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Evolution of Melanocortin-2 Receptor Activations: Studies on a Mammal and a Fish

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EVOLUTION OF MELANOCORTIN-2 RECEPTOR ACTIVATIONS:
STUDIES ON A MAMMAL AND A FISH

A Dissertation
Presented to
the Faculty of Natural Sciences and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Liang Liang
June 2013
Advisor: Dr. Robert M. Dores
ABSTRACT

The structure and function relationship between melanocortin-2 receptor (MC2R) and ACTH are the most complicated in melanocortin receptor gene family. A comparative study on the activation of human and rainbow trout MC2R will provide a useful model system for understanding how ACTH emerged as the sole ligand for the MC2R of bony vertebrates. This dissertation will discuss how studies utilizing analogs of hACTH(1-24) have revealed two critical amino acid motifs in this ligand (HFRW and KKRRP) which are required for the activation of MC2R. In addition, the KKRRP motif functioned as the unique binding site for MC2R that directly contributes to the ligand selectivity feature, as revealed from studies on an ACTH antagonist which exclusively targets MC2R. Finally, based on our model for the interaction of ACTH and MC2R, the amino acid residues within TM4, EC2, and TM5 domains responsible for ACTH ligand selectivity will be evaluated by site-directed mutagenesis studies.
ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my advisor, Dr. Robert Dores, whose expertise, understanding, and patience, added considerably to my graduate experience. Without his guidance and persistent help, this dissertation would not have been possible. I appreciate his unsurpassed knowledge and skills in so many areas, especially his great assistant in writing.

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I also thank undergraduate student Kristin Schmid, who did excellent work on rainbow trout melanocortin-2 receptor project as her honor thesis. Her work completes this comparative study on melanocortin receptor of human and bony fish.

In addition, I would like to thank my fellow graduate students Christina Reinick, Cinda Hill, Perry Davis, and Yesenia Garcia. They each enriched my experience in the PhD program and also for all the good time in the lab over these years.

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INTRODUCTION

Stress and Hypothalamus-pituitary-adrenal (HPA) Axis

The ability to evoke stress response to physiological and psychological stimuli is necessary to increase the survival rate of organisms. In mammals, common stress responses include neuroendocrine response and autonomic response, known as the two-pronged physiological defense mechanism (Pecoraro et al., 2006). The neuroendocrine stress response is regulated by hypothalamus-pituitary-adrenal (HPA) axis (Figure 1). In this circuit, stressful stimuli cause corticotropin releasing hormone (CRH) to be released from hypothalamic paraventricular nucleus (PVN) to pituitary portal circulation, and then stimulate the secretion of adrenocorticotropic hormone (ACTH) from corticotropes in the anterior pituitary. The melanocortin peptide ACTH then induces the production of glucocorticoids in the adrenal cortex, via the activation of the ACTH receptor or the melanocortin 2 receptor (Chan et al., 2011). Consequently, glucocorticoids launch both rapid and prolonged effects on its target cells throughout the system, influencing metabolism, feeding behavior, and energy expenditure (Pecoraro et al., 2006; Mormede et al., 2011). Meanwhile, the HPA axis is under the negative feedback regulation of the circulating glucocorticoids at the level of the hippocampus, hypothalamus and pituitary (Jacobson and Sapolsky, 1991; Keller-Wood and Dallman, 1984; Cole et al., 2000).
In teleosts, steroidogenesis occurs in the interrenal tissues of the head kidney instead of adrenal gland. Therefore, the hypothalamus-pituitary-interrenal (HPI) axis mediates their neuroendocrine stress response. CRH neurons whose cell bodies are located at nucleus preopticus and nucleus lateralis tuberalis regions of the hypothalamus directly synapse to pituitary adrenocorticotropic cells (Thomas, 2008). As the result of CRH stimulation, ACTH is released from the pituitary to the systemic circulation. Thereafter, ACTH targets the melanocortin 2 receptors (MC2R) expressed on interrenal steroidogenic cells, and activates steroidogenesis which could be sustained for several hours in some fish species (Liu et al., 2006; Mommsen et al., 1999).

**Melanocortin Receptor Family**

Melanocortin receptors (MCRs) are G protein-coupled receptors (GPCRs), in the rhodopsin/β adrenergic like family A. GPCRs are single chain polypeptides forming seven membrane spanning alpha-helical regions that are linked by extracellular and intracellular domains (Devi, 2005). Conserved structural features of GPCRs found in MCRs include consensus N-linked glycosylation sites near the extracellular amino terminus and a palmitoylation site in the intracellular C-terminus (Yang, 2011; Gantz and Fong, 2003). Melanocortin receptors have short N- and C-terminus and a small second extracellular loop, therefore, they are the smallest GPCRs known. So far, five melanocortin receptors have been identified in vertebrates (Cone, 2006). Their physiological functions and ligand preference are summarized in Table 1.
**MC1R:** Human MC1R (317 amino acids) is predominantly expressed in melanocytes and melanoma cells, and it couples to the cyclic adenosine monophosphate (cAMP) signaling pathway (Chhajlani and Wikberg, 1992). Because MC1R mediates pigmentation, it is a major determinant of skin phototype and sensitivity to ultraviolet (UV) light induced skin damage (García-Borrón et al., 2005). In addition, MC1R is also found in fibroblasts, endothelial cells, monocytes, dendritic cells, neutrophils, granulocytes, osteoclasts, Leydig and lutein cells etc., suggesting its wide spectrum of physiological functions, such as anti-inflammatory and anti-pyretic actions (García-Borrón et al., 2005).

**MC2R:** MC2R (297 amino acids in humans) is also known as the ACTH receptor, since ACTH is the only ligand for this receptor (Table 1). As one of the key components of HPA/I axis, MC2R mediates the expression of steroidogenic enzymes via cAMP and PKA signaling pathway. As a result of MC2R activation, cortisol (the glucocorticoid in human and most teleosts) is secreted and elicit stress response on its target tissues. Mutations of MC2R account for approximately 25% of the cases of an autosomal recessive disease, familial glucocorticoid deficiency (FGD) (Chan et al., 2008). Other than the zona faciculata of the adrenal cortex, MC2R is also expressed in skin (Slominski et al., 1996) and adipocytes (Boston and Cone, 1996).

**MC3R:** Human MC3R is a 361 amino acid protein, which is mainly expressed in hypothalamic and limbic regions of brain (Roselli-Rehfuss et al., 1993). *Mc3r/-* mice showed accelerated weight gain and accumulation of extra fat mass, indicating its role in peripheral metabolism and energy balancing (Butler, 2006). Moreover, MC3R is the
only receptor in the melanocortin receptor family that can activate both cAMP, inositol
triphosphate (IP₃) and Ca²⁺ signaling pathways (Konda et al., 1994; Wachira et al., 2003).
Expression of MC3R is also identified in the placenta, stomach and pancreas (Gantz et al.,
1993).

**MC4R:** Another receptor in this family that regulates energy homeostasis is
MC4R (322 amino acids in humans). It is predominantly expressed in the brain,
autonomic nervous system and spinal cord (Mountjoy and Wild, 1998). Compared to
MC3R knockout mice, the obesity syndrome was far more sever in *MC4R*⁻⁻ mice, and is
associated with hyperphagia, hyperinsulinemia, hyperleptinemia (Huszar et al., 1997). In
addition to energy metabolism regulation, MC4R is also found to be involved in erectile
dysfunction (Martin and MacIntyre, 2004) and pain (Starowicz and Przewlocka,
2003). MC4R transduces a signal by coupling to the heterotrimeric Gs protein and
activating adenylate cyclase (Gantz et al., 1993).

**MC5R:** MC5R (325 amino acids) is the last melanocortin receptor to be cloned,
and is expressed in skin, adrenal gland, adipose tissue, kidney, lymph nodes, liver,
skeletal muscle and exocrine glands (Cooray and Clark, 2011). MC5R is involved in the
production of sebaceous lipids (Chen et al., 1997), protein and tear secretion by the
lacrimal gland (Entwistle et al., 1990). Activation of MC5R leads to the increase of
intracellular cAMP and Ca²⁺ (Hoogduijn et al., 2002).
Melanocortins

The natural ligands of melanocortin receptors are melanocortin stimulating hormones (MSHs) (α-MSH, β-MSH and γ-MSH) and ACTH, which are all derived from a common precursor pro-opiomelanocortin (POMC). In vertebrates, the POMC gene is expressed in hypothalamic neurons, anterior and intermediate pituitary, immune system and skin (Bertagna, 1994). Depending on the type of tissue, there are two outcomes in the posttranslational processing of POMC: (1) in the corticotropes of the anterior pituitary, POMC is endoproteolytically cleaved by proprotein convertase 1/3 (PC1/3). As a result, the major product is ACTH; (2) in the melanotropes of intermediate pituitary, melanocytes in skin, and certain neurons, PC1/3 and proprotein convertase 2 (PC2) work together to produce α-MSH, β-MSH, and γ-MSH (Figure 2A). Other end-products cleaved from the same precursor include CLIP, β-lipotropin (LPH), γ-LPH, and β-endorphin (Eipper and Mains, 1980).

Although individual preference does exist, MC1R, MC3R, MC4R and MC5R can be activated by any forms of the melanocortin peptides (see Table 1). Hence, people have identified a common sequence of melanocortins known as the “HFRW” motif (shown in Figure 2B) as the binding and activation site for these receptors (Schwyer, 1977). However, “HFRW” motif is not sufficient to allow MSHs to activate MC2R, since ACTH is the only ligand for this receptor. Because α-MSH [N-acetyl-ACTH(1-13)amide] is cleaved from ACTH by PC2 but unable to bind to MC2R. It appears that MC2R requires an additional binding site on the C-terminus of ACTH, which is not present in MSHs (Veo et al., 2011).
Melanocortin 2 Receptor Accessory Protein (MRAP)

While the heterologous expression of MC1R, MC3R, MC4R and MC5R has been successfully achieved in mammalian cell lines, studies found that only mammalian MC2R could not be functionally expressed in heterologous cells lines unless those cell lines were derived from the adrenal cortex (Forti et al., 2006; Rached et al., 2005). In addition, it was reported that a subset of patients exhibiting FGD syndrome had functional MC2 receptors, but were resistant to ACTH treatment (Metherell et al., 2005). Collectively, these observations led to the discovery that adrenal cortical cells express melanocortin receptor accessory proteins (Metherell et al., 2005).

In the genomes of many vertebrates, there are two paralogous mrap genes (mrap1 and mrap2) (Hinkle and Sebag, 2009; Webb and Clark, 2010). MRAPs are single chain polypeptides with a single membrane spanning domain. Both MRAP1 and MRAP2 can form antiparallel homodimers in the ER (Hinkle and Sebag, 2009), and both types of homodimers will couple to MC2R in the ER and facilitate the trafficking of mammalian MC2Rs to the plasma membrane (Figure 3). However, only MRAP1/MC2R complexes on the plasma membrane can be efficiently activated by ACTH, whereas the efficacy of ACTH activation is greatly diminished for MRAP2/MC2R complexes (Hinkle and Sebag, 2009; Webb and Clark, 2010). Furthermore, the role of MRAP1 in the trafficking and activation of MC2R is not limited to mammals, but also has been confirmed in orthologs of zebra fish (Danio rerio), rainbow trout (Oncorhynchus mykiss), and a frog (Xenopus tropicalis) (Agulleiro et al., 2010), suggesting that all vertebrate MC2Rs require MRAP1 for functional expression (Liang et al., 2011).
Evolution of MCR and MRAP

Five melanocortin receptor coding gene were found in the genomes of tetrapods, whereas none was identified in non-chordate metazoans (Vastermark and Schioth, 2011), indicating the evolution of melanocortin receptor gene family appeared during the evolution of phylum Chordata. Thus, the evolution of this gene family would have been influenced by the two generally accepted genome duplication events (2R hypothesis) that occurred during the radiation of this phylum (Ohno et al., 1968; Lundin, 1993) and then at least one localized gene duplication event (Schioth et al., 2005). Using the evolution of HOX genes as a model (Holland et al., 1994), the generally accepted scenario (shown in Figure 4A) is that the ancestral gene of MCR undergo first duplication event and yield first two paralogous genes (MCa and MCb receptors) in agnathan vertebrates, and then the second duplication event led to four paralogous genes in the ancestral gnathostomes (Vastermark and Schioth, 2011). The fifth paralogous melanocortin receptor coding gene that is present in the genomes of teleost Danio rerio (Logan et al., 2003), and the tetrapods Xenopus tropicalis and Mus musculus (Cone, 2006) provides the evidence for the localized gene duplication in the early radiation of the gnathostomes (Schioth et al., 2005).

In the search for MRAP orthologs, it was noticed that the ortholog of MRAP2 but not MRAP1 is present in the genomes of sea lamprey and elephant shark, whereas Fugu (Takifugu rubripes) has both paralogous genes (Vastermark and Schioth, 2011). As a result, mrap2 gene is considered as the ancestral gene of MRAP, and it appeared relatively early in the evolution of the vertebrates, but prior to the second genome
duplication event that occurred with the emergence of the gnathostomes (Ohno et al., 1968). At a later stage, the timeline of the appearance of \textit{mrap1} gene overlaps with MC2R orthologs (Figure 4B). Phylogenetic and functional expression analysis suggests that the co-evolution of MRAP1/MC2R has diverged to yield a bony fish trend and a tetrapod trend with respect to the interaction between MC2R and MRAP1 during the radiation of the vertebrates (Liang et al., 2011).

\textbf{Specific Aims:}

Working from these observations, this study had two objectives. The first objective was to answer the question: which regions in ACTH are responsible for the MC2R activation? We hypothesized that three zones of ACTH were involved in the MC2R activation. ACTH(1-24) consists of twenty-four residues of ACTH(1-39) that are conserved across most species and are capable of activating MC2R, and was used as the wild type ligand (Figure 2)(Costa et al., 2004). The first zone in ACTH(1-24), named Zone A for the purposes of the experiment, is where residues 6-9, HFRW, are found. This sequence appears in the sequences of $\alpha$- and $\beta$-MSH and is necessary for the activation of the other MCRs (Cone, 2006). The second region of interest consisted of residues 10-14, GKPVG (Glycine-Lysine-Proline-Valine-Glycine), and was named Zone B. The third region of interest consisted of residues 15-19, KKRRP (Lysine-Lysine-Arginine-Arginine-Proline), and was dubbed Zone C (Figure 2). The hypothesized mechanism of activation of MC2R by ACTH(1-24) is viewed as a multi-step process. The C-terminal region, Zone C, of ACTH(1-24) would make contact with MC2R at a site different from the HFRW-binding site, which would cause the opening of the HFRW-
binding site. This event would allow the HFRW motif on the ligand to bind and cause
the subsequent conformational changes to the receptor and activation of the G-protein
(Baron et al, 2009). The approach to validate this hypothesis was to test the activation of
analogs of ACTH in Zones A, B, and C of wild type MC2R.

The second objective was to answer the question: which regions of MC2R are
responsible for ACTH ligand selectivity (Figure 5)? The hypothesized model entails a
docking pocket for the C-terminal residues of ACTH(1-24), Zone C, and a separate
binding site for HFRW, Zone A, with a central adaptor region of ACTH, Zone B, to
accommodate a better fit into the two pockets. The second docking pocket was
hypothesized to be created by transmembrane regions 4 and 5 and extracellular loop 2.
Individual residues in TM4, EC2, and TM5 were replaced with an alanine residue and
each single mutant construct was stimulated with ACTH in a dose-dependent manner.
Figure 1. Hypothalamus-Pituitary-Adrenal axis in mammals (Adapted from Lightman and Conway-Campbell, 2010)
Table 1. Localization, physiological functions and ligand preference of melanocortin receptors 1 through 5 in mammals (Adapted from Cone, 2006).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Sites of expression</th>
<th>Known physiological functions</th>
<th>Agonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1-R</td>
<td>Melanocytes</td>
<td>Pigmentation (regulation of the eumelanin-pheomelanin switch)</td>
<td>$\alpha$-MSH $&gt;$ $\beta$-MSH $&gt;$ ACTH $&gt;$ $\gamma$-MSH</td>
</tr>
<tr>
<td>MC2-R</td>
<td>Adrenal cortex</td>
<td>Adrenocortical steroidogenesis</td>
<td>ACTH</td>
</tr>
<tr>
<td>MC3-R</td>
<td>CNS, GI tract, kidney</td>
<td>Energy homeostasis, natriuresis</td>
<td>$\gamma$-MSH $&gt;$ $\alpha$-MSH $&gt;$ $\beta$-MSH $&gt;$ ACTH</td>
</tr>
<tr>
<td>MC4-R</td>
<td>CNS</td>
<td>Energy homeostasis, erectile function</td>
<td>$\alpha$-MSH $&gt;$ $\beta$-MSH $&gt;$ ACTH $&gt;$ $\gamma$-MSH</td>
</tr>
<tr>
<td>MC5-R</td>
<td>Exocrine cells</td>
<td>Synthesis and secretion of exocrine gland products</td>
<td>$\alpha$-MSH $&gt;$ $\beta$-MSH $&gt;$ ACTH $&gt;$ $\gamma$-MSH</td>
</tr>
</tbody>
</table>
Figure 2. Melanocortin peptides in humans. A) Selective processing of POMC by enzymes PC1/3, and PC2. Some KR cleavage sites are suspected to be selected by PC2. B) Alignment of amino acid sequences of melanocortin peptides.
Figure 3. Melanocortin 2 Receptor Accessory Protein (MRAP) forms antiparallel homodimer in ER, and then facilitates the trafficking of MC2R to plasma membrane (Adapted from Webb and Clark, 2010).
Figure 4. Modeling the evolution of melanocortin receptors in phylum chordata. A) Hypothetical scheme for the radiation of the melanocortin receptors during the evolution of the chordates. Ancestral MCR—predicted MCR gene in protochordates. MCR’ and MCR”—predicted MCR genes formed after the first genome duplication event. MC5R/MC2R—predicted ancestral gene for MC5R and MC2R. B) Phylogeny of melanocortin receptors in extant vertebrates. (MCR) refers to the predicted ancestral MCR gene in protochordates. [] indicates some genes that is predicted to have been secondarily lost in some species. * indicates MC2R in these species that can only be activated by ACTH. (Adapted from Baron et al., 2009).
METHODS AND MATERIALS

Tissue Culture

Chinese Hamster Ovary (CHO) cells (obtained from ATCC, VA) were used in all experiments performed. Cells were grown in Kaighn’s Modification of Ham’s F12K media (ATCC) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml normacin, and maintained in a humidified incubator with 95% air and 5% CO₂ at 37°C. When reaching 80% confluence, CHO cells were split into subcultures using 0.05% trypsin/0.53 mM EDTA.

DNA Constructs

Human MC2R (accession no. AA067714.1) and rainbow trout MC2R (accession no. EU119870.1), were synthesized by GenScript (Piscataway, NJ) with an N-terminal V-5 epitope tag and inserted into a pcDNA3.1+ vector. Mouse (Mus musculus) MRAP1 (accession no. NM_029844), zebrafish (Danio rerio) MRAP1 (accession no. XR_117835), were individually synthesized by GenScript with an N-terminal FLAG epitope tag and were separately inserted into pcDNA3.1+ vectors. cAMP reporter construct CRE-Luc (Chepurny and Holz, 2007) was provided by Dr. Patricia Hinkle (University of Rochester, NY). All DNA constructs were inserted in pcDNA3.1+ vector individually.
Mutations were introduced to receptor constructs by site-directed mutagenesis with individual alanine substitutions (Performed by GenScript, Piscataway, NJ). Positions in hMCc2R and rtMC2R examined by this study were shown in Figure 5.

ACTH Analog Peptides

Human adrenocorticotropic hormone ACTH(1-24) and NDP-α MSH was purchased from Sigma-Aldrich Inc.(St. Louis, MO). The rest of hACTH(1-24) analogs including alanine substitutions and truncated peptides were synthesized by New England Peptide Inc. (Boston, MA). The amino acid sequences of hACTH(1-24) analogs used in this study are listed in Table 2.

Immunocytochemistry

CHO cells were seeded in 8-well chamber slides (2.5×104 cells/well), and after 24 hours, transfected with 1µg of DNA construct using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and OptiMEM (Mediatech Inc, Herndon, VA). Cells were allowed to express the receptors/MRAPs for 24 hours and fixed with 4% PFA for 15 minutes, washed, and then half of the wells were permeablisized with 0.3% triton X-100 for 10 minutes. Primary antibodies (mouse anti V5 and/or rabbit anti FLAG, 1:500 dilution) were applied to the cells for 1 hour at 37°C. After three washes with PBS, cells were incubated with secondary antibodies (donkey anti mouse conjugated with Alexa388 and/or donkey anti rabbit conjugated with Alexa555, 1:800 dilution) for 45 minutes at 37°C. After three washes with PBS, chambers were removed from slides. Coverslips
were mounted with Vecta-shield (Vector Laboratories Inc, Burlingame, CA), and nuclei were stained with DAPI (blue). Slides were imaged using 100X oil immersion objective with a fluorescent Zeiss Axioplan 2 microscope equipped with Hamamatsu digital camera. All images were analyzed with SlideBook (www.slidebook.com) software.

**cAMP Reporter Assay**

For the cAMP assay, 2.5×10^6 CHO cells were cotransfected with MC2R, MRAP, and CRE-Luc (Chepurny and Holz, 2007) DNA constructs (2µg each) using Amaxa Cell Line Nucleofector II system (Lonza, MD) with solution T and program U-23. After 10-minute recovery, cells were then seeded in white 96-well plate with a final density of 1×10^5 cells/well. 48 hours after transfection, cells were stimulated with ACTH analogs diluted within serum-free CHO media for 4 hours. Analogs were tested at doses ranging from 10^{-6}M to 10^{-14} M, and each dose was tested in triplicate against a wild type ACTH(1-24) control. Then stimulating media were removed, Luciferase substrate reagent Bright GLO (Promega, WI) was applied to the wells, and incubated at room temperature for 5 mins. Luminescence was measured by Bio-Tek Synergy HT plate reader (Winooski, VT). To determine the basal levels of cAMP production, transfected CHO cells stimulated with vehicle were measured along with each experiment group individually. Luminescence readings were first subtracted by their basal activity and then fit to Michaelis-Menton equation to obtain EC_{50} values. Data were analyzed using Kaleidograph software (www.synergy.com)
cAMP Enzyme Immunoassay

Cells transiently transfected with MC2R and MRAP (Amaza nucleofection kit T) were plated in 6-well plates at a density of $3 \times 10^5$ cells/well and allowed to recover from transfection for 48 hours. ACTH analogs were diluted as needed in standard extracellular solution (10mM D-glucose, 2mM KCl, 1mM MgCl$_2$, 5mM CaCl$_2$, 140mM NaCl, 10mM HEPES and 0.5% BSA). 1mM 3-Isobutyl-1-methylxanthine (IBMX) was used in solutions to prevent the degradation of intracellular cAMP from phosphodiesterase. Analogs were tested at concentrations ranging from $10^{-6}$M to $10^{-10}$ M, and each dose was tested in triplicate against a wild type ACTH(1-24) control. After 15-minute incubation at 37°C, analogs were removed from the wells, and the cells were lysed and collected within 0.1M HCl after well scraping. Lysate was centrifuged at 4.5RCF for 5 minutes, and the supernatant containing cAMP was assayed immediately using a direct cAMP EIA kit (Assay Design, Ann Arbor, MI) and measured with Bio-tek Synergy HT plate reader (Winooski, VT).

Radioligand Binding Assay

CHO Cells transiently transfected with MC2R and MRAP (Amaza nucleofection kit T) were plated in 12-well plates at a density of $2 \times 10^5$ cells/well. Assay was performed after for 48 hours after transfection for cell recovery and receptor expression. To stabilize these cells, binding buffer (10mM Glucose, 1mM MgCl$_2$, 10mMHEPS, 5mM CaCl$_2$, 140mM NaCl, 2mM KCl, with 0.1% BSA) was applied to each well, incubated for 20 min at 37°C and then removed. $1 \times 10^5$ cpm ACTH(1-39)Tyr23$I^{125}$ (Perkin Elmer,
Waltham, MA) was used in combination of non-radiolabeled ACTH analogs within binding buffer. After 1 hour incubation at 37°C, binding reaction was terminated by removing the binding buffer and followed by three washes with cold PBS on ice. Cells were then solubilized with 0.1% SDS for 30 minutes at room temperature. After well scraped, cell lysate was collected and the radioactivity was quantified by a gamma counter.

**Cell Surface ELISA**

CHO cells were seeded in 24-well plates (5×104 cells/well), and after 24 hours, transfected with 250ng of each DNA construct using lipofectamine 2000 (Invitrogen, CA). Cells were allowed to express the receptors for 24 hours and fixed with 4% PFA. 0.3% triton X-100 was applied to half of the wells to permeabilize the cells. Then blocking buffer (5% dry milk in PBS supplemented with 10% FBS and 5% normal goat serum) was applied to the well overnight at 4°C. cells were incubated with primary antibodies mouse anti V5 and/or rabbit anti FLAG(1:2000) for 1 hour at 37°C, washed three times for 5 minutes, followed with secondary antibody goat anti-mouse conjugated with HRP (1:2000) for 45mins at 37°C. Wells were again washed three time for 5 minutes, and allowed to dry before adding 200 µL TMB (Invitrogen, MD) and allowed to incubate for 10 minutes at room temperature. The reaction was halted by adding 200 µL 1N HCl and gently swirling. 300 µL from each well was transferred to a 96-well plate and read immediately at absorption 450nm by Bio-Tek Synergy HT plate reader (Winooski, VT). All experiments were performed in triplicates with a GFP transfected
control. Data were normalized to GFP control and analyzed using Student’s t-test for equal variance with Kaleidograph software (www.synergy.com).

**Statistical Analysis**

Data points are expressed as the mean with standard error of values obtained from experiments which were performed in triplicates. Differences between experimental treatments with corresponding controls were determined using unpaired two-tailed Student’s t-test for equal variance. Significance was set at $P \leq 0.05$. 
Figure 5. Two-dimensional structures of the hsMC2R (A) and rtMC2R (B). Mutant positions examined in this study are highlighted in grey.
Table 2. hACTH(1-24) analogs used in this study. Alanine substitutions in amino acid sequences are underlined.

<table>
<thead>
<tr>
<th>ACTH Analogs</th>
<th>Amino Acid Sequences</th>
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<tbody>
<tr>
<td>Wild Type</td>
<td>ACTH(1-24) SYSMEHFWGKPVGKRRPVKVYP</td>
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<tr>
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<td>A4/HFRW SYSMEAAAAGKPVGKRRPVKVYP</td>
</tr>
<tr>
<td></td>
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<td>HARW SYSMEHARWGPVGGKRRPVKVYP</td>
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<td></td>
<td>HFRA SYSMEH FRAGKPVGGKRRPVKVYP</td>
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<td>Zone B</td>
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</tr>
<tr>
<td></td>
<td>A10/14 SYSMEHFWKA KPVAKKRRPVKVYP</td>
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<td>KP SYSMEHFWGAAVGGKRRPVKVYP</td>
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<tr>
<td></td>
<td>P12 SYSMEHFWGKA VGGKRRPVKVYP</td>
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<td></td>
<td>ACTH(1-22) SYSMEHFW KVAKKRRPVKVYP</td>
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<td></td>
<td>ACTH(1-21) SYSMEHFWGKKRRPVKVYP</td>
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<td>Zone C</td>
<td>A5/Tetra SYSMEHFWGKPVGAAAAAVKVYP</td>
</tr>
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<td></td>
<td>AARRP SYSMEHFWGKPVGAARRPVKVYP</td>
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<td>AAW SYSMEHFWGKPVGKRAAWKVYP</td>
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<td>Y23 SYSMEHFWGKPVGKKRRPVKYP</td>
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<td>P24 SYSMEHFWGKPVGKKRRPVKYP</td>
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<td>α-MSH Analogs</td>
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<td>ACTH(4-10)</td>
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<td>KPV Ac-GKKRRP</td>
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CHAPTER ONE

Ligand Selectivity Properties of Human Melanocortin-2 Receptor

The ligand selectivity feature makes melanocortin 2 receptor a very special member in its gene family. Unlike other melanocortin receptors, mammalian MC2Rs, and perhaps all bony vertebrate MC2Rs, can only be activated by ACTH, but not by any MSHs (Cone, 2006). This chapter will focus on identifying the critical amino acid motifs that are responsible for hMC2R ligand selectivity by analyzing various regions of hACTH sequence.

As noted in the Introduction, the full length of ACTH peptide in most species of vertebrates is 39 amino acid residues (Dores and Lecaude, 2005). However, earlier studies reviewed by Schwyzer (1977) indicated that the functional domain required for activating the adrenal “ACTH” receptor was located within the first 24 residues of this hormone. Hence, in this study, hACTH(1-24) was used in all the experiments representing mammalian adrenocorticotropic hormone. As summarized by Schwyzer (1977), truncated analog studies have uncovered two functional motifs within ACTH: the HFRW motif and the KKRR motif, which will be reevaluated in this study using alanine substitution analogs in order to retain the secondary structure of ACTH as much as possible.
Since the HFRW motif is present in all of the melanocortin peptides (Nakanishi et al., 1979), the key to understanding the ligand selectivity of an MC2R would appear to lie in residues 14 to 24 in the sequence of ACTH(1-24). As a result, the importance of the C-terminal domain will be carefully examined utilizing alanine substitution analogs of hACTH(1-24). Based on the sequence of interest, ACTH analogs examined in this chapter were grouped into four categories (Table 2): (1) Zone A analogs—$H^6F^7R^8W^9$; (2) Zone B analogs—$G^{10}K^{11}P^{12}V^{13}G^{14}$; (3) Zone C analogs—$K^{15}K^{16}R^{17}R^{18}P^{19}$; (4) Zone D analogs—$V^{20}K^{21}V^{22}Y^{23}P^{24}$.

**Results**

**Ligand Selectivity of hMC2R**

**Zone A Analogs**

To determine the importance of each amino acid residue in HFRW motif, we designed and examined five Zone A analogs (See sequences in table 2) on CHO cells transiently transfected with hMC2R and mMRAP1 (Figure 6A). Consistent with previous studies, A4 analog which has four alanine substitutions at HFRW motif cannot stimulate the hMC2R activation. In order to test how these positions of this region contribute to the activation of receptor, analog AFRW, HARW, HRAW, and HFRA was applied individually to stimulate the transfected cells. Analog HFRA was unable to activate hMC2R in the same manner of A4 analog, indicating that the tryptophan residue plays an essential role in this motif. Moreover, although analog AFRW, HARW, and HRAW
were able to activate hMC2R, the shifts in EC$_{50}$ values of their activation curves were 8-fold, 8000-fold, and 440-fold as compared to ACTH(1-24) (Table 3). Student t-test analysis showed that alanine substitution on phenylalanine (HARW) and arginine (HFAW) significantly diminished receptor activation. As a result, the order of the importance of these positions is W$^9$>F$^7$>R$^8$>H$^6$.

**Zone B Analogs**

The region between HFRW and KKRRP was also first examined using four ACTH(1-24) analogs with alanine substitutions at G$^{10}$K$^{11}$P$^{12}$V$^{13}$G$^{14}$, G$^{10}$G$^{14}$, K$^{11}$P$^{12}$, and P$^{12}$ (Analog A10-14, AKPVA, GAAVG, and GKAVG in Table 2) on transfected CHO cells. Analog A10-14 showed significantly reduced activation activity with a 900-fold increase in the EC$_{50}$ value, whereas other analogs substituted with single or double alanines in this region did not show any obvious decline as compared to ACTH(1-24) (Figure 6B and Table 3). Nevertheless, when we tested two truncated analogs ACTH(1-21) and ACTH(1-22) that skip position G$^{10}$G$^{14}$ or K$^{11}$P$^{12}$V$^{13}$ within this domain, ACTH(1-21) complete lost the stimulatory effect to hMC2R while the ACTH(1-22) response was undetectable until 10$^{-7}$M (Figure 6C).

**Zone C Analogs**

Three ACTH analogs with alanine substitutions (See sequences in Table 2) were synthesized in order to evaluate the importance of KKRRP motif in terms of hMC2R activation on transfected CHO cells. Replacing KKRRP with five alanines (A5 analog)
greatly reduced the activation of receptor by 3000-fold shifting in EC$_{50}$ (Figure 6D and Table 3). Further, position K$_{15}$K$_{16}$ and R$_{17}$R$_{18}$P$_{19}$ were tested separately using analog AARRP and KKAAA. The EC$_{50}$ values of both analogs were significantly increased as compared to ACTH(1-24), while analog KKAAA is a less potent ligand compared with AARRP (Table 3).

**Zone D Analogs**

Albeit alanine substitution at KKRRP motif introduced a significant right shift in ACTH dose response curve, note that it was still able to stimulate a weak response at concentration of 10$^{-7}$M (Figure 6B). This observation raise the possibility that the subsequent sequence V$_{20}$K$_{21}$V$_{22}$Y$_{23}$P$_{24}$ may play a role in facilitating ACTH(1-24) binding to the receptor. To test this hypothesis, six Zone D analogs (A20-23, V20, K21, V22, Y23 and P24) were examined on hMC2R (see sequences in Table 2), whereas all of these analogs did not affect the activation curve as compared to ACTH(1-24) (Figure 7).

**Binding Affinity Analysis**

In addition to receptor activation assay, analogs from each region that showed great reduction in activation activities were further examined with radioligand binding assay (Figure 8A). A right shift in binding curve showed in all three analogs (A4, A5 and A10-14), while only the K$_i$ value of analog A5 was significantly greater as compared to ACTH(1-24) (P<0.05).
Discussion

The HFRW motif is present in all of the melanocortins (Nakanishi et al., 1979). N-terminally truncated analogs of mammalian ACTH(1-24) in which this region of the polypeptide has been eliminated lack the ability to activate any melanocortin receptor (Eberle and Schwyzer, 1975; Schwyzer, 1977). Hence, the HFRW motif has been identified as the core sequence of all melanocortin peptides, and consistently in this study, alanine substitutions in this motif (A4 analog) completely abolished the activation of hMC2R as indicated in Figure 6A. However, not all four positions in this motif are equally important for the activation of hMC2R. It appears that tryptophan\(^9\) plays a prominent role in the functioning of this motif, since alanine substitution on this position caused a complete loss in activation as analog A4 did. Assuming that the HFRW motif in ACTH(1-24) also forms a reverse \(\beta\)-turn as observed for \(\alpha\)-MSH (Ying et al., 2003), the backbone structure held by the stacking of histidine\(^6\) and phenylalanine\(^7\) aromatic rings would still be intact in analog HFRA. Thus, the side chain of tryptophan\(^9\) was probably interacting with certain sites of hMC2R which is critical for activation. Other than tryptophan\(^9\) and phenylalanine\(^7\) which is the central residue in the reverse \(\beta\)-turn, alanine substitution at arginine\(^8\) greatly affected receptor activation as well. As reported by Lee and colleagues (1998), the same arginine\(^8\) in \(\alpha\)-MSH analog was responsible for the electrostatic interactions between the ligand and the complementary residues of the receptors (e.g., the TM3 residue D122 in hMC4R). When Zone A analogs were applied to human MC4R, similar effects showed in the activation curves (Figure 8B). The activation
of hMC4R required the presence of tryptophan$^9$, which appears to be the most significant residue in HFRW motif as well. However, every residue in this motif was important for hMC4R activation, and the order of importance of other residues was: arginine$^8$ > phenylalanine$^7$ > histidine$^6$ (Table 4). Based on these observations, it is reasonable to postulate that the HFRW motif is an important binding site for hMC2R, but at least in terms of activation it is slightly different with the mode of hMC4R. According to Pogozheva et al. (2005), of the twelve residues in TM2, TM3, TM6 and TM7 of hMC4R that are involved in the binding of HFRW motif, ten amino acids are conserved residues in melanocortin receptors (Figure 9). Six amino acids are identical in all five melanocortin receptors, while four amino acids (F129 in TM3, V262 and V265 in TM6, and G288 in TM7) are unique in MC2R. The differences in residues may lead to a broader HFRW binding site for MC4R, hence, alanine substitution at HFRW motif had a greater effect on MC2R activation as compared to MC2R.

As reviewed by Schwyzer (1977), the shortest analog of ACTH(1-24) that still retains some level of biological activity is ACTH(1-16), and conversely analogs longer than ACTH(1-16) are progressively stronger agonists. Costa et al. (2004) conducted a series of studies that focused on residues in the motif KKRRP in the sequence of hACTH(1-24). Alanine substitutions at any of these sites lowered the biological activity of the ACTH(1-24) analog with varying degrees of efficacy. Collectively, the experiments using C-terminally modified analogs of ACTH(1-24) point to the KKRRP motif in mammalian ACTH(1-24) as an important region for the activation of MC2R. In agreement with this hypothesis, the replacement of the KKRRP motif in hACTH(1-24)
with alanines (A5 analog) produced an analog with a greatly diminished capacity for activating MC2R (Figure 6B). This reduction in activation was probably resulting from the significant drop of ligand binding affinity (Figure 8A). With a closer look in KKRRP motif, the R\textsuperscript{17}R\textsuperscript{18}P\textsuperscript{19} motif appears to be more critical than the K\textsuperscript{15}K\textsuperscript{16} motif (Figure 6B and Table 3). Although analog A5 introduced a right shift in activation curve, it was still able to bind to and stimulate hMC2R at concentrations higher than 10\textsuperscript{-7} M (Figure 6B), implying the involvement of the subsequent sequence VKVYP at C-terminal of ACTH(1-24). Albeit alanine substitution at this domain didn’t affect hMC2R activation curves (Figure 7), truncated analog KKRRP partially lost its competitive ability as compared to KKRRP analog (See Chapter Two Figure 12B), probably due to a reduction of binding affinity. Taken together, these observations supported the hypothesis that KKRRP motif functions as a secondary binding site of ACTH for the activation of hMC2R.

Accepting the premise that there is an HFRW binding site as well as a KKRRP binding site on hMC2R, we next wished to address the question of the relative distance between these two binding sites. As indicated in Figure 7, substitution with five alanines at this region greatly reduced activation activities of hMC2R, while the truncated Zone B analog, neither ACTH(1-21) nor ACTH(1-22), was able to stimulate this receptor. Based on these observations, it seems that the G\textsuperscript{10}K\textsuperscript{11}P\textsuperscript{12}V\textsuperscript{13}G\textsuperscript{14} sequence, especially the distance between HFRW and KKRRP motif, would play a critical role in positioning the ligand into the corresponding binding sites of these two motifs. Assuming that initial contact with the hMC2R occurs at the proposed KKRRP binding site, and this binding event would then lead to the docking of the HFRW domain into its corresponding binding
site of the receptor, altering the secondary structure of the GKPVG region of hACTH(1-24) would cause the HFRW motif unable to be properly positioned into the HFRW binding site on the receptor (Figure 10). Based on these assumptions, this model would explain the inability of α-MSH to activate MC2R, or even to act as a competitive inhibitor of ACTH(1-24) (Buckley et al., 1981). It would appear that since α-MSH lacks the KKRRP motif, this ligand cannot be properly oriented for inserting into the HFRW binding site in melanocortin-2 receptor. On the other hand, α-MSH can successfully activate MC1R, MC3R, MC4R, and MC5R, even without a complete KKRRP sequence (Cone, 2006). In this regard, it would be reasonable to propose that these MCRs may have a flexible binding site for HFRW motif, which allow both α-MSH and ACTH(1-24) to activate MC1R, MC3R, MC4R, and MC5R.
Figure 6. Activation curves of hMC2R by the stimulation of hACTH(1-24) analogs: A) Zone A analogs; B) Zone B alanine substitution analogs; C) Zone B truncated analogs; D) Zone C analogs
Figure 7. Activation curves of hMC2R by the stimulation of hACTH(1-24) Zone D analogs.
Figure 8. A) hMC2R ligand binding analysis. B) Activation curves of hMC4R by the stimulation of hACTH(1-24) Zone A analogs.
<table>
<thead>
<tr>
<th>ACTH Analogs</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; ± SEM (pM)</th>
<th>P Values</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; ± SEM (nM)</th>
<th>P Values</th>
</tr>
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<tbody>
<tr>
<td>ACTH(1-24)</td>
<td>4.2 ± 1.3</td>
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<td>11.8 ± 8.4</td>
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<tr>
<td>Zone A</td>
<td></td>
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<tr>
<td>A4</td>
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<td></td>
<td>148.2 ± 79.8</td>
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<tr>
<td>AFRW</td>
<td>36 ± 11</td>
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<td>HARW</td>
<td>33505 ± 2559***</td>
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<tr>
<td>HFAW</td>
<td>1845 ± 467**</td>
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<tr>
<td>HFRA</td>
<td>NA</td>
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</tr>
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<td>Zone B</td>
<td></td>
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<tr>
<td>A10-14</td>
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<td>AKPVA</td>
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<tr>
<td>GKAVG</td>
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<td>ACTH(1-22)</td>
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<td>Zone C</td>
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<tr>
<td>A5</td>
<td>12214 ± 4745*</td>
<td>0.03</td>
<td>459.7 ± 466.1*</td>
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<tr>
<td>AARRP</td>
<td>14.4 ± 3.3*</td>
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<tr>
<td>KKAAA</td>
<td>120 ± 24.5**</td>
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EC<sub>50</sub> values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (*:P<0.05, **:P<0.005, ***: P<0.005). NA, No activation
Table 4. Effects of ACTH(1-24) Zone A analogs on hMC4R activation

<table>
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<tr>
<th>ACTH Analogs</th>
<th>EC$_{50}$ ± SEM (nM)</th>
<th>P Values</th>
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<tr>
<td>ACTH(1-24)</td>
<td>0.2 ± 0.85</td>
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<td>NA</td>
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<tr>
<td>AFRW</td>
<td>36 ± 8.9*</td>
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<td>HARW</td>
<td>3128 ± 424**</td>
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<td>HFAW</td>
<td>5284 ± 580***</td>
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<tr>
<td>HFRA</td>
<td>NA</td>
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</table>

EC$_{50}$ values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (*:P<0.05, **:P<0.005, ***: P<0.005). NA, No activation
Figure 9. Human MCRs sequence alignment. Shaded residues indicate sites in which at least 60% of the amino acids at that position are identical for all sequence. *: Positions involved in the binding of HFRW motif in MC4R (Pogozheva et al., 2005).
Figure 10. Schematic diagram for the model of hMC2R activation. A) ACTH(1-24) can bind to and activate hMC2R. B) ACTH(1-22) can bind to hMC2R but not activate it. C) α-MSH cannot bind to hMC2R.
CHAPTER TWO

Identification of an ACTH Antagonist

The primary role of ACTH is to stimulate the synthesis of glucocorticoids in adrenal cortex via melanocortin-2 receptor (Chan et al., 2011). Due to the presence of HFRW motif, which is the core sequence of melanocortin peptides (Schwyer, 1977), ACTH is capable of activating any other receptor in this gene family. As concluded in Chapter One, one of the distinct features of MC2R the need for both the HFRW and KKRRP motif in order to activate the receptor. By contrast, α-MSH [Acetyl-ACTH(1-13)-amide] has the HFRW motif, but lacks the KKRRP motif, and as a result this ligand cannot activate MC2R. Collectively these data point to the region at position 14 to 24 of ACTH as a possible motif that could function as a competitive inhibitor of ACTH for MC2R. In this chapter, ligand competition and binding assays will be utilized to examine the potential of several truncated C-terminal analogs as ACTH antagonist that could selectively target hMC2R.
Results

hMC2R cAMP Response Analysis

To evaluate the direct response of activated receptor in transfected CHO cells, cAMP measurement was used in the experiments of this chapter. To begin with, we examined the cAMP response after 15-minute stimulation of ACTH(1-24), A5 analog, and KKRRP analog. In the presence of IBMX, these analogs were introduced to the cells individually, and then the cell lysate was harvested and assayed immediately using the direct cAMP enzyme immunoassay kit. As indicated in Figure 11A, the cAMP production in response to 100nM A5 analog was 200pmol, which was only 42% of the amount of cAMP produced by ACTH(1-24) stimuli (P<0.5). The cAMP level was undetectable when the cells were stimulated with 1nM A5 analog. Meanwhile, due to the lack of HFRW motif, analog KKRRP was unable to induce a cAMP response at concentration of 1nM or 100nM.

To examine whether HFRW and KKRRP motif that are present in separate peptide fragments are sufficient to activate hMC2R, the transfected CHO cells were incubated with 100nM KKRRP and 1µM α-MSH or 100nM KKRRP and 1µM ACTH(4-10). There was no receptor activation detected based on the level of cAMP production (Figure 11B).

Ligand Competition cAMP Assay

As discussed in Chapter One, the KKRRP motif is the secondary binding site of ACTH(1-24) for hMC2R. Consequently, ACTH(1-24) analogs containing KKRRP motif
but lacking HFRW motif should be able to function as a competitive inhibitor of this receptor. A serial of C-terminus analogs (Sequences shown in Table 2) were synthesized and tested at a concentration of 1µM with the co-incubation of ACTH(1-24) ranging from $10^{-11}$ to $10^{-7}$M. We first examined analog KPV, KKRRP and RRP (ACTH position 11-24, 15-24, and 17-24). The co-incubation of these analog with ACTH(1-24) led to the reduction of cAMP production at various degrees (Figure 12A). Analog KPV and RRP decreased the maximal cAMP response by 30% and 20% respectively, whereas analog KKRRP completely abolished the ACTH(1-24) induced cAMP response. Additionally, the KKRRP analog fully kept this inhibitory effect when the analog concentration applied to the cells was reduced from 1µM down to 100nM; at the concentration of 10nM, KKRRP could still block 35% of ACTH(1-24) induced cAMP response (Figure 12C). Thereafter, the inhibitory effect of two modified forms of KKRRP analogs was evaluated as shown in Figure 12B. Attachment of an acetyl group at the N-terminus of KKRRP analog (Ac-KKRRP) was not as an efficient antagonist as compared to KKRRP analog, but it retained 30% inhibition ability, while alanine substitution at position 20-24 (KKRRPAs) caused this analog to lose the ability to compete with ACTH(1-24) (Figure 12B).

**Ligand Binding Affinity Analysis**

To determine the binding affinity of these truncated analogs, radioligand binding assay was performed using $^{125}$I labeled ACTH(1-39) (Figure 12D). Compared with ACTH(1-24) ($K_c = 1.2 \times 10^{-8}$M $\pm 8.4 \times 10^{-9}$M), a right shift in ACTH(1-39) binding curve
displayed with the co-incubation of analog RRP ($K_i = 3.0 \times 10^{-7} \text{M} \pm 8.8 \times 10^{-8} \text{M}$) and KKRRPAs ($K_i = 8.7 \times 10^{-8} \text{M} \pm 3.2 \times 10^{-8} \text{M}$), indicating their low binding affinity. In agreement with cAMP response analysis, analog KKRRP can bind to hMC2R most efficiently ($K_i = 2.5 \times 10^{-8} \text{M} \pm 1.5 \times 10^{-8} \text{M}$) among these analogs.

**Discussion**

The overproduction of ACTH(1-39) by the anterior pituitary gland, as a result of Cushing’s Syndrome or due to chronic stress, leads to the over stimulation of MC2R on adrenal cortical cells and the subsequent overproduction of cortisol (Cushing, 1932; Arnaldi et al., 2003). In addition to the surgical resection of the underlying tumor, medications currently used in the therapies of Cushing’s syndrome primarily target adrenocortical steroidogenesis (e.g., ketoconazole and metyrapone), ACTH secretion (e.g., bromocriptine and octreotide) or glucocorticoid receptor (e.g., mifepristone) (Tritos and Biller, 2012). Nevertheless, due to the lack of selectivity, administration of these medicines may induce many side-effects such as gastrointestinal toxicity, nausea, and hypertension (Tritos and Biller, 2012). In the development of effective medication for Cushing’s Syndrome, one strategy to prevent systemic effects and enhance the selectivity to HPA axis would be to block the activation of MC2R by introducing an ACTH antagonist.

Previous studies reported that truncated analog ACTH(6-24) could bind to hMC2R that was expressed on OS3 adrenal cells, but completely lost the ability to
activate the receptor (Chen et al., 2007); ACTH(11-24) can act as a competitive inhibitor of ACTH(1-24) (Seelig et al., 1975). These observations shed light to the identification of a ACTH antagonist that embedded within the sequence of ACTH(1-24). As discussed in Chapter One, HFRW motif is required in ACTH(1-24) to activate all melanocortin receptors; therefore, the absence of this domain is necessary in the sequence of ACTH antagonist. Moreover, α-MSH (acetyl-ACTH(1-13) amide) is sufficient to activate MC1R, MC3R, MC4R and MC5R, but not to MC2R. In order to properly bind to MC2R, KKRRP motif (position 15-19) is required to be present in the ligand. Clearly, position 14-24 of ACTH(1-24) is a critical region to study that would lead to the selectivity of antagonist to MC2R. As a result, we evaluated the ability of competitive inhibition of several truncated ACTH analog in CHO cells expressing hMC2R and mMRAP1. Surprisingly, ACTH(15-24)/KKRRP was working as the best competitive inhibitor compared with ACTH(11-24)/KPV and ACTH(17-24)/RRP, at the concentration as low as $10^{-8}$ M (Figure 12A, 12C). This finding was further supported by ligand binding affinity analysis (Figure 11A). Although alanine substitution at position 20-23 in ACTH(1-24) didn’t affect the activation of hMC2R (Chapter One), same replacement performed in analog ACTH(15-24) did diminish the competition and binding ability of this analog (Figure 12B; 11), suggesting the last five position may play a role in the secondary structure of analog KKRRP that facilitates its binding to the receptor. Collectively, these observations suggested that ACTH(15-24) can act as an antagonist of ACTH(1-24) in our *in vitro* system.
Over the past decade, many pharmaceutical and biotech companies have drawn their attention to peptide drugs due to the distinctive advantages of this class of medications, such as high bioactivity and specificity, minimal drug-drug interactions, as well as low tissue accumulation and toxicity (Ayoub and Scheidegger, 2006). On the other hand, stability of peptides is their major disadvantages that limited the success of peptide drugs. The intrinsic properties of peptides could result in short half-life and low bioavailability issues when administered in vivo (Ayoub and Scheidegger, 2006). ACTH(15-24) is a short peptide with only ten amino acid residues. Hence, the main challenge it may encounter in serum or plasma would be the peptidase degradation predominantly by exopeptidases. To overcome this problem, the common approach is to protect the free N- and C-terminus with minor modifications. For example, one of the naturally modified hormones is α-MSH; under the protection of both amino-acetylation and carboxy-amidation, it can only be degraded by endopeptidases in vivo (Marks et al., 1976). In the case of somatostatin analogs, N-terminal acetylation alone was able to expand the half-life from 3 minutes up to 400 minutes (Benuck and Marks, 1976). As a result, we evaluated the competition ability of ACTH(15-25) when attached with an acetyl group (analog Ac-KKRRP). Although this adjustment at N-terminus caused a partial lose in its antagonist function (Figure 12B), whether this analog could make a good drug candidate in in vivo system is waiting for further examinations.
Figure 11. cAMP response in transfected CHO cells after 15-minute stimulation of A). ACTH(1-24), A5, or KKRRP; B). KKRRP/α-MSH or KKRRP/ACTH(4-10)
Figure 12. ACTH(1-24) dose response curves with co-incubation of C-terminal truncated analogs: A) 1µM of KPV, KKRRP and RRP; B) 1µM of KKRRP, Ac-KKRRP and KKRRPAs; C) 1nM to 1µM KKRRP. D) Binding affinity of ACTH analogs at hsMC2R.
CHAPTER THREE

Site-directed Mutagenesis of TM4, EC2, TM5 in Human MC2R

Extensive studies have been performed to examine the molecular basis responsible for ligand binding and signaling in MC1R, MC3R, and MC4R (Yang et al., 2000; Chen et al., 2006), and conserved residues in transmembrane domains of these receptors have emerged as key elements that facilitate both α-MSH binding and receptor activation (Haskell-Luevano et al., 1996). As reported by Pogozheva and colleagues, ten residues in hMC4R that are involved in the binding of HFRW motif distributed in TM2, TM3, TM6 and TM7 (Table 5 and Figure 13). Note that 90% of these residues are also present at the same position in other melanocortin receptors of humans (MC1R, MC3R and MC5R), and at least 70% of these residues are conserved in human, frog and rainbow trout MC2R orthologs (Table 5). Since HFRW motif is required for the activation of all melanocortin receptors (Schwyzer, 1977), it is reasonable to propose that MC2Rs use a similar binding mechanism (TM2, TM3, TM6 and TM7) for the HFRW motif of ACTH(1-24).

As shown in the sequence alignment in Figure 13, the differences in primary sequence between human MC2R and MC4R are primarily in two aspects: 1). The amino acid sequences of N-terminus, C-terminus, and IC3 in MC4R are significantly longer as
compared with MC2R. 2). Primary sequence identity of TM1, EC1, TM4, EC2, and TM5 is less than 45% between MC4R and MC2R. These observations are consistent with Pogozheva’s hypothesis that none of the proposed HFRW binding regions fall into these domains. The low sequence identity of these domains may be the key to understand the recognition mechanism which determines the ligand selectivity of MC2R—a secondary binding site other than the HFRW motif to bind to, that allows this receptor to exclude α-MSH while accepting ACTH(1-24).

Assuming that the 3-dimentional structure of MC2R orients the seven transmembrane regions in a barrel conformation as shown in the schematic diagram in Figure 14, then TM2, TM3, TM6, and TM7 would be in close proximity to interact with HFRW motif, and EC2 could be positioned in the KKRRP binding site flanked by TM4 and TM5. After a careful comparison between the primary sequences of these domains in difference species, we found that between the highly conserved intracellular loop2 and the cytosolic region of TM5, there is a sharp dichotomy distinguishing MC2R primary sequence from all other MCRs (Sequences alignment shown in Figure 15). Due to the extremely low primary sequence identity of this continuous domain in MC2R orthologs, EC2 with the flanking regions in TM4 and TM5 is likely the responsible domain for creating a unique binding site for KKRRP motif in MC2Rs.

In this Chapter, 23 mutant hMC2R were synthesized for evaluating the importance of this domain from G162 to P183 (See diagrams in Figure 5), in terms of both receptor activation and plasma membrane expression levels. Since alanine is the generally accepted amino acid of choice for mutagenesis substitution in this type of
analysis (Yang et al., 2000), individual alanine substitution was used for replacing original amino acid residues in mutagenesis in order to avoid disrupting receptor tertiary structure. In the presence of mMRAP1, cAMP luciferase reporter assay was performed to analyze the activation activity of these mutant hMC2Rs. All mutant receptors were tested using hACTH(1-24) at concentrations ranging from 10-13M to 10-6M. A wild type hMC2R was used as the positive control for each mutant receptor experiment. The basal luciferase activity of each receptor was subtracted from the luminescence readings individually. Then the dose response curves were plotted on a logarithmic graph using the Michaelis-Menton equation (Kaleidograph software). EC\textsubscript{50} values of ACTH(1-24)-mediated response curves were used as the parameter to compare receptor activation levels. Statistical difference of EC\textsubscript{50} values between wild type hMC2R and mutants were determined using unpaired Student t-test with equal variances. Significant difference was confirmed when P<0.05. After ACTH signaling defect mutants were identified, cell surface ELISA was performed on these receptors in the presence of mMRAP1 to evaluate their trafficking efficiency.

Results

Effects of Single Alanine Substitution in hMC2R for ACTH Signaling

In this study, alanine substitution was individually introduced to positions of interest in hMC2R, including six amino acid residues in TM4 (G162-I167), seven residues in TM5 (T177-P183), and the entire EC2 (F168-I176). We expressed the mutant
receptor in CHO cells in the presence of mMRAP1, and the functions of these mutants were evaluated using cAMP responsive reporter assay. As summarized in Table 6, in 22 single mutant hMC2Rs we examined, 16 mutant receptors showed no changes in the ACTH(1-24)-mediated response curves as compared with wild type hMC2R, suggesting that alanine substitution at these positions did not affect hMC2R/ACTH interaction and receptor activation. Meanwhile, we identified six mutant receptors that displayed significant decreases in ACTH(1-24)-mediated responses (P<0.05). These positions are T164, F168, S169, H170, F178 and L181 (Table 6).

Within six TM4 mutants we examined, only one position, T164, had a significant negative effect on ACTH dose response curve (Figure 16). The EC\textsubscript{50} of ACTH(1-24) for mutant T164 was (7.5±1.59)×10\textsuperscript{-12} M as compared to a EC\textsubscript{50} of (2.7±1.23)×10\textsuperscript{-12} M for wild type hMC2R (P=0.038) (Table 6). Of 9 residues in the domain of EC2, alanine substitution on three successive positions that are located at the very beginning of this loop displayed significant reduction in ACTH(1-24) responses (Figure 17A). The EC\textsubscript{50} values for these mutants, F168, S169 and H170, were (1.4±0.4)×10\textsuperscript{-11} M, (1.7±0.5)×10\textsuperscript{-11} M, and (6.1±2.0)×10\textsuperscript{-11} M, which were approximately 4-fold, 5-fold, and 16-fold of the EC\textsubscript{50} of the wild type hMC2R (P=0.03, 0.02, and 0.01, respectively) (Table 6). Clearly, H170 was the most important site in EC2 that mediated receptor activation. In addition, positions F178 and L181 identified from TM5 mutants had even greater effects than all positions tested in TM4 and majority in EC2 (Figure 18). As indicated in Table 6, the EC\textsubscript{50} values for F178 and L181 were (6.6±1.0)×10\textsuperscript{-10} M and (3.7±1.4)×10\textsuperscript{-11} M, which were approximately 174-fold and 10-fold of wild type hMC2R EC\textsubscript{50}. Hence, mutant F178
in TM5 produced the greatest right-shift in ACTH(1-24) dose response curve (Figure 18A), and this position is likely one of the core residues that mediate the receptor/ligand interaction of hMC2R.

Next, we put alanine substitutions on both significant phenylalanine positions F168 and F178, and examined the activity function of this mutant (Figure 19A). Albeit EC$_{50}$ of mutant FF was $(9.2\pm2.1)\times10^{-8}$ M and ACTH dose response curve was greatly shift to the right, this receptor kept full activation ability when stimulated with $10^{-6}$ M ACTH, indicating that double substitution of F168 and F178 cannot completely block ACTH signaling. Hence, there are other positions involved in this binding event. Finally, we substituted all six significant positions (T164, F168, S169, H170, F168 and F178) with alanine residues to make an hMC2R 6A mutant, and this mutant completely lost the activation activity (Figure 19B)

**Effects of Single Alanine Substitution in hMC2R for Receptor Trafficking**

In order to eliminate the possibility of trafficking defects introduced by alanine mutations, we co-transfected CHO cells with mMRAP1 and individual activation defect mutant hMC2R identified by cAMP luciferase reporter assay, and performed cell surface ELISA to evaluate the plasma membrane expression levels of these mutants. All experiments were executed in triplicates. Data were first subtracted by the background signals, converted to the ratio of plasma membrane expression to total expression, and then normalized to wild type hMC2R. As indicated in Figure 20, albeit mutant S169 and L181 showed a lower level of surface expression as compared with wild type hMC2R,
Student t-test analysis showed that none of these activation defect mutants demonstrated significant trafficking impairment.

Discussion

By virtue of the functionally important residues that were previously identified in MC1, MC3, and MC4R, nine residues in MC2R were found to be responsible for ACTH binding and signaling (Chen et al., 2007). Within these positions, seven residues are located in TM2, TM3, TM6 and TM7, which is the proposed HFRW motif binding site identified from MC4R (Pogozheva et al., 2005). Not surprisingly, the other two residues in these positions, F168 in EC2 and F178 in TM5, are both unique amino acids in MC2R, and their position fall out of the proposed binding pocket for HFRW motif (Chen et al., 2007). As discussed in the ligand binding studies in Chapter One, the recognition sites for HFRW and KKRRP motif on ACTH(1-24) must span two portions in the receptor. Based on the 3-D structure of receptor (Figure 14), if the HFRW motif is positioned between TM2, TM3, TM6 and TM7, then the logical location for the KKRRP motif to bind must be present in TM4, EC2, and TM5. These observations supported our hypothesis that TM4, EC2 and TM5 may indirectly interact with the KKRRP motif, which in turn determine the ligand selectivity of MC2R.

In this Chapter, we systematically characterized the functional expression of 22 single-mutation hMC2Rs from position G162 to P183, which cover the entire EC2 and the flanking regions in TM4 and TM5. As indicated in Table 6 and Figure 16-18, 6
mutants, T164, F168, S169, H170, F178, and L181, caused significant decrease in ACTH response curve while trafficking defects were undetectable in plasma membrane expression analysis (Figure 20). In a previous study based on type 1 FDG mutant MC2R cases, Chung and colleagues identified six trafficking-competent mutations with ACTH signaling defects (D20N, 144M, D103N, D107N, R128C, and H170L) from 24 naturally occurring missense mutations found in these patients (Figure 21A; Chung et al., 2008). D107N and R128C are located in TM3, providing the evidence for the importance of this transmembrane domain in the interaction between MC2R and ACTH. These findings also supported Pogosheva’s hypothesis that the binding site for HFRW motif in MCRs is between TM2, TM3, TM6 and TM7. Note that only one position (H170L) was detected in the region we are focusing on (TM4/EC2/TM5). Other than residue H170, two phenylalanine residues F168 and F178 were the previously reported by Chen’s group (Chen et al., 2007). Our receptor activation results from double phenylalanine mutation (FF) indicated that removing these residues at the same time cannot completely block ACTH signaling (Figure 19A). Hence, there must be more residues involved in this process, and it appears to be position T164, S169 and L181 as we identified from our target region. In addition, these residues were the first time to be discovered that impaired ACTH(1-24) signaling without disrupting human MC2R trafficking in the presence of mMRAP1.

Taken together, the distribution of these essential residues is as follows: one position in TM4, two discrete positions in TM5, and three successive positions located at the beginning of EC2 (Summarized in Figure 21B). Notably, hMC2R has a relatively
short extracellular loop 2, which is composed of only nine amino acids. Thus, 33% residues of EC2 are involved in the binding of ACTH, suggesting a critical role of this loop playing in the proposed KKRRP motif binding region. When these positions were substituted by alanine residues at the time, the ACTH(1-24) was completely blocked. In order to confirm this effect was solely induced by the inactivated binding sites but not due to the trafficking defects of this mutant, the receptor cell surface expression analysis needs to be done in the future.

As described in Chapter One, our model for the binding of ACTH to MC2R is that the ligand first makes contact with the receptor at a KKRRP binding site and then activation is possible when the HFRW motif of the ligand is inserted into the corresponding binding site on the receptor. If the primary binding site for KKRRP motif is disabled in MC2R, then ACTH(1-24) would be rejected by this receptor just as α-MSH. Would this alternation completely prevent the binding of ACTH(1-24) to the receptor? Would it eliminate the ACTH response of MC2R, or just take away its ligand selectivity? In this regard, a mutant hMC2R with alanine substitutions at all six residues identified in this Chapter will be examined in future studies.
Table 5. Proposed HFRW binding sites in MC4R and corresponding residues of MC2R.

<table>
<thead>
<tr>
<th>AA</th>
<th>Region</th>
<th>MC4R</th>
<th>MC2R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>X</td>
</tr>
<tr>
<td>E100</td>
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<td>D122</td>
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<td>I129</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>L288</td>
<td>TM7</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

H: Human; X: *Xenopus tropicalis*; RT: Rainbow trout (*Onchorynchus mykiss*)
Figure 13. Sequence alignment of human MC2R and MC4R. Positions shaded indicate identical amino acids in these receptors. * indicates proposed amino acids that are involved in HFRW binding sites in MC4R as reported by Pogozheva.
Figure 14. Schematic diagram of MC2R proposed binding sites for HFRW motif and KKRRP motif.
Figure 15. MCRs sequence alignment from IC2 to TM5. Positions shaded in black indicate sites in which 75% of the amino acids at that position are identical for all sequence; positions shaded in white indicate sited in which at least 75% of the amino acids are identical in MC1R, MC3R, MC4R and MC5R. h: human; x: Xenopus tropicalis; r: rainbow trout (*Oncorhynchus mykiss*); z: zebra fish (*Danio rerio*).
Figure 16. ACTH(1-24) dose response curves of TM4 mutant hMC2Rs in the presence of mMRAP1.
Figure 17. ACTH(1-24) dose response curves of EC2 mutant hMC2Rs in the presence of mMRAP1.
Figure 18. A-C) ACTH(1-24) dose response curves of TM5 mutant hMC2Rs in the presence of mMRAP1.
Figure 19. A) ACTH(1-24) dose response curves of hMC2R FF mutant (F168 & F178). B) ACTH(1-24) dose response curves of hMC2R 6A mutant.
Table 6. EC$_{50}$ values of mutant hMC2R.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Alanine Substitution</th>
<th>EC$_{50}$ ± SEM (10$^{-12}$M)</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>N/A</td>
<td>3.8 ± 0.3</td>
<td>N/A</td>
</tr>
<tr>
<td>TM4</td>
<td>G162</td>
<td>4.5 ± 1.79</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>I163</td>
<td>4.3 ± 1.15</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>T164</td>
<td>7.5 ± 1.59*</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>M165</td>
<td>4.6 ± 0.98</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>V166</td>
<td>7.2 ± 1.6</td>
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</tr>
<tr>
<td></td>
<td>I167</td>
<td>3.4 ± 0.8</td>
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</tr>
<tr>
<td>EC2</td>
<td>F168</td>
<td>14.1 ± 4.6*</td>
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</tr>
<tr>
<td></td>
<td>S169</td>
<td>17.2 ± 5.1*</td>
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</tr>
<tr>
<td></td>
<td>H170</td>
<td>60.9 ± 20.3*</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>H171</td>
<td>5.0 ± 0.61</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>V172</td>
<td>3.3 ± 3.87</td>
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</tr>
<tr>
<td></td>
<td>P173</td>
<td>4.8 ± 1.02</td>
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</tr>
<tr>
<td></td>
<td>T174</td>
<td>5.9 ± 1.7</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>V175</td>
<td>4.4 ± 2.4</td>
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</tr>
<tr>
<td></td>
<td>I176</td>
<td>1.7 ± 0.8</td>
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</tr>
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<td>TM5</td>
<td>T177</td>
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</tr>
<tr>
<td></td>
<td>F178</td>
<td>660 ± 100**</td>
<td>0.0014</td>
</tr>
<tr>
<td></td>
<td>T179</td>
<td>10 ± 2.7</td>
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<tr>
<td></td>
<td>S180</td>
<td>8.1 ± 7.4</td>
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</tr>
<tr>
<td></td>
<td>L181</td>
<td>37 ± 14*</td>
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<td></td>
<td>F182</td>
<td>4.2 ± 1.5</td>
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</tr>
<tr>
<td></td>
<td>P183</td>
<td>9.1 ± 3.3</td>
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</tr>
<tr>
<td>Double</td>
<td>F168 &amp; F178 (FF)</td>
<td>92000 ± 21000*</td>
<td>0.006</td>
</tr>
<tr>
<td>Mutation</td>
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<td></td>
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<tr>
<td>Sextuple</td>
<td>T164, F168, S169, H170, F168, F178 (6A)</td>
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</table>

EC$_{50}$ values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (*:P<0.05, **:P<0.005). N/A: No activation.
Figure 20. Cell surface expression analysis of ACTH signaling defect mutants.
Figure 21. Schematic plot of the MC2R mapping the locations of A) 6 trafficking-competent mutations identified from Type 1 FDG (Chung et al., 2008). B) 6 residues identified in this study for the proposed KKRRP motif binding site.
CHAPTER FOUR

Ligand Selectivity Properties of Rainbow Trout Melanocortin-2 Receptor

According to the 2R hypothesis, the first gene duplication of ancestral MCR gene yielded two paralogous genes (MCa and MCb receptors) in agnathan vertebrates, and then the second duplication event produced four paralogous genes (MC1R, MC5/2R, MC3R and MC4R) in ancestral gnathostomes (Vastermark and Schioth, 2011). As to the fifth paralogous gene MC2R, which could be activated only by ACTH, appeared in the ancestral bony fishes and has since radiated throughout the extant bony vertebrate classes (Schioth et al., 2005). Recent studies revealed that a MC2R ortholog cloned from cartilaginous fish Elephant shark could be transported to plasma membrane in the absence of MRAP1, and it is the only MC2R currently identified that can be activated by ACTH and MSHs (Reinick et al., 2012). These observations indicated that MC2R dependence on MRAP1 for functional activation and the exclusive selectivity of this melanocortin receptor for ACTH are features that emerged after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes more than 400 million years ago. Therefore, understanding the ligand selectivity features of a bony fish MC2R would be an essential component in modeling the evolution MC2R activation. In this study, rainbow trout (Oncorhynchus mykiss) MC2R (rtMC2R) was selected for demonstrating
teleost MC2R activation. Like the mammalian MC2Rs, rMC2R requires the presence of MRAP1 to function properly (Liang et al., 2011). In the co-evolution of MC2R and MRAP1 during the radiation of vertebrates, it appears that at least a tetrapod branch and a bony fish branch have emerged with respect to the interaction between MC2R and MRAP1 (Liang et al., 2011). Since we started this project when the genes encoding rainbow trout MRAPs had not been identified yet, zebrafish MRAP1 (zfMRAP1) was co-expressed with rMC2R within CHO cells in order to obtain the optimal ACTH responses.

In the analysis of hACTH(1-24) and hMC2R interactions (Chapter One), amino acid residues within Zone A, Zone B, and Zone C were involved in ACTH binding and signaling to varying degrees. To evaluate the functions of these domains in the activation activities of rMC2R, we did a comparative study using the same analogs as Chapter One (See sequences in Table 2): (1) Zone A analogs—H\textsuperscript{6}F\textsuperscript{7}R\textsuperscript{8}W; (2) Zone B analogs—G\textsuperscript{10}K\textsuperscript{11}P\textsuperscript{12}V\textsuperscript{13}G; and (3) Zone C analogs—R\textsuperscript{15}K\textsuperscript{16}R\textsuperscript{17}R\textsuperscript{18}P\textsuperscript{19}. Note that the position 15 of rACTH(1-24) as well as many other vertebrate species is an arginine (R\textsuperscript{15}), instead of a lysine (K\textsuperscript{15}) in hACTH(1-24) (Figure 22), hence it is referred as R/KKRRP motif for this Chapter. Both of these basic amino acids are generally considered to be interchangeable and able to readily substitute for one another. In addition, it has been reported that hACTH(1-24) can fully stimulate rMC2R that are expressed in CHO cells in the presence of zfMRAP1 (Liang et al., 2011). Hence, hACTH(1-24) was used in this study as the wild-type rACTH(1-24).
Results

Ligand Selectivity of rtMC2R

Zone A analogs

Five Zone A analogs (See sequences in table 2) were applied individually to CHO cells transiently transfected with rtMC2R and zfMRAP1, and CRE-Luc activity was measured after 4 hours of stimulation (Figure 23A). Consistent with the parallel experiment on hMC2R, replacing the entire HFRW motif abolished rtMC2R response completely. Nevertheless, the importance of the four positions in Zone A with respect to the activation of rtMC2R was different as compared to hMC2R. Firstly, all four analogs (AFRW, HARW, HRAW, and HFRA) greatly diminished rtMC2R activation with a 23-fold, 49-fold, 1400-fold, and 681-fold increase in EC\textsubscript{50} values (Table 7), whereas for hMC2R, ARFW analog did not significantly alter the dose response curve. Secondly, analog HFRA was able to stimulate weak rtMC2R activation at the concentrations higher than $10^{-7}$M, whereas the same analog failed to activate hMC2R. Finally, the order of effectiveness of these positions affecting rtMC2R activation is $R^8 > W^9 > F^7 > H^6$.

Zone C analogs

To determine the role of R/KKRRP motif on the activation of rtMC2R, CHO cells expressing rtMC2R and zfMRAP1 were challenged with three Zone C analogs respectively (See sequences in Table 2). In the absence of R/KKRRP motif, analog A5 failed to promote any stimulatory effect on rtMC2R even at concentration of $10^{-6}$M
(Figure 23B), clearly indicating that this motif is equally critical as HFRW motif. Additionally, position R\textsuperscript{15}K\textsuperscript{16} and R\textsuperscript{17}R\textsuperscript{18}P\textsuperscript{19} were tested separately using analog AARRP and KKAAA. The EC\textsubscript{50} values of KKAAA and AARRP were significantly increased up to 1112-fold and 615-fold as compared with hACTH(1-24) (Table 7).

**Zone B analogs**

The importance of the region between HFRW and KKRRP was first examined using four ACTH(1-24) analogs with alanine substitutions at G\textsuperscript{10}K\textsuperscript{11}P\textsuperscript{12}V\textsuperscript{13}G\textsuperscript{14}, G\textsuperscript{10}G\textsuperscript{14}, K\textsuperscript{11}P\textsuperscript{12}, and P\textsuperscript{12} (Analog A10-14, AKPVA, GAAVG, and GKA AVG in Table 2). Generally, alanine substitution at Zone B had greater effects on rtMC2R activation curves than hMC2R. As indicated in Figure 23C and Table 7, both A10-14 and AKPVK significantly reduced activation activity by 376-fold and 67-fold increase on EC\textsubscript{50} values, while analog GAAVG, and GKA VG also introduced a right shift in activation curve, albeit the EC\textsubscript{50} values were not statistically larger than hACTH(1-24). Two truncated Zone C analogs, ACTH(1-21) and ACTH(1-22), were also tested on rtMC2R. Note that skipping position G\textsuperscript{10}G\textsuperscript{14} or K\textsuperscript{11}P\textsuperscript{12}V\textsuperscript{13} in Zone B induced a complete loss in hMC2R activation (Figure 6C). Similar scenario was observed in rtMC2R activation curves. Receptor activation in response to both ACTH(1-21) and ACTH(1-22) were undetectable when analog concentration was lower than 10\textsuperscript{-7} M (Figure 23D).
Discussion

As the core sequence of hACTH(1-24), HFRW motif plays an essential role in activating hMC2R, with the tryptophan at the last position that predominantly contributes to the function of this motif (Figure 6A). In the case of rtMC2R, A4 analog did not have any stimulatory effect even at concentration of $10^{-6}$M (Figure 23A), hence, HFRW motif is also required for the activation of rtMC2R. Nevertheless, the importance of each position within this region results in a slightly different scenario. Replacement of a single alanine at any of these positions significantly diminished the receptor activation activity, whereas none of them was able to completely abolish the stimulatory effect at higher doses (Figure 23A). It appears that histidine$^6$ and phenylalanine$^7$ that establish the reverse $\beta$-turn structure in $\alpha$ MSH (Ying et al., 2003) are actually less important than tryptophan$^9$ and arginine$^8$ in the interaction of hACTH(1-24) and rtMC2R (Table 7). These observations are mostly consistent with hMC2R indicating that the side chains of tryptophan$^9$ and arginine$^8$ may play an essential role interacting with rtMC2R.

Next, we examined the effect of alanine substitutions at R/KKRRP motif on rtMC2R activation. As discussed in Chapter One, hMC2R recognizes the R/KKRRP motif in hACTH(1-24) as a significant binding site that determines its ligand selectivity property. Not surprisingly, alanine substitutions at entire KKRRP motif caused analog A5 fully lost the ability to stimulate rtMC2R (Figure 23B). Note that replacing the entire R/KKRRP motif with alanine is not sufficient to eliminate the stimulatory effect in human MC2R at concentration of $10^{-6}$M, whereas in rtMC2R ligand activation study, the
absence of receptor activity acquired from A5 analog dose response curve has been
clearly supporting the secondary binding site hypothesis. Additionally, it appears that
R$^{15}$K$^{16}$ motif and R$^{17}$R$^{18}$P$^{19}$ motif are equally contributing to this binding site (Figure 23A
and Table 7).

Having confirmed the same activation sites HFRW motif and R/KKRRP motif for
rtMC2R, we evaluated whether the G$^{10}$K$^{11}$P$^{12}$V$^{13}$G$^{14}$ sequence between these two motifs
is as critical as it is in hMC2R. As compared to hMC2R, alanine substitutions at entire
Zone B had less negative effects on rtMC2R activation curve, whereas individual
substitution caused a greater right-shift in each curve (Figure 23C and Table 7). With
regard to Zone B truncated analogs, albeit a weak activation activity appeared at
concentration higher than 10$^{-7}$M, both ACTH(1-22) and ACTH(1-21) greatly diminished
rtMC2R responses (Figure 23D). Collectively, the relative distance between HFRW motif
and R/KKRRP motif plays a more important role than the sequence of this region.

According to the proposed model for the interaction between ACTH(1-24) and
MC2R (Figure 10), R/KKRRP motif first binds to MC2R, and HFRW motif is then
positioned in its corresponding binding site, leading to the activation of receptor. In
agreement of this hypothesis, our results obtained from rtMC2R indicated that R/KKRRP
motif, HFRW motif, as well as the amino acid sequence in between are absolutely
required for the activation of the activation of rtMC2R (Figure 23). As compared to the
influence of these analogs on hMC2R activation, individual residues within these motifs
may play a slightly different role in terms of the interaction with certain residues of the
receptor, whereas the importance of three motifs was consistent in these receptors. A
comparative analysis between human and rainbow trout MC2R will be discussed in next chapter.
Figure 22. Amino acid sequence alignment of ACTH in different species. Residues shaded in grey are sites at least 83% identical in all sequence.
Figure 23. Activation curves of rtMC2R by the stimulation of hACTH(1-24) analogs: A) Zone A analogs; B) Zone C alanine substitution analogs; C) Zone B alanine substitution analogs; D) Zone B truncated analogs.
Table 7. Effects of ACTH(1-24) analogs on rtMC2R activation.

<table>
<thead>
<tr>
<th>Analogs</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; ± SEM (10&lt;sup&gt;-11&lt;/sup&gt; M)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>hACTH(1-24) 3.3 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Zone A</td>
<td>A4 N/A</td>
<td></td>
</tr>
<tr>
<td>AFRW</td>
<td>77 ± 20*</td>
<td>0.01</td>
</tr>
<tr>
<td>HARW</td>
<td>163 ± 54.4*</td>
<td>0.02</td>
</tr>
<tr>
<td>HFAW</td>
<td>4614 ± 1869*</td>
<td>0.03</td>
</tr>
<tr>
<td>HFRA</td>
<td>2250 ± 1400*</td>
<td>0.03</td>
</tr>
<tr>
<td>Zone B</td>
<td>A10-14 1242 ± 161**</td>
<td>0.0008</td>
</tr>
<tr>
<td>A10/14</td>
<td>222 ± 56*</td>
<td>0.01</td>
</tr>
<tr>
<td>KP</td>
<td>27.8 ± 12.3</td>
<td>0.06</td>
</tr>
<tr>
<td>P12</td>
<td>8.1 ± 2.0</td>
<td>0.06</td>
</tr>
<tr>
<td>ACTH(1-22)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>ACTH(1-21)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Zone C</td>
<td>A5 N/A</td>
<td></td>
</tr>
<tr>
<td>AARRP</td>
<td>2030 ± 489*</td>
<td>0.007</td>
</tr>
<tr>
<td>KKA AAA</td>
<td>3668 ± 778**</td>
<td>0.0046</td>
</tr>
</tbody>
</table>

EC<sub>50</sub> values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (*:P<0.05, **:P<0.005). NA, No activation
CHAPTER FIVE

Site-directed Mutagenesis of TM4, EC2, TM5 in Rainbow Trout MC2R

When ancestral teleosts diverged from ancestral cartilaginous fishes over 400 million years ago, their melanocortin-2 receptor acquired two distinct features that were retained in MC2Rs in amphibians (Liang et al., 2011) and mammals (Cone, 2006): MRAP1 dependence and melanocortin ligand selectivity (Reinick et al., 2012). While these common features are shared by teleosts and tetrapods, during the co-evolution of MC2R, MRAP1 and ACTH, subtle variations could have arose. Take as an example a comparison of the MC2R circuit in humans and the rainbow trout. First examining the ligands, 92% of amino acid residues in the sequence of rainbow trout ACTH(1-24) are identical to human ACTH(1-24). However, the primary sequence identity of melanocortin 2 receptor of these two species drops to 47% (Figure 24).

Analogs of ACTH(1-24) in the three zones had adverse effects on the activation of both human and rainbow trout receptors, and there were subtle differences in the ways that zone A, B and C analogs interacted with these receptors. From the mutant hMC2R experiment discussed in Chapter Three, we have identified six significant sites within TM4/EC2/TM5 region which was responsible for the proposed binding domain for KKRRP motif. In order to find out whether rtMC2R use similar positions or residues as
hMC2R for the interaction with ACTH(1-24), we designed mutant constructs of rtMC2R with alanine substitutions within the same region as the hMC2R mutant study. Since TM4/EC2/TM5 region (22 positions) of rtMC2R contains three neutral, non-polar alanine residues (A156, A160 and A164), we eventually made 19 mutant rtMC2R for receptor activation evaluation using cAMP luciferase reporter assay. For these experiments the wild-type and single alanine mutant constructs of rtMC2R were co-expressed in CHO cells with zfMRAP1 as described in Chapter Four. Luminesce data for receptor activation levels were analyzed as described in Chapter Three.

Results

Effects of single alanine substitution in rtMC2R for ACTH signaling

As performed the comparative experiments for hMC2R mutants study, the corresponding mutated regions of rtMC2R are V153-A160 in TM4, EC2 (F161-I169), and F170-F175 in TM5. A total of 19 mutant rtMC2Rs were individually co-expressed with zfMRAP1 in CHO cells, and receptor functions were examined by cAMP responsive reporter assay. These mutant receptors included 5 mutants at TM4, 8 mutants at EC2, and 6 mutants at TM5. As summarized in Table 8, of 19 residues we examined, 11 residues did not affect ACTH(1-24)-mediated response curve when replaced with alanine. In other words, there were 8 mutant receptors (V157, M158, V159, F161, K168, F171, I172, and F175) that displayed significant decrease in ACTH(1-24)-mediated responses as compared with wild type rtMC2R (P<0.05), whereas in the same region
examined in hMC2R, we only identified 6 significant positions (See Chapter Three, Table 7).

\[ EC_{50} \text{ value of ACTH(1-24) on wild type rtMC2R was } (4.6\pm0.7)\times10^{-11}\text{M}, \]
which was the positive control for all mutant rtMC2Rs. Compared with wild type rtMC2R, three successive positions in TM4 mutants, V157, M158, and V159, had significant negative effects on ACTH dose response curve (Figure 25). The \( EC_{50} \) values of these mutants were \((1.9\pm0.6)\times10^{-10}\text{M}, (1.5\pm0.3)\times10^{-10}\text{M}, \) and \((5.1\pm1.2)\times10^{-9}\text{M}, \) which were approximately 4-fold, 3-fold, and 110-fold of the wild type hMC2R \( EC_{50} \) value \((P=0.04, 0.03, \text{and } 0.01, \text{respectively})\) (Table 8). Note that there was only one significant residue identified from TM4 mutants of hMC2R. Albeit the amino acid residue was threonine \((T164)\) in human receptor instead of valine \((V157)\) in rainbow trout MC2R, they are located at the same position of TM4, indicating a critical role of this position in both human and rainbow trout ACTH/MC2R interaction and receptor signaling.

From eight EC2 mutant receptors, we identified two residues that displayed significant reduction in ACTH(1-24) responses (Figure 26): F161 with a \( EC_{50} \) of \((2.2\pm0.7)\times10^{-10}\text{M} \) \((P=0.045)\) and K169 with a \( EC_{50} \) of \((8.2\pm0.2)\times10^{-11}\text{M} \) \((P=0.02)\) (Table 8). Mutation at these residues induced a 5-fold change in \( EC_{50} \) of F161 and a 2-fold increase in \( EC_{50} \) of K169. Compared with the three significant positions \((F168, S169 \text{ and } H170)\) identified from hMC2R EC2 mutants, position F161 was conserved and also important in rainbow trout and human receptor \((F168)\), while position K169 was significant for ACTH interaction with rtMC2R but not for hMC2R.
Three out of six TM5 mutants greatly diminished ACTH(1-24) responses as compared with wild type rtMC2R (Figure 27A-B), including F171 (EC$_{50}$ = (2.1±0.8)×10$^{-9}$ M), I172 (EC$_{50}$ = (2.6±0.8)×10$^{-10}$ M) and F175 (EC$_{50}$ = (4.6±2.0)×10$^{-9}$ M) (Table 8). EC$_{50}$ values of these mutants were approximately 47-fold, 6-fold, and 100-fold of wild type rtMC2R EC$_{50}$ respectively (P= 0.03, 0.03, 0.04). In addition, position F171 (which is F178 in hMC2R) is a conserved residue in MC2Rs but different with all other MCRs. Mutation on this position greatly altered ACTH(1-24) response in both hMC2R and rtMC2R. These observations indicated that this position is involved in the ligand selectivity of MC2Rs.

Further, we replaced seven significant residues (V157, M158, V159, F161, F171, I172, and F175) with alanine residues in one mutant rtMC2R. The substitutions caused this mutant receptor to lose ACTH(1-24) response completely (Figure 27C). In order to rule out the possible defects on receptor trafficking that might be introduced by alanine substitutions, cell surface ELISA will be performed on rtMC2R mutants in the future.

Discussion

As discussed in Chapter Three, within TM4/EC2/TM5 region (G162-P183) of human MC2R, we identified six positions that were involved in ACTH(1-24) signaling but not the trafficking of receptors, including T164, F168, S169, H170, F178, and L181. The corresponding 22 amino acid residues in rainbow trout MC2R are from V153 to F175, and we found 8 residues that significantly decreased ACTH(1-24) responses,
which are V157, M158, V159, F161, K168, F171, I172, and F175. Since rtMC2R has a shorter N-terminus than hMC2R, the same position we examined in these two receptors was numbered differently. Therefore, we marked all 14 significant residues with the degrees of EC\textsubscript{50} fold change on TM4/EC2/TM5 sequence alignment diagram for a better comparison (Figure 28). As compared with the EC\textsubscript{50} of wild type MC2R, residues that had significant larger EC\textsubscript{50} were shaded in red in this diagram, suggesting that ACTH(1-24) response were substantially diminished in these mutants. The sequence alignment clearly revealed that three significant sites that were involved in hMC2R/ACTH interaction were important for rtMC2R signaling as well. These residues are T164, F168 and F178 in hMC2R, and V157, F161, and F171 in rtMC2R. At the site T164/V157 of TM4, albeit the threonine in hMC2R was replaced by a valine in rtMC2R, these two amino acids are structurally very similar to each other. The other two positions are both phenylalanine residues F168/F161 and F178/F171, which are located at the beginning of EC2 and TM5. In addition, these phenylalanine residues are both conserved in human, frog, and bony fish MC2Rs but distinct with all other melanocortin receptors (Figure 15). Collectively, these observations indicated that position T164/V157, F168/F161, and F178/F171 are involved in the interacting sites for MC2R to bind ACTH(1-24), specifically on the KKRRP motif of this peptide.

In addition, majority of these significant residues were different between these two receptors, reflecting that the tertiary structure of TM4/EC2/TM5 in these receptor may interact with KKRRP motif in different ways. First, three significant hMC2R residues (S169, H170 and L181) turned out to be unimportant for rtMC2R. Note that
S169 and H170 together with F168 are the first three amino acids in hMC2R EC2. These residues accounts for 33% of entire EC2, and 50% of significant residues identified in hMC2R TM/EC2/TM5 domains. Hence, it appears that the extracellular loop 2 of hMC2R plays a dominant role in terms of the function of this region. Second, newly identified positions from rtMC2R that did not appear in hMC2R mutants were M158, V159, K168, I172, and F175. Putting all 8 significant sites in rtMC2R together, there are three successive positions in TM4, three relatively close positions in TM5, and two discrete positions in EC2. 6 out of 8 positions are present in TM4 and TM5, suggesting the predominant domains for rtMC2R interacting with ACTH(1-24) are TM4 and TM5 instead of EC2.

Based on the fold-change in the EC$_{50}$ values of mutants as compared with wild type MC2R, these sites were further sorted into three categories (As shown in Figure 28): 1) EC$_{50}$ larger than 100-fold of wild type MC2R, including F178 of hMC2R, V159 and F175 of rtMC2R (Positions labeled with *); 2) EC$_{50}$ larger than 10-fold of wild type MC2R, including H170 of hMC2R and F171 of rtMC2R (Positions labeled with °); and 3) EC$_{50}$ smaller than 10-fold of wild type MC2R. In this figure, 5 out of 14 residues caused the EC$_{50}$ values of mutants to increase 10-fold than the wild type control. Note that position F178/F171 in TM5 was the only one position that is identical in these two receptors, restating the importance of this phenylalanine residue. Meanwhile, non-conserved positions in these 5 residues are H170 in EC2 of hMC2R EC2, and V159 and F175 in TM4 and TM5 of rtMC2R. Hence, in addition to the same phenylalanine at TM5, hMC2R and rtMC2R actually use different domains, which are EC2 or TM4/TM5.
In conclusion, the comparative analysis revealed that position T164/V157 in TM4, F168/F161 in EC2, and F178/F171 in TM5 are responsible for the interaction of ACTH(1-24) KKRRP motif, and the importance of these positions are consistent for hMC2R and rtMC2R. However, these receptors have slightly different preference within the overall interaction site. hMC2R uses EC2 as the predominant domain with three successive significant residues, whereas rtMC2R mainly relies on its TM4 and TM5 regions.
Figure 24. Human and rainbow trout MC2R sequence alignment. Positions shaded in black indicate identical sites in these receptors. h: human; r: rainbow trout (Oncorhynchus mykiss).
Figure 25. ACTH(1-24) dose response curves of TM4 mutant rtMC2Rs in the presence of zfMRAP1.
Figure 26. ACTH(1-24) dose response curves of EC2 mutant rtMC2Rs in the presence of zfMRAP1.
Figure 27. A-B) ACTH(1-24) dose response curves of TM5 mutant rtMC2Rs in the presence of zfMRAP1. C) rtMC2R 7A mutant ACTH(1-24) activation curve.
Table 8. EC\textsubscript{50} values of 19 mutant rtMC2R with single alanine substitution.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Alanine Substitution</th>
<th>EC\textsubscript{50} ± SEM (10\textsuperscript{-11}M)</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>N/A</td>
<td>4.6 ± 0.7</td>
<td>N/A</td>
</tr>
<tr>
<td>TM4</td>
<td>V153</td>
<td>3.4 ± 0.46</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>G155</td>
<td>14 ± 6.0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>V157</td>
<td>19 ± 5.9*</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>M158</td>
<td>15 ± 3.4*</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>V159</td>
<td>510 ± 124*</td>
<td>0.007</td>
</tr>
<tr>
<td>EC2</td>
<td>F161</td>
<td>22 ± 7.3*</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>C162</td>
<td>17 ± 6.4</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>D163</td>
<td>1.7 ± 0.59</td>
<td>0.098</td>
</tr>
<tr>
<td></td>
<td>T165</td>
<td>5.8 ± 2.1</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>V166</td>
<td>5.6 ± 2.0</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>I166</td>
<td>2.4 ± 1.1</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>K168</td>
<td>8.2 ± 1.5*</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>I169</td>
<td>1.5 ± 0.9</td>
<td>0.43</td>
</tr>
<tr>
<td>TM5</td>
<td>F170</td>
<td>5.8 ± 0.52</td>
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<tr>
<td></td>
<td>F171</td>
<td>214 ± 84*</td>
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<tr>
<td></td>
<td>I172</td>
<td>26 ± 8.2*</td>
<td>0.027</td>
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<tr>
<td></td>
<td>V173</td>
<td>3.9 ± 0.68</td>
<td>0.19</td>
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<tr>
<td></td>
<td>L174</td>
<td>16 ± 5.6</td>
<td>0.081</td>
</tr>
<tr>
<td></td>
<td>F175</td>
<td>460 ± 200*</td>
<td>0.043</td>
</tr>
<tr>
<td>Septuple Mutation</td>
<td>V157, M158, V159, F161, F171, I172, F175 (7A)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

EC\textsubscript{50} values are expressed as mean ± SEM (n=3). Statistical differences were assessed by student t-test compared with ACTH(1-24) (*:P<0.05, **:P<0.005). N/A: No activation. Position A154, A156, A160 and A164 were not tested.
Figure 28. Positions (Red) that significantly affected ACTH signaling in human and rainbow trout MC2R TM4, EC2 and TM5 domains. A) Fold increase of mutant EC\textsubscript{50} values as compared with wild type MC2R. B) *: residues that had an EC\textsubscript{50} over 100-fold of wild type MC2R; °: residues that had an EC\textsubscript{50} over 10-fold of wild type MC2R; Positions shaded in gray indicate identical sites of these receptors. h: human; r: rainbow trout (Oncorhynchus mykiss).
The melanocortin-2 receptor has two functional activation features which are distinct from the other melanocortin receptors (e.g., MC1R, MC3R, MC4R, MC5R): MRAP1 dependency and ligand selectivity for ACTH (Hinkle and Sebag 2009; Webb and Clark, 2010). These features of MC2R had appeared since the ancestral ray-finned fishes diverged from ancestral cartilaginous fishes over 400 million years ago (Reinick et al., 2011). This study focused on the important sites in both ACTH(1-24) and MC2R that are responsible for receptor activation, and whether this pattern has changed between mammal (human) and bony fish (rainbow trout) MC2Rs.

In the sequence of melanocortin peptides, the HFRW motif is required in order to activate melanocortin receptors (Figure 2B). Previous studies on human MC4R identified ten residues located in the TM2, TM3, TM6 and TM7 regions that are involved in the binding of the HFRW motif (Pogozheva et al, 2005). In human MC1R, MC3R, MC4R and MC5R 90% of these residues are identical, and as a result all of these receptors can be activated by ACTH or any type of MSHs. In MC2Rs, 70% of these residues are conserved in human, frog and rainbow trout MC2R orthologs as well (Figure 24). As a result, the HFRW motif binding site for MC2R in human and rainbow trout should be at the same positions (i.e., TM2, TM3, TM6, and TM7). Zone A analog stimulation results
(see Chapter 1) indicated that the HFRW motif was critical for both hMC4R and MC2R (human and rainbow trout) with a subtle difference predicted at the tertiary structure level for the two receptors (Table 3 and Table 7). The tryptophan (W⁹) was the most important position in the HFRW motif for the activation of both hMC2R and hMC4R, whereas in rainbow trout MC2R, the arginine (R⁸) seems to be more important than tryptophan (W⁹). In addition to the requirement for the HFRW motif, the KKRRP motif was also essential for MC2R activation, especially with respect to the rainbow trout receptor (Table 7). In addition, residues R¹⁷R¹⁸P¹⁹ are more important than K¹⁵K¹⁶ in both hMC2R and rtMC2R (table 3 and Table 7). Finally, the sequence between the HFRW motif and the KKRRP motif is GKPVG. Replacement of individual residues of this region did not adversely affect receptor activation of both hMC2R and rtMC2R. Nevertheless, this region should be important, because incubation of receptor-transfected CHO cells with two peptides each containing HFRW motif and KKRRP motif respectively (i.e., α-MSH and the KKRRP analog), was unable to activate hMC2R (Figure 11B). This controversy was resolved by testing two truncated analog ACTH(1-22) and ACTH(1-21) which were also unable to activate hMC2R and rtMC2R as well (Table 3 and Table 7). Collectively, it is now clear that the sequence of the HFRW motif and the KKRRP motif in ACTH(1-24) can activate hMC2R and rtMC2R as a result of the sequence length in between these two motifs (i.e., GKPVG) that properly orients the ligand to its binding sites in the receptor.

After conducting studies on the features of ACTH(1-24), this thesis then addressed questions relating to the mechanism for activation of MC2R. Although both HFRW and KKRRP motif are required in MC2R activation, they play different roles in
the binding of ACTH(1-24) to MC2R. Schwyzer (1977) had proposed that the KKRRP motif in ACTH played a critical role in the binding of the ligand to the receptor. As indicated in Chapter 2, ACTH(15-24), the KKRRP motif analog, significantly reduced ligand binding affinity, whereas analog A4, the HRFW motif analog, did not show any effect on the binding curve (Table 3). Furthermore, a short peptide (KKRRPVKVYP) only containing the last ten amino acid residues in ACTH(1-24) was able to bind to hMC2R to the same degree as full length ACTH(1-24) (Figure 12D). These observations led to the discovery of an ACTH(1-24) antagonist (KKRRPVKVYP) that specifically targets hMC2R instead of other melanocortin receptors. In our in vitro system, the application of this antagonist can block ACTH(1-24) activation completely at the concentration of 100nM, and it can reduce ACTH(1-24) activation by 35% when applied at concentration of 10nM (Figure 12C). These observations lend support to the hypothesis presented in Figure 10 that ACTH first binds to MC2R at the KKRRP motif, and then the HFRW motif in the ligand is positioned into HFRW binding site on the receptor because of the “bridging” action of the GKPVG motif in the ligand.

The studies on the ACTH antagonist set the stage for the characterization of the KKRRP binding site on the receptor (Chapter 3). The KKRRP binding site in MC2R appears to be the predominant region that is not only defining its ligand selectivity, but also initiating the binding of ACTH(1-24). The 3-dimensional model for MCRs (Figure 14) predicts that the transmembrane domains for HFRW binding site (TM2, TM3, TM6 and TM7) are at close proximity and form the space to allow HFRW motif to sit inside. Based on that orientation, it would follow that the KKRRP motif of ACTH should be
positioned within the region close to TM4, TM5 and EC2 (Figure 14). In order to test this hypothesis, we made single-alanine mutant receptors at this region in both human and rainbow trout. Although the sequence identity between hMC2R and rtMC2R for this region is only 32% (Figure 28), the importance of certain positions in TM4, EC2, and TM5 for the activation of receptor became apparent. It appears that TM5 was important for both receptors (F178 hMC2R/F171 F175 rtMC2R), whereas EC2 (H170) of hMC2R and TM4 (V159) of rtMC2R were also involved in receptor activation (Figure 28). Of all the residues in this region that adversely affected the EC$_{50}$ by a factor of 5 fold or greater, only F178 in hMC2R and F175 in rtMC2R are at the same relative position in their respective receptor. Clearly the 3-dimensional structure of this binding pocket must be the feature that human receptor and the rainbow trout receptor share in common.

In the next step in this research it would be informative to use ACTH(15-24) to probe the proposed KKRRP binding site. For these experiments, the iodinated competitive inhibitor (KKRRPVKVYP) would be made in order to support the conclusion that these sites in MC2R are involved in the binding of KKRRP motif of ACTH. When applied to the mutant receptors, the prediction would be that the competitive inhibitor should cause a decline in the binding curves as compared to the wild-type receptor. Furthermore, 3D structure modeling analysis of MC2R should be done in the future to obtain the detailed description of receptor/ligand relationship.
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Minireview

Observations on the ligand selectivity of the melanocortin 2 receptor

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ABSTRACT

The melanocortin 2 receptor (MC2R) is unique in terms of ligand selectivity and in vitro expression in mammalian cell lines as compared to the other four mammalian MCs. It is well-established that ACTH is the only melanocortin ligand that can activate the ACTH receptor (i.e., melanocortin 2 receptor). Recent studies have provided new insights into the presence of a common binding site for the HPRW motif common to all melanocortin ligands. However, the activation of the melanocortin 2 receptor requires an additional amino acid motif that is only found in the sequence of ACTH. This mini-review will focus on these two topics and provide a phylogenetic perspective on the evolution of MC2R ligand selectivity.

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1. Introduction

Although the pituitary hormones α-MSH and ACTH were characterized over 50 years ago [25,26], the biosynthetic relationship between these two polypeptides as well as their common mode of action would not become apparent until the initial characterization of the mRNA that codes for the proopiomelanocortin, POMC [32], and the discovery of the melanocortin receptor gene family (see for review [7]). The initial enigma with respect to these two polypeptides was their common amino acid sequence. α-MSH is the first thirteen amino acids in the sequence of ACTH(1–39) for every vertebrate species that has been studied [12] with the exception of the lampreys [43]. Furthermore, ACTH(1–39) is a major end-product in the corticotrophic cells of the anterior pituitary, whereas, α-MSH is a major end-product in the melanotropic cells of the intermediate pituitary [4]. The explanation for the differential distribution of these two polypeptides was resolved when it was discovered that these polypeptides are derived from the prohormone precursor, POMC [32]. Although ACTH(1–39) is an end-product in the corticotrophic cells of the anterior pituitary, in the melanotropic cells of the intermediate pituitary this same polypeptide functions as a biosynthetic intermediate and gives rise to α-MSH (for review see [13]). The subsequent discovery of the proopiomelanocortin convertases, PC1/3 and PC2 (for review see [39]), provided the mechanism to explain the tissue specific posttranslational processing of POMC in the anterior and intermediate lobes of the pituitary [10,28,46].

Viewing the POMC system from a physiological perspective, ACTH(1–39) serves as a chemical signal in the hypothalamo-pituitary-adrenal-renal axis (HPA/A), whereas, α-MSH plays no physiological role in this circuit [4,6]. By comparison, α-MSH (α-melanocyte-stimulating hormone) regulates physiological color change through the activation of pigment granule translocation in dermal chromatophores [6,16]. More recent studies indicate that α-MSH can also function as a neuromodulator in the hypothalamus and plays a role in the regulation of appetite/feeding behavior in mammals and perhaps in all vertebrates [6,35]. Given the level of primary sequence identity between α-MSH and ACTH(1–39), were these polypeptides acting through the same receptor on target cells or were there distinct "ACTH" and "α-MSH" receptors? The characterization of the melanocortin receptors (MCR) from the genome of the mouse [7] and the subsequent characterization of these GPCR genes from all the major classes of vertebrates resolved this question [40], but also raised new issues.

2. Phylogeny of MCRs

The phylogenetic distribution of MCRs in various vertebrate classes is summarized in Fig. 1. The discovery of two melanocortin-related receptors in the lamprey genome [17] coupled with the presence of five MCR genes in the genome of tetrapods points to the role of genome duplication events in the radiation of this GPCR gene family during chordate evolution. Since it is assumed that an ancestral MCR gene originated in some early chordate lineage, the two genome duplication events that are predicted to have occurred during chordate evolution [21,27,33] should account for the presence of four paralogous melanocortin receptor genes in gnathostomes, yet five paralogous genes are present in tetrapods [7] and some bony fish genomes [45]. In order to explain these observations, one scenario proposed that the initial outcome of the two rounds of genome duplication events gave rise to MC1R, MC3R, MC4R genes and a proposed proto-MC5R/MC2R gene [5]. Synonym studies [22] would appear to support the assumption that a local gene duplication of a putative proto-MC5R/MC2R gene give
mouse MCIR gene [31], attempts to study this unique ligand/receptor relationship were hampered by another unique feature of the bony vertebrate MCIR orthologs.

4. MCIR and the MRAFs

Although the MCIR, MC3R, MC4R, and MC5R genes can be functionally expressed in a variety of cell lines, the MC2R gene could only be functionally expressed in cell lines derived from the adrenal cortex [14,30,36,38] or cell lines, like the rat INS-1 β-cells [2], which like mouse and human β-cells express the MC2R gene. Recent studies have revealed that cells which are capable of functionally expressing MC2R also co-express the melanocortin receptor accessory protein (mrap) gene. In mammals and perhaps many non-mammalian vertebrate species there are two paralogous mrap genes (mrap1 and mrap2). The mammalian MRAF protein genes have been extensively studied and the salient features of these two proteins have been recently reviewed [20,43]. The properties of these proteins can be summarized as follows. The MRAF protein products are single chain polypeptides with a single transmembrane spanning domain, following synthesis at the KER, MRAF1 monomers form a homodimer in which the two polypeptide chains are oriented in an antiparallel manner. MRAF2 also forms a homodimer (20,44). When the mc2r gene is transfected into cells that do not endogenously express the mrap1 or the mrap2 genes, the trafficking of newly synthesized MC2R from the ER to the plasma membrane will not occur. In addition, cells transfected with the mc2r gene and the mrap2 gene, trafficking of the newly synthesized MC2R to the plasma membrane will occur, but the MC2R on the plasma membrane cannot be subsequently activated by ACTH. However, in cells that are transfected with the mc2r gene and the mrap1 gene, not only does trafficking of the newly synthesized MC2R to the plasma membrane occur, these receptors can also be activated by ACTH. Collectively, these observations indicate that MRAF1 and MRAF2 are intracellular chaperones that facilitate the movement of MC2R to the plasma membrane. In addition it appears that MRAF1 must be in contact with MC2R at the plasma membrane in order for the receptor to be in the proper conformation to bind ACTH and be activated by this ligand. Finally a recent study indicates that at the plasma membrane MC2R is present as a homodimer. The MC2R homodimer is combined with two MRAF1 homodimers to make a hexameric complex. The hexameric complex, then, is the functional "ACTH" receptor on the plasma membrane of the transfected cells [8]. In summary, the functional expression of MC2R in cells in culture requires the co-expression of a mc2r gene construct and a mrap1 gene construct. Is the interaction between MRAF1 and MC2R an ancestral feature of the mc2r gene? It would appear so. Recent studies on the activation of zebrafish MC2R indicate that a zebrafish mrap1 construct must be co-transfected into HEK 293 cells in order for the zMC2R receptor to be activated by ACTH [1].

5. Binding and activation sites on MC4Rs

Given the distinctive features of MC2R in terms of ligand activation and functional expression, what properties does MC2R share in common with MC1R, MC3R, MC4R, and MC5R? Since all five melanocortin receptors can be activated by ligands that have the amino acid motif, HHRW, it would be reasonable to assume that all vertebrate melanocortin receptors share a common HHRW binding site. That hypothesis was tested by Pogozheva et al. [34] using a complicated, yet elegant strategy to model the putative HHRW binding site in the human MC4 receptor. The model identified TM 2, 3, 6, and 7 as the most likely regions to form a binding site for the HHRW motif of mammalian α-MSH. The intriguing feature of the model was the identification of a site that could accommodate the β-hairpin turn in the HHRW motif [19]. The model was tested by performing single alanine substitutions to selected residues in TM 2, 3, 6, and 7 regions that could potentially have ionic interactions or form H-bonds with residues in the HHRW motif of α-MSH. The single-alanine mutant forms of human MC4R were transfected into HEK 293 cells and the production of AMP in these cells was compared to HEK 293 cells transfected with the wild-type form of human MC4R. The results of those experiments are summarized in Fig. 2. In the sequence of human MC4R there are 21 amino acids distributed between TM2, TM3, EC2, TM6, and TM7 that are involved in the α-MSH binding site. It is interesting that the majority of these sites are predicted to make contact with the phenylalanine residue in the HHRW motif of α-MSH. Ten of these residues are present in the sequences of Squamoso acanthinus MC4R and Silurana tropicalis MC4R (Fig. 2). Several of these same residues are also found in the melanocortin receptors cloned from the lamprey genome [5], this binding pocket must have emerged early in the evolution of the vertebrates.

For at least MC1R, MC3R, MC4R, and MC5R, the HHRW binding site is also the activation site for these receptors [43]. Since the phenylalanine and the arginine residues in the HHRW motif form the reverse beta-turn in this portion of the melanocortin core sequence, would alanine substitution of either of these residues eliminate activation of the MC4 receptor? To test this possibility, the CT1–7 cells [29] that endogenously express the mouse mc4r gene were used [42]. As shown in Fig. 3A, these cells can be stimulated by either α-MSH or ACTH[1–24]. The C1–7 cells were then challenged with analogs of ACTH[1–24] in which the HHRW motif was either: (a) replaced with four alanine residues [A4 analog]; (b) the F7 residue was replaced with an alanine residue [HARW analog]; or (c) the K8 residue was replaced by an alanine residue [HFARW analog]. As shown in Fig. 3B, at concentrations of either 10^{-6} or 10^{-5} M none of these analogs of ACTH[1–24] activated the MC4 receptor on the CT1–7 cells, whereas cells incubated with ACTH[1–24] were clearly activated at both concentrations. Since the HHRW motif in a melanocortin ligand is responsible for both binding to and activating the receptor [41], the results presented in Fig. 3B would appear to indicate that none of the alanine-substituted ACTH[1–24] analogs may have bound to the MC4 receptor; hence no activation. However, when the A4, HARW, and HFARW analogs, at a concentration of 10^{-5} M were individually co-incubated with ACTH[1–24] at concentrations of either 10^{-9} or 10^{-10} M, there was a significant drop in activation as compared to cells stimulated with either concentration of ACTH[1–24] without the analogs present (Fig. 3C). These results indicate that for lower concentrations of ACTH[1–24] the alanine-substituted analogs may be acting as competitive inhibitors which would imply that residues either N-terminal or C-terminal to the HHRW motif are also playing a role in the positioning of the melanocortin ligand at the HHRW binding sites on the receptor.

Given these observations for the MC4R, and it is assumed that MC1R, MC3R, and MC5R would respond in a similar manner, what are the implications for the melanocortin 2 receptor? As indicated in Fig. 2 the rat MC2 receptor has six of the eleven residues found in the HHRW binding site for MC4Rs. However as already noted, mammalian MC2Rs, and perhaps all bony vertebrate MC2Rs, can only be activated by ACTH[1–24], but not by α-MSH [N-acetyl-ACTH[1–13]amide]. The key, then, to understanding the activation of an MC2 receptor would appear to lie in residues 14 to 24 in the sequence of ACTH[1–24]. As reviewed by Schouyer [41], the shortest analog of ACTH[1–24] that still retains some level of biological activity is ACTH[1–16], and conversely analogs longer than ACTH[1–16] are progressively stronger agonists. Costa et al. [9] conducted a series of studies that focused on residues in the motif K2(D/I)3HHRW in the sequence of NACHT[1–24]. Alanyl substitu-
tions at any of these sites lowered the biological activity of the ACTH(1–24) analog with varying degrees of efficacy. Collectively, the experiments using C-terminally modified analogs of ACTH(1–24) point to the KKKRRP motif in mammalian ACTH(1–24) as important for the activation of MC2R. If this conclusion is correct, the prediction would be that replacement of the KKKRRP motif in mammalian ACTH(1–24) with alanines (A5 analog) should produce an analog with a greatly diminished capacity for activating MC2R. As observed in Fig. 3B, the A5 analog of ACTH(1–24) can activate the MC4 receptor on GT1–7 cells. Hence, these substitutions do not adversely affect the conformation of the ACTH(1–24) with respect to interaction with MC4R at the HHRW binding site of this receptor. However, when rat INS-1 cells, which endogenously express the MC2 receptor [2], were incubated with the A5 analog, there was a significant drop in the activation of the MC2 receptor as compared to stimulation by ACTH(1–24) (Fig. 4). This result would support the importance of the KKKRRP motif in mammalian ACTH(1–24) for the activation of the MC2 receptor and also suggests that residues at positions 20–24 may play some role in the activation of the receptor.

The model that emerges to explain the activation of MC2R by ACTH(1–24) appears to be dependent on the presence of two sites in the receptor: an HHRW binding and activation site and a KKKRRP binding site. The former site is not accessible to MSH-sized ligands, whereas binding of ACTH(1–24) to the latter site would appear to properly position ACTH(1–24) so that the N-terminal of the ligand can make contact with the HHRW binding site on MC2R to activate the receptor. Intrinsic to this two step model is the interaction between MC2R and MRAFP1 at the plasma membrane. Since zebrafish MC2R has the same requirements for activation as mammalian MC2R (presence of MRAFP1 and activation by ACTH(1–24)), but not by α-MSH [1]), this mechanism must be present in the common ancestor to the ancestral bony fishes and the ancestral lobe-finned fishes. These two requirements (MRAFP1 dependence
Fig. 3. Activation of MC4R on GT1-7 cells by α-MSH and ACTH(1-39), GT1-7 (G418 resistant) cells endogenously express the mouse MC4R [26,43]. The cells were maintained as described in [26,42]. GT1-7 cells were seeded at a density of 6 x 10⁴ cells/well in a 6-well plate. (A) After 48 h, GT1-7 cells were stimulated with either NEMI-MSH (white histograms) or ACTH(1-39) (gray histograms) (New England Peptide) at doses ranging from 10⁻⁵ to 10⁻¹ M. The ligand was delivered in "stimulating solution" that had the composition: 140 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl₂, 5.0 mM CaCl₂, 10 mM HEPES, 10 mM glucose, 0.1% BSA, and supplemented with 1 mM BSA (pH adjusted to 7.2). Cells were then lysed using 0.1 M HCl. The CAMP kinase in the samples were measured using the Direct-CAMP ELISA kit (Enzo Life Sciences Inc.) and analyzed on an Bio-Tek Synergy HT plate reader. Data are expressed as mean ± SEM (n = 3 wells of GT1-7 cells.) In the negative control wells an equal number of GT1-7 cells were incubated with the stimulation solution without ligand. Data were normalized to the maximal response for the saturating doses of NEMI-MSH. B) In separate wells with GT1-7 cells at a density of 6 x 10⁴ cells/well were stimulated with either CAMP (1-39) (gray histograms), the Aα analog [SYNVEAAGVPGKSSRPVYPY] (1), the Aβ analog [SYNVEAAGVPGKSKRRPSVYYP] (3), the Cα analog [SYNVEARWAGVPGKSKRRPSVYYP] (5), or the Cα analog [SYNVEAARWAGVPGKSKRARPSVYYP] (4) at concentrations of either 10⁻¹ for 10⁻³ M as described for (A). After a 15 min stimulation period, the cells were lysed and AMP levels in the samples were measured using the Direct-cAMP ELISA kit (Enzo Life Sciences Inc. Data are expressed as mean ± SEM (n = 3 wells of GT1-7 cells). Data were normalized to the maximal response for the saturating doses of ACTH(1-39). Analog 1, 2, and 3 did not activate the M4 receptor (P < 0.05). (C) In separate wells GT1-7 cells were incubated for 15 min with either ACTH(1-39) alone at a concentration of 10⁻⁸ M (gray histogram) or co-incubated with ACTH(1-39) at concentrations of 10⁻⁸ M and analogs 1, 2, and 3 at concentration of 10⁻⁶ M. Each experiment was conducted as described in the legend for (A). Data were normalized to the maximal response for ACTH(1-39) alone. In the negative control wells an equal number of GT1-7 cells were incubated with the stimulation solution without ligand. Analog 1, 2, and 3 significantly inhibited the activation of the MC4 receptor in the presence of 10⁻⁶ and 10⁻⁸ M ACTH(1-39) (P < 0.05).

6. Conclusions and future questions

The functional expression of a melanocortin 2 receptor requires interaction with MRAPI in order for activation of MC2R to be possible. Is there a common site on vertebrate MC2Rs for interaction with MRAPI? Can a mammalian MRAPI facilitate the activation of a fish MC2R?

When did the MRAPI/MC2R interaction appear in vertebrate evolution? Perhaps this question can be addressed by studies on cartilaginous fishes. Studies on Squalus acanthias yielded orthologs of MC2R and MC4R that could be functionally expressed in HEK 293 cells, however an ortholog of MC5R could not be expressed in these cells and as a result the ligand selectivity properties of this MC2R have not been determined [23,37]. Does Squalus acanthias MC2R have a requirement for MRAPI, and can this MC2R be selectively activated by ACTH, but not by the shark MSH ligands?

The functional definition of a melanocortin 2 receptor is that it is a MC2R that can only be activated by ACTH, but not by any of the MSHs. When did MRAPI evolve a conformation that would accommodate ACTH as a ligand, but exclude α-MSH? A recent phylogenetic study has identified an apparent ortholog of MC2R in the genome of the elephant shark, Callorhinus milhi [44]. Will the putative C. milhi MC2R have a requirement for MRAPI and what are the ligand selectivity properties of this CPCR?

Finally, as predicted by Schwyzer [45], positions 16 through 19 in ACTH(1-38) are important for the activation of MC2R.
binding of C-terminal analogs like ACTH(15–24) activate MC1R alone or can the binding of C-terminal analogs of ACTH make the receptor amenable to activation by α-MSH? Finally, where in MC1R is the binding site for the ACTH(15–19) motif of ACTH?

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References

APPENDIX 2
Functional expression of frog and rainbow trout melanocortin 2 receptors using heterologous MRP1s

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Abstract

Analysis of the functional expression of the melanocortin 2 receptor (MC2R) from a rather broad spectrum of vertebrates indicates that MC2R is sexually selective for the ligand, ACTH, and the melanocortin receptor accessory protein 1 (MRAP1) is required for high affinity ACTH binding and activation of MC2R. A phylogenetic analysis of MRAP1 suggested that tetrapod sequences and bony fish sequences may represent two distinct trends in the evolution of the mrap1 gene. To test this hypothesis, a frog (Xenopus tropicalis) MC2R was expressed in CHO cells either in the presence of a tetrapod (mouse) MRAP1 or a bony fish (zebrafish) MRAP1. The response of frog MC2R to different concentrations of human ACTH(1–24) was more robust in the presence of mouse MRAP1 than in the presence of zebrafish MRAP1. Conversely, the cAMP response mediated by the rainbow trout (Oncorhynchus mykiss) MC2R was almost twofold higher and occurred at 1000-fold lower ACTH concentration in the presence of zebrafish MRAP1 than in the presence of mouse MRAP1. Collectively, these experiments raise the possibility that at least two distinct trends have emerged in the co-evolution of MC2R/MRAP1 interactions during the radiation of the vertebrates.

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1. Introduction

The biosynthesis of transmembrane proteins, such as the G-protein coupled receptors (GPCRs), involves the translation of the GPCR mRNA at the rough endoplasmic reticulum (ER), followed by trafficking of the GPCR through the endoplasmic reticulum (ER) and Golgi, leading to incorporation of the GPCR into the lipid bilayer of a secretory vesicle at the trans Golgi. The eventual merging of the secretory vesicle with the plasma membrane will result in the positioning of the GPCR on the plasma membrane [28]. Since this pathway is present in all eukaryotic cells many GPCRs have been functionally expressed in a variety of eukaryotic cells. In the case of the melanocortin receptors (MCs), GPCRs that can be activated by the melanocortin ligands (i.e., ACTH, α-MSH, β-MSH, and γ-MSH) [6], MC1R, MC3R, MC4R, and MC5R can be functionally expressed in, for example, HEK293 cells. The heterologous expression of these MCs in a mammalian cell line has been achieved not only for human and mouse orthologs of these receptors (for review see [5]), but also for the MC orthologs of two species of bony fish [4,14], and the MC orthologs of a cartilaginous fish [15,22]. However, there are two exceptions to this generalization. The MC5R ortholog from the spiny dogfish Squalus acanthias has not been successfully expressed in HEK293 cells [15]. In addition, neither the mouse MC2R ortholog [18], nor for that matter any vertebrate MC2R ortholog has been functionally expressed in HEK293 cells when the MC2R ortholog alone was transfected into these cells [12,31]. This study will focus on the heterologous expression of MC2R orthologs.

The open reading frame encoding MC2R (ACTH receptor) was initially isolated by screening a human genomic library [18], and a MC2R cDNA construct was subsequently functionally expressed in Cloudman S91 melanoma cells. Later studies found that mammalian MC2R constructs could not be functionally expressed in heterologous cell lines unless those cell lines were derived from the adrenal cortex [9,17,21,24]. In addition, it was observed that a subset of patients exhibiting familial glucocorticoid deficiency type 2 syndrome had functional MC2 receptors, yet were resistant to ACTH treatment [17]. Collectively, these observations led to the discovery that adrenal cortical cells express the melanocortin receptor accessory protein (mrp1) gene [17]. In the genomes of many vertebrates there are two paralogs mrp genes (mrp1 and mrp2) [12,31]. As shown in Fig. 1, MRP1 and MRP2 are single chain polypeptides with a single membrane spanning domain. Both paralogs form antiparallel homodimers in the ER [25–27], and this unique feature appears to be associated with the residues located...
between positions 43 and 55 in Fig. 1. In addition, each paralog has a domain located between positions 21 and 38 to facilitate the trafficking of MC2R from the ER to the plasma membrane [30]. Given these common features, the homodimers of both MRAF1 and MRAF2 will form a complex with MC2R in the ER and facilitate the trafficking of mammalian MC2Rs to the plasma membrane.
However, only MRA11/MCR2 complexes on the plasma membrane can be efficiently activated by ACTH, whereas the efficacy of ACTH activation is greatly diminished for MRAF2/MCR2 complexes [12,21]. Finally, a recent study indicates that when a human mnrp1 cDNA construct and a human mcr2 cDNA construct are co-transfected into HEK293 cells, the functional ACTH receptor is a hexameric complex composed of a MCR1 homodimer and two MRA11 homodimers [7].

The role of MRA11 in the trafficking and activation of MCR2 is not limited to mammals. A recent study indicates that zebrasfish MCR2 can be activated only when a zebrasfish mcr2 cDNA construct is co-transfected with a zebrasfish mnrp1 construct in HEK293 cells [1]. Collectively these studies, on widely divergent vertebrate species (mammals and a bony fish), would suggest that all vertebrate MCR2s require MRA11 for functional expression. It also appears highly likely that all vertebrate MCR2s can only be activated by ACTH but not by any of the MSH-related melatoninergic ligands [5]. Thus, in vitro studies to understand the evolution of the unique ligand selectivity properties of MCR2 in the various lineages of bony vertebrates (e.g., ray finned fishes, lobe finned fishes, anamniotes, and amniotes) should be considered.

2. Materials and methods

2.1. MCR2 and MRA11 constructs

Rainbow trout (Oncorhyncus mykiss) [2], accession No. EU119887, and frog (Xenopus tropicalis) MCR2, accession No. XP001342801.2, were synthesized by GenScript with a FLAG epitope tag and inserted into pcDNA3.1 vector, respectively. Zebrasfish (Danio rerio) MRA11, accession No. XP001342801.2, was synthesized by GenScript with a FLAG epitope tag and inserted into pcDNA3.1 vector. The human (h) MCR2 cDNA was obtained from the Missouri S&T DNA Resource Center. The mouse (m) MRA11 was provided by Dr. Adrian Clark at Queen Mary University (London, United Kingdom).

Rainbow trout MCR2 was cloned from adrenocortical tissue using the following primers: forward primer contains Nhel site, V5 epitope tag sequence, and Kozak sequence: 5'-TATATATATATACTTACGACCACTGGTGAGCTGTTTCTCGGCTTGATAGTCGTGTTG- CAAAGTACCTGAAACC-3', the reverse primer with a MfeI site and no stop codon: 5'-TATATATATATACTTACGACCACTGGTGAGCTGTTTCTCGGCTTGATAGTCGTGTTG- CAAAGTACCTGAAACC-3'. The amplicon agreed with the published X. tropicalis MRA11 sequence (ENSXETG00000018157).

The Plasmid product was cloned into a pcDNA Neo vector containing a 3xFLAG epitope sequence followed by a stop codon in frame with a Nhel site.

2.2. Cell growth

Most of the experiments were done in transiently transfected CHO cells. The CHO cells were grown at 37° in a humidified 5% CO2 incubator in DMEM/F12 with 5% fetal calf serum. X. tropicalis MCR2 was also transiently transfected in an A6 Xenopus kidney fibroblast cell line kindly provided by Dr. Jacques Robert at the University of Rochester Medical Center (Rochester, NY). A6 cells were grown at 27° in a humidified 5% CO2 incubator in basic amphibian serum-free medium supplemented with 10% fetal calf serum as described [23].

2.3. Immunocytochemical staining of CHO cells expressing MCR2 and MRA11

CHO cells were grown in 8-well chamber slides (5 × 10^4 cell/well) for 24 h prior to transfection. The cells were transfected with 1 μg of either the rainbow trout or frog mcr2 cDNA construct either in the presence or absence of the mouse mnrp1 cDNA construct using lipofectamine 2000. After 24 h, the cells were fixed with 4% paraformaldehyde and either permeabilized with 0.3% Triton X-100 (Fig. 7A) or left unpermeabilized (Fig. 7B-D). The primary antibodies were diluted 1:500 in PBS + 1% BSA solution. A mouse monoclonal anti-V5 antibody was used to detect MCR2 receptors. A rabbit polyclonal anti-FLAG antibody was used to detect mMRA11. Cells were incubated with primary antibodies for 1 h at 37° C. Following a wash step the cells were incubated with secondary antibodies for 45 min at 37° C. The secondary antibodies were diluted 1:800 in PBS + 1% BSA solution. A donkey anti-mouse antibody linked to Alexa Fluor 488 was used to visualize MCR2 receptors (green). A donkey anti-rabbit antibody Alexa Fluor 555 was used to visualize mMRA11 (red). For localization of MCR2 and MRA11, unpermeabilized cells were incubated with both primary antibodies, followed by incubation with both secondary antibodies (Fig. 7C and D). As a control, untransfected CHO cells were stained with the V5 or donkey anti-mouse antibody and no reactions was observed. In addition, CHO cells transfected with the MCR2 construct were incubated with secondary antibody, but not primary antibodies. Once again no reaction was observed. For all staining experiments, coverslips were mounted onto slides using Vectashield and nuclei were stained with DAPI (blue). Images were acquired using a 100X oil immersion objective (Zeiss Plan-Neofluar) with a fluorescence microscope equipped with a Hamamatsu digital camera. All images were analyzed using SlideBook software.

2.4. cAMP and expression assays

For the functional expression of the hM2 receptor and the frog hM2 receptor a cAMP-reporter assay was used. Cells were plated in 96 well dishes and transfected with 40 ng/well local DNA using FuGENE HD according to manufacturer's instructions. Transfections typically were performed using 13.3 ng/well of a cAMP-dependent luciferase reporter containing multiple copies of the cAMP response element (CRE) from the rat insulin promoter [5] provided by Dr. George Holz (Syracuse, NY), 21.3 μg/ml receptor DNA and 5.3 μg/ml MRAF or GFP DNA. After 24 h, the medium was replaced with 40 μl fresh DMEM/F12 containing 0.1% BSA with no additions, 20 μM forskolin or ACTH [1–24] from Phoenix Biochemicals at the concentration shown. After 5 h, the cells were harvested and One Step Luciferase Assay Reagent (Nanolight Technologies) was added. Luminescence was read on a BioTek plate reader. Values shown represent the mean and SEM from triplicate wells in a representative experiment.

Cell surface expression of hM2 receptors, which contained an extracellular, N-terminal triple HA tag, and total expression of
V5-tagged MRAPs were measured by whole cell ELISA as previously reported [25]. Significance of differences between pairs was evaluated by Student’s unpaired t-test and between groups by ANOVA with Tukey’s test. The functional expression of rainbow trout MC2R was also done in CHO cells. For these experiments, 2 µg of rainbow trout MC2R, MRAP and CRE-3xLuc reporter plasmid were transfected using a Cell Line Nucleofector Kit (Amaxa Inc.) with solution T and program U-23, and seeded on 96-well plate at a density of 10^5 cell/well. 48 h after transfection, cells were stimulated with appropriate ligands in serum-free CHO media for 4 h at 37 °C. At the end of the incubation, 100 µl of Bright-Glo luciferase assay reagent (Promega Inc.) were applied to each well and incubated for 5 min at room temperature. Luminescence was measured with Bio-Tek Synergy HT plate reader. All experimental treatments were performed in triplicates, and statistical differences were determined using unpaired Student’s t-test for equal variance with Kaleidagraph software. Significance was set at P < 0.05.

2.5. Phylogenetic analysis of MRAP sequences

The amino acid sequences of MRAP1 and MRAP2 were manually aligned [8]. The web site http://www.ensembl.org/Multi/blastview was used to search for MRAP-related sequences in the following species: Human - Homo sapiens, Monkey - Macaca mulatta, Lemur - Microcebus murinus, Rat - Rattus norvegicus, Mouse - Mus musculus, Chicken - Gallus gallus, Lizard - Anolis carolinensis, Frog - Xenopus (Siluroidea), Bony Fish - Danio rerio and Takifugu rubripes. The Oreochromis niloticus MRAP1 (ENSMUSG00000108774) was reported by Aguileira et al. [1]. The web site http://eshark.gnome.imcb.a-star.edu.sg/, was used to search for MRAP-related sequences in the genome of the cartilaginous fish, Callorhinus milii [29].

Maximum Parsimony Analysis was done using the PAUP 4.0 program (Sinauer). The data set was analyzed using the exhaustive search mode algorithm, and the bootstrap values, based on 1000 replicates, are shown in Fig. 2.

3. Results

3.1. Phylogenetic analysis of vertebrate MRAPs

Fig. 1 presents the alignment of a representative set of MRAP1 and MRAP2 amino acid sequences. In order to align the two sets of paralogs, 23 gaps were required. This is an exceptionally large number for two paralogs gene products. The alignment revealed: (a) sites with high primary sequence identity (at least 70%) in both paralogs (gray), (b) sites with high primary sequence identity (at least 66%) in MRAP1 orthologs only (yellow), (c) sites with high primary sequence identity (at least 80%) in mammalian MRAP1 orthologs only (pink); and (d) sites with high primary sequence identity (at least 75%) in MRAP2 orthologs only (green). Although the overall sequence identity of the two paralogs was only 14%, it should be noted that both paralogs have a N-linked glycosylation site near the C-terminal (position 9; Fig. 1), the amino acid motif Y/WEY in the domain requiring for trafficking MC2R to the plasma membrane, a reasonable degree of sequence identity in the domain required for forming an ortholog specific homodimer (positions 43–54), and 46% of the positions in the transmembrane spanning domain were highly conserved. By contrast, the C-terminal region of the MRAP1 orthologs is highly divergent, and a construct of mammalian MRAP1 lacking the C-terminal domain still was found to be functional [12]. In the C-terminal of the MRAP2 orthologs there are several conserved amino acid motifs (positions shaded in green; Fig. 1). Although the functional significance of these amino acid motifs is unknown, these conserved regions were useful for the alignment of MRAP2 sequences.

Maximum parsimony analysis of the MRAP2 sequences yielded a single most parsimonious tree with a set of branching clades that corresponded to the phylogeny of the vertebrates (Fig. 2A). The bootstrap values for the amniote tetrapod clades were robust. However, the maximum parsimony analysis of the MRAP1 sequences was more complex. This analysis yielded three equally parsimonious trees, and the consensus tree is presented in Fig. 2B. The sequences split into a mammalian clade, a bony fish clade, and the chicken MRAP1 clade did not group with either clade. The bootstrap values for the mammalian clade were strong, and this clade could be further divided into a rodent clade (mouse and rat MRAP1 orthologs) and a primate clade (lemur, monkey, and human MRAP1 orthologs). While the bony fish clade was clearly distinct from the mammalian clade, it was not possible to resolve the phylogenetic relationships of the three bony fish MRAP1 sequences using this algorithm. The position of the chicken MRAP1 sequence is problematic, and was probably influenced by the absence of other non-mammalian tetrapod MRAP1 sequences. Although the Xenopus laevis (http://genome.jgi-psf.org/Xent4/Xent4.home.html) and Anolis carolinensis (http://www.ensembl.org/Anolis.carolinensis) genome projects would appear to be likely sources to augment this analysis, a search of both genome data bases for MRAP1 has not revealed an ortholog for either species. Apart from this caveat, Fig. 2B would appear to indicate a tetrapod/mammalian trend and a bony fish trend in the evolution of MRAP1. From a functional standpoint these trends diverge at positions 30–31 in Fig. 1. The LDL1 motif in mammalian MRAP1 is essential for facilitating the activation of mammalian MC2 receptors following an ACTH binding event [26]. Note that this motif is absent in all MRAP2 sequences (Fig. 1), and is altered in the teleost fish MRAP1 sequences. These observations would suggest that the
activation of a tetrapod MC2 receptor (e.g., frog MC2R) may be more robust in the presence of a tetrapod MRAF1, than in the presence of a fish MRAF1, and the converse may be true for the functional expression of a fish MC2 receptor (rainbow trout). The following experiments were done to test this hypothesis.

3.2. Functional activation of frog MC2 receptor in CHO cells

For this study the functional expression of frog MC2 receptors in CHO cells in the presence of a heterologous mouse MRAF1 (mMRAF1) was compared to the functional expression of the human receptor (hMC2R) in the presence of mMRAF1. For all experiments the CHO cells were transfected with a cAMP-responsive reporter construct, CRE-luciferase, and the MC2 receptor of interest was expressed either in the presence or absence of an MRAF construct. Cells were then challenged with different concentrations of human ACTH[1–24] [hACTH(1–24); Fig. 3A]. In agreement with published data [27], hMC2 receptors responded to subnanomolar concentrations of hACTH(1–24) with a robust cAMP response when hMC2 receptors were co-expressed with mMRAF1.

Since an mMRAF1 ortholog has not been detected in the X. tropicalis genome but an MRAF2 ortholog is present, we tested the hypothesis that the frog receptor (xtMC2R) may only require the Xenopus MRAF2 (xtMRAF2) ortholog for functional expression. Co-transfection of xtMC2 receptors with xtMRAF2 generated a weak response when challenged with different concentrations of hACTH[1–24], and the MC2 receptor behaved in a similar manner when co-transfected with xtMRAF2 (Fig. 3A). However, when xtMRAF2 was co-expressed in CHO cells with mMRAF1, a strong cAMP response was observed. It should be noted that almost 10-fold higher concentrations of hACTH[1–24] were required to activate the xtMC2 receptor as compared to the activation of the mammalian MC2 receptor under similar conditions (Fig. 3A).

The weak response of the hMC2 receptor in the presence of xtMRAF2 could be the result of fewer xtMC2 receptors trafficking to the plasma membrane in the presence of xtMRAF2 as compared to mMRAF1. However, as indicated by the cell surface ELISA (Fig. 4), both mMRAF1 and xtMRAF2 were expressed at equivalent levels on the surface of CHO cells, and both accessory proteins strongly increased the density of hMC2 receptors on the plasma membrane. We assume that xtMC2 receptors were also present on the plasma membrane in equivalent numbers in the presence of either mMRAF1 or xtMRAF2. As shown in Fig. 7D, xtMC2 receptors could be visualized on the surface of CHO cells when co-expressed with mMRAF1.

It is also conceivable that the lower sensitivity of xtMC2 receptors expressed in CHO cells for hACTH(1–24) is an indication that the mammalian cells might lack some unidentified chaperone required for effective targeting by the xtMC2 receptor. To test this hypothesis, we repeated the experiments presented in Fig. 3A in an established Xenopus cell line, the A6 kidney fibroblast line (Fig. 3B) [10]. A6 cells did not transfet well and responses were relatively small, but the results were quite similar to those obtained in the mammalian cells. The xtMC2 receptors responded better to hACTH[1–24] when they were co-transfected with mMRAF1 than with xtMRAF2. Unlike CHO cells, the Xenopus A6 cells displayed a small cAMP response to hACTH[1–24]; in the absence of a transfected receptor or MRAF, suggesting that hACTH(1–24) can activate some endogenous receptors. Responses of xtMC2 receptors in CHO and A6 cells to 10 μM ACTH, presumably a maximally effective concentration, are summarized in Fig. 4B.

Finally, when the xtMC2 receptor was co-expressed with a fish MRAF1 (zebrafish MRAP1; xtMRAP1), there was a decline in the absolute amount of cAMP produced by CHO cells expressing xtMC2 receptors and xtMRAP1, as compared to CHO cells expressing xtMC2 receptor and mMRAF1 (Fig. 5A). In addition, xtMC2

Fig. 3. Functional expression of frog MC2R in CHO cells. Either CHO cells (A) or Xenopus A6 kidney fibroblasts (B) were transfected with cDNA encoding CRE-luciferase with or without hMC2 receptor, xtMC2 receptor, mMRAF1 and/or xtMRAF2. The next day cells were incubated for 5 h with different concentrations of ACTH before measurement of luciferase activity. For expression of MRAF and nMC2R in CHO cells, the EC₅₀ values for ACTH were 1.17 × 10⁻¹³ M and 4.45 × 10⁻¹¹ M with mMRAF1 and xtMRAF2, respectively. In CHO cells expressing xtMC2 receptor, the EC₅₀ was 8.05 × 10⁻¹⁴ M with mMRAF1 but could not be established with no MRAP or xtMRAF2. In Xenopus A6 cells, EC₅₀ values were 7.06 ± 1.11 × 10⁻⁷ M and 6.63 ± 0.22 × 10⁻⁸ M ACTH for xtMC2 receptor expressed with no accessory protein, mMRAF1 and xtMRAF2, respectively.
Fig. 4. ELISA measurements of MRAp and 2MC2R on CHO cells. (A) MRAp and 2MC2R receptor expression. CHO cells were transfected as shown. The levels of HA-2MC2 receptor on the cell surface and total V5-tagged MRAp were measured by ELISA. *P < 0.01 vs. control with GFP alone. (B) Maximal responses of 2MC2 receptors expressed in CHO or Xenopus A6 cells. Cells were transfected as shown, grown overnight, and incubated for 5 h with 10 µM ACTH before measurement of luciferase activity. *P < 0.01 vs. no ACTH.

Fig. 5. Functional expression of frog MCR2 with mMRAP1 or zMRAP1. (A) To determine how the xMC2R would interact with zebrafish (z) MRAp1, equal numbers of CHO cells were either co-transfected with the CRE-luciferase cDNA, xMC2R cDNA, and zMRAP1 cDNA or co-transfected with CRE-luciferase cDNA, xMC2R cDNA, and mMRAP1 cDNA. After 48 h in culture, the cells were incubated with different concentrations of ACTH (1-24) and luciferase activity was measured after 4 h of incubation. The IC50 value for xMC2R/zMRAP1 was nearly 3.5-fold greater than xMC2R/mMRAP1, and the maximum response for xMC2R/zMRAP1 was lower than the response in cells expressing xMC2R/mMRAP1. (B) The levels of V5-tagged xMC2 receptor on the cell surface in the presence of either mMRAP1 or zMRAP1 using an immunofluorescent cell surface assay procedure [36].
receptors showed a 2.5-fold drop in sensitivity to hACTH 1–24 when in the presence of zmMRAP1, as compared to nMRRAP1. The drop in xMGR stimulation by ACTH in cells co-expressing zebrafish MRRAP1 was not due to fewer receptors on the plasma membrane as indicated by the cell surface ELISA analysis (Fig. 5B).

3.3. Functional activation of rainbow trout MC2 receptor in CHO cells

As expected, when a rainbow trout mc2r cDNA construct was transfected into CHO cells in the absence of an mrna construct, there was no functional expression of the receptor (Fig. 6A). An immunocytochemical analysis indicated that rainbow trout mc2r cDNA was expressed in CHO cells, but the immunopositive MC2 receptors were restricted to the cytoplasm, and no MC2 receptor immunoreactivity was apparent on the plasma membrane (Fig. 7A). However, when the rainbow trout mc2r cDNA construct was co-transfected with a mouse mrrap1 cDNA (Fig. 6A), the transfected cells produced cAMP in a dose-dependent manner when stimulated with increasing concentrations of human ACTH 1–24. In agreement with this observation, it was possible to detect patches of rainbow trout MC2 receptor immunoreactivity on the plasma membrane of unpermeabilized transfected CHO cells (Fig. 7C). Note that the distribution of immunoreactive xMGR/zmMRAP1 (yellow) was not uniform on the plasma membrane (Fig. 7C) as compared to the distribution of xMGR/zmMRAP1 (Fig. 7D). However, mouse MRAP1 did facilitate the trafficking of the rainbow trout MC2 receptors to the plasma membrane, and the receptor could be activated by human ACTH 1–24. The EC50 (8.9 × 10⁻⁸ M) for this dose response curve was relatively high.

This outcome may be a function of using a mammalian ACTH 1–24 analog to stimulate the cells instead of a teleost ACTH analog. However, a more plausible explanation may lie with the selection of the MRAP in the co-transfection experiment. When the rainbow trout mc2r cDNA construct was co-transfected with a zebrafish mrrap1 cDNA construct (Fig. 6A) both the EC50 (7.9 × 10⁻¹⁰ M) and ligand saturation levels were significantly higher in response to stimulation by hACTH 1–24, as compared to the co-transfection of CHO cells with rainbow trout MC2 receptor and mouse MRAP1. The dose response curve was in the same range as the co-expression of human MC2 receptor and mouse MRAP1 (Fig. 3). In addition, the rise-in stimulation of xMGR by ACTH in cells co-expressing zebrafish MRRAP1 was not due to an increase in the number of xMGR receptors on the plasma membrane as indicated by the cell surface ELISA analysis (Fig. 6B).

4. Discussion

During the evolution of the MC2 receptor two properties have emerged that set this GPCR apart from the other members of the melanocortin (MC) receptor gene family: exclusive ligand selectivity for ACTH, and the requirement for interaction with MRAP1 in order to achieve full functional activation. The origin of these unique properties has not been resolved. While it is generally accepted that MC receptors are found only in the chordates, and that the ancestral MC receptor gene most likely emerged in an ancestral chordate lineage that predates the origin of the agnathan fishes, no MC receptor orthologs have been found in the genomes of extant protochordates [29]. However, two MC receptor orthologs have been characterized from the genome of an agnathan fish [11], but neither MC appears to be an ortholog of xMGR. Both lamprey MC receptors can be functionally expressed in HEK293 cells in the absence of MRAP, and both MC receptors can bind ACTH, as well as α-MSH and be activated by these ligands [11]. Hence the origin of the mc2r gene must have occurred after the emergence of the ancestral gnathostomes and as a consequence of the genome duplication event that demarcates this stage in the evolution of the chordates [20]. Since unique ligand and MRAP requirements are features common to the MC2 receptor in at least two species of bony fishes [1,16], an amphibian (X. tropicalis), and in mammals,

![Image](https://example.com/fig6.png)

**Fig. 6.** Functional expression of rainbow trout MC2R in CHO cells. (A) To determine the activation parameters of rainbow trout (r) MC2R in the presence of either zMRRAP1 or nMRRAP1, equal numbers of CHO cells were either co-transfected with the CRE-luciferase cDNA, rMCR2 cDNA, and zMRRAP1 cDNA or co-transfected with CRE-luciferase cDNA, xMGR cDNA, and zMRRAP1 cDNA. After 48 h in culture, cells were incubated with different concentrations of ACTH 1–24 and luciferase activity was measured after 4 h of stimulation. The EC50 value for the rMC2R/zMRRAP1 was 300-fold greater than the xMC2R/xMRRAP1, and the ACTH 1–24 response was robust for the rMC2R/zMRRAP1. As a control rMC2R was transfected into CHO cells in the absence of either MRRAP1 cDNA. (B) The levels of VS-tagged rMC2 receptor on the cell surface in the presence of either zMRRAP1 or nMRRAP1 using an immunofluorescent cell surface assay procedure [38].
[6], these traits must have been acquired prior to the divergence of the ancestral Actinopterygii (ray-finned fishes) and the ancestral Sarcopterygii (lobe-finned fishes and tetrapods) over 400 million years ago. An understanding of the transitions that occurred in a proto-MC2R ortholog may still be apparent from studies on the MC receptors in extant cartilaginous fishes [29] or perhaps in the older lineages of ray-finned fishes, such as the extinct chondrosteans and holostean fishes, or in an extant lineage of the lobe-finned fishes, such as the lungfishes [19]. At present, then, it would be reasonable to propose that some, as yet, undetermined feature in the tertiary structure of bony vertebrate MC2 receptors prevents α-MSH and the other MSH ligands from binding and activating the MC2 receptor but accommodates ACTH for this purpose. In addition, and coincidentally, an as yet undetermined feature in the tertiary structure of bony vertebrate MC2 receptors allows for a positive interaction between the MC2 receptor and MRAF1 resulting in activation following an ACH binding event. This study has focused on this MRAF1/MC2R interaction.

The presence of a MRAF2 ortholog in the lamprey genome [29] would indicate that the mrap gene appeared relatively early in the evolution of the vertebrates, but prior to the second genome duplication event that occurred with the emergence of the gnathostomes [20]. Given this time line, the presence of mrap1 and mrap2 paralogs in representatives of the bony fishes, an avian species (Gallus gallus) [29] and in several mammalian genomes is expected (Fig. 8). What was not expected is the apparent absence of an mrap1 ortholog in the genomes of the cartilaginous fish, Callorhinchus milii, the amphibian Xenopus tropicalis [29], and the reptile Anolis carolinensis. Presumably in all three of these lineages an mrap1 orthologous gene has been secondarily lost. As indicated in this study, the assumption of a secondary loss of the mrap1 ortholog in some gnathostome lineages would appear to be, from a functional standpoint, an unsatisfactory explanation as will be discussed presently.

What does emerge from Figs. 1 and 8 is that following the duplication of the ancestral mrap gene, the MRAF1 and MRAF2 paralogs have retained high primary sequence identity in the N-terminal domains required for trafficking MC2R to the plasma membrane (positions 26–29; Fig. 1), in the homodimer domain (43–50; Fig. 1), and in the transmembrane spanning domain (50–68; Fig. 1). Hence as has already been shown for mammals [12,31] and for the zebrafish [1], the trafficking of MC2R from the ER to the plasma membrane can be achieved through an interaction with either MRAF1 or MRAF2, and does not occur if either MRAF paralog...
was not present. Therefore, there must be a domain on mammalian and zebrafish MC2R that blocks with the MRAP2 paralog in the ER, and as a result allows for the trafficking of MC2R out of the ER. Furthermore for full functional activation, the MC2R must be in contact with MRAP1 at the plasma membrane [1,12,31], and this feature appears to reside in the amino acid motif at positions 30–33 (Fig. 1) that is present in the MRAP1 orthologs, but absent in the MRAP2 paralogs.

Given these observations, the apparent absence of an MRAP1 ortholog from the X. tropicalis genome may indicate that: (a) the X. tropicalis (or) MC2 receptor does not require MRAP for trafficking to the plasma membrane and for activation, or (b) X. tropicalis MRAP2 facilitates these functions. However, as revealed in Fig. 3A, expression of xMC2R in CHO cells in the absence of an MRAP results in no activation by hACTH(1–24) because trafficking of xMC2R to the plasma membrane has been blocked (data not shown). In this regard, the X. tropicalis MC2 receptor is behaving in the same manner as a bony fish or mammalian MC2 receptor. In addition when xMC2R was co-expressed with X. tropicalis MRAP2, the receptor could only be marginally activated at the highest dose of hACTH(1–24). In this experiment it appears that X. tropicalis MRAP2 facilitated the trafficking of xMC2R to the plasma membrane, but because X. tropicalis MRAP2 lacks the LDVYI motif this MRAP could not enhance the interaction between xMC2R and hACTH(1–24). However, when xMC2R was co-expressed with mouse MRAP1, trafficking of the receptor to the plasma membrane was effectively achieved (Fig. 7D), and xMC2R responded to hACTH(1–24) in a dose dependent manner (Fig. 5). Furthermore, the X. tropicalis MC2 receptor could only be activated by hACTH(1–24) and not by NDPLYVSH as doses as high as 10–6 M when co-expressed with mouse MRAP1 (data not shown). Clearly the X. tropicalis MC2 receptor has the predicted domain required for interacting with mouse MRAP1. Hence, the combination of xMC2R/mMRAP1 can be used in heterologous expression studies in mammalian cells to analyze the interactions that occur between ACTH and the X. tropicalis MC2 receptor during an activation event. These observations however raise the question of how does the X. tropicalis MC2 receptor function in X. tropicalis interrenal cells if an MRAP1 ortholog is not in the frog genome. Assuming that the X. tropicalis genome project is complete, some other accessory protein may have taken over the function of MRAP1. Other GPCR accessory proteins such as the RAMPs and KTPs have been identified [3,13] and some of these chaperones appear to share a common origin. Thus the intercession of a novel accessory protein is not unprecedented. The experiment with the frog A6 kidney cells was an attempt to address this question. The assumption was that since the frog A6 kidney cells are of mesodermal origin and have a common embryonic origin with the frog adrenocortical cells, perhaps the A6 cells express a similar subset of genes as the frog adrenocortical cells. However, as shown in Fig. 7B, expression of xMC2R in A6 cells in the absence of an endogenous MRAP construct did not enhance the activation dose response curve for xMC2R. Future studies should focus on the characterization of MRAP-related mRNAs from X. tropicalis adrenocortical cells.

When considering the results presented in Fig. 3A, was the activation of xMC2R in the presence of mMRAP1 optimal? The human MC2 receptor was much more sensitive to stimulation by hACTH(1–24) in the presence of mMRAP1 as compared to the xMC2R. This difference in sensitivity may reflect how well xMC2R interacted with the mouse MRAP1. It is noteworthy that when xMC2R was co-expressed with zebrafish MRAP1 in CHO cells (Fig. 5) there was a drop in both sensitivity for hACTH(1–24) and the amplitude of the dose response curve as compared to the xMC2R/mMRAP1 co-transfection. This is another question that could be resolved from studies on frog adrenocortical cells.

The extremes in functional activation patterns were even greater for the heterologous expression of the rainbow trout MC2 receptor. As expected, the rainbow trout MC2 receptor could not be functionally expressed when transfected into CHO cells in the absence of an MRAP construct (Fig. 6); Immunocytochemical analysis of these cells indicated that the rTM2C2R immunoreactivity was restricted to compartments in the cytosol and did not traffic to the plasma membrane (Fig. 7A). In the presence of mouse MRAP1, the rTM2C2R could be activated by hACTH(1–24) in a dose dependent manner, and immunoreactive rTM2C2R could be detected on the plasma membrane of the transfected CHO cells (Fig. 7C). However, the distribution of staining over the surface of the rTM2C2R/mMRAP1 transfected cells (Fig. 7C) was more diffuse than the staining
pattern observed for the xMC2R/mIRAP1 transfected cells (Fig. 7D). It would appear that the interaction between the xMC2R and the mammalian MIRAP1 is functional, but not optimal. In support of this conclusion, the co-transfection of rainbow trout MC2 receptor and the zebrafish MIRAP1 in CHO cells yielded a robust dose response curve for hACTH (1-24) (Fig. 6).

Collectively, these experiments would suggest that the co-evolution of the xMC2R gene and the MIRAP1 gene has diverged to yield a bony fish trend with respect to the interaction between xMC2R and MIRAP1 and a tetrapod trend with respect to the interaction between xMC2R and MIRAP1. A corollary to this hypothesis is that there may be a further bifurcation of the tetrapod MIRAP2/MIRAP1 trend between non-mammalian and mammalian tetrapods. Establishing the critical regions in bony fish and tetrapod MC2Rs that contribute to the ligand selectivity properties of these MC2Rs and their respective interactions with their lineage specific MIRAP1 can be effectively explored through the heterologous expression in mammalian cells. For each MC2R studied using expression in heterologous mammalian cells, it will be important to analyze ACTH sensitivity by the adrenal/interrenal cells of that species. The possible presence of novel MC2R accessory proteins, and the potential interaction between MC2R and MCR5 in some adrenal/interrenal cells, as documented for the barfinnounder kidney [16], add to the complex interactions involved in the regulation of the hypothalamic/pituitary/adrenal/interrenal axis.

Finally, it should be noted that well characterized, heterologous expression systems such as CHO cells are widely used to study receptor signaling. Advantages include relatively easy transfection procedures, high levels of expression of receptor, accessory proteins and reporters, and strong CAMP-dependent responses. The ACTH responses detected in CHO cells in this study were entirely dependent on the transfected receptors introduced into the CHO cells. Homologous cell lines are not available for several of the species of receptors and accessory proteins tested in this study. Hence these studies using CHO cells have provided insights into how xMC2R and xMC5R could potentially perform when endogenously expressed in interrenal cells, and are useful for designing experiments to evaluate endogenous expression in frog and fish interrenal cells, respectively.

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References

APPENDIX 3
Identification of an MRAP-Independent Melanocortin-2 Receptor: Functional Expression of the Cartilaginous Fish, Callorhinchus milii, Melanocortin-2 Receptor in CHO Cells

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Phylogenetic analyses indicate that the genome of the cartilaginous fish, Callorhinchus milii (elephant shark), encodes a melanocortin-2 receptor (MC2R) ortholog. Expression of the elephant shark mc2r cDNA in Chinese hamster ovary (CHO) cells revealed that trafficking to the plasma membrane and functional activation of the receptor do not require coexpression with an exogenous melanocortin receptor-2 accessory protein (MRAP) cDNA. Ligand selectivity studies indicated that elephant shark MC2R-transfected CHO cells produced cAMP in a dose-dependent manner when stimulated with either human ACTH (1–24) or [Nle9,α-Phε9]-MSH. Furthermore, the order of ligand selectivity when elephant shark MC2R-transfected CHO cells were stimulated with cartilaginous fish melanocortins was as follows: ACTH (1–24) > γ-MSH > α-MSH > β-MSH. Elephant shark MC2R is the first vertebrate MC2R ortholog to be analyzed that does not require melanocortin receptor-2 accessory protein 1 for functional activation. In addition, elephant MC2R is currently the only MC2R ortholog that can be activated by either ACTH- or MSH-sized ligands. Hence, it would appear that MC2R dependence on melanocortin receptor-2 accessory protein 1 for functional activation and the exclusive selectivity of this melanocortin receptor for ACTH are features that emerged after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes more than 400 million years ago. (Endocrinology 153: 0000–0000, 2012)
fishing, one of the stem ancestral gnathostome groups (13). Studies on the shark, *Squalus acanthias* (14, 15), revealed the presence of orthologs of MC3R, MC4R, and MC5R in the *S. acanthias* genome. However, a recent analysis of the genome project for the holoccephalan cartilaginous fish, *Galeorhinus miliaris* (elephant shark) uncovered orthologs only for MC1R, MC2R, and MC3R (10). The identity of the elephant shark MC2Rs was based on a maximum likelihood analysis, which placed each elephant shark sequence in a clade with the corresponding human MCR ortholog (10). In this regard, the detection of a putative elephant shark MC2R was of particular interest since previous studies on two species of elasmobranch sharks had not detected an MC2R ortholog in the genomes of these cartilaginous fishes (14, 15, 16). Maximum parsimony analysis of elephant shark MC2R, human MC4Rs, and zebrafish MCRs (Fig. 1; Supplemental Fig. 1) published on The Endocrine Society’s Journals Online website at http://endo.endojournals.org also placed the elephant shark MC2R in the same clade with human and zebrafish MC2R.

A comparison of several gnathostome MC2R sequences (Supplemental Fig. 2) indicates that elephant shark MC2R has several features in common with the gnathostome MC2R including a short N-terminal domain with two N-linked glycosylation sites, a short C-terminal domain, and at least 50% primary sequence identity in transmembrane regions 6 and 7, intracellular loops 1 and 2, and extracellular loop 3. However, these sequence comparisons do not reveal whether elephant shark MC2R has the same pharmacological properties as teleost or tetrapod MC2 receptors. Working on the assumption that all vertebrate MC2R orthologs are MRAPI dependent and can be activated only by ACTH but not by α-MSH, a V-5 epitope-tagged elephant shark mc2r cDNA was transiently transfected into a heterologous mammalian cell expression system [Chinese hamster ovary (CHO) cells] in the presence and absence of either mammalian or teleost MRAPI orthologs or an elephant shark MRAP2 ortholog, and ligand selectivity analyses were done with either mammalian melanoctinins or cartilaginous fish melanocinins. These experiments revealed that the elephant shark MC2R is an MRAP-independe MCR that can be activated by either ACTH or MSH-sized ligands with varying degrees of efficacy.

### Materials and Methods

**MC2R and MRAPI constructs**

Elephant shark (*C. miliaris*) MC2R (accession no. FAA704.1) and human MC2R (accession no. AA067714.1) were synthesized by GenScript (Piscataway, NJ) with an N-terminal V-5 epitope tag and inserted into a pcDNA3.1+ vector. Mouse (Mus musculus) MRAPI (accession no. XM_028644), zebrafish (Danio rerio) MRAPI (accession no. XM_117835), and elephant shark (*C. miliaris*) MRAPI2 (accession no. BR000861) were individually synthesized by GenScript with an N-terminal FLAG epitope tag and were separately inserted into pcDNA3.1+ vectors.

**Tissue culture**

The experiments were done in transiently transfected CHO cells (American Type Culture Collection, Manassas, VA). The CHO cells were grown at 37 °C in a humidified 5% CO2 incubator in Kainh's modification of Ham's F-12 (2 mM glutamine, 1500 mg/liter bicarbonate) with 10% fetal bovine serum, 5 ml penicillin/streptomycin, and 1 ml normocin.

**Immunofluorescence microscopy**

CHO cells were grown in two-well chamber slides (1 × 10^5 cells/well) for 24 h before transfection. The cells were transfected with 1 μg of *elephant shark mc2r* cDNA construct using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and Opti-MEM (Cellgro, Manassas, VA). After 24 h in culture, the cells were fixed with 4% paraformaldehyde for 15 min, and the immunofluorescence analysis was done on permeabilized and nonpermeabilized cells (Fig. 2) as described previously (8). The primary antibody, a mouse monoclonal anti-V5 antibody, was diluted 1:100 in PBS + 1% BSA solution. The cells were incubated with the primary antibody for 1 h at 37 °C. After a wash step, the cells were incubated with the secondary antibody, donkey anti-mouse antibody linked to Alexa Fluor 488 (1:400 in PBS + 1% BSA) for 45 min at 37 °C to visualize MC2R immunofluorescence (Fig. 2, green). Coverslips were mounted onto slides using Vectashield (Vector Laboratories, Burlington, CA), and nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) (Fig. 2, blue). The
control for this experiment was the absence of immunofluorescence in permeabilized transfected cells following incubation with the V5-specific secondary antibody only (Fig. 3A).

To demonstrate that the clone of CHO cells used in this study lacks endogenous MRAP, cells were transfected with a human MC2R cDNA (1 μg) as described above, and immunofluorescence was done on nonpermeabilized cells (Fig. 3B) and permeabilized cells (Fig. 3C). Finally, to demonstrate that exogenous MRAP can be functionally expressed in the clone of CHO cells used in this study, cells were cotransfected with a human MC2R cDNA (1 μg) and a mouse MRAP cDNA (1 μg) as described previously (8), and immunofluorescence was done on nonpermeabilized cells (Fig. 3D–F). Images were obtained using a ×100 oil immersion objective (Zeiss Plan-NEOFUAR; Carl Zeiss, New York, NY) with a fluorescence microscope equipped with a Hamamatsu digital camera (Hamamatsu, Bridgewater, NJ) and a Sutter excitation filter wheel with Semrock filters (Sutter Instruments, Novato, CA). All images were analyzed using SlideBook software (Intelligent Imaging Innovations, Inc, Denver, CO).

Functional expression assays

CHO cells were transfected with 2 μg each of elephant shark mc2r cDNA and cre-luc reporter plasmid (17) either with an mrap cDNA construct or in the absence of an mrap cDNA construct using a cell line transfection kit (Amresco, Inc, www.amos-cco.com) with solution C and program U-023. The cells were then plated on a white, 96-well plate at a density of 1 × 10^5 cells/well. After 48 h in culture, the transfected cells were stimulated with melanocortin ligands in serum-free CHO media for 4 h at 37 C at concentrations ranging from 10^-17 to 10^-22 M. After the incubation period, 100 μl of Bright-Glo luciferase assay reagent (Promega Inc, Madison, WI) was applied to each well and incubated at room temperature for 5 min. Luminescence was then measured with a Bio-Tek Synergy HT plate reader (Winooski, VT). The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: human ACTH(1–24), [Nle6, d-Phe1] (N-terminal) α-MSH, and the dogfish (S. acanthias) melanocortins ACTH(1–24), α-MSH, β-MSH, γ-MSH, and δ-MSH (18).

Data analysis

All experimental treatments were performed in no less than triplicate and then corrected for control values, which were obtained by using transected cells that were left unstimulated. In each of the assays, maximal activation levels were between 3 and 10 times the control levels. The values in the luciferase assays were normalized to the average readings for cells treated with 1 μM human ACTH(1–24) (Figs. 4 and 5A and Supplemental Fig. 3) or 1 μM dogfish ACTH(1–24) (Fig. 5B). Average values and SEM were graphed using KaleidaGraph software (Synergy Software, Reading, PA), and the EC50 value for each ligand was determined. The EC50 values were compared by using the Student’s t test.

Maximum parsimony analysis

Maximum parsimony analysis was done using the branch and bound analysis algorithm (PAUP* 4.0). The following MCR amino acid sequences were analyzed: human MCR (AA441335.1), MC2R (AA677714.1), MC5R (AA727261.1), MC4R (AA092061.1), MC3R (NP_003590.1), and zebrafish MCR1 (AA162848.1), MC2R (NP_775386.1), and MC3R (NP_775386.1). The sequence of Lampetra fluviatilis MCR (lamprey: ABB36647.1) was used as the outgroup. The bootstrap values in Fig. 1 were derived from 1000 replications.

Results

Immunofluorescence analysis of elephant shark MC2R expressed in CHO cells

To determine the parameters for expressing elephant shark MC2R in CHO cells, the V5-labeled elephant shark mc2r cDNA construct was transfected into CHO cells and V5 immunofluorescence was detected in permeabilized cells (data not shown). We also checked for V5 immunofluorescence in cells in which the transfected cells were not permeabilized. The operating assumption was that no immunofluorescence would be detected on the surface of the nonpermeabilized transfected CHO cells. However, as indicated in Fig. 2, V5 immunofluorescence was detected on the surface of the transfected CHO cells. Because CHO cells do not express an endogenous mrap gene (19), and no exogenous mrap cDNA was cotransfected with the elephant shark mc2r cDNA construct, it appears that the trafficking of the elephant shark MC2 receptor from the endoplasmic reticulum to the plasma membrane does not involve interaction with an MRAP. As an indication of the specificity of the V5 secondary antibody, no immunofluorescence was detected when elephant shark MC2R-transfected CHO cells were incubated with the V5-specific secondary antibody alone (Fig. 3A). To demonstrate that our clone of CHO cells does not express endogenous mrap
genes, cells were transfected with a human mc2r cDNA, and no immunofluorescence was detected on the surface of nonpermeabilized cells (Fig. 3B). However, immunofluorescence was detected in permeabilized cells, which indicated that the hmc2r cDNA was expressed (Fig. 3C).

Finally, to demonstrate that exogenous MRAP can be functionally expressed in our clone of CHO cells, cells were cotransfected with a hmc2r cDNA and a mouse mrap1 cDNA. This experiment indicated that colocalization of the immunoreactive human MC2R and the mouse MRAP1 was apparent on the plasma membrane of nonpermeabilized cells (Fig. 3, D–F).

**Coexpression of elephant shark MC2R with MRAP cDNA constructs**

To determine whether coexpression with MRAP1 would affect the activation of elephant shark MC2R, CHO cells were first cotransfected with an *elephant shark* mc2r cDNA construct and either a mouse mrap1 (mMRAP1) cDNA construct or a zebrafish mrap1 (zMRAP1) cDNA construct (Fig. 4). The mouse and zebrafish MRAP1 were used in this experiment due to the fact that a MRAP1 ortholog has not been found in the elephant shark genome database (10), yet mMRAP1 and zMRAP can be used for functional activation of teleost MC2R (6, 8). The transfected cells were stimulated with human ACTH (1–24) in a dose-dependent manner (Fig. 4). Note that coexpression of elephant shark MC2R with either MRAP1 cDNA had no effect, either positive or negative, on activation. In this experiment the EC50 value for elephant shark MC2R expressed alone was 5.2 ± 1.0 × 10−10 M, and in presence of mMRAP1 or zMRAP1 the EC50 values were 5.6 ± 1.1 × 10−10 M and 8.9 ± 1.9 × 10−10 M, respectively. CHO cells were also cotransfected with the *elephant shark* mc2r cDNA construct and an *elephant shark* mrap1 cDNA construct and stimulated with human ACTH (1–24) (Supplemental Fig. 3). However, the presence of the elephant shark MRAP2 had no effect, either positive or negative, on the EC50 value.

**Ligand selectivity of elephant shark MC2R**

In a subsequent experiment, CHO cells were cotransfected with the *elephant shark* mc2r cDNA construct and stimulated with either human ACTH (1–24) or NDP-MSH. The operating assumption was that NDP-MSH
would have no effect on the elephant shark MC2R ortholog. However, NDP-MSH activated the elephant shark MC2R in a dose-dependent manner (Fig. 5A). The EC$_{50}$ value for the human ACTH (1–24)-stimulated cells was 6.3 $\pm$ 2.5 $\times$ 10$^{-10}$ M, and the EC$_{50}$ value for the NDP-MSH-stimulated cells was 1.3 $\pm$ 0.6 $\times$ 10$^{-7}$ M. Although human ACTH (1–24) was clearly the more potent ligand ($P < 0.05$), it is unprecedented for a MC2R ortholog to have any sensitivity for an MSH-sized ligand.

The potency of various cartilaginous fish melanocortins was also tested on CHO cells transfected with elephant shark MC2R. For this experiment the S. acanthias (dogfish) melanocortins ACTH (1–25), $\alpha$-MSH, $\beta$-MSH, $\gamma$-MSH, and $\delta$-MSH (18) were tested at concentrations ranging from 10$^{-5}$ to 10$^{-11}$ M (Fig. 5B). The EC$_{50}$ values for the dogfish melanocortins were as follows: ACTH (1–25), 4.3 $\pm$ 1.4 $\times$ 10$^{-10}$ M; $\gamma$-MSH, 6.3 $\pm$ 1.8 $\times$ 10$^{-9}$ M; $\delta$-MSH, 1.1 $\pm$ 0.2 $\times$ 10$^{-8}$ M; $\alpha$-MSH, 1.4 $\pm$ 0.5 $\times$ 10$^{-7}$ M; and $\beta$-MSH, 4.7 $\pm$ 1.4 $\times$ 10$^{-7}$ M. Although the EC$_{50}$ values for ACTH (1–25), $\gamma$-MSH, and $\delta$-MSH were not statistically different, ACTH (1–25) was clearly a more potent ligand than either $\alpha$-MSH or $\beta$-MSH ($P < 0.01$). The order of sensitivity for the dogfish melanocortins was ACTH (1–25) = $\gamma$-MSH = $\delta$-MSH > $\alpha$-MSH = $\beta$-MSH.

### Discussion

The distribution of melanocortin receptor paralogs and a MRP2 paralog in extant cartilaginous fishes presents a number of unresolved questions. The model for the evolution and radiation of melanocortin receptor paralogs predicts that five MCR genes may have been present in the ancestral gnathostomes (10). Among the extant groups of gnathostomes (i.e. cartilaginous fishes, ray finned fishes, lobe finned fishes, and tetrapods), the presence of five MCR paralogs within individual species in each group has been confirmed with the exception of the cartilaginous fishes. Among these gnathostomes, cDNA were cloned from the genome of the elasmobranch, S. acanthias, which corresponded only to orthologs of MC3R, MC4R, and MC5R, respectively, whereas, an in silico analysis of the genome of the holoccephalan, C. mili (elephant shark), revealed only the presence of orthologs of MC1R, MC2R, and MC3R, respectively. If in fact the ancestral gnathostomes had five paralogous MCR genes, then it would appear that gene loss has played a significant role in the selection of MCR paralogs in extant cartilaginous fishes.

Previous studies on the elasmobranch (sharks and rays) MCR (i.e. MC3R, MC4R, and MC5R) indicated that ACTH is more potent than any of the elasmobranch MSH (i.e. $\alpha$-MSH, $\beta$-MSH, $\gamma$-MSH, and $\delta$-MSH) at activating these receptors (20, 21). It is known that corticotropic cells of the anterior pituitary of elasmobranches express the pome gene, and these cells synthesize ACTH (18, 22). It is also known that melanotropic cells of the intermediate pituitary of elasmobranches also express the pome gene yet make the various MSHs (18, 23). Because elasmobranch MCR can be activated by either ACTH or the MSH, the mechanism for regulating the production of glucocorticoids by the interrenal glands of sharks and rays appears to be more complex than in the bony vertebrates. The receptor on the elasmobranch interrenal gland responsible for initiating glucocorticoid synthesis has not been identified. However, based on the current possibilities (i.e. MC3R, MC4R, or MC5R), activation of interrenal (HPA/I) cells cannot be regulated by a single melanocortin as seen in hypothalamus-pituitary-adrenal/hypothalamus-pituitary-interrenal axes of teleosts and tetrapods (3, 6, 24). Because agouti gene-related peptide (AGRP) have been detected in the genome of at least one cartilaginous fish (10), perhaps these polypeptides play a role in regulating glucocorticoid synthesis. Alternatively, another polypeptide/G protein-coupled receptor combination may regulate glucocorticoid synthesis in elasmobranches. In this regard, a recent study on an angiotensin II receptor expressed by stingray head kidney cells (interrenal tissue) is relevant (25). Activation of this receptor will...
result in glucocorticoid production. It would appear that multiple mechanisms may be present in elasmobranch interrenal cells to regulate the production of glucocorticoids. However, studies to delineate these mechanisms have not been done.

The current study has focused on the pharmacological properties of an MC2R ortholog in the genome of the holoceratophalan cartilaginous fish, C. mili. The identity of this receptor as a MC2R ortholog was based on a maximum likelihood analysis (10) and was supported by maximum parsimony analysis of a similar data set of MCR amino acid sequences (Fig. 1). However, the elephant shark MC2R ortholog has properties that are very different from either teleost or tetrapod MC2R orthologs. Immunofluorescence analyses and functional expression assays done in CHO cells indicated that the elephant shark MC2 receptor does not require MRAP for either trafficking to the plasma membrane (Fig. 2) or functional activation by human ACTH (1–24) (Fig. 4). Indeed, cotransfection with teleost or tetrapod MRAP or an elephant shark MRAP2 ortholog had no effect, either positive or negative, on the functional activation of the receptor (Fig. 4 and Supplemental Fig. 3). Although these data do not reveal a function for the elephant shark MRAP2 ortholog, collectively these results indicate that the elephant shark MC2 receptor is an MRAP-independent MC2R ortholog.

This conclusion is not unprecedented. As presented in the introductory text, the working hypothesis for the evolution of the melanocortin receptor gene family (9, 10) is that the ancestral melanocortin gene emerged in an ancestral, and now extinct, protostome lineage. Following the first chordate genome duplication event (1R), two paralogous MCR-like genes are predicted to have been present in the ancestral agnathans, and following the second chordate genome duplication event (2R), four paralogous melanocortin receptor genes are predicted to have been present in the ancestral gnathostomes. A local gene duplication of one of the paralogous melanocortin receptor genes in the ancestral gnathostome genome is predicted to account for the presence of a fifth paralogous melanocortin receptor gene in the stem gnathostome classes (i.e., the cartilaginous fishes, the ray finned fishes, and the lobe finned fishes and tetrapods) (10). In support of this hypothesis, two paralogous MCR cDNAs (MCa and MCb) have been characterized from the lamprey genome (26). Phylogenetic analyses indicate that MCa is a MC1R ortholog and MCb is a MCR ortholog. The lamprey MCa receptor has been functionally expressed in human embryonic kidney-293 EBNA cells without the need for cotransfection with an exogenous mrap cDNA. Note that no MC2R orthologous gene has been detected in the lamprey genome.

With respect to the other cartilaginous fish melanocortin receptors (i.e., dogfish MC3R, MC4R, and MC5R), all of these receptors can be functionally expressed in heterologous mammalian cells without cotransfection with an exogenous mrap cDNA (14, 15, 21). When all these observations are taken collectively, two hypotheses emerge: 1) the MC2R gene most likely originated with the ancestral gnathostomes before the radiation of the three stem classes of gnathostomes; and 2) melanocortin receptors in agnathans and in the ancestral gnathostomes were MRAP independent. Based on these hypotheses, we predict that the MRAP-independent nature of the elephant shark MC2R was an ancestral feature rather than a derived trait.
brane and for functional activation of the MC2 receptor must have evolved after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes. Current understanding of the dependence of the MC2R on MRAP indicates that a complex forms between two MRAP homodimers and a MC2R homodimer (27). Although the MC2R trafficking motif and functional activation motif on MRAP have been identified (28, 29), the corresponding contact site on the MC2 receptor for interaction with MRAP has not been determined for either teleost or tetrapod MC2R.

Another major difference between elephant shark MC2R and teleost and tetrapod MC2R is with respect to ligand selectivity. There is an extensive literature on the exclusive selectivity of the adrenal cortex (i.e., MC2R) receptor for ACTH and the inability of α-MSH to activate this receptor (30) or even to act as a competitive inhibitor of ACTH binding to the receptor (31). Although ACTH (1-24) was a more potent stimulator of elephant shark MC2R than any of the MSHs that were tested, the fact remains that the receptor could be activated by NDP-MSH or the dogfish fish MSH in a dose-dependent manner (Fig. 5, A and B). A similar preference for ACTH-sized ligands compared with MSH-sized ligands has been observed for the lamprey melanocortin-a (MCa) receptor (26), and the S. acanthias (dogfish) MC3R, MC4R, and MC5R (14, 15, 21).

Clearly the HFRW binding site on the elephant shark MC2R can accommodate either ACTH or the MSH. This is a feature that has been observed for teleost and tetrapod MC1R, MC3R, MC4R, and MC5R but not for a teleost or tetrapod MC2R ortholog. Some understanding of the ligand selectivity of the elephant shark MC2R receptor ortholog relative to the teleost and tetrapod MC2R orthologs may be apparent from a comparison of the amino acid sequences of human MC2R, human MC4R, and elephant shark MC2R (Fig. 6A).

The mammalian and cartilaginous fish lineages last shared a common ancestor well over 400 million years ago (32); hence, it would be assumed that the number of identical positions in the human MC4R sequences relative to the teleost and tetrapod MC2R sequence would be few. In fact, only 33% of the amino acid positions in this alignment are identical in all three sequences (Fig. 5A, residues shaded gray). As indicated by the red shaded amino acid positions, an additional 24 positions are identical in the human and teleost and tetrapod MC2R orthologs. However, there are 29 positions (shaded blue; Fig. 6A) that are identical for human MC4R and elephant shark MC2R. Clearly these positions did not influence the positioning of the teleost and tetrapod MC2R in the maximum parsimony analysis (Fig. 1) but may play a role in ligand selectivity. There are some unexpected similarities between human MC4R and elephant shark MC2R that are worth noting.

All melanocortin receptors have a binding site to accommodate the HFRW motif of the melanocortin ligands (for review see Ref. 3). Pogorzhea et al. (33) used a computer modeling strategy and a site-directed mutagenesis paradigm to identify 11 critical amino acids in human MC4R that form the HFRW binding site. These residues are marked with a star in Fig. 6A. Note that the human MC2R sequence has eight of these conserved amino acid positions. However, the, 123
The additional conserved amino acid positions are found in transmembrane region 3 at residues 132 and 133. These two residues are also present at the same position in nearly every MC1R, MC3R, MC4R, and MC5R that has been characterized (16), including the two lamprey melanocortin receptor paralogs (26). Hence, it would be reasonable to assume that residues in transmembrane (TM) 2, TM3, TM6, and TM7 were also present in the ancestral vertebrate MCR. However, these two amino acid positions are not found in MC2R orthologs (Supplemental Fig. 2)). Furthermore, ACTH binding to a teleost or tetrapod MC2R ortholog also requires accommodation of the K/RKRR motif (29), which is present in the ACTH sequence of gnathostomes (34).

Assuming that the transmembrane regions of melanocortin receptors are oriented as shown in Fig. 6B to form the HPRW binding site, then the likely site for the K/RKRR binding site would be in the TM4, extracellular loop 2 (EC2), or TM5 portion of the receptor. In these regions of the receptor (Fig. 6A, blue box), it appears that the elephant shark MC2R is more MC4R-like than MC2R-like. Amino acid substitutions in the TM4/EC2/TM5 during the early radiation of ancestral bony fishes could have altered the three dimensional shape of the receptor to create the K/RKRR binding site. Thus, studies on some of the older lineages of the bony fishes may shed light on these changes. However, the ability of the teleost and tetrapod MC2 orthologs to exclude the MSH indicates that these receptors must have a more dynamic activation mechanism, which involves a confirmation change when the appropriate ligand (i.e. ACTH) makes contact with the receptor. In this regard, characterization of the K/RKRR binding on MC2R orthologs will be important in deciphering the mechanism of activation.

Although the phylogenetic analyses indicate that the elephant shark MC2R is orthologous to the teleost and tetrapod MC2R, from a functional perspective, elephant shark MC2R has properties that are distinct from these bony vertebrate MC2R orthologs. It would appear that after the divergence of the ancestral cartilaginous fishes and the ancestral bony fishes, significant changes occurred to the ancestral bony fish MC2R ortholog, which have influenced the regulation of interrenal cells and adrenal cortex cells in teleosts and tetrapods.

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Functional expression of *Squalus acanthias* melanocortin-5 receptor in CHO cells: Ligand selectivity and interaction with MRAP

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**A B S T R A C T**

The melanocortin-5 receptor (MC5) of the dogfish *Squalus acanthias* (SocMC5 receptor) can be functionally expressed in CHO cells in the absence of the co-expression of an exogenous MRAP cDNA. Both human ACTH(1-24) and dogfish ACTH(1-25) were much better stimulators of the SocMC5 receptor than any of the mammalian or dogfish MSH ligands that were tested. The order of ligand selectivity for the dogfish melanocortin was ACTH(1-25) > α-MSH > β-MSH > γ-MSH. Unlike mammalian MC5 receptors, the functional expression of the SocMC5 receptor was not negatively impacted when the receptor was co-expressed with a cartilaginous fish (Callionymus milii) MRAP2 cDNA. However, co-expression with either mouse mMRAP1 or zebrafish zMRAP1 increased the sensitivity of SocMC5 receptor for ACTH(1-24) by at least one order of magnitude. Hence, SocMC5 receptor has the potential to interact with MRAP1 orthologs and in this regard behaved more like a melanocortin MC5 receptor ortholog than a melanocortin MC5 receptor ortholog. These observations are discussed in light of the evolution of the melanocortin receptor gene family in cartilaginous fish, and the physiological implications of these observations are considered.

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1. Introduction

The functional expression of melanocortin receptors in heterologous mammalian cells is a useful procedure for analyzing the ligand selectivity and trafficking requirements of these G-protein coupled receptors. In mammals there are five receptors in the melanocortin receptor gene family (MC1 receptor, MC2 receptor, MC3 receptor, MC4 receptor, and MC5 receptor) and these receptors are activated with varying degrees of efficacy by the melanocortin peptides (ACTH, α-MSH, β-MSH, and γ-MSH) (Conn, 2006). Orthologs of both mammalian and fish MC6 receptor, MC7 receptor, MC8 receptor, MC9 receptor, and MC10 receptor have been functionally expressed in mammalian HEK293 cells (Agulliero et al., 2010; Conn, 2006; Klovins et al., 2004a; Ringholm et al., 2002). Hence, following the cloning of orthologs of MC5 receptor, MC6 receptor, and MC8 receptor from the genome of the cartilaginous fish, *Squalus acanthias* (Sac), cDNA constructs of dogfish melanocortin receptors were individually transfected into HEK293 cells (Klovins et al., 2004a; Ringholm et al., 2003). Although this approach resolved the ligand selectivity properties of the SacMC5 receptor and the SacMC6 receptor (Hatina et al., 2007a; Klovins et al., 2004b; Ringholm et al., 2003), the SacMC8 receptor-transfected HEK293 cells could not be stimulated by any melanocortin ligand (Ringholm et al., 2003). Subsequent transfection of a SacMC5 receptor-EGFP fusion protein cDNA construct into HEK293 cells revealed that the fusion protein did not move to the plasma membrane, but was retained in intracellular compartments (Klovins et al., 2004b).

The inability of the SacMC5 receptor to move to the endoplasmic reticulum to the plasma membrane in HEK293 cells may have been the result of improper folding of the SacMC5 receptor in the endoplasmic reticulum (Tan et al., 2003). However, a comparison of the SacMC5 receptor amino acid sequence to other vertebrate MC5 receptor sequences (Fig. 1A) does not immediately reveal any obvious alterations in the primary sequence of the SacMC5 receptor that might have induced improper folding of this receptor, and minimum parsimony analysis (Fig. 1B) indicated that SacMC5 receptor is the sister sequence to a clade of bony vertebrate MC5 receptor sequences. It is possible that the functional expression of the SacMC5 receptor in HEK293 cells may require interaction with an accessory protein to facilitate trafficking to the plasma membrane in a manner analogous to the interaction between mammalian and bony fish melanocortin-2 receptors (MC2 receptors) and melanocortin receptor accessory protein (MRAP) (Agulliero et al., 2010; Mithierr et al., 2005; Roy et al., 2007; Sebag and Hindle, 2007, 2009a; Webb et al., 2009). However, studies on the interaction between mammalian MC5 receptor and MRAP indicated that trafficking of this melanocortin receptor to the plasma membrane is actually inhibited following co-transfection with either MRAP1 or MRAP2 (Sebag and Hindle, 2009a).

Given these various possibilities, the following study was done to determine whether the SacMC5 receptor could be functionally expressed in Chinese Hamster Ovary (CHO) cells. The recent discovery of an MRAP2 ortholog in the genome of the cartilaginous fish, *Squalus acanthias*,
Fig. 1. Alignment of vertebrate M3 receptor amino acid sequences. A) The amino acid sequences of Homo sapiens (mMCLR; NP_005904), Mus musculus (mMCLR; AA007211), Galago gallar (cSERS; NP_001268386), Xenopus tropicalis (xSERS; NP_001268386), Andinus flavescens (cSERS; AAI24736), and Squalus acanthias (sSERS; AA052990) were aligned as described previously (Smith et al., 1996). Amino acid positions shaded in gray were identical in 80% of the taxa. N-linked glycosylation sites are underlined. (*) denotes an amino acid position that is in the proposed HR2W binding site of the receptor (Pogosheva et al., 2005). Abbreviations: TM—transmembrane spanning domain; EC—extracellular loop; IC—intracellular loop. B) Maximum parsimony analysis was done using the exhaustive analysis algorithm (PAUP 4.0). The sequence of Lagenorhynchus cruciger receptor (LMCAG; AAI26647.) was used as the outgroup. The bootstrapping values in bold were derived from 1000 replications.
Callorhinchus milii (Cm) (Vastermark and Schüth, 2011) provided the opportunity to test the hypothesis that co-transfection of a SacMCR receptor cDNA with a CmMRAP2 cDNA would influence the functional expression of the SacMCR receptor in CHO cells. Finally, once the conditions for expressing the SacMCR receptor in CHO cells were resolved, the primary objective of this study was to determine the ligand selectivity of the SacMCR receptor using both mammalian and dogfish metanol cortin ligands.

2. Materials and methods

2.1. MC$_5$ receptor and MRAP constructs

Spiny dogfish (S. acanthias) MC$_5$ receptor, accession number AY562212, was synthesized by GenScript (Piscataway, NJ) with a V-5 epitope tag, and the cDNA was inserted into a pcDNA3.1 vector. Mouse (Mus musculus) mMRAP1, accession number NM_029644, zebrafish (Danio rerio) zMRAP1, accession number XR_177835, and elephant shark (C. milii) CmMRAP2, accession number BR000866, were also synthesized by GenScript with a FLAG epitope tag, respectively, and each cDNA was separately inserted into pcDNA3.1 vectors.

2.2. Tissue culture

The experiments were all done in transiently transfected CHO cells (ATCC, Manassas, VA). The CHO cells were grown at 37 °C in a humidified 5% CO$_2$ incubator in Kainoh’s Modification of Ham’s F-12 (2 mM glutamine, 1500 mg/l bicarbonate; ATCC, Manassas, VA) with 10% fetal bovine serum, 5 ml pen/strep, and 1 ml normocin.

2.3. Immunocytochemistry

CHO cells were grown in 2-well chamber slides (1 × 10$^5$ cells/well) for 24 h prior to transfection. The cells were transfected with 1 μg of dogfish $mc_5$ receptor cDNA construct using lipofectamine 2000 (Invitrogen; www.invitrogen.com) and Opti-MEM (Gelgro, Manassas, VA). Twenty-four hours post-transfection, the cells were fixed with 4% paraformaldehyde for 15 min then permeabilized using 0.3% Triton for 10 min (Fig. 2A) or left unpermeabilized (Fig. 2C). The primary antibody was diluted 1:100 in PBS + 1% BSA solution. A mouse monoclonal anti-V5 antibody was used to detect SacMCR. The cells were incubated with the primary antibodies for 1 h at 37 °C. After a wash step, the cells were incubated with secondary antibody for 45 min at 37 °C. A donkey anti-mouse antibody linked to Alexa Fluor 488 was used to visualize SacMCR (green). The secondary antibody was diluted 1:400 (donkey anti-mouse) in PBS + 1% BSA. Cover slips were mounted onto slides using Vectashield (Vector Lab, Burlingame, CA), and nuclei were stained with DAPI (blue). The controls for this experiment were the absence of staining in non-transfected cells following incubation with the primary and secondary antibodies (Fig. 2B), and the absence of staining in transfected cells following separate incubation with the secondary antisera (Fig. 2D). Images were obtained using a 100× oil immersion objective (Zeiss Plan-NEOFAR) with a fluorescence microscope equipped with a Hamamatsu digital camera. All images were analyzed using Slidebook (www.slidebook.com) software.

2.4. Functional expression assays

CHO cells were transfected with 2 μg each of SacMCR receptor, MRAP, and CRE-Luc reporter plasmid (Chepurin and Holt, 2007) using a Cell Line Nucleofector Kit (Amaxa Inc.; www.amaxa.com) with solution T and program U-023. Experiments were also done in which CHO cells were only transfected with SacMCR receptor and the CRE-Luc reporter plasmid. The cells were then plated on a white, 96-well plate at a density of 1 × 10$^5$ cells per well. Forty-eight hours after transfection, cells were stimulated with the appropriate ligands in serum-free CHO media for 4 h at 37 °C at concentrations ranging from 10$^{-11}$ M to 10$^{-8}$ M. After a four hour incubation period, 100 μL of Bright-Glo luciferase assay reagent (Promega Inc., Madison, WI) was applied to each well and incubated at room temperature for 5 min. Luminescence was then measured with a Bio-Tek Synergy HT

![Fig. 2. CHO cells expressing the SacMCR receptor. A) The V5-tagged SacMCR receptor is seen in the cytoplasm of permeabilized cells (arrow). B) Non-transfected CHO cells shown no reaction when incubated with the primary and secondary antisera. C) The V5-tagged SacMCR receptor was detected on the cell surface of unpermeabilized CHO cells (white arrow). D) Transfected CHO cells incubated with only the secondary antisera did not show any reaction. Green = SacMCR receptor (Alma-Human). Blue = nuclei (DAPI). Space bar = 10 μm.](image-url)
plate reader (Winooski, VT). The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: human ACTH(1–24), NDP-α-MSH, and the dogfish melanocortin: ACTH(1–25), α-MSH, β-MSH, γ-MSH, and δ-MSH (Amenyi et al., 1999).

2.5. Data analysis

All experimental treatments were performed in no less than triplicate, and then corrected for control values, which were obtained by using transfected cells that were left unstimulated. In each of the assays, maximal activation levels were between three to ten times the control level. Corrected values were normalized to the average readout for cells treated with 1 μM ACTH (for the luciferase assay). Average values and standard errors of the mean were graphed using KaleidaGraph software (www.synergy.com), and the EC_{50} value for each ligand was determined. The curves were not constrained through the 0.0 origin. The EC_{50} values were compared by using the student’s t-test.

2.6. Maximum parsimony analysis

Maximum parsimony analysis was done using the exhaustive analysis algorithm (PAUP 4.0, paup.cst.sunysb.edu). The following Mc2 receptor amino acid sequences were analyzed: Homo sapiens (human; NP_055904.1), Macaca mulatta (monkey; AA100721.1), Gallus gallus (chicken; NP_001202385.1), Xenopus tropicalis (KF_031562922.1), Talpidae rubripes (fugu fish; AA032475.1), D. rerio (zebrafish; NP_775389.1), and S. acanthias (dogfish; AA676890). The sequence of Lampetra fluviatilis (lamprey; AB819647.1) GαiR was used as the outgroup. The bootstrap values in Fig. 1B were derived from 1000 replications.

3. Results

3.1. Immunocytochemical staining of CHO cells expressing the SacMC2 receptor

In order to evaluate the expression of the SacMC2 receptor in CHO cells, the cells were transfected with a sacMC2 receptor cDNA construct that encoded a V5 epitope tag located at the N-terminal of the receptor. Two days post-transfection, as expected, permeabilized cells showed a positive reaction when incubated with the V5 epitope antibody (Fig. 2A). What was not expected was that immunocytochemical staining of a parallel well of unpermeabilized transfected cells showed a positive reaction on the surface of the transfected cells when incubated with the V5 epitope antibody (Fig. 2C). In the controls for this experiment, non-transfected cells showed no reaction when incubated with the primary and secondary antibodies (Fig. 2B), as well as transfected cells incubated with the secondary antibody alone (Fig. 2D).

3.2. Ligand selectivity of the SacMC2 receptor

Once it was established that the SacMC2 receptor was trafficking to the plasma membrane of CHO cells, it seemed expedient to determine whether the SacMC2 receptor could be activated by melanocortin ligands. For these experiments, the sacMC2 receptor cDNA and the cre-hsc CAMP reporter cDNA were co-transfected into CHO cells, and two days post-transfection cells were stimulated with either human ACTH(1–24) or mammalian NDP-α-MSH at concentrations ranging from 10^{-5} to 10^{-10} M (Fig. 3A). Both mammalian ligands stimulated the transfected CHO cells in a dose dependent manner. The EC_{50} for hACTH(1–24) was 5.4 ± 1.1 × 10^{-8} M as compared to an EC_{50} for the mammalian NDP-α-MSH of 9.8 ± 2.3 × 10^{-8} M. While it appeared that hACTH(1–24) might be a more potent ligand than mammalian NDP-α-MSH, stimulation by these two ligands was not statistically different (P > 0.8). Next the SacMC2 receptor transfected CHO cells were stimulated with dogfish melanocortins at concentrations ranging from 10^{-2} to 10^{-10} M (Fig. 3B). In these experiments, dogfish ACTH(1–25) was the most potent ligand, whereas dogfish β-MSH induced minimal activation only at a concentration of 10^{-8} M. The EC_{50} values for each ligand were: ACTH(1–25) (4.6 ± 0.9 × 10^{-8} M), α-MSH (1.8 ± 0.3 × 10^{-7} M), γ-MSH (1.5 ± 0.3 × 10^{-8} M), and δ-MSH (2.0 ± 0.3 × 10^{-8} M), and the P value for ACTH(1–25) potency relative to the potency of each MSH was 0.05 (α-MSH), 0.005 (γ-MSH), and 0.05 (δ-MSH). The order of sensitivity for the dogfish melanocortins was ACTH(1–25) > γ-MSH > δ-MSH > α-MSH > β-MSH.

3.3. SacMC2 receptor co-expression with MRAps

Since SacMC2 receptor could be functionally expressed in CHO cells in the apparent absence of an accessory protein(s), we then tested the hypothesis that the functional activation of the SacMC2 receptor would be inhibited by co-expression with MRAps in a manner analogous to interaction between mammalian Mc2 receptors and the MRAps (Webb and Clark, 2010). In the first experiment, the effect of a C. milli MRAp2 (CMmpMRAp2) on the functional expression of the SacMC2 receptor in CHO cells was tested (Fig. 4A). However, this MRAp had no effect, either positive or negative, on the activation of the SacMC2 receptor. The next experiment was to determine whether the activation of the SacMC2 receptor might be affected by co-expression with MRAp. For this experiment the sacMC2 receptor cDNA construct was
either: a) expressed alone in CHO cells; b) co-expressed with mouse MRAPI (mMRAPI); or c) co-expressed with zebrafish MRAPI (zMRAPI). As indicated in Fig. 4B, rather than inhibiting the activation of the SacMC9 receptor, the vertebrate MRAPI significantly improved the sensitivity of the receptor for hACTH (1–24) (P = 0.005). While the EC50 value for the dose response curve when the SacMC9 receptor was expressed alone was 3.4 ± 1.4 × 10−8 M, when the SacMC9 receptor was co-expressed with mouse MRAPI the EC50 value was 6.0 ± 2.0 × 10−8 M, and when the dogfish receptor was co-expressed with zebrafish MRAPI the EC50 value was 1.1 ± 0.5 × 10−8 M. Fig. 4B also shows that while co-transfection of the SacMC9 receptor with zebrafish MRAPI led to a lower EC50 value, it ultimately resulted in a significantly lower maximum activation value than the SacMC9 receptor co-expressed with mouse MRAPI or when the SacMC9 receptor was expressed alone.

4. Discussion

Based on the results of the current study it would appear that the inability to functionally express the SacMC9 receptor in HEK293 cells has been an intrinsic feature in the primary sequence of the SacMC9 receptor construct that affected the folding of the receptor in the endoplasmic reticulum of HEK293 cells. Since several melanocortin receptors, from a rather wide range of vertebrates, have been functionally expressed in HEK293 cells (Agulheiro et al., 2010; Cone, 2006; Klovins et al., 2004b; Ling et al., 2004; Ringlehin et al., 2002), this is possibly an anomaly unique to the SacMC9 receptor.

4.1. Evolution of MC9 receptor

The immunocytochemical analysis and the ligand selectivity studies indicate that the SacMC9 receptor can be functionally expressed in a heterologous cell line (CHO cells) without co-expression of an exogenous MRPAP DNA, a feature common to teleost and mammalian MC9 receptors (Cone, 2006; Schlotthauer et al., 2009). Since the origin of the MC9 receptor gene and the other paralogous genes in the melanocortin receptor family appears to be the result of two predicted genome duplication events that occurred during the evolution of the chordates (Holland et al., 1994; Lundin, 1993; Ohno, 1968), and at least one local gene duplication that most likely occurred early in the evolution of the ancestral gnathostomes (Baron et al., 2009; Klovins et al., 2004a; Schlothauer et al., 2008), this scenario would account for the presence of the MC9 receptor ortholog in all of the vertebrate gnathostome lineages (e.g., cartilaginous fishes, ray-finned fishes, and lobe-finned fishes; for reviews see Vasternark and Schlothauer, 2011; Vee et al., 2011). During the radiation of the gnathostomes some of the functional properties of the MC9 receptor orthologs have diverged, and this study has focused on two aspects of this functional evolution: ligand selectivity and interaction with MRPAP.

4.2. Phylogeny of melanocortin receptor ligand selectivity

With respect to ligand selectivity, recent studies on the two lamprey melanocortin receptors and on the MC9 receptor and MC3 receptor orthologs present in the dogfish genome indicate that these melanocortin receptors have a higher affinity for ACTH than for the MSHs (Haitai et al., 2007a). These observations have led to the conclusion that ACTH might have been the primary ligand for the ancestral melanocortin receptors (Haitai et al., 2007b). The results of this study indicate that the SacMC9 receptor has retained this apparent ancestral feature. As shown in Fig. 3, the SacMC9 receptor has a higher sensitivity for ACTH (either human ACTH [1–24] or dogfish ACTH [1–24]) than any of the dogfish MSHs. In addition, the order of ligand selectivity for the dogfish melanocortins was ACTH (1–24) > ov-MSH > β-MSH > α-MSH > γ-MSH > δ-MSH; the same ligand selectivity profile observed for the SacMC9 receptor and the SacMC9 receptor functionally expressed in HEK293 cells (Haitai et al., 2007a). The SacMC9 receptor and the SacMC9 receptor also have a higher affinity for δ-MSH than β-MSH, and δ-MSH was a more potent ligand for the SacMC9 receptor as compared to β-MSH.

Finally these observations on non-mammalian MC9 receptor orthologs are in sharp contrast to the ligand selectivity of mammalian MC9 receptor orthologs. For mammalian MC9 receptors the order of ligand selectivity is α-MSH > ACTH > β-MSH > γ-MSH (Cone, 2006). All of the melanocortin peptides activate the melanocortin receptors through contact with the HIRF motif present in these ligands (Cone, 2006), and the critical amino acids in TM2, TM3, TM6, and TM7 that form the proposed HIRF binding site (Pogosheva et al., 2005) are rigorously conserved in the MC9 receptor sequences presented in Fig. 1. Thus, there must be some tertiary feature in non-mammalian MC9 receptors to allow ACTH to fit into the HIRF binding site with greater efficacy than α-MSH.

4.3. Phylogeny of melanocortin receptor interaction with MRPAP

Another distinctive feature of mammalian MC9 receptor is the interaction with the MRPAPs. The MRPAPs (MRAP1 or MRAP2) facilitate the trafficking of mammalian MC9 receptor to the plasma membrane.
In addition, MRAPI is required for full activation of the MC2 receptor following an ACTH binding event (Hinkle and Sebag, 2009; Webb and Clark, 2010). MRAPIs have an opposite effect on mammalian MC3 receptors. For this receptor, the MRAPIs interfere with MC3 receptor dimerization and trafficking of the receptor to the plasma membrane (Chan et al., 2009; Sebag and Hinkle, 2009b). Although the MC3 receptor is the predominant melanocortin receptor on mammalian adrenocortical cells (Cone, 2006; Mountray et al., 2002), the MC3 receptor is also expressed in adrenocortical cells of some mammals (van der Kraan et al., 1998). It would appear that the co-expression of MRAPI and MRA2 in adrenocortical cells selectively enhances the functional expression of the MC2 receptor, and inhibits the functional expression of the MC3 receptor to ensure that adrenocortical cells are only stimulated by ACTH, but not by any of the other melanocortins (Hinkle and Sebag, 2009; Metherell et al., 2005; Roy et al., 2007; Webb and Clark, 2010).

Interactions between the MC2 receptor and the MRAPIs have only been analyzed for some mammalian MC2 receptor orthologs. As a result, it is not known at which point or points in the phylogeny of the vertebrates the interaction between the MC2 receptor and the MRAPIs emerged. However, there have been no reports on the characterization of MRAPI orthologs from the dogfish. Although, a MRAPI ortholog had been identified in the genome of the holocellulian cartilaginous fish, C. milii (Cm) (Vastermark and Schött, 2011). Since an MRA2 ortholog has also been detected in the genome of the lamprey Petromyzon marinus (Vastermark and Schött, 2011), it is likely that the genome of the dogfish, might also have an mrap2 gene. We tested the hypothesis that co-expression of the SacmC3 receptor with CmMRAPI2 would result in a relationship analogous to the mammalian MC2 receptor/MRAPI interaction. As indicated in Fig. 4A, CmMRAPI2 had no apparent effect, either negative or positive, on the functional expression of the SacmC3 receptor.

The apparent absence of an interaction between the SacmC3 receptor and CmMRAPI2 would suggest that the tendency for the MC2 receptor orthologs and the MRAPIs to form a multimeric complex emerged after the divergence of the ancestral cartilaginous fish and the ancestral bony fish. However, the unexpected observation that mouse mraipi1 and zebrafish zbrasip1 had an effect on the functional expression of the SacmC3 receptor (Fig. 4B) would indicate that the SacmC3 receptor has the capability to interact with an MRAPI. Both mraipi1 and zbrasip1 increased the sensitivity of the receptor for ACTH. Since it is assumed that the dogfish genome lacks an mrap1 gene, the effects observed in Fig. 4B are pharmacological, and the apparent increase in sensitivity may have been due to the MRAPIs interfering with the internalization of ligand-bound MC3 receptor. Yet these observations may point to an ex-adaptation in the primary sequence of the SacmC3 receptor that may be fundamental to understanding the origin of this melanocortin receptor paralog. Alternatively, these observations may be the result of functional convergence.

5. Conclusions

It is perplexing that only three melanocortin receptor paralogs have been detected in the two eel loaches (S. aurantius and Hermodonius junctus) (Baron et al., 2009; Kollins et al., 2004a; Ringholm et al., 2003) that have been studied. It would appear that melanocortin receptor gene loss may have occurred during the radiation of the eel loaches since theoretically at least four melanocortin receptor paralogs should have been present in the genome of the ancestral cartilaginous fishes (Baron et al., 2009), and it is quite possible that all five melanocortin receptor paralogs were present in the genome of the ancestral cartilaginous fishes (Vastermark and Schött, 2011). Earlier studies on eel loaches indicated that injection of ACTH results in the release of a glucocorticoid from the head kidney (Delbos and Delbos, 1992). In addition, MC2 receptor expression has been detected in extracts of the interrenal tissue (Baron et al., 2009). Hence it is possible that the HPI axis in eel loaches can utilize the MC2 receptor on the interrenal cells to induce glucocorticoid production. Given the ligand selectivity of the SacmC3 receptor does this mean that products from either the anterior pituitary or the intermediate pituitary can induce the release of glucocorticoids, and if so what mechanisms determine which lobe of the pituitary will be involved? The use of receptor expression in heterologous cells can provide useful information on the ligand selectivity of a receptor, however, to resolve questions related to the physiological role of the SacmC3 receptor in the dogfish, in vivo studies are required.

Acknowledgments

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APPENDIX 5
Using the human melanocortin-2 receptor as a model for analyzing hormone/receptor interactions between a mammalian MC2 receptor and ACTH(1-24)

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1. Introduction

ACTH (adrenocorticotropin) was one of the first pituitary hormones to be characterized [26], and although this 39-amino acid polypeptide is relatively small, the biochemical nature of this hormone is more complex than the other melanocortin peptides (i.e., α-MSH, β-MSH, γ-MSH). Studies conducted over 30 years ago established that all of the melanocortin-related peptides are derived from the proopiomelanocortin precursor, POMC (proopiomelanocortin) [21]. In addition, it was found that melanocortins are synthesized in the corticotrophic cells of the pars distalis, and the melanotrophic cells of the pars intermedia [2]. Finally, in these pituitary cell types, differential posttranscriptional processing of POMC occurs to yield ACTH as the major melanocortin end-product of the melanotropes [17]. In terms of function, studies on mammals established over 40 years ago that ACTH is the sole ligand for the “ACTH” receptor present on adrenal cortex cells [38]. A later study would reveal that the “ACTH” receptor is in fact the melanocortin-2 receptor (MC2R) [30]; a G-protein coupled receptor (GPCR) in the rhodopsin family [10]. Subsequent studies have discovered that the melanocortin-2 receptor has functional activation features which are distinct from the other melanocortin receptors (e.g., MC1R, MC3R, MC4R, MC5R) [23,42]. Hence, it appears that the co-evolution of ACTH and MC2R has been shaped by selection pressures which have led to the one-on-one relationship between the ligand and the receptor that is observed in the hypothalamus/pituitary/adrenal axis of mammals. This review will use studies on mammalian melanocortin-2 receptors (principally human MC2R) to evaluate the structure/function features of ACTH, and to recapitulate the unique functional expression features of mammalian MC2Rs. In addition, a review of the mutations that have been detected in the human mc2r gene will underscore the value of using the human melanocortin-2 receptor as a model for comparative studies on orthologs of this receptor in other gnathostomes.

2. Structure/function analyses of ACTH(1-24)

As noted in Section 1, ACTH is a 39 amino acid polypeptide in most species of vertebrates [14]. However, earlier studies reviewed by Schwyzer [38] indicated that the functional domain required for activating the adrenal “ACTH” receptor was located within the first 24 residues of this hormone. Perhaps it is not a surprise that this region of ACTH is highly conserved among the gnathostomes [14]. For example as shown in Table 1 when the primary amino sequence of human ACTH(1-24) and dogfish (Squalus acanthias) ACTH(1-24) are compared, the sequence identity is 88%. As summarized by Schwyzer [38], analogous studies using mammalian ACTH(1-24) have uncovered a functional motif within ACTH: the HHK motif and the HKRR motif. Utilizing alanine substitution
### Table 1

Comparison of ACTH sequences.

<table>
<thead>
<tr>
<th>Species</th>
<th>ACTH (1-39)</th>
</tr>
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<tbody>
<tr>
<td>HsACTH</td>
<td>YSYQREHRNGKKQIQEEFVSQGERDEESAEAPFLEP</td>
</tr>
<tr>
<td>XLACTH</td>
<td>YSYQREHRNGKKQIQEEFVSQGERDEESAEAPFLEP</td>
</tr>
<tr>
<td>OmACTH</td>
<td>YSYQREHRNGKKQIQEEFVSQGERDEESAEAPFLEP</td>
</tr>
<tr>
<td>SsACTH</td>
<td>YSYQREHRNGKKQIQEEFVSQGERDEESAEAPFLEP</td>
</tr>
</tbody>
</table>

The K/KKRKRK proposed binding site is underlined. Positions where substitutions have occurred are shaded in black. Hs (Human ACTH: Accession No. C9G666625.1), Xi (Eustomus tropicalis: Accession No. WP_001151181.1), Om (Oncorhynchus mykiss: Accession No. WP_001181090), Ss (Squalus acanthias: Accession No. F011197).

### Fig. 1

Comparison of the amino acid sequence of human melanocortin receptors. The amino acid sequences of human MC1R (Accession No. AAK26525), MC3R (Accession No. AA867742), MC4R (Accession No. AA867741), and MC5R (Accession No. AA867741) were aligned as described previously [15]. Potential N-linked glycosylation sites are underlined. Positions that are identical in at least four of the five sequences are shaded in gray. The primary sequence identity of the five paralogues vs. S25. The (+) indicates positions in the human melanocortin-4 receptor that are found in the HHRW binding site of BMCR [32]. Note the high level of primary sequence conservation at these sites in MC1R, MC3R, MC4R, and MC5R. Nine of the 10 sites are identical in these four paralogues, and six of the 10 sites are conserved in BMCR.
analogs of hACTH(1–24), each of these motifs has been evaluated as described below.

The HHRW motif is present in all of the melanocortins [31]. N-terminally truncated analogs of mammalian ACTH(1–24) in which this region of the polypeptide has been eliminated has been shown to activate any melanocortin receptor [10,33]. Conversely, all melanocortin receptors have an HHRW binding site. The location of this site was determined by a modeling and site-directed mutagenesis analysis of the human melanocortin-4 receptor [32]. As shown in Fig. 1, there are 10 critical amino acid positions distributed among TM2 (transmembrane domain 2), TM3, TM5, and TM7 of hMC4R which form this site. These amino acid positions are highly conserved in hMC1R, hMC2R, hMC4R, and hMC5R (Fig. 1). It is important to note that these four melanocortin receptors can be activated by α-MSH as well as ACTH(1–24). Secondary structure analyses indicate that the HHRW motif in α-MSH [N-acetyl ACTH(1–13)amide] forms a reverse β-turn [22] that is then inserted into the corresponding binding site on the melanocortin receptor [22]; the result is activation.

Although the tertiary structure of hACTH(1–24) has not been determined, it is assumed that the HHRW motif in this ligand would also form a reverse β-turn as observed for α-MSH. However, in the human MC2R sequence (Fig. 1) only six of the 10 residues in the proposed HHRW binding site are conserved as compared to hMC4R. Thus, the HHRW binding site on all melanocortin receptors may not be identical, or to look at this issue from a different perspective, perhaps some residues in the HHRW motif of hACTH(1–24) play a more or less prominent role in the activation of the human melanocortin-2 receptor as compared to the activation of the human melanocortin-4 receptor. Previous studies had used N-terminally truncated analogs of ACTH(1–24) [38] to address this question, but these analogs may have altered secondary structure. To minimize this issue, we tested alanine analogs of the HHRW motif in hACTH(1–24). As compared to hACTH(1–24) (Fig. 2A; Table 2), the AFRW analog with an alanine substitution at H5 displayed a right shift on the activation curve. This resulted in an increase of EC50 value from 4.2 ± 1.3 to 36.0 ± 11 pm (P = 0.057) as compared to stimulation by the wild type hACTH(1–24) (Table 2). Although the AFRW analog was ninefold less potent at activating the hMC2R, the P value (Table 2) was just below significance. By comparison, substitution at F7 (analog HAFW) and R8 (analog HFAW) significantly reduced the potency of the ligands by nearly 1000- and 440-fold, respectively. The EC50 values and P values for these analogs are shown in Table 2. Interestingly, the analog HFAA, in which W10 was replaced by an alanine, failed to stimulate hMC2R even at the highest concentration (10−5 M) tested (Fig. 2A). The same observation was obtained when the entire HHRW motif was replaced by alanines (analog A4). These observations would indicate that tryptophan is the most important residue in the HHRW motif and is absolutely required for activation of the receptor.

When the same hACTH(1–24) analogs were used to stimulate the human melanocortin-4 receptor, a slightly different profile was observed (Fig. 2B). For this receptor no activation was observed when the receptor was incubated with either analog A4 or analog HFAA (Table 2). In addition, the EC50 value of AFRW increased by about 100-fold as compared to hACTH(1–24) (Table 2), and this shift was statistically significant (P < 0.05). Furthermore, stimulation with the HAPW analog and HFAW analog was extremely weak (15,000 and 26,000 increase in EC50, respectively) as compared to stimulation by hACTH(1–24) (Table 2). As shown in Fig. 2B, alanine substitutions to the HHRW motif at F7 and R8 of hACTH(1–24) have a more pronounced negative effect on the activation of hMC4R as compared to hMC2R. Collectively these results point to 3-dimensional differences in the HHRW binding site of hMC2R as compared to hMC4R.

Given these observations we thought it would be reasonable to re-evaluate the KRRR motif in hACTH(1–24) (Table 1) by analyzing alanine-substitution analogs in this region of hACTH(1–24). The importance of this motif became apparent from studies on C-terminally truncated analogs of hACTH(1–24) [4,5,38]. These analyses indicated that hACTH(1–16) was the shortest analog which could

![Fig. 2. Activation of hMC2R and hMC4R by analogs in the HHRW motif of hACTH(1–24).](image)
Table 2
Summary of the ACTH(1–24) alanine substitution study.

<table>
<thead>
<tr>
<th>Analogs</th>
<th>Amino acid sequences</th>
<th>hMC2R EC50 ± SEM (nM)</th>
<th>P values</th>
<th>hMC4R EC50 ± SEM (nM)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH(1–24)</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>4.2 ± 1.3</td>
<td>0.057</td>
<td>36 ± 8.5</td>
<td>0.008</td>
</tr>
<tr>
<td>A6</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ARWF</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>36 ± 11</td>
<td>0.057</td>
<td>36 ± 8.5</td>
<td>0.008</td>
</tr>
<tr>
<td>HRRW</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>33505 ± 2559</td>
<td>0.0001</td>
<td>31864 ± 2427</td>
<td>0.0009</td>
</tr>
<tr>
<td>HRFW</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>1845 ± 607</td>
<td>0.008</td>
<td>5384 ± 260</td>
<td>0.0004</td>
</tr>
<tr>
<td>HREW</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>A10-14</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>3606 ± 381</td>
<td>0.0004</td>
<td>61 ± 1.7</td>
<td>0.2</td>
</tr>
<tr>
<td>A5FVA</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>3.1 ± 1.4</td>
<td>0.2</td>
<td>3.7 ± 1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>GAANG</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>12214 ± 4745</td>
<td>0.03</td>
<td>144 ± 2.3</td>
<td>0.02</td>
</tr>
<tr>
<td>GAAPF</td>
<td>SYMMEHRKVRGKVRFRPVKYPY</td>
<td>120 ± 24.5</td>
<td>0.004</td>
<td>120 ± 24.5</td>
<td>0.004</td>
</tr>
</tbody>
</table>

EC50 Values are expressed as mean ± SEM (n = 3). Statistical differences were assessed by Student t-test. NA, No activation.

* P < 0.05.
** P < 0.005.

Still induce a minimal biological response when incubated with rat adrenal cortex cells in culture [7] or in a mammalian OS3 adrenal cell line transfected with a hMC2R cDNA [5]. While the progressive loss of biological activity of the C-terminally truncated hACTH(1–24) analogs could be attributed to a conformational change in the analogs, the observation that the analog KPVGCRRRPVKNKYPY can act as a competitive inhibitor of ACTH(1–24) [41] provided evidence for a KRRR binding site in MC2R in addition to the HRRW binding site. A later study that utilized alanine substitutions in hACTH(1–24) underscored the importance of P13 in this proposed binding site [12]. In Fig. 3A, we looked at the relative importance of the K11/R14 motif as compared to the R11/R14 motif in the activation process. As indicated in Table 2, both analogs showed a statistically significant increase in EC50 values as compared to hACTH(1–24). Stimulation with the A10-14 analog resulted in a threefold increase in EC50 relative to the positive control. Although this shift in EC50 was statistically significant (Table 2), the fold shift appears to be marginal. However, stimulation with the KAAA analog resulted in a 29-fold increase in the EC50 value (Table 1). When all the positions in this motif were replaced with alanines (A5 analog) there was a 3000-fold increase in the EC50 relative to the positive control (Table 2, Fig. 3A). However, it was interesting that the A5 analog did not totally block activation of the hMC2R; whereas, substitution of alanines in the HRRW motif of the ligand (A4 analog; Fig. 2A) completely inhibited activation of the receptor. Perhaps positions 20–24 in ACTH(1–24) also play a role in the activation of the receptor. This possibility should be explored. Accepting the premise that there is an HRRW binding site as well as a KRRR binding site on hMC2R, we next wished to address the question of the relative distance between these two binding sites. The operating hypothesis was that binding of hACTH(1–24) at the proposed KRRR binding site would position the HRRW domain of hACTH(1–24) into its corresponding binding site on the receptor. A corollary to this hypothesis would be that the C11/R14 motif would play a critical role in the positioning of the ligand in these two binding sites. To test this hypothesis, the activation activity of analogs with alanine substitutions in this region of hACTH(1–24) was tested (Fig. 3B). Analogs with either

Fig. 3. Activation of hMC2R by analogs of hACTH(1–24) in the KRRR motif and the GKPVC motif. (A) CHO cells were transfected with the following plasmids as described for Fig. 2A: hmc2r, mouse mgrp, and crenov. Activation assays were done as described for Fig. 2A. The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: the hACTH(1–24), hACTH(1–24)A11, hACTH(1–24)A14, hACTH(1–24)A11A14, hACTH(1–24)A11A14, and hACTH(1–24)A11A14. N = 3. (B) CHO cells were transfected with the following plasmids as described for Fig. 2A: hmc2r, mouse mgrp, and crenov. Activation assays were done as described for Fig. 2A. The following ligands, all synthesized by New England Peptide (Boston, MA), were tested: the hACTH(1–24), hACTH(1–24)A11, hACTH(1–24)A14, hACTH(1–24)A11A14, hACTH(1–24)A11A14, and hACTH(1–24)A11A14. N = 3.
single alanine or double alanine substitutions did not affect the activity curves of BM2CR (see EC_{50} values in Table 2). However, when all amino acid positions in this motif were replaced with alanines (A10–14 analog), there was a significant reduction in potency resulting in a 900-fold right shift in the activation curve.

It would appear that a change in the secondary structure of the A(10–14) analog affected activation of the receptor. A model summarizing these observations is presented in Fig. 4. This model assumes that initial contact with the BM2CR occurs at the predicted KKKRP binding site. The model assumes that the secondary structure of the C^{N\text{His}-C^{\text{His}}-C^{\text{His}}-C^{\text{His}}-C^{\text{His}}}-region of BM2CR(1–24) properly positions the HHRW motif of the ligand into the HHRW binding site on the receptor (Fig. 4A). Altering the secondary structure of the GPRP motif could misorient the N-terminal of the ligand in the HHRW binding site on the receptor (Fig. 4B) or interfere with binding at the proposed KKKRP binding site on the receptor.

Based on these assumptions, this model would explain the inability of α-MSH to activate MC2R (Fig. 4C) or even to act as a competitive inhibitor of ACTH(1–24) [44]. Since α-MSH lacks the KKKRP motif, this ligand cannot be properly oriented for insertion at the HHRW binding site on the melanocortin-2 receptor. However, it should be noted that either α-MSH or ACTH can enter the HHRW binding site in MC2R, MC3R, MC4R, or MC5R and activate these receptors [10]. Clearly, the linear sequence data provided in Fig. 1 does not reveal the 3-dimensional features which distinguish the HHRW binding site in MC2R from the corresponding site in MC1R, MC3R, MC4R, or MC5R. It should be noted that ACTH stimulation of target cells not only results in an increase in intracellular cAMP, but also leads to an inward Ca\(^{2+}\) influx [8,13,20,21,25]. The possibility that the ACTH analogs used in this study are affecting Ca\(^{2+}\) influx was not tested.

3. Role of MRA1P1 in the activation of the melanocortin-2 receptor

Among the actinopterygian and sarcopterygian gnathostomes perhaps the most enigmatic aspect of the ACTH/MC2R relationship has been the realization that this interaction requires the insertion of the accessory protein, MRA1P1 (melanocortin-2 receptor accessory protein 1) [28]. It should be noted that the early structure/function studies utilizing analogs of ACTH(1–24) were done on explants of mammalian adrenal cortex tissue [38] or isolated adrenal cortex cells [3]. The characterization of the mouse melanocortin-2 receptor by Mueller et al. [30] was done in Cloudman S91 melanoma cells. However, attempts to functionally express mammalian mc2r cDNAs in mammalian cell lines such as HEK-293 cells, CHO cells, or COS cells were completely unsuccessful, even though the other melanocortin receptor paralogs (i.e., MC1, MC3R, MC4R, MC5R) could be functionally expressed in these cell lines [18,24,31,35]. It appeared that some cell types, such as adrenal cortex cells and adipose cells, had an additional factor which facilitated the functional expression of the melanocortin-2 receptor.

Support for this hypothesis came from studies on Familiar Glucocorticoid Deficiency (FGD). FGD is a congenital syndrome in humans which results in a life-threatening underproduction of cortisol by adrenal cortex cells. For some patients the problem is an error in the biosynthetic pathway for making cortisol from cholesterol [29]. For other patients the cortisol deficiency is due to mutations in the melanocortin-2 receptor [8]. However, for a subset of patients the defect which resulted in FGD is not a mutation in the glucocorticoid biosynthetic pathway or the melanocortin-2 receptor, but rather a mutation in an accessory protein expressed in adrenal cortex cells (Type 2: [28]). The accessory protein is MRA1P1 [28].

MRA1Ps are single chain polypeptides with a single membrane spanning domain [28]. Two MRA1P paralogs have been characterized from the genomes of vertebrates [23,42], and are designated MRA1P1 and MRA1P2. Both paralogs have the distinctive feature of forming homodimers in which the two proteins are oriented in an antiparallel manner in the membrane of the endoplasmic reticulum [39,40]. Both the MRA1P1 homodimer and the MRA1P2 homodimer appear to independently form a complex with the melanocortin-2 receptor at the endoplasmic reticulum. While both the MRA1P1/MC2R and the MRA1P2/MC2R complexes will move to the plasma membrane, only the MRA1P1/MC2R complex can efficiently bind ACTH and be activated [23,42]. The reason for the differences in the functions of mammalian MRA1P1 and MRA1P2 resides in the amino acid motif, LD(T)I (40). This amino acid motif is present in the N-terminal of mammalian MRA1Ps, but absent from the N-terminal of mammalian MRA2Ps, and is responsible for inducing
the conformation change in MC2R which makes the receptor functionally active. Finally, a study on the co-expression of human MRAP1 and human MC2R in mammalian HEK-293 cells indicated that the MRAP1/MC2R complex is a hexameric oligomer consisting of two MRAP1 homodimers and one MC2R homodimer [11,37]. In Figs. 2, 3 and 6 the functional expression of human MC2R was done by also co-expressing mouse MRAP1 in the CHO cells. A previous study indicated that there was no evidence that MRAPs are expressed in the CHO cells used in this study [34].

The significance of the interaction between ACTH, MC2R, and MRAP1 cannot be understated. As reviewed recently [23,42], when the melanocortin-2 receptor is expressed alone, the receptor is folded improperly. As a result, the misshaped receptor will be retained in the endoplasmic reticulum and eventually degraded. Hence, while Fig. 1 shows the amino acid positions in the linear sequence of human MC2R that most likely form the HFRR binding site, unless the melanocortin-2 receptor forms a complex with MRAP1, the receptor will not be in the appropriate 3-dimensional conformation required for activation. In addition, without MRAP present to serve as a trafficking chaperone, the MC2 receptor will be unable to leave the endoplasmic reticulum.

For humans the various types of Familial Glucocorticoid Deficiency can be treated by taking cortisol supplements. Could this syndrome occur in a non-mammalian bony vertebrate? The answer is, most likely yes. Recent studies indicate that the melanocortin-2 receptor for the zebrafish, Danio rerio [1] and the frog, Xenopus tropicalis [27] both require co-expression with an MRAP1 in order to be functionally activated. Hence, if mutations occur in a non-mammalian bony vertebrate in either the mc2r gene which affect either trafficking or activation, or if mutations occur in the corresponding sites in the mrap1 gene that affect either trafficking or activation of MC2R, the outcome would most likely be deleterious to the fitness of the organism.

This conclusion is not surprising after comparing the linear sequences of human, zebrafish, and X. tropicalis melanocortin-2 receptors as shown in Fig. 5A. Note that all three orthologous MC2R sequences have identical sequences at positions 103, 156, 130, 260, 263, and 266 in the proposed HFRR binding site (red boxes), and variable sequences at positions 133, 134 (TM3), and 291, 294 (TM7) as compared to hMC4R.

In this regard, naturally occurring mutations which inhibit activation of human MC2R, resulting in FGD, have been detected at the conserved positions 126 and 130 in TM3 (Fig. 5A). Tracking other naturally occurring activation inhibiting mutations may point to other functional domains in the receptor that are conserved among the bony vertebrates [7,19]. For example, an activation inhibiting mutation has been detected at R159 in the DRY motif. This motif is critical for proper interaction between the receptor and the G-Protein which leads to the activation of adenyl cyclase [7]. An apparently perplexing activation inhibiting mutation has also been observed at R159 in extracellular loop 2 (EC2) [7] of human MC2R. Extracellular loop 2 is outside of the HFRR binding site. Assuming that the 3-dimensional structure of MC2R orients the seven transmembrane regions in a barrel conformation as shown in Fig. 5B.
then TM2, TM3, TM6, and TM7 which form the HHRW binding site of MC2R [7], would be in close proximity. In this model EC2 could be positioned in the proposed KKKRP binding site flanked by TM4 and TM5.

To test this hypothesis alanine substitution mutants were made in TM4, EC2, and TM5, respectively, and the ability of hACTH(1–24) to activate these hMC2R mutants was tested (Fig. 6). Receptor activation by hACTH(1–24) for all three mutants was significantly reduced, but to different degrees, as compared to the wild type hMC2R. The EC2 mutant displayed a 10,000-fold right shift, suggesting an essential role of extracellular loop 2 in the binding of ACTH to the receptor. Meanwhile, TM4 and TM5 mutants showed a 10- and 100-fold increase in EC50 values, respectively. While the outcome of this experiment is intriguing, the actual roles of these three regions in the activation of hMC2R will need to be tested by analyzing single alanine mutations in these regions of the receptor.

4. Conclusions

When considering the factors which distinguish the human melanocortin-2 receptor from the other human melanocortin receptors (i.e., MC1R, MC3R, MC4R, MC5R), three features stand out. First, while the primary sequence of the human melanocortin-2 receptor indicates that many of the amino acid positions found in the HHRW binding site of the human melanocortin-4 receptor are present (Fig. 1), these two binding sites do not operate in an identical manner. As indicated in the ligand activation studies, analysis of the HHRW motif of hACTH(1–24) indicated the following order of importance for activation of hMC2R: W□ > R□ > F□ > H□. However, for hMC4R the order of importance of the residues in the HHRW motif of ACTH(1–24) is W□ > H□ > F□ > R□ (Fig. 2). These observations would suggest that the 3-dimensional structure of the "HHRW" binding sites in hMC2R and hMC4R may be similar, but not identical. While there has already been shown that α-MSH cannot activate the zebrafish melanocortin-2 receptor [1] or the X. tropicalis melanocortin-2 receptor [27], it is not known whether these non-mammalian MC2Rs react to the HHRW analogs of hACTH(1–24) in the same manner as the human melanocortin-2 receptor.

Another unique feature of the human melanocortin-2 receptor is that activation by ACTH(1–24) is dependent on the KKKRP motif in this ligand. The operating model (Fig. 4) is that the ligand first makes contact with the receptor at a proposed KKKRP binding site and then activation is possible when the HHRW motif of the ligand is inserted into the corresponding binding site on the receptor. Within this region of ACTH(1–24), the KKR motif is more critical that the KK motif. In several non-mammalian vertebrates the sequence at position 16–19 in ACTH(1–24) is KKR (Table 1) [14]. The prediction would be that the substitution of an arginine residue for a lysine residue at position 16 of ACTH(1–24) will not interfere with the activation of non-mammalian MC2Rs.

Finally, during the initial synthesis of the human melanocortin-2 receptor at the rough endoplasmic reticulum the receptor is in an inactive conformation that cannot move independently to the plasma membrane [40]. However, if MRAF1 or MRAF2 are present at the rough endoplasmic reticulum, either of these accessory proteins can rescue the melanocortin-2 receptor, and facilitate the trafficking of the receptor to the plasma membrane [23,36,37,42]. However, of the two MRAF paralogs, only MRAF1 is capable of facilitating both the trafficking of hMC2R to the plasma membrane and the functional activation of the receptor at the plasma membrane. Identical observations have been made for the functional activation of zebrafish MC2R [1] and X. tropicalis MC2R [27]. Although the sample set is limited to three species (i.e., human, frog, and zebrafish), the phylogenetic positions of these organisms would suggest that in the ancestral bony fishes a functionally mutated melanocortin-2 receptor was rescued by an MRAF that was co-synthesized in cells expressing the ancestral mc2r gene. The preceding scenario begs the question of whether there is an MRAF1 independent melanocortin-2 ortholog in any extant gnathostome. Studies on melanocortin-2 orthologs in non-bony fishes or in cartilaginous fishes will resolve this question.
Evolution of melanocortin receptors in cartilaginous fish: Melanocortin receptors and the stress axis in elasmobranches

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ABSTRACT

There is general agreement that the presence of five melanocortin receptor genes in tetrapsods is the result of two genome duplications that occurred prior to the emergence of the gnathostomes, and at least one local gene duplication that occurred early in the radiation of the ancestral gnathostomes. Hence, it is assumed that representatives from the extant classes of gnathostomes (i.e., Chondrichthyes, Actinopterygi, Sarcopterygi) should also have five paralogous melanocortin genes. Current studies on cartilaginous fishes indicate that while there is evidence for five paralogous melanocortin receptor genes in this class, to date all five paralogs have not been detected in the genome of a single species. This mini-review will discuss the ligand selectivity properties of the melanocortin-3 receptor of the elephant shark (subclass Holocephali) and the ligand selectivity properties of the melanocortin-3 receptor, melanocortin-4 receptor, and the melanocortin-5 receptor of the dogfish (subclass Elasmobranchii). The potential relationship of these melanocortin receptors to the hypothalamus/pituitary/interrenal axis will be discussed.

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1. Introduction

The presence of five melanocortin receptor-coding genes in the genomes of tetrapsods coupled with the absence of melanocortin-related receptors in non-chordate metazoaans [48] suggests that the evolution of this family of G-protein coupled receptors occurred during the evolution of phylum Chordata. As a result, the evolution of this gene family would have been influenced by the two generally accepted genome duplication events that occurred during the radiation of this phylum [14,38], and at least one localized gene duplication event [43]. Using the evolution of HOX genes as a model [25], the generally accepted scenario for the evolution of the melanocortin receptor gene family is that successive genome duplications in first the ancestral protostomes, and subsequently in the agnathan vertebrates, would have led to four paralogous melanocortin receptor-coding genes in the ancestral gnathostomes [48]. The presence of five paralogous melanocortin receptor-coding genes (i.e., mcr1, mcr2, mcr3, mcr4, mcr5) in the genomes of the teleost Danio rerio [32], and the tetrapsods Xenopus tropicalis (http://www.genome.jgi-psf.org/Xen4/Xen4.home.html) and Mus musculus [10] provide support for the fifth melanocortin-coding gene resulting from a localized gene duplication [43]. Given these observations it was expected that five melanocortin receptors (MCRs) would be present in all of the extant classes of gnathostomes (i.e., Chondrichthyes, Actinopterygi, and Sarcopterygi; [36]. However, while studies on the cartilaginous fishes (class Chondrichthyes) have uncovered five paralogous mcr genes in representatives from the two subclasses of extant cartilaginous fishes (i.e., Holocephali and Elasmobranchii), all five mcr genes were not detected in the same species. Fig. 1 provides the current view of the phylogeny of the extant cartilaginous fishes, and the distribution of these mcr genes. A molecular phylogenetic analysis places the emergence of the ancestral cartilaginous fishes at approximately 445 MYA [142]. This analysis supports the monophyletic origin of subclass Holocephali and subclass Elasmobranchii. A subsequent evolutionary developmental biology study [18] and another molecular phylogenetic study [27] place the divergence of the two subclasses between 420 and 410 MYA. As shown in Fig. 1, an analysis of the genome database for the elephant shark, Callorhinus milii (subclass Holocephali), revealed the partial amino acid sequence of a MCR ortholog, and the full length sequences of orthologs of MCR2 and MCR3, respectively [48]. However, within the genome of the dogfish, Squalus acanthias (subclass Elasmobranchii), only cDNAs corresponding to MCR1, MCR4, and MCR5 were cloned and sequenced from this species [2841]. It is possible that additional melanocortin receptor paralogs may be detected in the C. milii genome when this genome sequencing project is completed. In addition, sequencing of the S. acanthias genome is needed to determine whether additional melanocortin receptor paralogs are present in that genome.

Since the deduced amino acid sequence of the elephant shark MCR1 ortholog is still incomplete, and the analysis of the elephant shark MCR2 ortholog is in progress, this mini-review will focus on...
MC1R  
MC2R  
MC3R  
MC4R  
MC5R  
Calliurbation milli  
Squalus acanthias

HOLOCEPHALI  
ELASMOBRANCHII  

ANCESTRAL CARTILAGINOUS FISHES

Fig. 1. Phylogeny of cartilaginous fish melanocortin receptors. In class Chondrichthyes, melanocortin receptors have been characterized from the dogfish, Squalus acanthias (subclass Holocephali) [25,49], and the elephant shark, Calliurbation milli (subclass Holocrophali) [48]. Abbreviations: MC1R = melanocortin-1 receptor; MC2R = melanocortin-2 receptor; MC3R = melanocortin-3 receptor; MC4R = melanocortin-4 receptor; and MC5R = melanocortin-5 receptor.

the ligand selectivity of the MCR paralog common to both species, MC3R, and on the properties of the dogfish MCR4 and MCR5 orthologs. Observations on these melanocortin receptors will be used to consider the organization of the hypothalamic/pituitary/interrenal axis in elasmobranches.

2. Elephant shark and dogfish MCR orthologs

Fig. 2 presents an alignment of the amino acid sequences of elephant shark MCR3 and dogfish MCR3. In these two receptor sequences, Intracellular Loop 1 (IC1), Transmembrane Domain 2 (TM2), and Intracellular Loop 2 (IC2) are identical, and the overall primary sequence identity of the two receptors is 83%. Dogfish MCR3 has been functionally expressed in HEK-293 EBNA cells, and stimulation with dogfish ACTH(1-25) resulted in a robust production of cAMP in the transfected cells, whereas stimulation with dogfish γ-MSH was 100-fold weaker [20]. In this study stimulation of dogfish MCR3 transfected cells by dogfish γ-MSH was not tested in the functional expression assay. However, ligand binding assays were done for all of the dogfish melanocortins, and based on the binding assay analysis the order of ligand selectivity for dogfish MCR3 is ACTH(1-25) > α-MSH > γ-MSH > β-MSH. The efficacy of dogfish β-MSH on cAMP production by dogfish MCR3 transfected HEK-293 cells was not tested, and binding studies indicated that dogfish MCR3 was nearly 500-fold less sensitive to dogfish β-MSH binding as compared to the binding of dogfish α-MSH [20]. Dogfish MCR mRNA expression was detected in different regions of the dogfish brain including the hypothalamus, telencephalon, brain stem, and olfactory bulb [29], and this expression pattern is similar to observations on the mammalian central nervous system where MC3R has been implicated in the regulation of energy balance [8].

Ligand selectivity for elephant shark MCR3 was based on the functional expression of this receptor in a heterologous assay using CHO cells, a derived mammalian immortalized cell line (Fig. 3). Dogfish ACTH(1-25) was clearly the preferred ligand (EC50 = 7.4 ± 1.1 × 10⁻⁹ M), whereas stimulation by dogfish α-MSH (EC50 = 1.5 ± 0.05 × 10⁻⁵ M), β-MSH (EC50 = 4.7 ± 0.6 × 10⁻⁶ M), and γ-MSH (EC50 = 5.6 ± 0.3 × 10⁻⁵ M) was significantly weaker. Stimulation of elephant shark MCR3 by dogfish β-MSH was only detected at the highest dose tested (10⁻⁴ M). Hence, elephant shark MCR3 can be activated by either ACTH or the MSHs, but ACTH is clearly the more potent ligand (P < 0.0001), and the order of ligand selectivity is ACTH(1-25) > α-MSH > β-MSH > γ-MSH > β-MSH. Since it is expected that functional activation analyses should parallel ligand binding assays, it would appear that elephant shark MCR3 has a clear preference for ACTH, whereas activation of dogfish MCR3 should be achieved equally well using either dogfish ACTH(1-25) or α-MSH. In this regard, the ligand selectivity of dogfish MCR3 is similar to mammalian MCR3 [16]. The weak binding affinity of dogfish β-MSH by dogfish MCR3, and the lack of stimulation by this melanocortin at physiological concentrations when tested with elephant shark MCR3 are consistent with the observation that β-MSH is the most variable of the melanocortins detected in gnathostomes, and is most likely an evolutionary anachronism rather than a functional chemical signal at least in cartilaginous fishes [12]. MCR mRNA expression has not been investigated in the central nervous system of the elephant.

![Alignment of dogfish and elephant shark melanocortin-3 receptors. The amino acid sequences of dogfish MCR3 (accession number: AA664252.1) and elephant shark MCR3 (accession number: FA446025.1) were aligned as previously described [14]. One gap was inserted in this alignment. Positions that are identical are shaded. Underlined amino acids indicate putative N-linked glycosylation sites. A (x) indicates a residue that is found in the NR4A binding site of the human melanocortin-3 receptor [36].](image-url)
4. Properties of dogfish MCRS

Although a cDNA encoding the deduced amino acid sequence of dogfish MCRS was characterized over 7 years ago [28], the ligand selectivity of this dogfish MCR was only determined recently [40]. In the former study attempts to functionally express a dogfish mcr5 cDNA in HEK-293 cells were unsuccessful [29]. This observation was perplexing due to the fact that radioligand binding studies for other paralogous melanocortin receptor cDNAs from a variety of species have been successfully expressed in HEK-293 cells [19,28,31]. The exception to this generalization is the paralog 2 receptor accessory protein [35]. In many teleost and tetrapod species there are two mrap genes (1 and 2), and interaction between mammalian MCR2 and the MRAPI paralog not only facilitates the trafficking of mammalian MCR2 to the plasma membrane but is also required for functional activation of the mammalian MCR2 by ACTH [24,30]. Subsequent studies indicate that the functional expression of teleost or non-mammalian tetrapod MCR2 orthologs in either HEK-293 or in CHO cells also require co-transfection with a mrap1 cDNA [19,29].

Based on the preceding observations, it was possible that dogfish MCR5 also required an accessory protein to facilitate trafficking to the plasma membrane and for functional activation. However, in a recent study it was found that dogfish MCR5 could be visualized on the surface of CHO cells in the absence of the co-expression with mammalian or teleost MRAs [40]. As expected, dogfish MCR5-transfected CHO cells could be activated by dogfish ACTH[1–25] as well as dogfish α-MSH, γ-MSH, and β-MSH. However, in this study it was observed that the order of ligand selectivity was ACTH[1–25] > α-MSH > γ-MSH > β-MSH > MRAPI [38]. In fact, ACTH[1–25] was 10-fold more potent than α-MSH and nearly 100 fold more potent than either γ-MSH or β-MSH. Once again β-MSH produced a minimal response above background at a concentration of 10−6 M.

A RT-PCR analysis study to the distribution of dogfish MC5 receptors indicated that the expression of this receptor could be detected in the telencephalon and in the hypothalamus, but the MC5 expression was not apparent in peripheral organs [29]. However, a similar study on the distribution of mcr5 expression in the shark Heterodonus francisci did detect mcr5 expression in the head kidney [7]. This observation raises the possibility that MCR5 plays a role in the HPI axis of elasmobranchs. This possibility will be discussed in the next section.

5. Ligand selectivity of cartilaginous fish MCRs

Focusing exclusively on the pharmacological properties of the cartilaginous fish MCR receptors considered in this review (i.e., elephant shark MCR1, dogfish, MCR5, MCR4, MC5), a recent observation is the preference of these receptors for cartilaginous fish ACTH[1–25] as compared to any of the cartilaginous fish MSH sequences tested. This outcome is not unprecedented. The lamprey melanocortin-a receptor has higher affinity for mammalian ACTH[1–17] than any of the MSH sequences used in that study [21]. In addition, the rainbow trout MC4 receptor and the MCR5 receptor had a higher affinity for ACTH[1–24] than any of the MSH-related ligands tested [19]. Although the sample size is small, it appears that ACTH could be the more potent ligand for agustus, cartilaginous fish, and some teleost melanocortin receptors [20].
These observations support the conclusion that ACTH may have been the ancestral ligand for the melanocortin receptors [29].

6. Features of the HPI axis in cartilaginous fishes

The detection of melanocortin receptors in cartilaginous fishes raises a number of questions with respect to the role these receptors play in the hypothalamic/pituitary/interrenal axis in these fishes. There is evidence that the interrenal cells of cartilaginous fishes play a role in regulating stress, glucose homeostasis, and osmoregulation [17]. These glucocorticoid and mineralocorticoid actions appear to be mediated by 1α,25-dihydroxyvitamin D3 (1α,25(OH)D3), a vitamin D analog that inhibits the expression of the glucocorticoid receptor and is present in other vertebrates [26].

Is the secretion of 1α,25(OH)D by interrenal cells regulated by a hypothalamic/pituitary axis (HPI) in any cartilaginous fish? The answer to this question is most likely yes, but the evidence is fragmentary. The early discovery of ACTH, characterized from extracts of the anterior pituitary of the dogfish [33] provided the first evidence that corticotropic cells were present in the anterior pituitary of cartilaginous fishes [6]. The subsequent characterization of pituitary ACTH in the dogfish, *Squalus acanthias*, [2], and the ray, *Dasyatis akajei* [3] verified that the anterior pituitary of cartilaginous fishes has the capability for synthesizing ACTH and β-endorphin; whereas the intermediate pituitary of these fishes has the capability of synthesizing several MSH-related peptides (i.e., α-MSH, β-MSH, γ-MSH, δ-MSH) as well as β-endorphin. In an earlier physiological study on a shark it was observed that injection with mammalian ACTH resulted in hyperglycemia presumably as a result of the action of an endogenous glucocorticoid influencing glucoregulation [11]. Collectively, these observations provide support for the hypothesis that the corticotropic cells of the anterior pituitary interact with the interrenal cells in the head kidney of elasmobranches. However, the missing element in this proposed endocrine circuit has been an understanding of how neurons in the hypothalamus regulate the secretion of ACTH by elasmobranch corticotropic cells.

Although an urotensin I ortholog has been characterized from the caudal neurosecretory cells of the dogfish, *Squalus acanthias* [48], thus far no CRF-like ortholog has been characterized from the CNS of an elasmobranch. However, a recent study on the genome database of the holocentrid *Callorhynchus milii*, has uncovered two CRF-related genes, an urotensin I gene, and an urocortin 3 gene [37]. It is anticipated that it is only a matter of time before orthologs for some or all of these CRF/Urocortin-related genes will be uncovered in the genome of an elasmobranch.

Based on these observations it seems reasonable to assume that the HPI axis is present in cartilaginous fishes. The current dilemma is whether ACTH selectively activates the interrenal cells of cartilaginous fishes. As discussed in this review, the ligand selectivity properties of the three melanocortin receptors characterized from the genome of the dogfish, *Squalus acanthias*, clearly indicated that dogfish MC3R, MC4R, and MC5R can all be activated by ACTH as well as by the various MSH sequences derived from cartilaginous fish POMC. Hence, whether the MC5 receptor is exclusively expressed by elasmobranch interrenal cells, or if MC3R or MC4R are also expressed by these cells, the fact remains that melanocortins derived from either the anterior pituitary or the intermediate pituitary could activate these interrenal cells (Fig. 4). These observations raise the question of whether elasmobranchs have distinct hypothalamic/anterior pituitary and hypothalamus/intermediate pituitary axes that converge on the interrenal cells of the head kidney. If so, are these two neuroendocrine circuits activated in response to different physiological stimuli? To date these possibilities have not been explored.
the rectal gland [4], and increased drinking rate [5,22]. Since 1α-OHB appears to function as both the mineralocorticoid and glucocorticoid steroid for caragilous fishes, these observations raise the question of whether glucocorticoid effects occur as a result of angiotensin II stimulation of elastombranch interrenal cells.

7. Conclusions

Although it is generally accepted that the caragilous fishes have a hypothalamic/pituitary/interrenal axis [17], unlike teleosts and tetrapods, there does not appear to be a melanocortin receptor on elastombranch interrenal cells that can only be activated by ACHT [1,10]. To date all of the melanocortin receptors that have been functionally characterized from caragilous fishes can be activated by either ACHT or most of the MSH-related peptides derived from POMC. Hence, POMC-related melanocortins from either anterior pituitary cells or intermediate pituitary cells could activate peripheral target cells that express melanocortin receptors. Clearly additional work is needed on the mechanisms that regulate the secretion of melanocortins from elastombranch anterior pituitary and intermediate pituitary cells, and the physiological functions of these melanocortins.

The receptor binding assays that have been performed on elastombranch melanocortin receptors [20,29,40,41] and the functional expression assays that have been performed on elastombranch [20] and holoccephalan melanocortin receptors (Fig. 3) [40] expressed in heterologous mammalian cell lines indicate that ACHT is the preferred ligand for the activation of these melanocortin receptors. Based on the studies that have been done on lamprey [21] and caragilous fishes it would appear that ACHT may have been the ancestral ligand for the melanocortin receptors.

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References


