 was observed at any concentration of α-MSH (Fig. 5).

4. Discussion

In the present study, we have provided a molecular and functional characterization of an Mc2r from the most basal bony vertebrate studied to date, the Mc2r from a polypterus species, the Senegal bichir. As will be discussed, we have demonstrated that the sbMc2r was selectively activated by ACTH (compared to α-MSH) and dependent on the cooperative interaction of Mrap1 for functional activation. These results increase the resolution of our view of how derived Mc2r function arose throughout the radiation of the vertebrates, indicating that a major shift in Mc2r function occurred at the root of the radiation of class Osteichthyes.

Although many similarities exist across the five-member family of melanocortin receptors (Ramachandrappa et al., 2013), Mc2r has many unique features due to the considerably more rapid sequence divergence of this gene (Schiöth et al., 2005). All melanocortin receptors are coupled to cAMP/PKA pathways and activated by POMC-derived peptides, however only Mc2r has been shown to require chaperoning by Mrap1 and exclusive selectivity for ACTH (Ramachandrappa et al., 2013). Importantly, these unique functional properties of Mc2r are not universal and seem to only be present in the Mc2rs of the most derived chordates (Dores and Chapa, 2021).

Recent investigations in our lab and others have renewed an interest in studying basal vertebrates to better understand the evolutionary origins of a stress-responsive HPI axis responsible for corticosteroid production (Bouyoucos et al., 2021). Although it has been shown that jawless and cartilaginous fishes, as well as basal bony fishes, produce corticosteroids in response to stress (Close et al., 2010; McCormick et al., 2020; Rai et al., 2015; Ruiz-Jarabo et al., 2019; Shaughnessy et al., 2020; Shaughnessy and McCormick, 2021), whether an ACTH- and Mc2r-mediated HPI axis exists in these basal vertebrate lineages remains unknown (Bouyoucos et al., 2021; Roberts et al., 2014). Whereas the Mc2rs of all bony vertebrates studied to date appear to display Mrap1 dependance and ACTH selectivity, the Mc2r of cartilaginous fishes have variable Mrap1 dependance and no ACTH selectivity (Barney et al.,



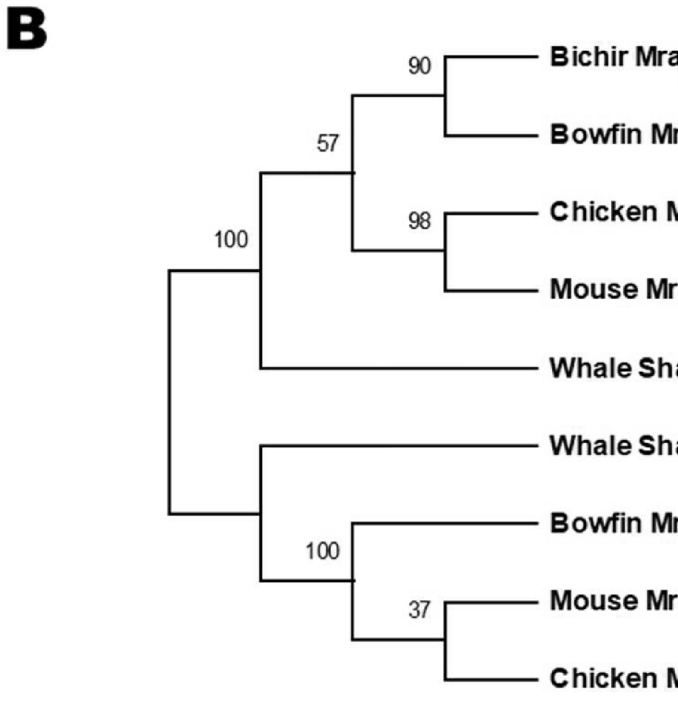


Fig. 3. Molecular characterization of Mrap amino acid sequences from a selection of jawed vertebrates, including bichir Mrap2. (A) Multiple sequence alignment of gnathostome Mrap1s and Mrap2s. Shading indicates identical (black) and molecularly similar (grey) residues. Horizontal bars indicate the activation motif (AM), reverse topology motif (RTM), and the membrane trafficking motif (MTM); arrows indicate continuation of the indicated regions. (B) Molecular phylogeny of gnathostome Mrap1s and Mrap2s. See Materials and Methods for details of analyses and sequence accession numbers.

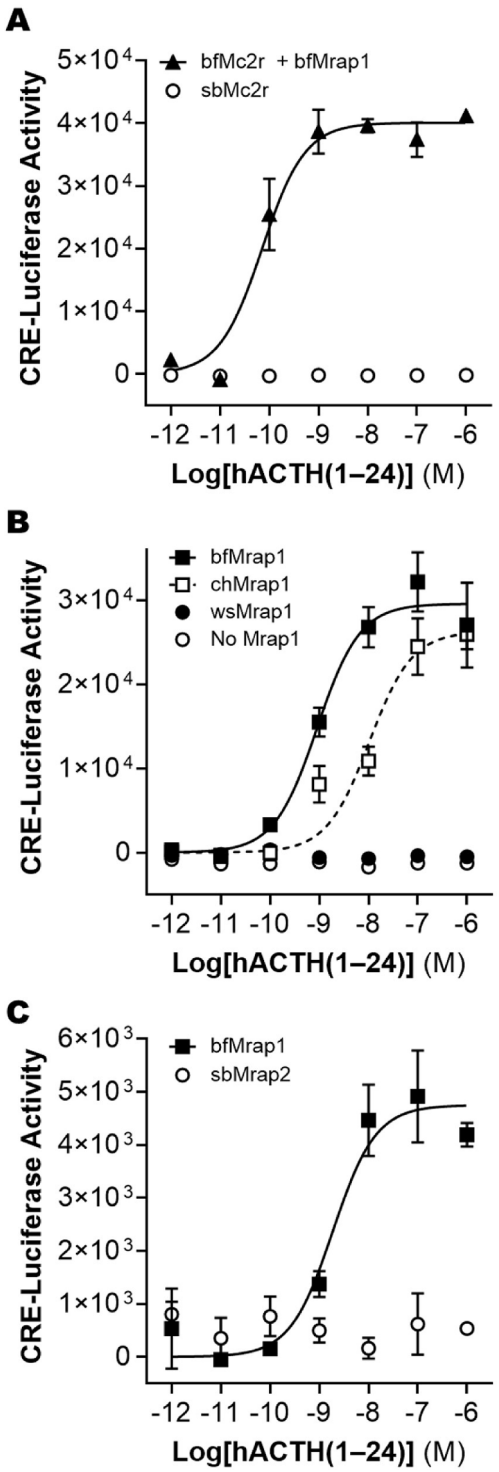


Fig. 4. Mrap1-dependance of bichir Mc2r. (A) Dose-response stimulation of bowfin (bf) Mc2r (co-expressed with bfMrap1) and bichir (sb) Mc2r (expressed without an Mrap) by human ACTH(1–24). (B) Dose-response stimulation by ACTH of sbMc2r expressed with and without various vertebrate Mrap1s (bfMrap1; chicken, chMrap1; whale shark, wsMrap1). (C) Dose-wise stimulation by ACTH of sbMc2r co-expressed with either bfMrap1 or sbMrap2. In all panels, data are presented as mean ± standard error ($n = 3$) and lines represent fitted dose–response curve (three-parameter polynomial).

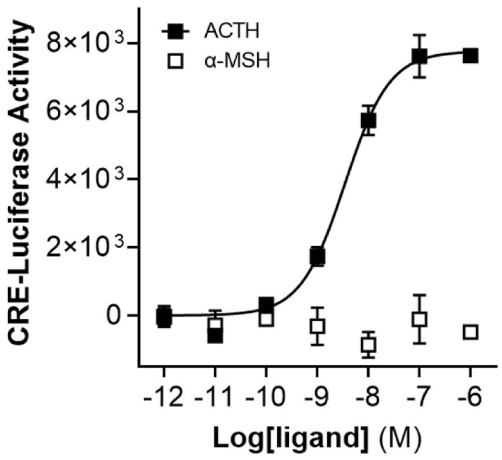


Fig. 5. ACTH selectivity of bichir Mc2r. Dose-wise stimulation by either human ACTH(1–24) or α-MSH of bichir Mc2r co-expressed with bowfin Mrap1. Data are presented as mean ± standard error ($n = 3$) and line represents fitted dose–response curve (three-parameter polynomial).

2019; Dores and Chapa, 2021; Hoglin et al., 2022). Thus, there appears to have been an important shift in Mc2r function during the emergence of bony vertebrates. By studying the Mc2r of the Sengal bichir in the present study, we aimed to determine whether sbMc2r, as the most basal bony vertebrate Mc2r studied to date, exhibited any ancestral (i.e., cartilaginous fish-like) features of Mc2r function, such as Mrap1 independence or lack of ACTH selectivity. In doing so, we aimed to further resolve the timing of acquisition of the derived features of Mc2r function in bony vertebrates.

Our analyses demonstrated that sbMc2r unambiguously exhibited only the derived features of Mc2r function, including an obligatory dependance on Mrap1 for activation and an exclusive selectivity for ACTH over α-MSH. These results indicate that the acquisition of these derived features of Mc2r function occurred at the root of the bony vertebrates, prior to the radiation of Polypteriformes. Indeed, the presence of these features of Mc2r function in both the actinopterygian and sarcopterygian lineages was already a good indication that these derived functional qualities of Mc2r would be represented in the common ancestor to all bony vertebrates (Dores and Chapa, 2021). However, functional analyses of the most basal members of these two primary bony vertebrate lineages have been needed to add support for this hypothesis. The present study provides such evidence from the actinopterygian lineage. It will be important to also analyze the functional properties of the Mc2r from the most basal members of Sarcopterygii, such as the lobe-finned fishes which include the lungfishes and the coelacanth.

The functional evolution of vertebrate Mc2r is likely related to the functional evolution of Mrap1. In the present study, it was not simply the case that sbMc2r could be activated when co-expressed with any vertebrate Mrap1. Whereas sbMc2r could be activated by ACTH when co-expressed with bowfin or chicken Mrap1, sbMc2r was unable to be activated when co-expressed with the Mrap1 of whale shark. This is likely explained by the putative activation motif $\delta^{18}D^{19}Y^{20}\delta^{21}$ (residue numbers indicated for mouse Mrap1) (Chan et al., 2009; Malik et al., 2015; Sebag and Hinkle, 2009; Webb and Clark, 2010). This activation motif is present in the bfMrap1 and chMrap1 but absent in wsMrap1. The δ-D-Y-δ motif is specific to osteichthyan Mrap1s, and directly follows the highly conserved Y-E-Y-Y motif contained in nearly all Mrap1 and Mrap2 sequence structures (Fig. 3A) (Dores and Chapa, 2021). In a previous study from our lab, we demonstrated that wsMc2r can be activated without co-expression with an Mrap, but only at supraphysiological concentrations of ACTH (Hoglin et al., 2022). This contrasts with the functional qualities of sbMc2r demonstrated in the present study, where sbMc2r could not be activated by any

concentration of ACTH without Mrap1 co-expression. Interestingly, sensitivity of wsMc2r to ACTH is substantially improved by co-expression with either wsMrap1 or wsMrap2, which are both missing a δ-D-Y-δ activation motif (Hoglin et al., 2022). This has been explained as wsMrap1 and wsMrap2 helping to increase the trafficking of the wsMc2r to the cell surface (Hoglin et al., 2022). It is due to the appearance in bony vertebrates of the requirement of Mrap1 co-expression for Mc2r activation by ACTH, coupled with the appearance of the δ-D-Y-δ activation motif in Mrap1 of bony vertebrates, that it has been suggested that Mc2r and Mrap1 have been co-evolving since the emergence of jawed vertebrates (Dores et al., 2016, 2014; Dores and Chapa, 2021; Västermark and Schiöth, 2011).

In the present study, the dependence of sbMc2r on co-expression with a bony vertebrate Mrap1 indicates that an *mrap1* gene containing a δ-D-Y-δ activation motif likely exists in the genome of *P. senegalus*, despite our current inability to detect any such *mrap1* in the published *P. senegalus* assembly. It is possible that the assembly of the bichir genome is either incomplete (less than 100 % coverage) or fragmented in such a way that makes the *mrap1* sequence impossible or difficult to detect. A similar phenomenon occurred with *Xenopus tropicalis*, for which an Mc2r was identified and functionally characterized as being Mrap1-dependant (Liang et al., 2011) many years before the genomic discovery of a *X. tropicalis* Mrap1 ortholog (Tai et al., 2022).

In summary, we have presented a molecular and functional characterization of the Mc2r of the Senegal bichir, which represents an Mc2r from the most basal bony vertebrate studied to date. The sbMc2r was obligatorily dependent on the cooperative interaction with a bony vertebrate Mrap1, and sbMc2r had exclusive selectivity for ACTH over α-MSH. These results indicate that an Mrap1 likely exists for *P. senegalus*, despite its apparent absence from the available genome assembly. Furthermore, our results add needed support to the hypothesis that the acquisition of Mrap1 dependence and exclusive ACTH selectivity with respect to Mc2r function occurred at the root of the radiation of the class Osteichthyes, though this hypothesis of ancestral Mc2r function requires more empirical testing from basal sarcopterygians.

Funding

This research was supported by the Long Research Endowment at the University of Denver to RMD, and by a National Science Foundation Postdoctoral Fellowship (DBI-2109626) to CAS.

CRediT authorship contribution statement

Ciaran A. Shaughnessy: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Mary F. Jensen:** Investigation, Writing – original draft, Writing – review & editing. **Robert M. Dores:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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