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Functional activation of cartilaginous fish melanocortin receptors

Christina Reinick
University of Denver

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FUNCTIONAL ACTIVATION OF CARTILAGINOUS FISH MELANOCORTIN RECEPTORS

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

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Christina Lee Reinick
June 2011
Advisor: Dr. Robert Dores
ABSTRACT

The evolution of the melanocortin receptor (MCR) gene family has been dictated by two genome duplication events (2R hypothesis). The gnathostomes are thus predicted to possess a minimum of four MCR genes. Previous studies on cartilaginous fish have shown evidence for the presence of only three MCRs. The purpose of this thesis is to functionally express the MC5R from the spiny dogfish (*Squalus acanthias*) and the putative MC2R and MC3R from the elephant shark (*Callorhinchus milii*); these receptors have not yet been fully characterized. In this study, SacMC5R was able to be expressed in CHO cells without the presence of an accessory protein, like MRAP. This receptor showed a preference for ACTH over α-MSH, and, even though SacMC5R showed no requirement for MRAP, co-transfection with mouse MRAP led to an increase in receptor sensitivity to ACTH. CmiMC3R was also found to have the highest sensitivity to ACTH. This receptor was able to be expressed without MRAP, but receptor activity was significantly enhanced in the presence of mouse MRAP. Functional analysis of CmiMC2R showed that this receptor was able to be trafficked to the cell surface and functionally expressed without MRAP; furthermore, the presence of MRAP did not appear to enhance activation. It was also found that while CmiMC2R had the greatest affinity for ACTH, it was also able to be stimulated by all of the MSHs. Analysis of the MCRs in these species suggests that cartilaginous fish have secondarily lost a MCR gene. The relationship between the MC2R and MC5R in cartilaginous fish is still unresolved.
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INTRODUCTION

Melanocortin Receptors and Peptides

The melanocortin receptors (MCRs) are a set of Rhodopsin family G protein-coupled receptors (GPCRs) (Schioth and Fredriksson, 2005). These receptors are all single chain polypeptides with seven membrane spanning α-helical domains. Each of the MCRs is associated with cAMP production through the stimulatory G protein, Gs, and adenylyl cyclase. There are five known MCRs (MC1R, MC2R, MC3R, MC4R, and MC5R), which are associated with a wide range of physiological functions in mammals (Cone, 2006). The MCRs are stimulated by the melanocortin peptides α-, β-, and γ-melanocyte-stimulating hormone (α-, β-, and γ-MSH) and adrenocorticotropic hormone (ACTH). These peptides are all derived from the proopiomelanocortin (POMC) gene and share the amino acid sequence His-Phe-Arg-Trp, which is necessary for activation of the MCRs (Nakanishi et al., 1979). Posttranslational processing of the POMC prohormone is tissue specific and carried out by the prohormone convertases PC1/3 and PC2 at dibasic cleavage sites (Figure 1; Eipper and Mains, 1980; Seidah and Chretien, 1999).
Figure 1: Overview of prohormone convertase endoproteolytic cleavage activity on the POMC gene. Posttranslational processing of the preprohormone is shown for the anterior (a) and intermediate pituitary (b) through to all of the possible end products.
The MC1R was the first melanocortin receptor to be cloned using a cDNA library from a human melanoma sample (Mountjoy et al., 1992) and is considered the classical \( \alpha \)-MSH receptor. It has subsequently been found to be expressed chiefly in cell types of the skin, specifically the cutaneous melanocytes, where it influences pigmentation (Gantz and Fong, 2003). The MC1R has, however, also been found in other tissues and cell types throughout the body, such as leukocytes, where it has an anti-inflammatory function (Chhajlani, 1996). This receptor binds \( \alpha \)-MSH and ACTH with equal affinity, followed by \( \beta \)-MSH, then \( \gamma \)-MSH (Table 1).

The cloning of the human ACTH receptor, MC2R, closely followed that of MC1R (Mountjoy et al., 1992). The MC2R is expressed largely in the zona reticularis and zona fasiculata of the adrenal cortex, and is known to influence steroidogenesis and steroid secretion. Unlike the other MCRs, which can be stimulated to some degree by any of the melanocortins, MC2R is highly selective and can only be stimulated by ACTH (Table 1). Mutations in this receptor are associated with the disorder familial glucocorticoid deficiency (FGD), which is characterized by low cortisol levels despite high plasma ACTH (Clark et al., 2005). Another unique feature of MC2R is that it is unable to be functionally expressed in most cell types without the addition of an accessory protein. The melanocortin 2 receptor accessory protein, MRAP, interacts directly with MC2R and is essential for trafficking the receptor from the endoplasmic reticulum (ER) to the cell surface (Metherell et al., 2005).

MC3R is expressed throughout the central nervous system (CNS) and in tissues such as the gastrointestinal tract and placenta (Gantz and Fong, 2003). MC3R-null mice
exhibit an obesity syndrome, suggesting a role for MC3R in energy homeostasis, but the specifics of this syndrome are not well understood (Cone, 2006). MC3R has been shown to have nearly equal affinity for all of the melanocortin peptides (Table 1). Among the MCRs, it is the most responsive to γ-MSH, which has the highest natriuretic activity of all of the MSH peptides; the MC3R may, therefore, also play a role in natriuresis (Ni et al., 1998).

MC4R is expressed primarily in the CNS. MC4R knockout mice show obesity, excessive hunger, and high insulin levels, indicating that this receptor influences food intake and energy homeostasis (Cone, 2006). It has also been shown that MC4R may play a role in sexual function (Gantz and Fong, 2003). α-MSH and ACTH are the most potent activators of MC4R, followed by β-MSH, then γ-MSH (Table 1).

MC5R is expressed in several peripheral tissues, but the only known function of this receptor in mammals is regulation of sebaceous gland secretion (Gantz and Fong, 2003). MC5R mRNA has also been found in several other exocrine glands, so it is possible that MC5R signaling may also influence synthesis or release of pheromones (Cone, 2006). MC5R binds α-MSH with the highest affinity, followed by ACTH, and then β-MSH and γ-MSH (Table 1).
Table 1: Binding affinities of mammalian melanocortin receptors for the melanocortin ligands. The antagonists for each receptor are also shown. (Gantz and Fong, 2003)

<table>
<thead>
<tr>
<th>RECEPTOR</th>
<th>POTENCY OF LIGANDS</th>
<th>ANTAGONISTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1R</td>
<td>$\alpha$-MSH = ACTH &gt; $\beta$-MSH &gt; $\gamma$-MSH</td>
<td>Agouti</td>
</tr>
<tr>
<td>MC2R</td>
<td>ACTH</td>
<td>Agouti</td>
</tr>
<tr>
<td>MC3R</td>
<td>$\alpha$-MSH = ACTH = $\beta$-MSH = $\gamma$-MSH</td>
<td>Agouti, AGRP</td>
</tr>
<tr>
<td>MC4R</td>
<td>$\alpha$-MSH = ACTH &gt; $\beta$-MSH &gt; $\gamma$-MSH</td>
<td>Agouti, AGRP</td>
</tr>
<tr>
<td>MC5R</td>
<td>$\alpha$-MSH &gt; ACTH &gt; $\beta$-MSH &gt; $\gamma$-MSH</td>
<td>Not Determined</td>
</tr>
</tbody>
</table>

*Gantz et al., 2003*
Melanocortin Evolution

Analyses of fossil records, gene sequences, and distribution of related proteins have led to the 2R hypothesis to explain the evolution of the chordate genome. The 2R hypothesis suggests that the chordate genome has undergone two genome duplication events (2R) over the past 500 million years (Ohno, 1970). The HOX gene clusters, which are involved in body plan development, serve as a model for these evolutionary events (Holland et al., 1994). There are four HOX gene clusters in mammals, while in protochordates, like Amphioxus, there is only one HOX gene cluster. Mapping of the HOX gene clusters across species has shown evidence for the duplication events that led from the one HOX gene cluster in Amphioxus to the four clusters found in mammals. The first genome duplication event is believed to have occurred within the early radiation of the protochordates at the emergence of the jawless vertebrates (the Agnatha), resulting in two HOX gene clusters in extant Agnathans, like the lamprey (Holland et al., 1994). A second genome duplication then occurred after the divergence between the Agnathans and Gnathostomes (jawed vertebrates), giving rise to four unique clusters in vertebrates (Holland et al., 1994).

Analysis of the melanocortin receptor gene family generally follows the pattern observed for the HOX gene clusters (Figure 2). The operating hypothesis is that there was a single, ancestral MCR gene in early protochordates. A genome duplication event then occurred that led to two MCR genes in the emerging agnathan vertebrates. A second
Figure 2: Schematic of the melanocortin receptor duplication events, as described by the 2R hypothesis.
genome duplication event at the emergence of the gnathostomes would then have led to four MCR genes. The models of the HOX gene clusters and the MCR genes diverge at this point as there are five MCR genes found in mammals rather than four. The appearance of a fifth MCR gene can be explained by adding a gene duplication event to the evolution of the gene family at some point in the radiation of the gnathostomes.

Support for this hypothesis is provided by analysis of the location of the melanocortin receptor genes on human chromosomes. In mammals, the MC2R and MC5R genes are very closely linked. Both genes are found on chromosome 18 and are separated by only 40 kb in humans and 67 kb in mice (Logan et al., 2003). This tight chromosomal linkage has led to the hypothesis that MC2R gave rise to MC5R through a gene duplication event. The locations of the other three MCR genes provide further evidence for the hypothesized evolution of the MCR gene family. MC1R and MC3R are found on completely different chromosomes (chromosome 16 and chromosome 20 respectively), and while MC4R is also found on chromosome 18, it is at a great enough distance from the position of the MC2R and MC5R genes that it is highly unlikely that it was involved in any gene duplication event, but may have rather been part of a separate chromosome that at some point fused to create one large chromosome 18 (Logan et al., 2003). The chromosomal locations of the MCR genes in mammals therefore appear to support the occurrence of two genome duplication events, followed by one gene duplication event.
Over the past two decades, studies have found the MC2R gene in the genome of several teleost fish, amphibians, reptiles, birds, and mammals (for review see Veo et al., 2011). However, the presence of a MC2R ortholog in the genome of a cartilaginous fish is currently a point of controversy.

**MCRs in Cartilaginous Fish**

Cartilaginous fish (chondrichthians) emerged during the late Silurian and early Devonian periods as one of the three major radiations of the gnathostomes, along with ray-finned bony fish and lobe-finned bony fish. The cartilaginous fishes diverged from the bony gnathostomes over 400 million years ago. These fish are characterized by cartilaginous skeletons, placoid scales, and pelvic claspers in males (Maisey, 1986). Cartilaginous fish can be classified into two distinct subclasses: Elasmobranchii (sharks, rays, and skates) and the Holocephali (chimaeras). True sharks were the first cartilaginous fish to appear. Major modifications to the basic body plan of the shark occurred in the Carboniferous Period, leading to the appearance of the holocephalans, and in the Jurassic Period, when the flattened rays appeared (Long, 1995).

The focus of this thesis will be on the MCRs in the spiny dogfish (Squalus acanthias), an elasmobranch, and the elephant shark (Callorhinus milii), a holocephalan. Cloning of MCRs in the spiny dogfish has shown evidence for the presence of three receptors: a SacMC4R (Ringholm et al., 2003), SacMC3R and SacMC5R (Klovins et al., 2004). According to the 2R hypothesis, cartilaginous fish
should possess a minimum of four MCRs, with the possibility of as many as five, so the presence of only three MCR genes in the spiny dogfish seems to present an enigma. However, cloning of the MCRs in this species was done without knowing the full sequence of the dogfish genome, so it is possible that another MCR gene is present, but was simply missed. The recent sequencing of the entire elephant shark genome has allowed this question to be addressed using another cartilaginous fish species (http://esharkgenome.imcb.a-star.edu.sg/). Even though the full sequence of the elephant shark is known, there are still only three MCRs that have been found in this genome database. These receptors have been characterized as CmiMC1R, CmiMC2R, and CmiMC3R (Vastermark and Schioth, 2011). While the MCRs in the elephant shark are different from those found in the spiny dogfish, the equal number of receptors confirms that cartilaginous fish appear to be an enigma in the evolution of the MCR gene family. This is further supported by studies on the horn shark (Heterodontus francisci), which have also found evidence for only three MCR genes (Baron et al., 2009). This raises the question: why are there only three MCRs in cartilaginous fish? One possible explanation for these observations is that one of the MCR genes was lost after the divergence of the ancestral gnathostomes into cartilaginous and bony fish. This thesis will attempt to address this question and the validity of the hypothesized gene loss by analyzing the ligand selectivity of the MCRs in the spiny dogfish and the elephant shark. Although the ligand selectivity of the SacMC3R and SacMC4R have been completed (Ringholm et al., 2003; Klovins et al., 2004), this study was unable to functionally express SacMC5R. This thesis will present new information on the functional expression of SacMC5R, and
consider the trends in the evolution of the MC5R gene. The studies conducted in this thesis on the elephant shark melanocortin receptors will focus on the ligand selectivity studies for the CmiMC3R, and address the question whether an MC2R analog (Vastermark and Schioth, 2011) is present in the elephant shark genome.
CHAPTER 1: Dogfish MC5R Functional Expression

INTRODUCTION

The MCRs of the spiny dogfish were the first to be cloned from a shark species (Ringholm et al., 2003; Klovins et al., 2004). These studies reveal three MCR genes in the genome of the spiny dogfish that corresponded to orthologs of MC3R, MC4R, and MC5R, respectively. However, when these orthologs were individually transfected into mammalian HEK293 cells, only SacMC3R and SacMC4R could be visualized on the plasma membrane using fluorescence microscopy; whereas MC5R was found to be retained in the endoplasmic reticulum. As a consequence, functional assays could only be performed for SacMC3R and SacMC4R (Ringholm et al., 2003; Klovins et al., 2004). SacMC4R has high sequence identity with human MC4R, and similar ligand selectivity to its human equivalent (Ringholm et al., 2003). SacMC3R also has high sequence identity with human MC3R, but has slightly higher affinity for α- and β-MSH, a significantly higher affinity for ACTH, and a lower affinity for γ-MSH than the human form (Klovins et al., 2004).

Receptor-EGFP fusion experiments have shown that SacMC3R and SacMC4R have the ability to be transported from the ER to the cell surface, but SacMC5R was
found to be retained in intracellular compartments when transfected into Chinese hamster ovary (CHO) cells and HEK293 cells (Klovins et al., 2004). Another MCR that exhibits this type of behavior is the MC2R (Webb and Clark, 2010). Since it has been hypothesized that MC2R may have given rise to MC5R through a gene duplication event, it is possible that SacMC5R may represent a proto-MC2R/MC5R. If this is the case, the inability of the receptor to translocate to the cell surface and show functional expression may be due to the need for an accessory protein, like MRAP.

MRAP is a small, single transmembrane domain protein that forms an antiparallel homodimer. MRAP has three distinct functional domains: the transmembrane domain (residues 36-61) is responsible for the physical MC2R/MRAP and MRAP/MRAP interactions, the cell-surface expression domain (residues 9-24) allows for effective trafficking of the MC2R to the cell surface, and the sequence just N-terminal to the transmembrane domain (residues 31-37) is required for adoption of the antiparallel homodimer structure (Webb and Clark, 2010). The cell-surface expression domain contains the sequence L/IDYI, which has been found to be required for effective MC2R ligand binding and signal transduction once the receptor has reached the cell surface (Sebag and Hinkle, 2009). A MRAP homologue, MRAP2, has also been found to facilitate trafficking of MC2R to the plasma membrane, but it lacks the L/IDYI motif and does not effectively facilitate signal transduction at the cell surface (Sebag and Hinkle, 2010).

MRAP is required for functional expression of MC2R, but it has been found to act as a bidirectional regulator of the MCR family due to its ability to inhibit some of the
other MCRs. MRAP and MRAP2 both have the ability to interact with all of the MCRs. This interaction does not alter the cell surface expression of MC1R or MC3R, but has been found to cause a small decrease in surface expression of MC4R and MC5R (Chan et al., 2009). This small reduction appears to be significant as cAMP generation mediated by these two receptors drops when MRAP and MRAP2 are also expressed (Chan et al., 2009). The MCR accessory proteins thus appear to modulate MCR activity throughout the body.

The mammalian MC5R is one of the least understood of the MCRs as it has a wide tissue distribution and yet its only confirmed function thus far is regulation of sebaceous gland secretion. This receptor has also been characterized in several other bony vertebrates like the zebrafish (Danio rerio) and the Japanese pufferfish (Takifugu rubripes), or Fugu. In the zebrafish, there are two MC5Rs that have been identified, DreMC5Ra and DreMC5Rb (Ringholm et al., 2002). These receptors both showed wide tissue distribution, high sequence identity to the human MC5R, and had similar affinity for the melanocortin ligands as the human MC5R. One unique characteristic of the MC5Rs in the zebrafish was the presence of DreMC5Rb in the heart; the only MCR found in the mammalian heart is MC3R (Ringholm et al., 2002). The MC5R in the Fugu was found to have less widely spread tissue distribution than in most species, but is still found in both central and peripheral tissues, which is in line with its tissue distribution in humans (Klovins et al., 2004). TruMC5R generally has a higher affinity for melanocortin ligands than the human receptor, especially for α-MSH; the Fugu MC5R has also been
found to be fairly unique in that the TruMC5R gene contains introns, which are not found in any mammalian MCRs (Klovins et al., 2004).

The objective of this research is to attempt to characterize the ligand selectivity of SacMC5R, which has thus far not been functionally expressed. Does this receptor, like MC2R, require an accessory protein, like MRAP, to be trafficked from the ER to the cell surface? Or, like several other MC5Rs that have been characterized, does MRAP inhibit receptor function? If functional expression of this receptor can be achieved, what kind of ligand selectivity does it have? One of the unique features of the melanocortin system in cartilaginous fish is that it contains a fifth melanocortin peptide, δ-MSH. Both human and dogfish peptides will, therefore, be used to understand ligand selectivity of SacMC5R and whether differences exist in the way that the receptor responds to ligands from different species. These findings will add to the understanding of the evolution and functional features of this particular receptor as well as the evolution of the melanocortin system in other vertebrates.
MATERIALS AND METHODS

MC5R and MRAP Constructs

Spiny dogfish (*Squalus acanthias*) MC5R, accession number AY562212, was synthesized by GenScript with a V-5 epitope tag and inserted into a pcDNA3.1 vector. Mouse (*Mus musculus*) MRAP1, accession number NM_029844, zebrafish (*Danio rerio*) MRAP1, accession number XR_117835, and elephant shark (*Callorhinchus milii*) MRAP2, accession number BR000861, were also synthesized by GenScript with a FLAG epitope tag and were separately inserted into pcDNA3.1 vectors.

Tissue Culture

The experiments were all done in transiently transfected CHO cells. The CHO cells were grown at 37°C in a humidified 5% CO₂ incubator in Kaighn’s Modification of Ham’s F-12 (2mM glutamine, 1500 mg/L bicarbonate) with 10% fetal bovine serum, 5 mL pen/strep, and 1 mL normocin.

Immunocytochemistry

CHO cells were grown in 2-well chamber slides (1x10^5 cells/well) for 24 hours prior to transfection. The cells were transfected with 1µg of dogfish *mc5r* cDNA
construct either in the presence or absence of mouse mrap cDNA using lipofectamine 2000 (Invitrogen) and Opti-MEM. 24 hours post-transfection, the cells were fixed with 4% paraformaldehyde for 15 minutes then either permeablized using 0.3% triton for 10 minutes or left unpermeablized. The primary antibodies were diluted 1:100 in PBS + 1% BSA solution. A mouse monoclonal anti-V5 antibody was used to detect SacMC5R, and a rabbit polyclonal anti-FLAG antibody was used to detect mouse MRAP. The cells were incubated with the primary antibodies for one hour at 37°C. After a wash step, the cells were incubated with secondary antibodies for 45 minutes at 37°C. A donkey anti-mouse antibody linked to Alexa-Fluor488 was used to visualize SacMC5R (green). A donkey anti-rabbit antibody linked to Alexa-Fluor555 was used to visualize MRAP (red). The secondary antibodies were diluted either 1:400 (donkey anti-mouse) or 1:800 (donkey anti-rabbit) in PBS + 1% BSA. Cover slips were mounted onto slides using Vectashield, and nuclei were stained with DAPI (blue). Images were obtained using a 100x oil immersion objective (Zeiss Plan-NEOFUAR) with a fluorescence microscope equipped with a Hamamatsu digital camera. All images were analyzed using SlideBook software.

**Functional Expression Assays**

CHO cells were transfected with 2µg of SacMC5R, MRAP, and Cre-Luc reporter plasmid using a Cell Line Nucleofector Kit (Amaxa, Inc.) with solution T and program U-023. Experiments were also done in which CHO cells were only transfected with SacMC5R and the Cre-Luc reporter plasmid. The cells were then plated on a white, 96-
well plate at a density of $1 \times 10^5$ cells per well. 48 hours after transfection, cells were stimulated with appropriate ligands in serum-free CHO media for four hours at 37°C. After the incubation period, 100µL of Bright-Glo luciferase assay reagent (Promega Inc.) were applied to each well and incubated at room temperature for 5 minutes. Luminescence was then measured with a Bio-Tek Synergy HT plate reader.

**Data Analysis**

All experimental treatments were performed in no less than triplicate (biological replicates), 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 readings for cells treated with 1µM ACTH (for the luciferase assay). Average values and standard errors were graphed using KaleidaGraph software. The built in 1:1 rectangular hyperbola function in KaleidaGraph was used to fit a curve to the data and provide $K_d$ values. Fits were unconstrained.
RESULTS

Since SacMC5R has thus far not been functionally expressed, the first step in the characterization of this receptor was to use immunocytochemistry to determine whether trafficking of this receptor to the plasma membrane was possible in CHO cells. Transfection of SacMC5R into CHO cells and subsequent staining show that the receptor is able to be detected in the cells without permeabilization, suggesting that SacMC5R is able to translocate from the ER to the cell surface (Figure 3).

Once it was established that the receptor was being trafficked to the cell surface, the next issue to address was whether SacMC5R could be functionally expressed. SacMC5R was stimulated with melanocortin peptides to test for activation of the receptor and efficient signal transduction, leading to an increased production of the second messenger cAMP. SacMC5R was again transfected into CHO cells, along with a CRE-Luc cAMP reporter cDNA, then stimulated using human ACTH (1-24) and the full range of dogfish melanocortin peptides [ACTH (1-25), α-MSH, β-MSH, γ-MSH, and δ-MSH] at concentrations ranging from $10^{-5}$ to $10^{-10}$ M. Figure 4 shows that SacMC5R did show some degree of activation with each of the six ligands. SacMC5R appears to have the greatest sensitivity to the two forms of ACTH (human and dogfish), followed by dogfish α-MSH, then γ- and δ-MSH; stimulation by β-MSH led to minimal activation of
SacMC5R, and only at the highest ligand concentration (Figure 4). These results show that even though SacMC5R is a relatively low affinity receptor, it is able to be functionally expressed in CHO cells without the presence of an accessory protein.
Figure 3: CHO cells expressing SacMC5R. V5 tagged SacMC5R is seen on the cell surface of unpermeablized CHO cells (white arrow). Green = SacMC5R (Alexa-Fluor488). Blue = nuclei (DAPI).
**Figure 4:** Functional expression of SacMC5R after stimulation with dogfish peptides. The combined results of two assays are presented with all data normalized to the activation of SacMC5R with 1µM dogfish ACTH. The red line represents stimulation with human ACTH (1-24) [denoted mACTH]; all other ligands used are from the spiny dogfish. (n=6 for 10^-6 ligand concentrations, n=3 for all others)
SacMC5R was also stimulated with various analogs of ACTH that contain alanine substitutions at one (HARW and HFAW) or all (HFRW/A4) of the positions in the motif HFRW (positions 6-9). This motif is present in all of the melanocortin peptides, and a HFRW binding site was predicted to be present in the primary sequence of SacMC5R (Baron et al., 2009). None of the three analogs tested induced any appreciable activation of the receptor (Figure 5), which is not especially surprising considering the receptor’s relatively low affinity for the natural melanocortin peptides and the essential role of the HFRW motif for activating MCRs.

After determining that SacMC5R could be functionally expressed in CHO cells, different forms of MRAP were co-transfected with the receptor to find out how SacMC5R would respond to the presence of this accessory protein. The literature shows that MC5R activity should be decreased by the addition of either MRAP or MRAP2 (Chan et al., 2009), so it was expected that the same trend would be observed when SacMC5R was challenged with various MRAPs. SacMC5R was first transfected with two different forms of MRAP1: one from a mammal (mouse) and the other from a bony fish (zebrafish). This experiment led to two interesting findings. The first was that the presence of MRAP seemed to increase receptor sensitivity to human ACTH (1-24), as the dose response curves for the mouse and zebrafish MRAPs were both shifted left from the curve representing SacMC5R alone (Figure 6). The curve for the receptor alone had a $K_d$ value of $9.41 \times 10^{-9}$, while the dose response curves for the receptor with the mouse MRAP and the receptor with zebrafish MRAP had $K_d$ values of $5.96 \times 10^{-9}$ and $1.12 \times 10^{-9}$ respectively. This finding suggests that SacMC5R does not behave the same way as its
human counterpart. Figure 6 also shows that while co-transfection of SacMC5R with zebrafish MRAP led to a lower $K_d$ value, it ultimately resulted in a significantly lower maximum activation value than SacMC5R with mouse MRAP or SacMC5R alone. This result is surprising since the dogfish is clearly much more closely related to the zebrafish than to the mouse.
Figure 5: Functional expression of SacMC5R with human ACTH(1-24) analogs. CHO cells were stimulated with human ACTH (1-24) [red] and various analogs of this ligand that contain alanine substitutions at position 7 (HARW; green), position 8 (HFAW; black), or at positions 6-9 (HFRW/A4; blue). The figure represents the activity of the CRE-Luc reporter after stimulation with the noted ligands. (n=4)
**Figure 6**: Functional expression of SacMC5R with mouse and zebrafish MRAP1. CHO cells were transfected with receptor alone or receptor with either mouse or zebrafish MRAP1, and then stimulated with human ACTH(1-24). (n=8)
The response of SacMC5R to MRAP1 was unexpected based on characterization of other MC5Rs, so the receptor was then further challenged by co-transfection with elephant shark MRAP2 to investigate whether its response to MRAP2 also differs from that of the human MC5R. Activation of SacMC5R alone and SacMC5R co-transfected with elephant shark MRAP2 were nearly identical when they were stimulated with ACTH (Figure 7). In cells stimulated with α-MSH, activation of SacMC5R with elephant shark MRAP2 appears slightly lower than SacMC5R alone (Figure 7), but the presence of elephant shark MRAP2 does not generally appear to significantly affect activation of SacMC5R. This result also differs from findings for the human MC5R, which has been shown to have decreased activation in the presence of MRAP2 (Chan et al., 2009).
Figure 7: Functional expression of SacMC5R with elephant shark MRAP2. CHO cells were transfected with either SacMC5R or SacMC5R and elephant shark MRAP2, and then stimulated with human ACTH(1-24) or α-MSH. (n=4)
DISCUSSION

This project utilized immunocytochemistry and functional expression assays to characterize the ligand selectivity of the SacMC5R. This receptor had previously been cloned and transfected into HEK293 cells, but was found to remain in intracellular compartments (Klovins et al., 2004). The inability to get the receptor to the cell surface also prevented any functional analyses from being performed for SacMC5R. One hypothesis to account for this observation that was presented in this thesis was that SacMC5R may represent a proto-MC2R/MC5R, and was unable to be properly trafficked and activated because it required the help of an accessory protein, like MRAP, to complete this task.

Initial visualization of SacMC5R in CHO cells with immunostaining revealed that, unlike the observations made by Klovins et al. (2004), this receptor was able to be identified on the cell surface of unpermeabilized cells (Figure 3). While this finding does not agree with what has previously been reported in the literature for this specific receptor, it does agree with what has previously been reported for other MC5Rs (for review see Cone, 2006). In its ability to traffic from the ER to the cell surface without MRAP, SacMC5R does, therefore, appear to function like other reported MC5Rs.
Successful translocation of SacMC5R to the plasma membrane in CHO cells then allowed for functional analysis of this receptor. MC5Rs are generally the least understood of the MCRs, but mammalian MC5R has been shown to have the highest affinity for α-MSH, followed by ACTH, then β-MSH, and finally γ-MSH (Gantz and Fong, 2003). Stimulation of SacMC5R with human ACTH and a full line-up of dogfish ligands revealed that this receptor is not only able to reach the cell surface, but is also functional and able to be stimulated by a variety of ligands when it gets there. SacMC5R showed a somewhat similar pattern of activation to its human counterpart, with ACTH (both human and dogfish) and α-MSH being the most potent activators of the receptor (Figure 4). Even though the human MC5R shows a preference for α-MSH over ACTH, many bony fish receptors have shown strong activation by human ACTH, so this observation appears to be in line with previous findings (Klovins et al., 2004; Cerda-Reverter et al., 2010). None of the dogfish receptors have thus far been stimulated with dogfish ligands; this is of special note because sharks have the melanocortin peptide δ-MSH, which is not produced in mammals. It was, therefore, interesting to find that δ-MSH was as potent of a ligand for SacMC5R as was γ-MSH (Figure 4). SacMC5R had a low affinity for these two ligands, but both provided strong maximal activation levels. It is also noteworthy that for SacMC5R, dogfish β-MSH appears to be a very poor ligand. It would be interesting to stimulate the other two dogfish MCRs with the dogfish melanocortin peptides to determine the role that δ-MSH plays in the melanocortin system of cartilaginous fish, and to see if the reduced role of β-MSH that was observed for SacMC5R is also seen in other cartilaginous fish MCRs. Overall, the ligand selectivity of
SacMC5R does not appear to vary dramatically from what would be expected in a MC5R.

The hypothesis that SacMC5R was unable to be expressed because of a need for MRAP was shown to be incorrect through activation with human ACTH and the dogfish ligands, but challenging the receptor by co-transfection with MRAP or MRAP2 was still an important step in the characterization of this receptor. Mammalian MC5Rs have been shown to be negatively affected by the presence of MRAP, likely due to a decrease in surface expression of the receptor (Chan et al., 2009). SacMC5R, however, showed somewhat mixed responses to co-transfection with MRAPs. Mouse MRAP appeared to increase sensitivity of the receptor, while zebrafish MRAP initially seemed to increase sensitivity of SacMC5R, but then resulted in significantly lower levels of total activation than the receptor alone (Figure 6). Furthermore, co-transfection with elephant shark MRAP2 did not seem to cause a significant change in receptor activation (Figure 7). The observed enhancement of SacMC5R signaling with mouse MRAP is a curious finding that suggests that this receptor does not fully behave like other bony vertebrate MC5Rs.

It would be helpful to further analyze surface expression of SacMC5R in the presence or absence of each of the forms of MRAP and MRAP2 to determine if, like observed in the human MC5R, the presence of MRAP alters the amount of receptor trafficking to the cell surface. This would provide more insight into whether the observed effects are due to changes in receptor trafficking, or if interaction with MRAP is affecting another aspect of receptor activity, like signal transduction.
CHAPTER 2: Functional Expression of Elephant Shark MCRs

INTRODUCTION

The MCRs of the spiny dogfish were the first to be studied in a cartilaginous fish (see chapter 1), and the finding that only three MCRs can be found in this species leads to several questions about the melanocortin system in cartilaginous fish. Do all cartilaginous fish have only three MCRs and are the same three receptors (MC3R, MC4R, and MC5R) found in other cartilaginous fish? The recent sequencing of the entire elephant shark genome has provided a new approach for investigating these questions. The cartilaginous fish can be separated into two subclasses: Elasmobranchii and Holocephali. The dogfish belongs to the former and the elephant shark belongs to the latter. Characterization of the MCRs in the elephant shark may thus be useful for understanding how the melanocortin systems among the cartilaginous fish compare to one another, which may also provide new insight into the evolution of this gene family.

There are four melanocortin-related genes that have been found in the elephant shark genome: three MCRs (Figure 8) and a MRAP2 (Figure 9). The results of a phylogenetic analysis were recently published and have identified the three elephant shark MCRs as CmiMC1R, CmiMC2R, and CmiMC3R (Vastermark and Schioth, 2011).
The sequence for the receptor identified as CmiMC1R has incomplete N- and C-termini, so this research will focus on the CmiMC2R and CmiMC3R. The objective of this study is to perform functional analyses on these two putative receptors and to determine whether they demonstrate the ligand selectivity that would be expected of a MC2R and MC3R respectively. Analysis of the CmiMC2R will be especially informative as this receptor should display very specific characteristics. Is this receptor only able to be activated by ACTH? MC2Rs have also been shown to require MRAP1 to be trafficked to the cell surface, but since the elephant shark genome lacks a MRAP1 gene will CmiMRAP2 be able to translocate the receptor and support ligand binding and signal transduction in its place? Functional analyses of the putative CmiMC2R and CmiMC3R will again be performed using both human and shark melanocortin peptides to provide a more complete picture of the melanocortin system in this species.
Figure 8: Alignment of putative elephant shark MCRs. The sequences of the three elephant shark MCRs are shown with labels for the transmembrane regions (TM), intracellular loops (IC), and extracellular loops (EC).
Figure 9: Elephant shark MRAP2 cDNA. The sequence for elephant shark MRAP2 is shown with the FLAG epitope tag (underlined).
MATERIALS AND METHODS

MCR and MRAP Constructs

Elephant shark (*Callorhinchus milii*) MC1R, accession number BR000855, MC2R, accession number BR000856, and MC3R, accession number BR000857, were synthesized by GenScript with a V-5 epitope tag and were separately inserted in pcDNA3.1 vectors. For information on the MRAP constructs used, refer to Chapter 1.

Tissue Culture

Refer to Chapter 1

Immunocytochemistry

Refer to Chapter 1

Functional Expression Assays

Refer to Chapter 1

Data Analysis

Refer to Chapter 1
RESULTS

MC3R Analysis

A recent phylogenetic analysis has identified one of the melanocortin genes in the elephant shark genome as CmiMC3R (Vastermark and Schioth, 2011). To further characterize this receptor and to determine if it behaves the way a MC3R would be expected to behave, both immunocytochemistry and functional assays were utilized. V5 tagged CmiMC3R was transfected into CHO cells, and immunostaining was used to visualize the receptor. CmiMC3R was visualized on the surface of unpermeablized cells, suggesting that this receptor is able to be properly trafficked from the ER to the cell surface without the need for the presence of an accessory protein, like MRAP (Figure 10).

Once it was established that translocation of CmiMC3R to the cell surface was taking place, the receptor was stimulated with human ACTH (1-24) and the full range of dogfish melanocortin ligands [i.e., ACTH (1-25), α-MSH, β-MSH, γ-MSH, and δ-MSH] at concentrations of 10^{-10} to 10^{-5} M to test for functionality and ligand selectivity. CmiMC3R was again transfected into CHO cells, along with a CRE-Luc reporter cDNA, and then stimulated with the selected melanocortin peptides. CmiMC3R showed the greatest activation following stimulation with dogfish ACTH (1-25) and human ACTH
(1-24); both forms of ACTH led to similar total levels of activity, but the receptor appears to be slightly more sensitive to the dogfish form of the peptide (Figure 11). The $K_d$ of the dose response curve for the receptor stimulated with dogfish ACTH (1-25) was $1.63 \times 10^{-8}$ as compared to a $K_d$ of $4.95 \times 10^{-8}$ for the receptor stimulated with human ACTH (1-24). Dogfish $\delta$-MSH and $\alpha$-MSH also activated CmiMC3R, although to a lower degree than the two forms of ACTH, while stimulation with dogfish $\beta$-MSH and $\gamma$-MSH resulted in minimal receptor activity (Figure 11). The results of this assay indicate that, consistent with findings for other MC3Rs, CmiMC3R is able to be stimulated by a variety of melanocortin peptides without the necessity for MRAP.
Figure 10: Expression of CmiMC3R in CHO cells. V5 tagged CmiMC3R is seen on the cell surface of unpermeablized CHO cells (white arrow). Green = CmiMC3R (Alexa-Fluor488). Blue = nuclei (DAPI).
Figure 11: Functional expression of CmiMC3R with dogfish ligands. The combined results of two assays are presented with all data normalized to the activation of CmiMC3R with 1µM dogfish ACTH. The red line represents stimulation with human ACTH (1-24) [denoted mACTH]; all other ligands used are from the spiny dogfish (DF). (n=3)
CmiMC3R was next stimulated by the various human ACTH analogs that contain alanine substitutions in the HFRW motif (positions 6-9). Figure 12 shows that alteration of this motif in either one position (HFAW or HARW) or in all four positions (HFRW/A4) led to a significant drop in receptor activation. This result appears to support previous findings that this motif is important for binding of the ligand to its receptor.

Of the four melanocortin genes that have been found in the elephant shark genome, three represent putative MCRs while the fourth is a gene for MRAP2. CmiMC3R was co-transfected with elephant shark MRAP2 to determine whether the presence of this accessory protein has any effect on the activation of the receptor. CHO cells transfected with both CmiMC3R and elephant shark MRAP2 did not show significant differences in total activation, but there did appear to be a slight increase in sensitivity to human ACTH in cells expressing both genes rather than the receptor alone (Figure 13). This is a surprising result since MRAP2 has not previously been found to have a significant effect on MC3R activity (Chan et al., 2009). The presence of elephant shark MRAP2 did not considerably alter the dose response curve for α-MSH stimulated cells as compared to cells transfected with the receptor alone.
Figure 12: Functional expression of CmiMC3R with ACTH analogs. CHO cells were stimulated with human ACTH (1-24) [red] and various analogs of this ligand that contain alanine substitutions at position 7 (HARW; green), position 8 (HFAW; black), or at positions 6-9 (HFRW/A4; blue). The figure represents the activity of the CRE-Luc reporter after stimulation with the noted ligands. (n=4)
Figure 13: Functional expression of CmiMC3R with elephant shark MRAP2. CHO cells were transfected with either CmiMC3R or CmiMC3R and elephant shark MRAP2, and then stimulated with human ACTH or α-MSH. (n=8)
CmiMC3R was then challenged with two forms of MRAP1 (mouse and zebrafish) to determine whether this accessory protein would alter receptor sensitivity or activity. CHO cells transfected with CmiMC3R alone or CmiMC3R with zebrafish MRAP displayed similar activation when stimulated with human α-MSH (Figure 14). Unexpectedly, co-transfection of CmiMC3R with mouse MRAP led to nearly a doubling of receptor activation; the dose response curve was also significantly shifted to the left, from an initial $K_d$ of $6.57 \times 10^{-8}$ for receptor alone to a $K_d$ of $1.60 \times 10^{-9}$ with the addition of MRAP, suggesting greater receptor sensitivity (Figure 14). This result suggests that CmiMC3R does not behave like its human equivalent when challenged with a mammalian MRAP. The observed effect of mouse MRAP on CmiMC3R is also unpredicted since no gene for MRAP1 has been found in the elephant shark genome.
**Figure 14:** Functional expression of CmiMC3R with mouse and zebrafish MRAP1. CHO cells were transfected with receptor alone or receptor with either mouse or zebrafish MRAP1, and then stimulated with human α-MSH. (n=4)
MC2R Analysis

The next receptor of interest that has been cloned from the elephant shark genome is the receptor that Vastermark and Schioth (2011) have identified as CmiMC2R. Identification as a MC2R suggests that the receptor should have two very distinct characteristics: it should require MRAP to be trafficked to the cell surface and it should only be activated by ACTH. Immunocytochemistry was used to test the first of these two assertions. V5 tagged CmiMC2R was transfected into CHO cells and immunostaining was utilized to test for presence of the receptor on the cell surface. CmiMC2R was visualized in unpermeablized cells in the absence of MRAP (Figure 15). This suggests that CmiMC2R is able to be translocated from the ER to the cell surface without the help of an accessory protein. There have thus far not been any MC2Rs characterized that are able to perform this action (Cone, 2006; Klovins et al., 2003; Ringholm et al., 2002).

Although the receptor was apparently able to make it to the cell surface without MRAP, it was not known whether the receptor would be functional on its own. Its identification as a MC2R led us to first attempt functional expression of CmiMC2R in the presence of MRAP and with ACTH(1-24) used for stimulation. As was expected, CmiMC2R showed activation with stimulation by human ACTH(1-24) when it was co-transfected with mouse or zebrafish MRAP1; however, CmiMC2R also showed similar activation by human ACTH(1-24) when no MRAP was present (Figure 16). Unlike human MC2R, CmiMC2R appears to be able to translocate to the cell surface and be stimulated by ACTH without interacting with any form of MRAP. Furthermore, the
presence of MRAP1 does not seem to significantly benefit the receptor in terms of maximum activation nor sensitivity to ACTH.
**Figure 15:** Expression of CmiMC2R in CHO cells. V5 tagged CmiMC2R is seen on the cell surface of unpermeabilized CHO cells (white arrow). Green = CmiMC2R (Alexa-Fluor488). Blue = nuclei (DAPI).
Figure 16: Functional expression of CmiMC2R with mouse and zebrafish MRAP1. CHO cells were transfected with CmiMC2R either alone (red) or with mouse (blue) or zebrafish (green) MRAP1. All cells were stimulated with human ACTH(1-24). (n=4)
CmiMC2R was not affected by MRAP1, but a MRAP1 gene has not been found in the elephant shark genome, so the receptor was next co-transfected with elephant shark MRAP2 to determine what effect, if any, the interaction with this accessory protein would produce. The MC2R/MRAP2 interaction has previously been found to help with trafficking to the cell surface, but the MC2R still lacks functionality in this scenario (Sebag and Hinkle, 2009). In addition to challenging CmiMC2R with MRAP2, the receptor was also stimulated with human α-MSH to determine if it possessed the ACTH selectivity that would be anticipated for a MC2R. This experiment led to two notable findings. The first was that CmiMC2R is able to be stimulated by both human ACTH(1-24) and α-MSH (Figure 17). This provides further evidence that CmiMC2R does not function like the human MC2R, as it is not ACTH specific. The second notable result was that co-transfection of CmiMC2R with elephant shark MRAP2 appears to decrease receptor maximal activation with stimulation by either ligand, and seems to slightly reduce receptor sensitivity when it is stimulated with α-MSH (Figure 17). This result is not unexpected as MRAP2 has not previously been found to enhance the activation of any of the MCRs (Sebag and Hinkle, 2009).
Figure 17: Functional expression of CmiMC2R with elephant shark MRAP2. CHO cells were transfected with either CmiMC2R alone or CmiMC2R with elephant shark MRAP2, and then stimulated with human ACTH (red and blue respectively) or α-MSH (green and black respectively). (n=8)
After determining that CmiMC2R was capable of being activated by human α-MSH, the receptor was stimulated by all five dogfish melanocortin peptides to determine its full range of ligand selectivity. CmiMC2R was found to be activated to some degree by all of the melanocortin ligands; it appears to be most sensitive to the two forms of ACTH (human and dogfish), followed by γ- and δ-MSH, α-MSH, and finally β-MSH (Figure 18). Although CmiMC2R seems to have a relatively low affinity for dogfish α-MSH, this ligand was able to reach a maximum activation level very similar to that of ACTH. It is also notable that this was the only receptor tested in this thesis that had any appreciable amount of stimulation by dogfish β-MSH. The results of this assay, once again, strongly argue against classification of this receptor as a MR2R.
Figure 18: Functional expression of CmiMC2R with dogfish ligands. CHO cells were transfected with CmiMC2R and stimulated with human ACTH (mACTH; red) or one of five dogfish melanocortin ligands (DF). The combined results of two assays are presented with all data being normalized to the average value for stimulation with 1µM human ACTH. (n=6 for 10^{-5} concentrations, n=3 for all others)
DISCUSSION

The purpose of this portion of the thesis was to characterize two newly identified MCRs from the elephant shark: CmiMC2R and CmiMC3R (Vastermark and Schioth, 2011). No elephant shark receptors had thus far been functionally expressed, so the goal was to determine whether these putative receptors functioned the way that a MC3R and MC2R would be expected to function. The two central issues that were addressed for each of the receptors were whether or not they required MRAP for activation and what type of ligand selectivity they displayed.

CmiMC3R

The operating assumptions if CmiMC3R is, in fact, an ortholog of the bony vertebrate MC3Rs were: a) this receptor would not require MRAP for trafficking to the plasma membrane, b) co-transfection with MRAP could have a negative effect on trafficking to the plasma membrane, and c) the elephant shark receptor could be activated by either ACTH or the MSHs. CmiMC3R was identified on the surface of unpermeablized CHO cells when transfected in the absence of MRAP (Figure 10), which confirmed its ability to translocate from the ER to the cell surface and allowed for further
analysis through functional expression assays. Stimulation of CmiMC3R with human 
ACTH(1-24) and all of the melanocortin ligands from the spiny dogfish showed that the 
receptor had the highest sensitivity to and greatest activation by the two forms of ACTH, 
with the dogfish form being a slightly better ligand than the human form. Dogfish α- and 
δ-MSH provided the next best receptor activation, while γ- and β-MSH provided the 
lowest levels of activation (Figure 11). The human MC3R has been shown to have fairly 
equal sensitivity to all of the melanocortin ligands and to be the most responsive MCR to 
gamma-MSH (Gantz and Fong, 2003). CmiMC3R thus appears to be more sensitive to ACTH 
and less sensitive to γ- and β-MSH than its human counterpart. This pattern of ligand 
selectivity has also been observed in the SacMC3R (Klovins et al., 2004). It is also 
interesting to note that dogfish δ-MSH was a moderately effective ligand since this form 
of MSH is not found in mammals and it is, therefore, not well established how a MC3R 
would respond to it.

It was clear that CmiMC3R was capable of being trafficked to the cell surface and 
activated by melanocortin ligands in the absence of MRAP, but co-transfection with 
various forms of MRAP and MRAP2 led to some interesting findings. MRAP has been 
shown to have a negligible effect on activation of mammalian MC3Rs (Chan et al., 
2009), but the presence of mouse MRAP significantly increased both the sensitivity and 
maximum activation levels of CmiMC3R when stimulated with human α-MSH (Figure 
14). Interestingly, the same effect was not observed when CmiMC3R was co-transfected 
with MRAP from the more closely related zebrafish, and only a slight increase in 
receptor sensitivity to human ACTH was observed when CmiMC3R was co-transfected
with elephant shark MRAP2 (Figure 13). It is unclear why mouse MRAP appeared to enhance CmiMC3R activation so dramatically, but it would be informative to perform surface expression assays to determine if perhaps an interaction between the two is leading to an increase in the amount of receptor present on the cell surface when compared to cells transfected with CmiMC3R alone.

The functional analyses carried out on CmiMC3R suggest that this receptor has some distinct differences from its human counterpart, but the results do not seem to argue against its classification as a MC3R. The differences observed may instead provide some insight into the function of earlier forms of the MC3R and provide new understanding of the way that the melanocortin system has evolved since the divergence of the cartilaginous fish and bony gnathostomes.

CmiMC2R

Among the MCRs, MC2R is arguably the most distinct. It is the only MCR that requires MRAP for translocation from the ER to the cell surface and subsequent activation and signal transduction (Metherell et al., 2005). It is also the one MCR that does not respond to any of the forms of MSH, but rather is activated by ACTH alone. For these reasons, the classification of one of the elephant shark melanocortin genes as CmiMC2R indicated that functional expression of this receptor would likely require a very specific set of conditions.
As was the case with both of the other MCRs characterized in this thesis, the first experiment conducted with this receptor was to transfect it into CHO cells and to use immunostaining to visualize the receptor in the cells. Surprisingly, the receptor was able to be seen in unpermeablized cells, suggesting that it was able to be trafficked to the cell surface without MRAP (Figure 15). This finding does not agree with what is reported in the literature for MC2Rs, but it also did not necessarily mean that the receptor on the surface would be functional without MRAP.

Functional expression was next attempted with the receptor alone and with two different forms of MRAP. This provided another unexpected result as the cells transfected with CmiMC2R were activated just as strongly by human ACTH as were the cells that had been transfected with CmiMC2R and MRAP, either mouse or zebrafish (Figure 16). This result once again does not agree with the literature as there have not previously been any MC2Rs reported that are functional in the absence of MRAP. The receptor was also co-transfected with elephant shark MRAP2, which led to a decrease in activation (Figure 17). This result was the first one that agreed with the literature, but the same pattern is expected in all of the MCRs (Chan et al., 2009).

It became incredibly evident that CmiMC2R does not function like a typical MC2R when the receptor was stimulated by the various forms of MSH. CmiMC2R was most sensitive to ACTH (human and dogfish), but it was activated to some degree by all of the melanocortin ligands from the spiny dogfish (Figure 18). Of all three receptors tested in the thesis, it was also the most sensitive to β-MSH. This data combined with the findings that CmiMC2R is able to be trafficked to the cell surface and stimulated without
MRAP all strongly suggest that this receptor either should not be classified as a MC2R or that the elephant shark possesses an early form of MC2R that has not evolved the unique characteristics that define the mammalian MC2R. While the latter option is a possibility, BLAST analysis suggests that the former is a more likely explanation. The top results in the BLAST analysis all represent MC5Rs and MC4Rs from various species. It, therefore, seems necessary for more work to be done on this receptor to ensure that it is correctly identified.
CONCLUSION

The 2R hypothesis suggests that the chordate genome has undergone two genome duplication events over the past 500 million years (Ohno, 1970). In terms of the melanocortin gene family, this suggests that there was one ancestral MCR in the early protochordates. A genome duplication event then gave rise to two MCR genes in the emerging agnathan vertebrates, and a second duplication event at the emergence of the gnathostomes would have led to four MCR genes. The appearance of a fifth MCR gene is hypothesized to be due to a gene duplication event at some point after the second genome duplication event. Ancestral gnathostomes should, therefore, have at least four MCR genes.

The cartilaginous fish are gnathostomes that could potentially have as many as five MCR genes, but because of early divergence from the lineage that gave rise to the bony vertebrates, it is possible that the ancestral cartilaginous fish may have had only four MCR genes. Extant cartilaginous fish are divided into two subclasses: Elasmobranchii (sharks, rays, and skates) and the Holocephali (chimaeras) (Long, 1995). MCRs have been detected in the genomes of at least one representative of each of these two major groups, the spiny dogfish and the elephant shark respectively, but at this point
only three MCR genes have been detected in each. To reconcile this observation with the expectations from the 2R hypothesis, it has been proposed that a gene loss has occurred.

The three MCR genes that have been found in the spiny dogfish are: SacMC4R (Ringholm et al., 2003), SacMC3R and SacMC5R (Klovins et al., 2004). This thesis focused specifically on the SacMC5R and it was found that this receptor, like bony vertebrate MC5Rs, was able to be stimulated by all of the melanocortin ligands in the absence of MRAP. SacMC5R did, however, show a preference for ACTH, whereas α-MSH is typically the most potent ligand for mammalian MC5Rs (Gantz and Fong, 2003). This preference for ACTH over α-MSH has also been observed in other bony fish receptors (Klovins et al., 2004; Cerda-Reverter et al., 2010). SacMC5R was distinct from other bony vertebrate MC5Rs in its enhanced activity when co-transfected with mouse MRAP. The interaction between MRAP and mammalian MC5Rs has been shown to reduce cell surface trafficking and signaling of the receptor, potentially through disruption of MC5R dimers (Sebag and Hinkle, 2009). SacMC5R, however, became more sensitive to ACTH when MRAP was present.

The three MCR genes that have been detected in the genome of the elephant shark have been identified as CmiMC1R, CmiMC2R, and CmiMC3R (Vastermark and Schioth, 2011). CmiMC1R was not analyzed in this thesis since the N- and C-termini of this receptor’s sequence are currently incomplete (Figure 8). CmiMC3R was found to be similar to previously studied MC3Rs in its ability to be activated by all of the melanocortin ligands in the absence of MRAP, but appeared to be slightly more sensitive to ACTH and less sensitive to γ-MSH than mammalian MC3Rs (Gantz and Fong, 2003).
This seems to be a pattern for cartilaginous fish MC3Rs, however, as a very similar trend of ligand selectivity has been reported for the SacMC3R (Klovins et al., 2004). CmiMC3R also differed from other bony vertebrate MC3Rs in its response to MRAP since the presence of mouse MRAP led to a dramatic increase in receptor activity. For mammalian MC3Rs, MRAP has previously been shown to have no significant effect on activation (Chan et al., 2009).

The final receptor analyzed during this study was the CmiMC2R, which proved to function in a vastly different way from bony vertebrate MC2Rs. Not only did this receptor not require MRAP to be trafficked to the cell surface and functionally expressed, but it was also able to be activated by all of the melanocortin ligands, not just ACTH. Co-transfection with MRAP, which would be expected to enhance receptor activity, did not seem to affect receptor function in any way.

As previously stated, one of the hypotheses for how bony vertebrates came to possess five MCR genes is that there was a local gene duplication event that took place, after the two genome duplication events, in which a proto-MC2R/MC5R gave rise to distinct MC2R and MC5R genes. Support for this hypothesis comes from research showing that in mammals these two genes are both located on chromosome 18 at a distance of only 40 kb from one another in humans and 67 kb from one another in mice (Logan et al., 2003). Synteny studies on the Fugu and zebrafish have shown that MC2R and MC5R are in close proximity in these species as well, separated by only 2.6 kb and 6.1 kb respectively (Klovins et al., 2003). Assuming that CmiMC2R has been correctly identified, the results of this thesis may also lend some support to this hypothesis. The
CmiMC2R did not function as would be expected based on our knowledge of other bony vertebrate MC2Rs. This receptor may, therefore, represent a type of proto-MC2R/MC5R that has not yet evolved the reliance on MRAP and the ACTH selectivity that has come to be associated with more modern forms of MC2R. The enhancement in SacMC5R when mouse MRAP was present may also suggest that the dogfish has a receptor that more closely resembles a proto-MC2R/MC5R than the bony vertebrate MC5R, which is inhibited by MRAP (Chan et al., 2009). Obtaining more information on the tissue distribution of MC2R and MC5R in cartilaginous fish would help provide more insight into whether this hypothesis is plausible. If the two genes are the product of a local gene duplication event, then it would be expected that their distribution would be more widespread than the mammalian forms of MC2R and MC5R. For example, there would likely be MC5R detected in the adrenal gland, which is usually an area where only MC2R is found. Further support of this gene duplication hypothesis would also be provided by more phylogenetic analyses of these two genes in cartilaginous fish. If they originated from a proto-MC2R/MC5R, then they should be closely related and share a great deal of sequence identity, especially in cartilaginous fish, which should represent fairly early forms of the receptors.

Another possibility is that CmiMC2R is not actually a MC2R. The results given by doing a BLAST analysis (http://www.ncbi.nlm.nih.gov/) all suggest that this receptor is either a MC4R or MC5R. It will, therefore, be important for the identity of this receptor to be more strongly supported before any real conclusions can be drawn from the CmiMC2R data presented in this study.
The studies on the cartilaginous fish receptors presented in this thesis provide some interesting new insight into the evolution of the melanocortin family, but they also highlight the need for the sequencing of a model cartilaginous fish species genome. The elephant shark genome has certainly provided new knowledge, but the limited availability of actual specimens for studies on MCR tissue distribution and functions does not make it an ideal model. New work is being done to sequence the genome of the skate, but this species may not be an ideal model either since it belongs to a branch of the cartilaginous fish that is believed to be more rapidly evolving than the others. The best candidate may, therefore, be the spiny dogfish. Knowing the full genome would add to the existing information on MCRs in this species and would dispel any doubt about whether any MCR genes have been missed.
BIBLIOGRAPHY


