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ANALYZING THE INTERACTION BETWEEN MELANOCORTIN 5 RECEPTOR,
MELANOCORTIN RECEPTOR ACCESSORY PROTEIN 1, AND MELANOCORTIN
2 RECEPTOR IN RAINBOW TROUT

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

Domicinda M. Hill

June 2012

Advisor: Dr. Robert M. Does

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Title: ANALYZING THE INTERACTION BETWEEN MELANOCORTIN 5 RECEPTOR, MELANOCORTIN RECEPTOR ACCESSORY PROTEIN 1, AND MELANOCORTIN 2 RECEPTOR IN RAINBOW TROUT

Advisor: Dr. Robert M. Does

Degree Date: June, 2012

ABSTRACT

Using CHO cells we sought to explore and characterize the functional relationship of rainbow trout MC5 receptor (rtMC5R) with zebrafish MRAP1 (zfMRAP1) and rainbow trout MC2 receptor (rtMC2R), as well as how the effect of such relationships may play a significant role in Hypothalamic-Pituitary-Adrenal/Interrenal axis activation. This research demonstrated that rtMC5R can be successfully expressed and functionally activated in CHO cells and, in a manner similar to mammals, α -MSH is the preferred ligand for rtMC5R. The presence of MRAP1 does not seem to inhibit the expression or function of rtMC5R, rather it appears that it may increase expression and total activation levels. In contrast, the presence of MRAP1 in conjunction with MC2R reduces expression and total activation levels of rtMC5R. Findings indicate that rtMC5R may function in three different ways in a cell, depending upon the presence of other local proteins. The interaction of rtMC5R with MRAP1 and MC2R, and the implications of such interactions will be described in this thesis.

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INTRODUCTION

The Melanocortin System

The melanocortin system is composed of melanocortin receptors, their accessory proteins, and their ligands. Melanocortin receptors (MCRs) are a family of small G protein-coupled receptors (GPCRs) (Gantz and Fong, 2003). MCRs are in subclass A-13 of the class-A, rhodopsin-like, GPCR family (Hinkle and Sebag, 2009). Like all GPCRs MCRs have 7 membrane spanning domains, and are coupled to an intracellular G-protein (Hinkle and Sebag, 2009). These receptors preferentially bind protein hormones derived from pro-opiomelanocortin (POMC). POMC is selectively processed to produce the melanocortins as well as β -endorphin and β -lipotropin (Eipper and Mains, 1980). The protein hormones known as melanocortins include the family of melanocyte stimulating hormones, α -MSH, β -MSH and γ -MSH as well as adrenocorticotrophic hormone (ACTH). The MCRs bind the melanocortins with varying levels of selectivity, as seen in table 1.

Activation of MCRs results in a similar cascade of events to other GPCRs. Upon ligand binding, the α -subunit of the G-protein is released which activates adenylyl cyclase. Adenylyl cyclase activation results in the production of the second messenger cyclic AMP (cAMP). Intracellular levels of cAMP regulate protein kinase A (PKA), and in our studies an increase in cAMP allows us to measure activity levels of our receptors (Chan *et al.*, 2011).

Receptor	Major sites of expression	Known physiological function	Preferred Ligands
MC1R	Melanocytes	Pigmentation	α -MSH = β -MSH = ACTH > γ -MSH
MC2R	Adrenal cortex	Adrenocortical steroidogenesis	ACTH
MC3R	CNS, GI tract, kidney	Energy homeostasis, natriuresis	α -MSH = β -MSH = ACTH = γ -MSH
MC4R	CNS	Energy homeostasis, sexual function	α -MSH = β -MSH = ACTH > γ -MSH
MC5R	Exocrine cells, adrenal cortex	Synthesis and secretion of exocrine gland products	α -MSH = β -MSH = ACTH > γ -MSH

Table 1 Localization, physiological function and ligand preference of melanocortin receptors 1 through 5 in mammals. Adapted from Cone, 2006

Five MCRs are expressed in the mammalian system, and are numbered by the order in which they were discovered. MCRs are expressed in differing patterns throughout the body, and each displays different physiological functions. Pigmentation is a main role of the melanocortin 1 receptor (MC1R), which is localized in cutaneous melanocytes, though it is also found in immune cells, keratinocytes, endothelial cells, fibroblasts, pituitary and testis (Gantz and Fong, 2003; Chhanjali, 1996). MC1R binds all POMC derivatives, showing equal preference for α -MSH, β -MSH and ACTH over γ -MSH (Cooray and Clark, 2011).

Responding to circulating ACTH as a result of corticotropin releasing hormone (CRH) release from the hypothalamus, MC2R is involved in the chronic stress response (Chan *et al.*, 2011). MC2R is also called the ACTH receptor, because it is the lone receptor that binds only ACTH (Gantz and Fong, 2003). MC2R is found in adrenal cells, as well as adipocytes, brain tissue, testis and the spleen (Chhanjali, 1996). The MC2R is involved in steroidogenesis, including synthesis of cortisol, corticosterone and aldosterone, and is also involved in the stress response as part of the Hypothalamic-Pituitary-Adrenal axis (HPA) (Figure 1) (Chan *et al.*, 2011).

Melanocortin 3 is localized in the brain, stomach, pancreas, heart, kidneys, skeletal muscle, intestine, placenta, testis and ovaries (Cone, 2006; Chhanjali, 1996). A major physiological role of MC3R is in energy homeostasis as well as inflammatory action (Cooray and Clark 2011). This receptor is unique due to the fact that it lacks a preference for any specific POMC derivative, and binds all melanocortins with near equal affinity (Cone, 2006).

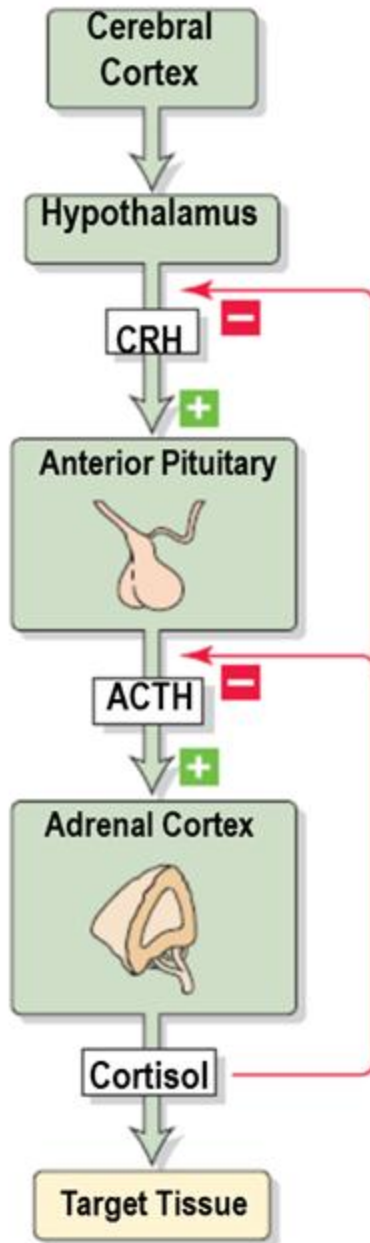


Figure 1 The Hypothalamic-Pituitary-Adrenal Axis. Activation begins in the cerebral cortex, which triggers release of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH stimulates the anterior pituitary to release ACTH, which then binds MC2R in the adrenal cortex. Stimulation of the adrenal cortex by ACTH results in cortisol release.

MC4R is predominantly localized in the nervous system, and binds α -MSH, ACTH and β -MSH with equal affinity (Cooray and Clark, 2011). This receptor is involved in sexual function and appetite regulation, as well as energy homeostasis similar to MC3R (Cooray and Clark, 2011).

The most recent MCR to be identified was MC5R, and knowledge of function and localization of this receptor is quickly growing. Possibly the most widely distributed MCR, MC5R is involved in exocrine function and therefore expressed in a variety of exocrine glands, including lacrimal, preputial and Harderian glands, as well as adipocytes, the adrenal gland, kidneys, testis, ovary, pituitary, and parts of the immune system (Cone, 2006; Gantz and Fong, 2003; Chhanjali, 1996). Results regarding the ligand preference of MC5R have been contradictory; some data supporting a preference for α -MSH over ACTH and vice versa. MC5R can also bind the rest of the melanocortins as well (Cone, 2006).

Activation of Mammalian MCRs

In mammals MC1R, MC3R, MC4R, and MC5R can bind and be activated to varying degrees by either ACTH or the MSHs (Gantz and Fong, 2005). In contrast, MC2R can only be activated by ACTH (Cooray and Clark, 2011). Expression and ligand preference studies for the MCRs were first performed in heterologous mammalian cell lines such as HEK293 cells, COS cells, HeLa cells and CHO cells. Though MC1R, MC3R, MC4R and MC5R were expressed, activated and characterized with little difficulty, performing the same task with MC2R was not so easily accomplished.

Scientists were eventually able to express MC2R in cell lines derived from the adrenal gland, it was not until 2005 that the melanocortin-2 receptor accessory protein (MRAP) was identified as being the protein critical for MC2R expression (Metherell *et al.*, 2005).

History and Knowledge of the MRAP System

To date there have been identified two accessory proteins that are part of this system, melanocortin receptor accessory proteins 1 and 2 (MRAP1 and 2, respectively). MRAPs are type 1 proteins with a single membrane spanning domain. These accessory proteins can both control the expression of MCRs on the plasma membrane, as well as assist in protein folding and trafficking (Sebag and Hinkle, 2008). MRAPs have been shown to immunoprecipitate with all five MCRs; however, MC2R requires MRAP in order to be functional, while MC1R, MC3R, MC4R and MC5R functionality is reduced by MRAP (Chan *et. al*, 2009). MRAPs have been found to form single pass, antiparallel homo- and heterodimers in which both termini protrude from the extracellular and intracellular face of the plasma membrane (Hinkle and Sebag, 2010).

In the human melanocortin system MRAP1 is required for the functional expression of MC2R (Figure 2A). MRAP involvement in activation of MC2R by ACTH is critical, and just a few point mutations in the MRAP sequence can result in MC2R being unable to be activated by ACTH, as shown by Sebag and Hinkle (2008). MRAP1 mutants with alanine substitutions were tested, and it was found that substitutions for residues other than 18-21 allowed for normal interaction with, and activation of MC2R. When alanines were substituted for residues 18-21, MC2R was still trafficked to the

plasma membrane; however the receptor could not be activated by ACTH. These studies indicated residues ¹⁸LDYI²¹ play a critical role in MRAP1 function. In addition, removal of residues 31-37 resulted in MRAP1 no longer forming antiparallel homodimers, and MC2R trafficking and dimerization were blocked (Sebag and Hinkle, 2008).

The two MRAP orthologs share significant homology, however, due to a few differences in amino acid sequence, the two proteins do not share identical function (Sebag and Hinkle 2008). Similar to MRAP1, MRAP2 is capable of trafficking MC2R to the plasma membrane. However, MRAP2 is incapable of enabling activation of MC2R once it reaches the plasma membrane. In their 2008 paper, Sebag and Hinkle showed that insertion of the LDYI motif into MRAP2 was enough to allow MC2R to be activated in the presence of MRAP2 only.

The requirement of MRAP for functional expression of MC2R is unique in the melanocortin family, though the interaction of MRAP with MC2R is not. As stated, MRAPs have been found to interact with all MCRs to differing degrees, with different results. Most notably for our experiments, when MC5R is expressed in the presence of MRAP it is no longer able to traffic to the plasma membrane (Figure 2B). Further studies showed a disruption in the formation of MC5R homodimers when MRAP was present (Sebag and Hinkle 2009).

MRAPs, MC2R and MC5R are all found in adrenal cortex cells, as well as the head kidney in fishes, and the inverse MCR/MRAP relationships provide cells with a well preserved mechanism by which to regulate receptor expression.

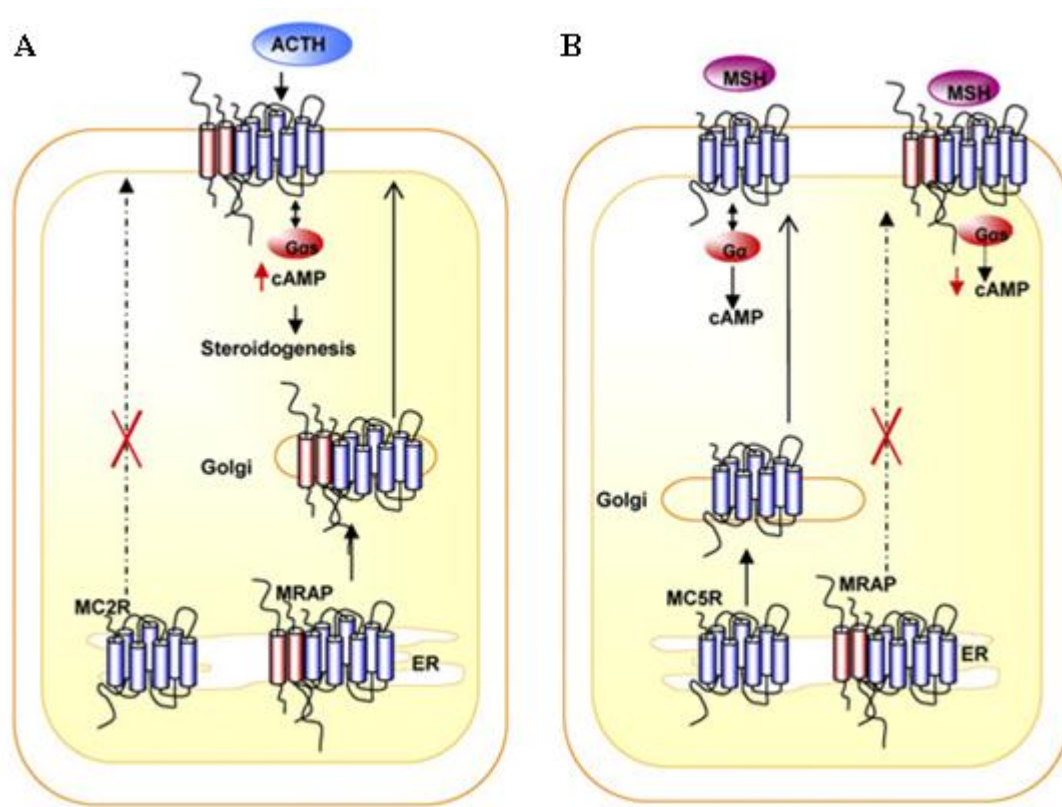


Figure 2 A) A schematic of the functional interaction between MRAP and MC2R. In the absence of MRAP, MC2R remains in the ER and is not trafficked to the plasma membrane. When MRAP is present, it forms an antiparallel homodimer which then interacts with MC2R and both are trafficked to the plasma membrane and MC2R can be activated with MRAP1. B) A schematic of the functional interaction between MRAP and MC5R. Alone MC5R is capable of being trafficked to the plasma membrane and being activated. The presence of MRAP prevents MC5R from being trafficked to the plasma membrane. Adapted from Clark, 2011

Teleosts MCR System

Teleost fish have a melanocortin system similar to mammals, with 5 subtypes of MCRs, and 2 MRAP orthologs, however there are some differences. Unlike mammals, teleost fishes do not have adrenal glands. Instead there is a head kidney which is a localization of adrenocortical cells in the most anterior portion of the kidney (Aluru and Vijayan, 2008). The head kidney acts in a manner similar to adrenal glands in mammals, responding to ACTH and releasing corticosteroids (Aluru and Vijayan, 2008). Another difference teleosts possess in contrast to mammals is a variance in the number of MCRs each species possess. *Takifugu rubripes* has a single copy of MCRs 1, 2, 4 and 5 and are missing a copy of MC3R (Västermark and Schiöth, 2011). Zebrafish on the other hand have six MCRs, possessing a single copy of receptors 1-4 and two copies of MC5R (Västermark and Schiöth, 2011). To date only the standard five MCRs have been identified in rainbow trout.

In rainbow trout MC5R has been found in the head kidney as well as the brain and ovary (Haitina *et al.*, 2004). In addition, MC2R is expressed in the head kidney of rainbow trout (Aluru and Vijayan, 2008). At the time of this thesis research, a rainbow trout MRAP ortholog had not been identified, though MRAP1 had been localized to the head kidney in zebrafish (Agulleiro *et al.*, 2010). Rainbow trout MC2R (rtMC2R) exhibits the same need for MRAP1 as observed in mammals, and zebrafish MRAP1 is sufficient to allow normal trafficking and function of the rtMC2R (Liang *et al.*, 2011).

The Hypothalamic-Pituitary-Adrenal Axis

The HPA is involved in a complex cascade of events known as the stress response, as well as many other actions. Activation of the HPA begins with the glucocorticoid sensing neurons located in the hippocampus. A drop in cortisol detected by these neurons will lead to the release of the polypeptide CRH from neurons in the hypothalamus. CRH will travel via the median eminence to the anterior pituitary where corticotrophic cells will be stimulated to release ACTH. This polypeptide hormone travels via the circulatory system to the adrenal cortex, binds to the MC2 receptor, and the result is the release of cortisol. Due to the lack of adrenal glands in teleosts, the HPA is instead referred to as the Hypothalamic-Pituitary-Interrenal axis (HPI; Norris, 2006).

Ligand Production

All ligands of melanocortin system originate from a single precursor protein known as pro-opiomelanocortin (POMC). POMC is produced and processed in many cells of the body, including the pituitary gland. Prohormone convertases 1/3 and 2 (PC1/3 and PC2) in the anterior and intermediate lobes of the pituitary gland, respectively, are responsible for the bulk of the processing of POMC to produce the melanocortins. Upon signaling from the hypothalamus these enzymes selectively cleave POMC to produce ACTH in the corticotroph cells of the anterior lobe of the pituitary, and α -MSH, β -MSH, and γ -MSH in melanotroph cells of the intermediate lobe of the pituitary (Figure 3).

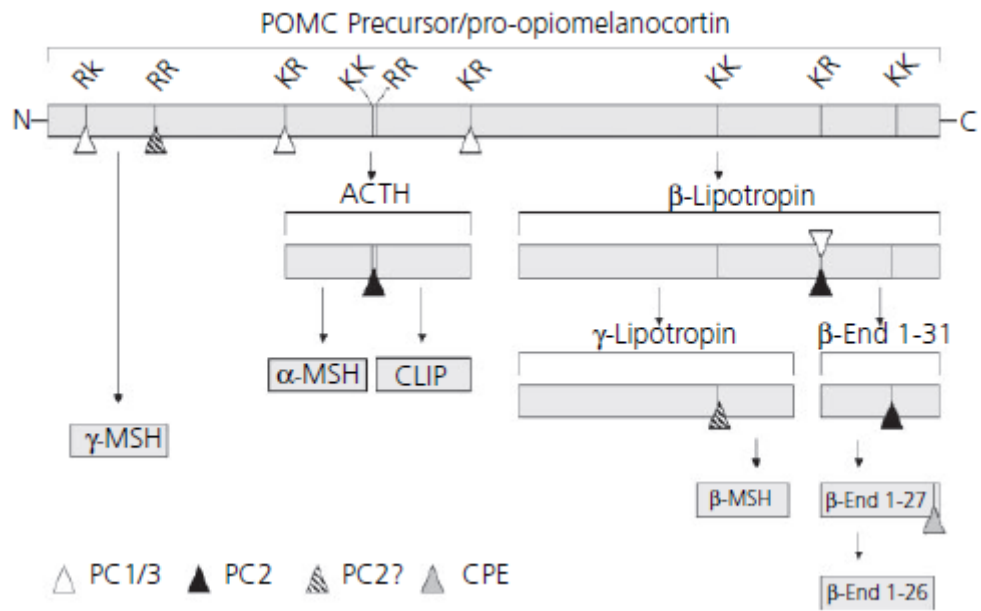


Figure 3 Selective processing of POMC by enzymes PC1/3, and PC2. Some KR cleavage sites are suspected to be selected by PC2, but have not been confirmed. Abbreviations: CPE = carboxypeptidase E, K = lysine, R = arginine. Figure from Helwig *et al.*, 2006

Experimental Question

In an effort to determine how far back the functional relationship between MC5R, MC2R and MRAP1 goes evolutionarily, we set out to determine ligand selectivity and functional activity of the rtMC5R and the relationship between MC5R, MRAP1 and MC2R in rainbow trout. More specifically we sought to answer whether rtMC5R is activated by either ACTH or α -MSH; is there a preference? In addition, is the functional expression of rtMC5R influenced by the presence of MRAP1, rtMC2R or a combination? In rainbow trout α -MSH release is involved in coloration changes. Our experiment will address how such a release could potentially affect head kidney stimulation. Thus addressing the question, is it possible that the α -MSH release activates MC5R in the head kidney and results in release of cortisol and activation of the stress response? Or does a MC5R/MRAP relationship exist similar to that found in mammals, resulting in a lack of MC5R expression in the head kidney?

Hypotheses and Experimental Design

Previous characterization of the MC5R, MRAP, MC2R relationships in mammals led us to hypothesize that rtMC5R can be functionally expressed on the plasma membrane on its own, but the addition of either rtMC2R or zfMRAP1 may inhibit trafficking and expression of MC5R. In addition we hypothesize that such a relationship is necessary to prevent activation of the HPI axis during coloration change events.

The present study uses immunofluorescence and cell surface ELISA to show plasma membrane expression of rtMC5R in CHO cells. Furthermore, the relationship

between rtMC5R, zfMRAP1, and rtMC2R is characterized through cyclic AMP functional assays in addition to immunoprecipitation and western blotting.

METHODS AND MATERIALS

Tissue Culture

The Chinese hamster ovary cell line (CHO), purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), was used for all experiments performed. Cells were maintained in F12K medium, supplemented with 10% heat inactivated fetal bovine serum (FBS), 100U/mL Penicillin and 100mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and 100 μ g/mL Normocin™ (InvivoGen, San Diego, CA, USA). Cells were grown at 37°C in 5%CO₂-95% air with a relative humidity of 70-90%. Media was changed 3 times weekly. At 70-80% confluence cells were subcultured using the following protocol. Medium was aspirated, cells rinsed in 1X phosphate buffered saline (PBS) and detached with trypsin EDTA 1X (Mediatecch, Manassas, VA, USA) for 3 minutes at 37°C. Media was added to inactivate trypsin. Subcultures were obtained by placing a small aliquot of trypsinized cells in a new flask with fresh media.

Immunofluorescence and Lipofection

Transient transfections of the CHO cell line were achieved using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and Opti-MEM (Cellgro, Manassas, VA) with standard protocol, and 1.5 μ g each cDNA or GFP vector.

Transfections were performed using cDNA for rainbow trout MC5R (sequenced by Haitina *et al.* 2004) with a HA epitope tag at the N-terminal, rainbow trout MC2R (accession no. 100136719) with a V-5 epitope tag at the N-terminal, and zebrafish MRAP1 (accession no. 100007319) or mouse MRAP1 (accession no. NP_084120.1) with a FLAG epitope tag at the N-terminal; all synthesized by GenScript (Piscataway, NJ). Construct combinations are listed below. Transfected cells were cultured on 8 chamber slides with 2.5×10^6 cells per well. At 24 hours post-transfection, cells were fixed with 4% paraformaldehyde in 1X PBS (PFA) 15 minutes, then permeabilized with 0.3% Triton-X in 1X PBS for 10 minutes. Cells were incubated for 1 hour at 37°C with monoclonal mouse anti-HA, monoclonal mouse anti-V-5 or polyclonal rabbit anti-V-5, and polyclonal rabbit anti-FLAG primary antibodies (1:500 in 1X PBS). Slides were rinsed in triplicate with 1X PBS, then incubated for 45 minutes with secondary antibody (1:800 in 1X PBS) at 37°C. Donkey anti-mouse antibody linked to Alexa-Fluor488, and donkey anti-rabbit antibody linked to Alexa-Fluor555 were used. Cover slips were mounted onto slides using Vectashield (Vector Lab, Burlingame, CA), and nuclei were stained with DAPI. Images were viewed under oil immersion at 100X on the Axioplan-2 fluorescence microscope (Zeiss, Ostalbkreis, Baden-Württemberg, Germany) using the slidebook imaging software (www.slidebook.com).

Experimental combinations were as follows: rtMC5R; rtMC5R and rtMC2R; rtMC5R and mMRAP1; rtMC5R, rtMC2R and zfMRAP1. Controls were performed as follows: non-transfected cells were treated with primary and secondary antibodies; or transfected were treated with secondary antibody.

Nucleofection and Cyclic AMP Reporter Assay

Transient transfections of the CHO cell line were achieved via nucleofection using the Nucleofector® II Device (Lonza, Walkersville, MD, USA). Cells were transfected using program U-023, solution T (Lonza), $\leq 3.0 \times 10^6$ cells per reaction and 2 μ g of each cDNA construct. Cells were allowed to recover for 10 minutes at 37°C in 500 μ L media, and plated. Media was changed 24 hours post transfection.

Transient transfections of the CHO cell line were performed using MC5R, MC2R and MRAP1 constructs in conjunction with a Cre-LUC reporter gene (compliments of Dr. Patricia Hinkle, Rochester, NY) in combinations as listed below. 2.5×10^6 cells were used per reaction, and cells were plated at a density of 10^5 cells per well in 96-well plates. Forty-eight hours post transfection media was removed and cells were stimulated for 4 hours with NDP α -MSH or ACTH(1-24) (Sigma-Aldrich, St. Louis, MO) at concentrations ranging from 10^{-12} to 10^{-6} in serum-free CHO media. Stimulation solutions were then removed and 100 μ l of Bright-Glo luciferase assay reagent (Promega Inc., Madison, WI) was applied to each well. Following a 5 minute incubation at room temperature, luminescence was measured using Bio-Tek Synergy HT plate reader (Winooski, VT). Experiments were performed in triplicate, and data were analyzed with Kaleidograph software using Student's t-test for equal variance. Significance was set at $p < 0.05$.

Experimental combinations were as follows: rtMC5R; rtMC5R and rtMC2R; rtMC5R and zfMRAP1; rtMC5R, rtMC2R and zfMRAP1; rtMC2R; rtMC2R and zfMRAP1. All combinations were cotransfected with a Cre-LUC reporter gene.

Cell Surface ELISA

Cells were transiently transfected using lipofection as previously described (Liang *et al.*, 2011), with different combinations of rtMC5R, rtMC2R and zfMRAP1 cDNA constructs. Transfected cells were cultured on 24-well plates with 5×10^5 cells per well. 24 hours post lipofection media was aspirated and cells were washed once with 1X PBS. Cells were then fixed with 200 μ L 4% PFA and half the wells were permeabilized with 200 μ L 0.3% Triton-X. Blocking buffer (5% non-fat dry milk in 1X PBS, supplemented with 10% FBS and 5% normal goat serum; 190 μ L/well) was added, and plates were incubated at 4°C overnight. Primary antibody against HA or V-5 at 1:2000 dilution was added 48 hours post lipofection, and allowed to incubate for 2 hours at room temperature. Wells were washed in triplicate with 1X PBS followed by a 45 minute incubation with secondary goat anti-mouse antibody conjugated with HRP (1:2000 dilution at room temperature). Wells were again washed in triplicate, and allowed to dry before adding 200 μ L TMB (Tetramethyl Benzidine; Invitrogen, Frederick, MD) and allowed to incubate for 10 minutes at room temperature. The reaction was halted by adding 200 μ L 1N HCl and gently swirling. 300 μ L from each well was transferred to a 96-well plate and immediately read at absorption 450nm by Bio-Tek Synergy HT plate reader. All experiments were performed in triplicate with a GFP transfected control. Data were normalized to GFP control and analyzed using Student's t-test for equal variance with Kaleidograph software. Significance was determined using Student's t-test for equal variance at $p < 0.05$.

Experimental combinations were as follows: rtMC5R; rtMC5R and rtMC2R; rtMC5R and zfMRAP1; rtMC5R, rtMC2R and zfMRAP1; rtMC2R; rtMC2R and zfMRAP1.

Immunoprecipitation

Transient transfections of the CHO cell line were achieved through nucleofection as described above (see Methods). Cells were detached using trypsin 48 hours post transfection, centrifuged at 5000 rpm for 10 minutes, and then media was removed. Cells were resuspended in 1X sterile PBS and spun at 2000 rpm for 10 minutes. Cells were lysed in 0.1% n-dodecyl- β -maltoside (Thermo, Rockford, IL) in 1X TBS, 1mM EDTA and 1% Triton, pH8, placed on ice for 15 minutes then spun at 10,000 rpm for 20 minutes. Supernatant was removed and tumbled overnight at 4°C with primary antibody against HA, using 1:1000 dilution. Magnetic protein A/G beads (Thermo) were added and lysate tumbled for 1 hour at 4°C. Pellet was washed twice in lysis buffer, then solubilized in 50 μ L western blot sample buffer and run. This procedure was provided by Dr. Patricia Hinkle (Rochester, NY).

Western Blot

Transient transfections of the CHO cell line were performed as previously described. Cells were detached with trypsin, spun at 5000 rpm for 10 minutes, resuspended in 1X sterile PBS and spun again at 2000 rpm for 10 minutes. Cells were lysed using 25-100 μ L NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA), placed on ice and sonicated briefly until no longer viscous. Lysate was divided into 25 μ L

aliquots and used or stored at -20°C. NuPAGE reducing buffer DTT (Invitrogen) was mixed with sample buffer LDS, 250µL and 584.5µL, respectively, and 8µL of mixture was added to 25µL of lysate. Lysate mixture was then boiled for 10 minutes. Boiled lysate mixture (10µL) or immunoprecipitations were run on NuPAGE 3-8% Tris-acetate (Life Technologies, Carlsbad, CA) gel in NuPAGE MES SDS running buffer (Invitrogen, Carlsbad, CA), diluted 20:1, loading every third well with a blank. Gels were run at 20mA constant current for 10-20 minutes followed 50mA constant current for the remainder. HiMark™ pre-stained high molecular weight protein standard (Life Technologies) and MagicMark™ XP Western Protein Standard were used. Blots were carried out in TGS buffer 1:10 (Bio-Rad, Hercules, CA), using a semi-dry preparation and run at 300mA constant current. Blots were rock incubated overnight at 4°C in primary antibody against HA, V-5 and FLAG 1:1000 in blotto with 0.5M sodium azide. The following morning, blots were rinsed and rocked in 1X TBST, three times for five minutes each. Alexa conjugated secondary antibodies were used at 1:1000 dilution in blotto + azide and incubated for 45 minutes, followed again by three washes in 1X TBST. Western blots were read using BioRad Molecular Imager FX and Quantity One® software (Bio-Rad).

RESULTS

Immunofluorescence

To determine if rtMC5R could be successfully transfected in CHO cells, cells were transfected with the rtMC5R construct and immunofluorescence staining was performed on permeabilized cells (Figure 4A). Positive staining for MC5R was observed throughout the cell, demonstrating successful transfection and expression of rtMC5R in the CHO cell line. In addition, unpermeabilized cells were stained (Figure 4B) and positive staining was observed on the plasma membrane, appearing as a green perimeter around the cell. Observation of a prominently stained perimeter in unpermeabilized cells confirmed proper trafficking and localization of rtMC5R to the plasma membrane in CHO cells. Controls of untransfected CHO cells with immunofluorescence staining, and rtMC5R transfected CHO cells subjected only to secondary antibody were performed; no sign of non-specific or background staining was observed (Figures 4C and 4D).

To test for interactions between rtMC5R, rtMC2R and MRAP, CHO cells were transfected with different combinations of cDNA constructs, and immunofluorescence staining was carried out on unpermeabilized cells. Due to difficulties with zfMRAP1 staining, a mouse MRAP1 construct was used in some cases. Cotransfection of rtMC5R with mMRAP1 showed signs of colocalization of the two proteins. Prominent, intense staining was observed for both rtMC5R (Figure 5A) and mMRAP (Figure 5B) along the

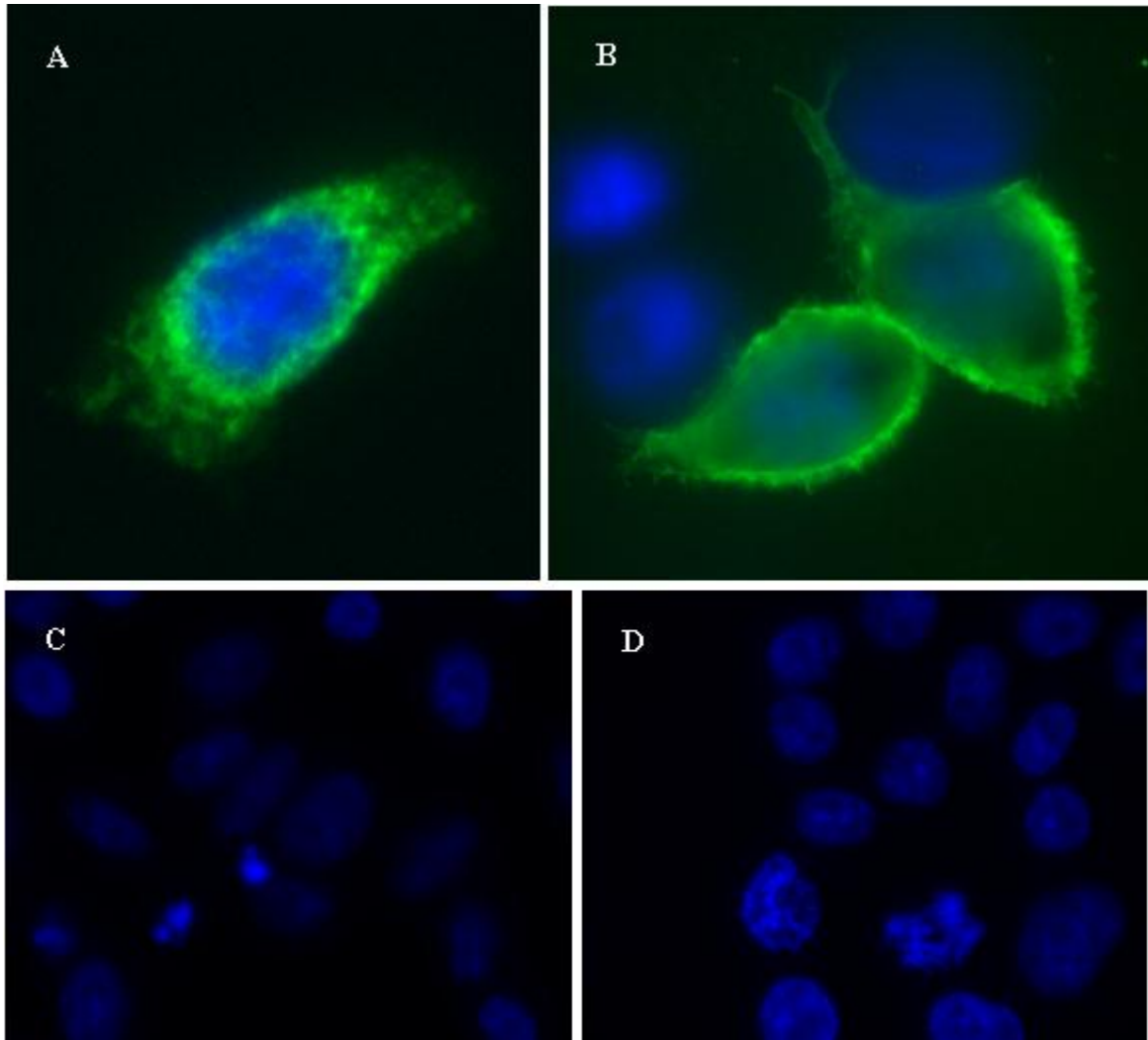


Figure 4 Immunofluorescence Staining of CHO cells A) A permeabilized CHO cell transfected with rtMC5R (green). Receptor can be visualized throughout the cell. B) Unpermeabilized CHO cells transfected with rtMC5R (green). Cells have an intense, green perimeter indicative of rtMC5R localization to the plasma membrane. C) Untransfected CHO cells with immunofluorescence staining using primary antibody against HA, V-5 and FLAG tag, and Alexa-conjugated secondary antibody. No non-specific staining can be seen. D) CHO cells transfected with rtMC5R were only incubated with Alexa-conjugated secondary antibody; no primary antibodies were used. There is no sign of non-specific staining. All cell nuclei were stained with DAPI (blue).

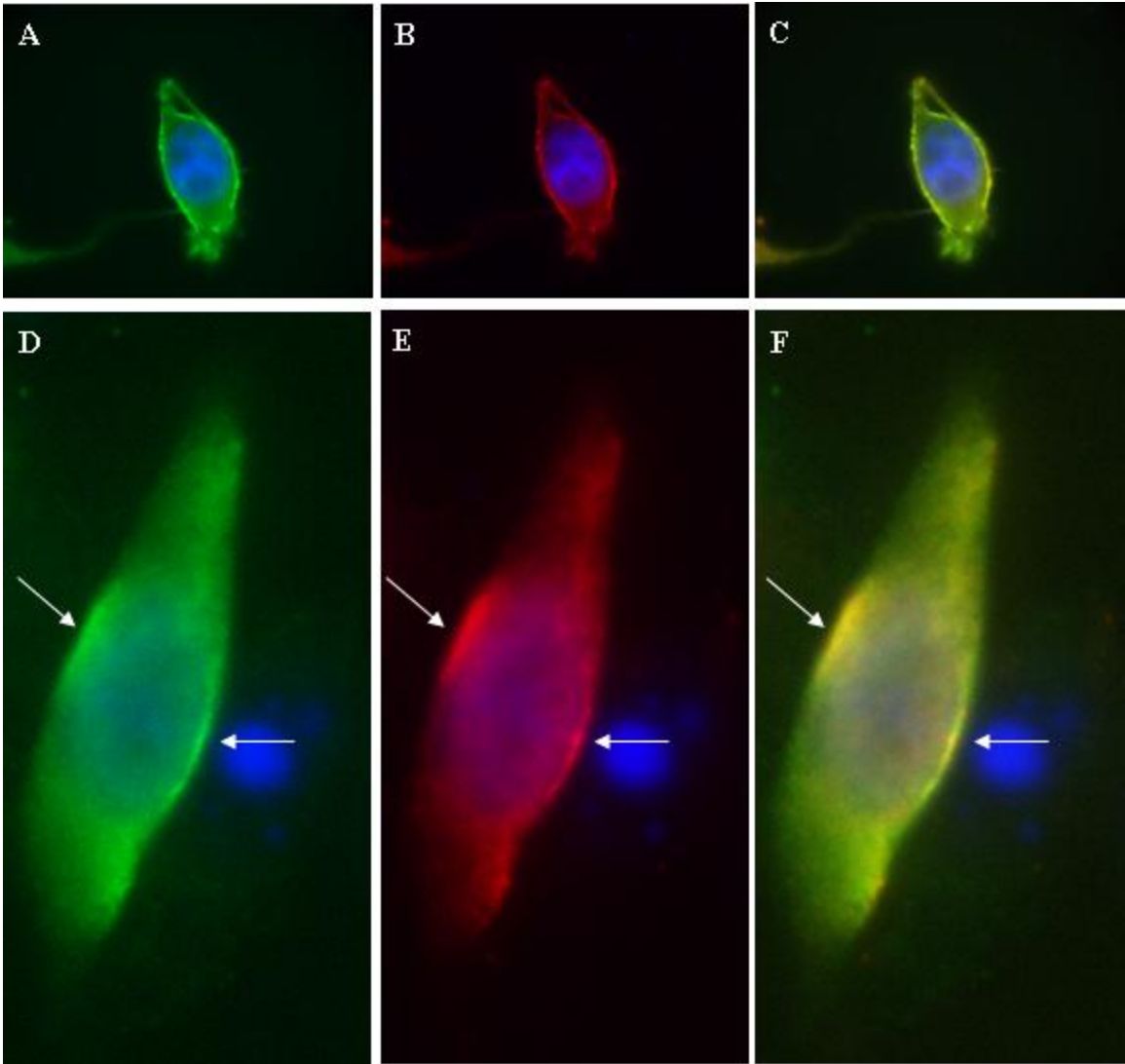


Figure 5 Images A-C are of CHO cells (unpermeablized) cotransfected with rtMC5R (green) and mMRAP1 (red). A) Intense staining shows rtMC5R on the plasma membrane. B) mMRAP appears localized on the plasma membrane. C) Overlay of A and B shows what appears to be colocalization of rtMC5R with mMRAP, indicative of a possible protein interaction. Images D-F are of unpermeablized CHO cells cotransfected with rtMC5R (green) and rtMC2R (red). Arrows indicate areas of strongest staining on the plasma membrane. D) Intense staining is less apparent than in (B), indicating a possible reduction in rtMC5R on the plasma membrane. E) Staining for rtMC2R with a small amount of slightly intense staining on areas of the plasma membrane. F) An overlay of images D and E shows a small amount of possible colocalization. All nuclei were stained with DAPI (blue).

cell perimeter. An overlay of the two images gave a strong indication of colocalization and a possible interaction between the two proteins (Figure 5C).

In cells cotransfected with rtMC5R and rtMC2R it appeared that rtMC5R was not being trafficked to the plasma membrane as efficiently. This is evidenced by the lack of intense staining for rtMC5R along most of the cell perimeter (Figure 5D). In addition, rtMC2R lacked intense staining along most of the cell perimeter (Figure 5E). An overlay of images showed evidence of possible colocalization of the two receptors on the plasma membrane (Figure 5F).

Finally, CHO cells were transfected with cDNA for all three peptides, and stained for rtMC5R with rtMC2R, or rtMC5R with zfMRAP1. When all three peptides were expressed in the cell, rtMC5R still appeared to localize to the plasma membrane, and staining with HA antibodies resulted in intense staining on the plasma membrane (Figure 6A). Staining for rtMC2R however, was not as prominent as for rtMC5R (Figure 6B). An overlay of images A and B showed slight evidence for colocalization of the two receptors, though still a large amount of rtMC5R did not appear to be colocalized with rtMC2R (Figure 6C). Triple transfection and staining for rtMC5R with zfMRAP1 showed strong evidence for colocalization. Rainbow trout MC5R shows prominent staining on the plasma membrane in figure 6D, as does zfMRAP1 in figure 6E. An overlay of the two images shows a large amount of colocalization, and suggests a possible interaction between the two proteins (Figure 6F). Simultaneous staining of rtMC5R, rtMC2R and zfMRAP1 was not performed.

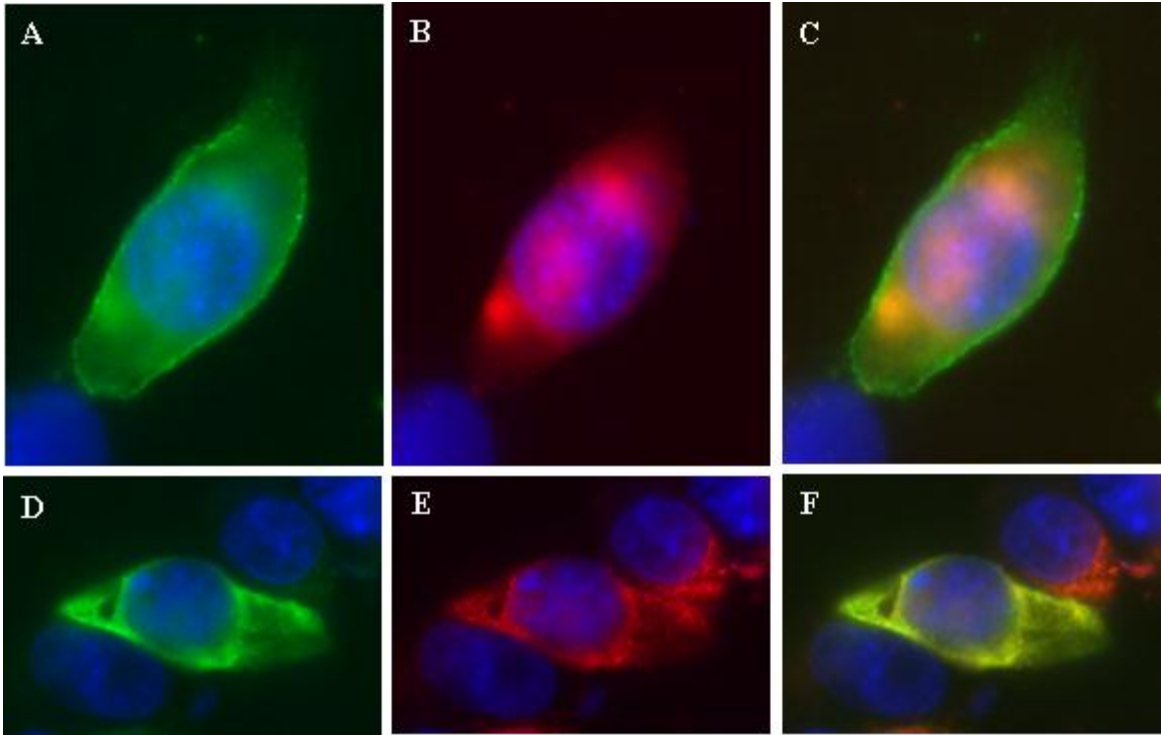


Figure 6 Images A-C are of CHO cells (unpermeablized) cotransfected with rtMC5R (green), rtMC2R (red) and zfMRAP1. A) Intense staining shows rtMC5R on the plasma membrane. B) Staining shows rtMC2R on the plasma membrane, though not as intensely as rtMC5R. C) Overlay of A and B shows what appears to be a small amount of colocalization of rtMC5R with rtMC2R, indicative of a possible protein interaction. Images D-F are of unpermeablized CHO cells cotransfected with rtMC5R (green), zfMRAP1 (red) and rtMC2R. D) Intense staining of rtMC5R is apparent on the plasma membrane. E) Zebrafish MRAP1 staining on areas of the plasma membrane indicates localization to the plasma membrane. F) An overlay of images D and E shows what appears to be a large amount of colocalization of rtMC5R with zfMRAP1. All nuclei were stained with DAPI (blue).

Ligand Selectivity of Rainbow Trout MC5 Receptor

Since cAMP is a second messenger produced when MCRs bind ligand, cAMP measurement was used to determine activation levels of receptors in CHO cells. Due to conflicting reports of ligand preference of MC5R, the initial assay carried out determined rtMC5R ligand preference by stimulating cells with both NDP- α -MSH and ACTH(1-24) (Reinick *et al.*, 2012; Ling *et al.*, 2004)(Figure 7A). The mammalian MC5R shows varying degrees of preference for α -MSH over ACTH (Gantz and Fong, 2003) so it was predicted that the rtMC5 receptor would show a similar preference. As expected, cAMP functional assays performed on CHO cells expressing rtMC5R displayed a significant preference for NDP- α -MSH ($EC_{50} = 5.0 \pm 2.4 \times 10^{-10}$ M) over ACTH(1-24) ($EC_{50} = 6.3 \pm 2.5 \times 10^{-9}$ M)($p = 0.041$) (Figure 7B). Rainbow trout MC5R was cotransfected with rtMC2R in the initial series of cAMP experiments, and resulted in a slight shift of activation curves; however the observed shifts were not significant (Figures 7C and 7D).

Functional Relationship Characterization

To continue characterizing the functional relationship between rtMC5R, rtMC2R and zfMRAP1, CHO cells were cotransfected with differing combinations of constructs and stimulated with NDP- α -MSH, the preferred ligand for rtMC5R. When expressing rtMC5R with combinations of zfMRAP1 and rtMC2R, there were differing levels of rtMC5R functionality (Figure 8A). As seen in the initial cAMP assay, when transfected alone, rtMC5R can be activated by NDP- α -MSH ($EC_{50} = 3.8 \pm 0.08 \times 10^{-10}$ M). When rtMC5R was co-transfected with zfMRAP1, it was unexpected that activation levels were

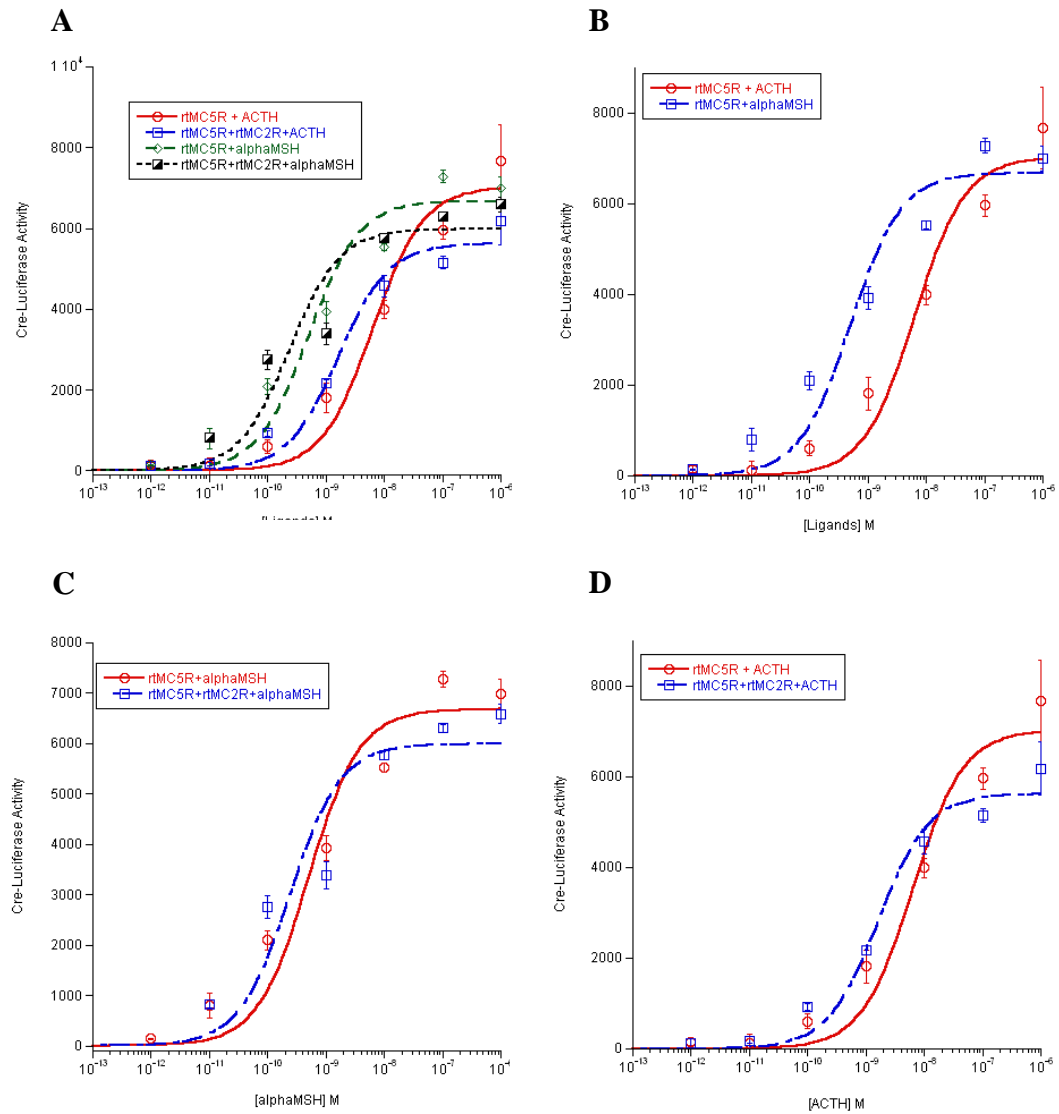


Figure 7 Preferred ligand curves for rtMC5R. A) CHO cells were transfected with rtMC5R and, cotransfected with rtMC5R and rtMC2R, then stimulated with NDP- α -MSH or ACTH 1-24. EC₅₀ values are as follows: rtMC5R + NDP- α -MSH = $5.0 \pm 2.4 \times 10^{-10}$ M, rtMC5R + rtMC2R + NDP- α -MSH = $2.3 \pm 1.4 \times 10^{-10}$ M, rtMC5R + ACTH(1-24) = $63 \pm 25 \times 10^{-10}$ M, rtMC5R + rtMC2R + ACTH(1-24) = $1.6 \pm 0.52 \times 10^{-9}$ M. Figures B-D are separate graphs of the same curves as seen in figure A. B) rtMC5R alone shows a significant preference for NDP- α -MSH over ACTH. $p=0.041$ C) Comparison of activation curves for rtMC5R transfected cells and rtMC5R/rtMC2R cotransfected cells stimulated with NDP- α -MSH. $p=0.206$ D) Comparison of activation curves for rtMC5R transfected cells, and rtMC5R and rtMC2R cotransfected cells stimulated with ACTH(1-24). $p=0.069$

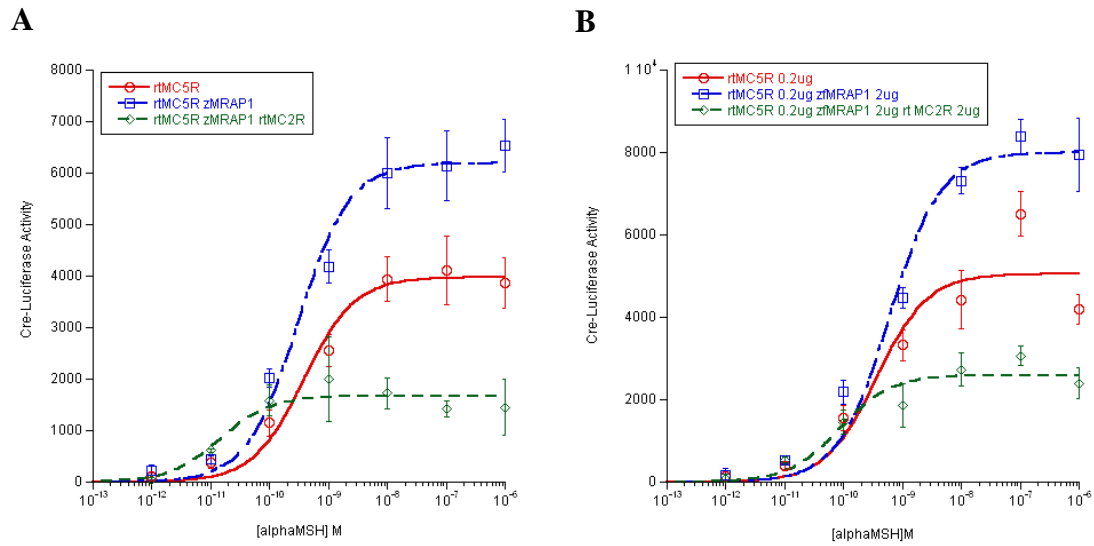


Figure 8: cAMP activity assay performed on CHO cells transfected with different combinations of rtMC5R, rtMC2R and zfMRAP and stimulated with NDP- α -MSH. A) Transfection of CHO cells with rtMC5R results in a standard activation curve ($EC_{50} = 3.8 \pm 0.8 \times 10^{-10}$ M). The addition of zfMRAP does not significantly change the EC_{50} ($3.0 \pm 1.0 \times 10^{-10}$ M) of the activation curve, though the total activation level is higher. Co-transfection of rtMC5R with zfMRAP and rtMC2R results in a significant change in EC_{50} ($0.15 \pm 0.08 \times 10^{-10}$ M) $p=0.01$ B) A decrease in the amount of rtMC5R cDNA transfected into cells results in similar set of activation curves to (A), however there is no significant difference between curves.

not significantly different, with an EC₅₀ value of $3.0 \pm 1.0 \times 10^{-10}$ M ($p = 0.28$). Though the EC₅₀ value is not significantly different from rtMC5R alone, the combination of rtMC5R with zfMRAP1 appears to increase rtMC5R total activation levels.

Simultaneous expression of rtMC5R, zfMRAP1 and rtMC2R significantly decreased MC5R activation by NDP- α -MSH to an EC₅₀ of $1.5 \pm 0.8 \times 10^{-11}$ M ($p=0.01$). Repetition of this experiment, with a reduced amount of rtMC5R cDNA vector, from 2 μ g to 0.2 μ g, resulted in similar activation curves; however, EC₅₀ values were not significantly different from each other (Figure 8B).

To further understand and characterize the interactions between rtMC5R, rtMC2R, and zfMRAP, a similar set of functional assays were performed, but cells were stimulated with ACTH(1-24) to assess rtMC2R activity (Figure 9). Results were as expected; when transfected alone, rtMC2R showed no activation. The addition of zfMRAP1 gave a normal activation curve with an EC₅₀ value of $1.8 \pm 1.0 \times 10^{-10}$ M; this curve served as the basis for comparison for the rest of the MC2R activation curves. Co-transfection of rtMC2R with rtMC5R resulted in a significant drop in cAMP levels (EC₅₀ = $3.6 \pm 1.0 \times 10^{-10}$ M) ($p=0.01$). Expression of all three peptides lowered the EC₅₀ value (EC₅₀ = $1.0 \pm 0.6 \times 10^{-10}$ M), but the decrease in activation was not statistically significant ($p = 0.45$).

Two possible scenarios provided a plausible explanation for the significant decrease in rtMC5R functionality when combined with both rtMC2R and zfMRAP1. First, the presence of rtMC2R along with zfMRAP causes a conformational change in the rtMC5R that prevents the receptor from effectively binding its ligand and thus being

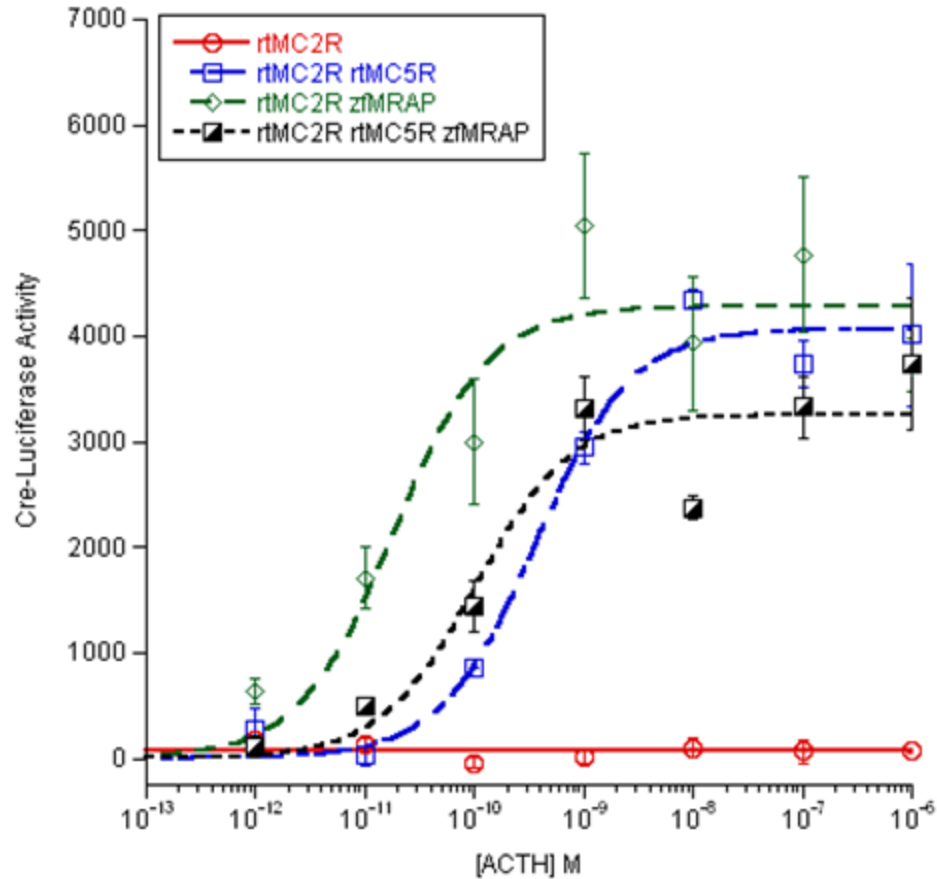


Figure 9 Cyclic AMP activity assays performed on CHO cells transfected with varying combinations of rtMC2R, rtMC5R and zfMRAP. Cells were stimulated with ACTH(1-24) to test for rtMC2R activity. Activity of cells cotransfected rtMC2R and zfMRAP1 served as the control in this assay. Cotransfection of all three cDNA constructs resulted in a significant drop in EC₅₀, though the cotransfection of rtMC2R with rtMC5R did not result in a significant shift in EC₅₀ from the control. P-values are as follows: between rtMC2R+zfMRAP1 and rtMC2R+rtMC5R p = 0.01; between rtMC2R+zfMRAP1 and rtMC2R+zfMRAP1+rtMC5R p=0.45; between rtMC2R+zfMRAP1+rtMC5R and rtMC2R+rtMC5R p=0.34.

activated. This explanation was unlikely since the presence of zfMRAP1 alone did not produce any significant changes in receptor functionality. A second, and more likely explanation, was that the majority of receptor was not reaching the plasma membrane, making it impossible to be activated by its ligand, and resulting in the significant decrease in activation. These two theories were tested by cell surface ELISAs on transfected CHO cells.

Cell Surface Expression of Rainbow Trout MC Receptors

Cell surface ELISA was performed on CHO cells transfected with the same combinations of rtMC5R, rtMC2R and zfMRAP1 as performed for cAMP assays. Initial assays used staining for the HA-epitope tag on rtMC5R (Figure 10). Results supported the idea that the decreased activation levels observed in cyclic AMP assays were a result of decreased expression of rtMC5R on the plasma membrane, rather than due a conformational change in the receptor which results in an inability of the receptor to functionally bind ligand. Cotransfection of rtMC5R with rtMC2R resulted in reduced surface expression of rtMC5R, though not significantly ($p=0.053$). This result coincides with the decrease in staining seen in the immunofluorescence assay. The combination of both receptors with zfMRAP1 however, does result in a significant decrease in rtMC5R cell surface expression ($p=0.005$). The co-expression of rtMC5R with zfMRAP1 did not result in a decrease in receptor expression ($p=0.75$), which coincides with the lack of a change in activation levels in the cAMP activity assays.

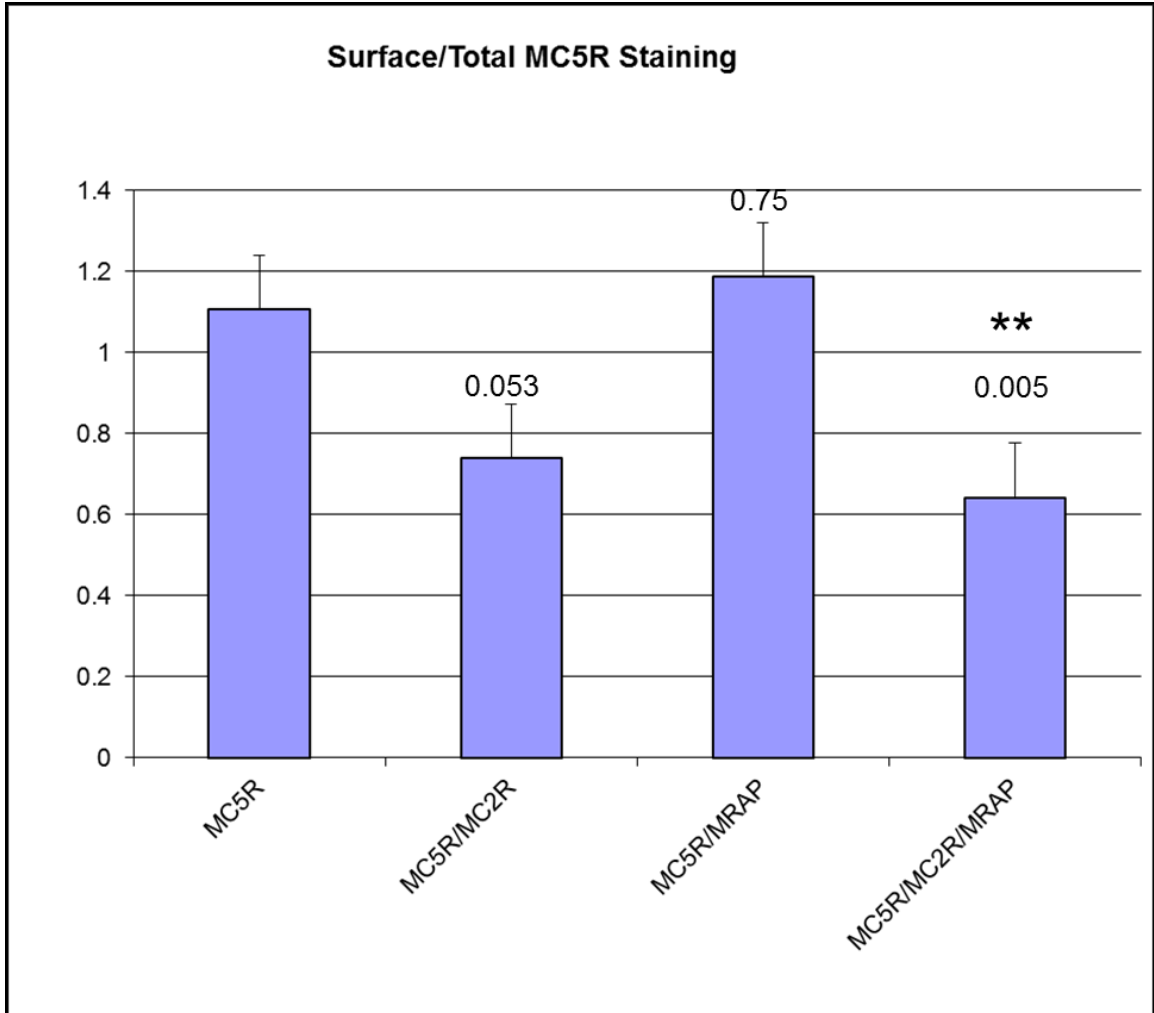


Figure 10 Cell surface ELISA, with primary staining for HA epitope tag on rtMC5R. Cotransfection of rtMC5R with rtMC2R decreased the amount of rtMC5R detected on the plasma membrane, $p=0.053$. Cotransfection with zfMRAP1 resulted in a slight increase in detected surface expression of rtMC5R, $p=0.75$. Cotransfection of all three cDNA constructs led to a significant decrease in detectable rtMC5R on the cell surface, $p=0.005$.

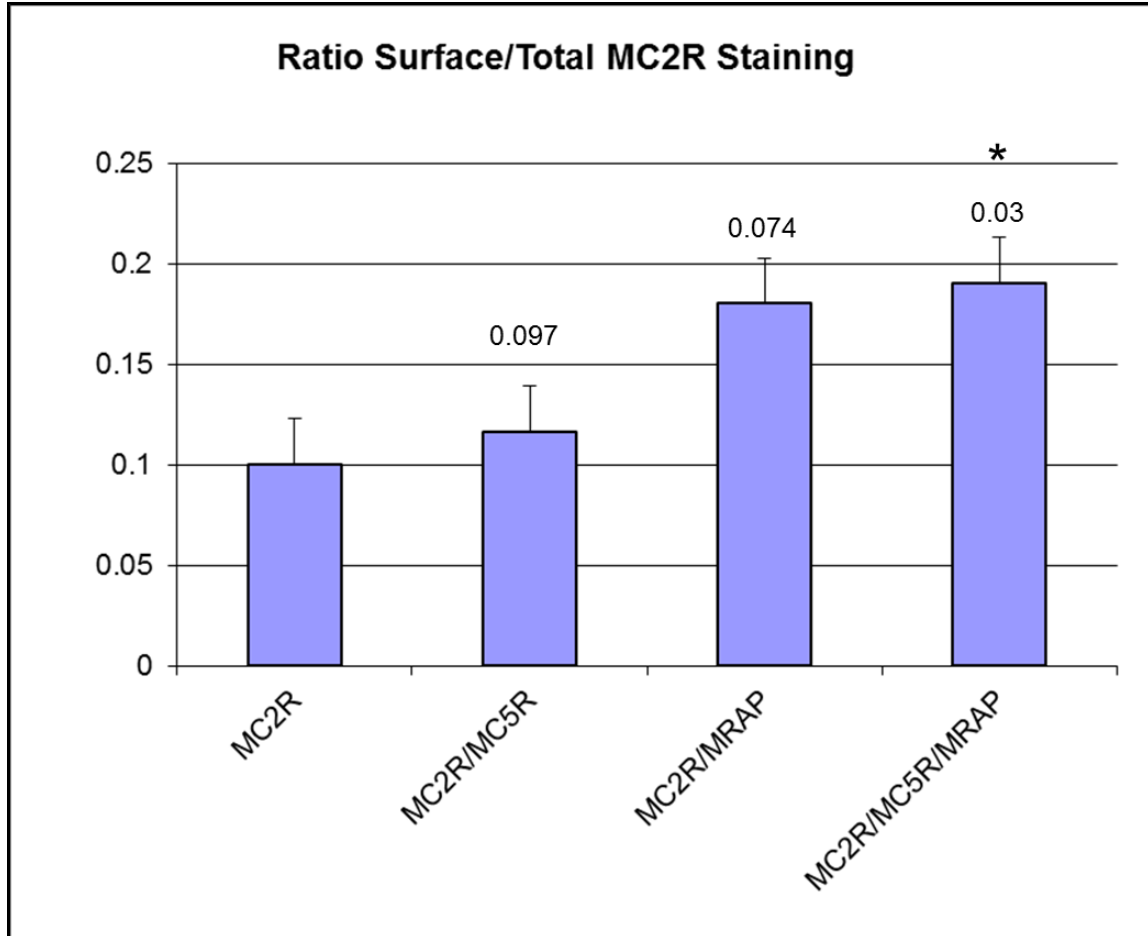


Figure 11 Cell surface ELISA, primary staining for V-5 epitope tag. Cotransfection of rtMC2R with rtMC5R resulted in no significant change in surface expression of rtMC2R, $p=0.074$. Cotransfection with zfMRAP1 resulted in an increase in detected surface expression of rtMC2R, however the increase was not significant, $p=0.097$. Cotransfection of all three cDNA constructs led to a significant increase in detectable rtMC2R on the cell surface, $p=0.03$.

When performing the same experiment, but staining for rtMC2R expression (Figure 11), the only significant change in expression levels observed was that the combination of rtMC2R, rtMC5R and zfMRAP1 ($p=0.03$), though the cotransfection of rtMC2R with zfMRAP1 did increase the surface expression of rtMC2R, ($p=0.097$). It should be noted that the difference between expression of all three peptides, and the expression of just rtMC2R with zfMRAP1 was not significant ($p=0.8$). It was not surprising that expression of rtMC2R with rtMC5R did not cause an increase in rtMC2R surface expression, as it has been established that MC2R cannot be trafficked to the plasma membrane in the absence of MRAP1. (Metherell *et al.*, 2005)

Decreased surface staining for both HA and V-5 epitope tags when all three proteins were simultaneously expressed provided strong evidence that the most likely explanation for the decrease in plasma membrane expression of MCRs was the formation an oligomer consisting of rtMC5R, rtMC2R and zfMRAP1, which cannot be efficiently trafficked to the plasma membrane. To test this theory, immunoprecipitation and western blotting assays were carried out.

Immunoprecipitation of Rainbow Trout MC5 Receptor

Initial immunoprecipitation and western blots were run in order to determine whether dimers could be expected to run through the gel without dissociating. rtMC5R is 341 amino acids long, so a single protein would be expected to result in bands at either 34kDa or 68kDa if protein had not been glycosylated; glycosylation would result in larger bands (Haitina *et al.*, 2004). The initial trial showed a large band at approximately

90kDa, indicating a glycosylated homodimer (Figure 12). Once it was confirmed that these dimers could stay intact in the gel, immunoprecipitation was carried out on CHO cells transfected with combinations of receptors and zfMRAP1. A control was employed in which no primary antibody was used during the immunoprecipitation process with cells singly transfected with rtMC5R. Faint bands were identified at approximately 90kDa in lanes run with immunoprecipitated rtMC5R transfected cells. Lanes run with cells that were cotransfected were ultimately inconclusive (Image not shown).

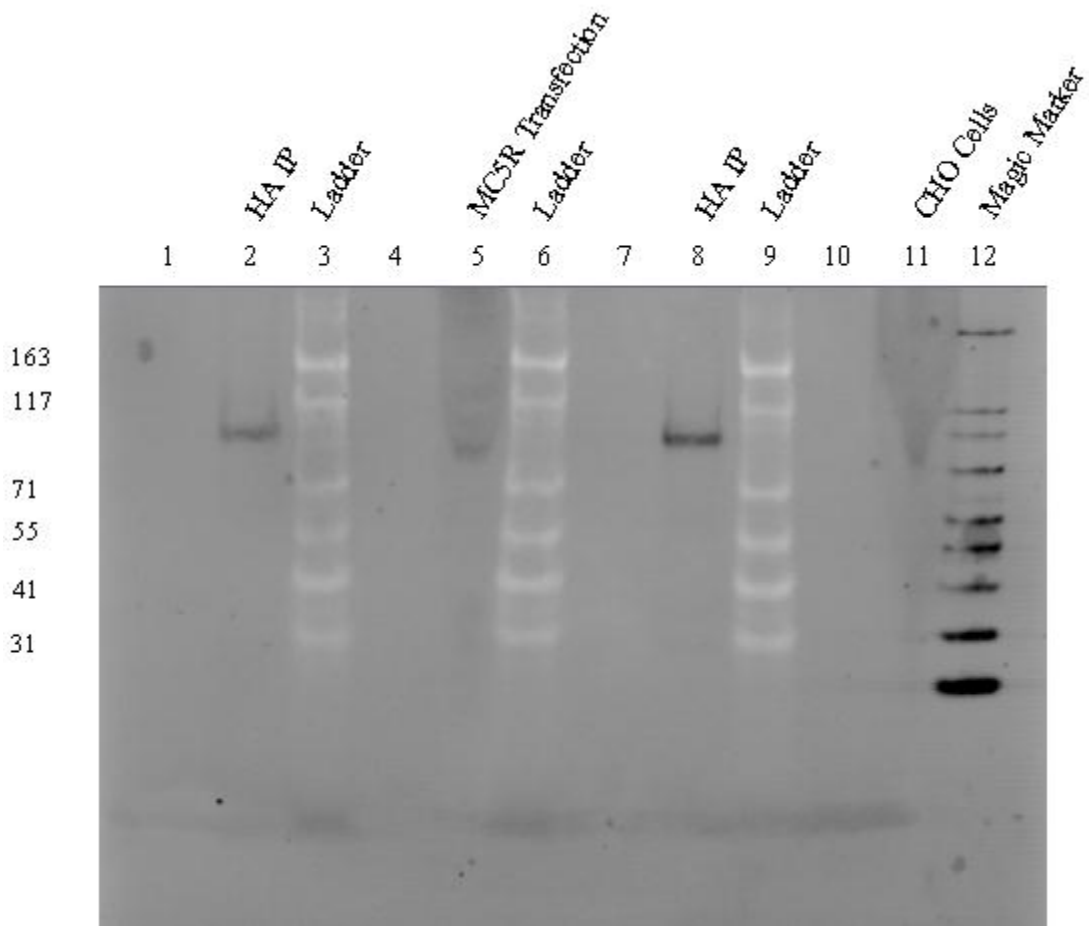


Figure 12 Western Blot Analysis of rtMC5R expressed in CHO cells. Lanes 2 and 8 were run with immunoprecipitated HA-tagged rtMC5R. A clear band is present in both lanes at approximately 90kDa, indicative of glycosylated homodimers. Lane 5 was run with CHO cell lysate from cells singly transfected with rtMC5R; a faint band is apparent at approximately 90kDa as well. Lane 11 was run with untransfected CHO cell lysate, no band was apparent. Every third lane was run blank.

DISCUSSION

The emergence of differential posttranslational processing mechanisms for the polypeptide hormone precursor, proopiomelanocortin (POMC) (Eipper and Mains, 1980), has resulted in the evolution of two distinct hypothalamus/pituitary axes: the hypothalamus/anterior pituitary/adrenal/interrenal axis (HPA/I), and the hypothalamus/intermediate pituitary/chromatophore/melanocyte axis (HPC/M). The former axis mediates the production of glucocorticoids by adrenal cortex or interrenal cells to facilitate the normal daily function of organ systems and to mediate chronic stress events (Norris, 2006). The later axis mediates either physiological color change (chromatophores) or the production of pigment granules in the integument (Norris, 2006). In the HPA/I axis, the critical end-product derived from the posttranslational processing of POMC is the melanocortin, ACTH (adrenocorticotropin). In the HPC/M axis, the critical end-products derived from POMC are the melanocortins, α -, β -, and γ -MSH (melanocyte stimulating hormone) (Figure 1). In addition, the functionality of both axes has been influenced by co-evolution with the melanocortin receptor gene family. In the HPA/I axis, adrenal cortex and interrenal cells express the melanocortin-2 receptor (MC2R), a GPCR that can only be activated by ACTH (Cone, 2006). In the HPC/M axis, chromatophores and melanocytes express the melanocortin-1 receptor (MC1R), a GPCR that can be activated by either ACTH or α -MSH with nearly equal efficacy (Gantz and

Fong, 2003). Hence, during a period of chronic stress, the release of high levels of ACTH will not only stimulate glucocorticoid synthesis, but also could result in a change in pigmentation in organisms like the teleost, *Oncorhynchus mykiss* (the rainbow trout). However, is the converse true? When a rainbow trout utilizes the HPC/M axis is there a rise in glucocorticoid release from interrenal cells? The initial response to this question after the characterization of the MC2 receptor in 1992 (Mountjoy *et al.*, 1992), was no; the ACTH receptor on adrenal cortex or interrenal cells can only be activated by ACTH, and not by any of the MSHs. However, with the subsequent identification of five melanocortin receptors (i.e., MC1R, MC2R, MC3R, MC4R, and MC5R; Cone, 2006), it has become apparent that target cells may express more than one melanocortin receptor at a time.

In the case of the HPA/I axis, the mechanism for regulating a response to ACTH became, on one hand more complex with the realization that the MC2 receptor requires interaction with the melanocortin-2 accessory protein 1 (MRAP1; Metherell *et al.*, 2005). Yet this interaction, at least in mammals, provided a mechanism for regulating the melanocortin receptor trafficking to the plasma membrane. It is now apparent that mammalian adrenal cortex cells will express MC2R, MC5R, and MRAP1 (Cooray and Clark, 2011). Previous studies had established that mammalian MC5R can be activated by ACTH or α -MSH (Gantz and Fong, 2003). However, in vitro analysis of the interactions between mammalian MC2R, MC5R, and MRAP1 revealed that the MC2R + MRAP1 interaction ensured proper trafficking of MC2R to the plasma membrane and facilitated activation of this receptor (Sebag and Hinkle, 2009). Conversely, MC5R

+MRAP1 interaction led to a decrease in the trafficking of MC5R to the plasma membrane (Sebag and Hinkle, 2009) and a corresponding decline in the production of cAMP when MC5R/MRAP1 transfected cells were stimulated with either ACTH(1-24) or NDP- α -MSH (Beleckis, 2012).

Based on these observations, coupled with the discovery that the interrenal cells of the rainbow trout also express MC2R, MC5R, and MRAP1 (Haitina *et al.*, 2004; personal communication Prof. Mathilakath Vijayan, University of Waterloo, Canada). The present study was conducted to address the hypothesis that the MC2R/MRAP1 and MC5R/MRAP1 interactions were ancestral features of the melanocortin receptors which emerged early in the radiation of the ancestral gnathostomes. These ancestral gnathostomes gave rise to the teleosts (rainbow trout) and the tetrapods (mammals). To test this hypothesis, three *in vitro* conditions were analyzed: 1) the expression and ligand selectivity properties of rainbow trout (rt) MC5R in a heterologous mammalian cell line (CHO); 2) the expression and ligand selectivity properties of rtMC5R and rtMC2R co-expressed in CHO cells; and 3) the expression and ligand selectivity properties of rtMC5R, rtMC2R, and zebrafish MRAP1 co-expressed in CHO cells. The first two analyses can provide a view of the pharmacological properties of the rtMC5 receptor, and it is highly likely that there are some rtMC5R target cells that either only express the MC5 receptor, or may express MC5R and MC2R. The third analysis provides an *in vitro* reconstruction of receptor and accessory protein conditions in rainbow trout interrenal cells and may be the most physiologically relevant of the three analysis paradigms. A summary of these analysis paradigms follows.

Rainbow Trout MC5R expression in CHO cells: Single receptor expression studies

The following comments summarize the properties of rtMC5R either expressed alone or in co-expression with zfMRAP1. Immunofluorescence analysis of rtMC5R expressed in CHO cells revealed that this cDNA construct could be expressed in CHO cells and the receptor could successfully traffic to the plasma membrane without the necessity for an accessory protein. Rainbow trout MC5R was clearly visualized both inside the cell (permeablized cells; Figure 4A) and on the plasma membrane (unpermeablized cells; Figure 4B). These results are consistent with mammalian MC5R behavior (Sebag and Hinkle, 2009).

When CHO cells were co-transfected with rtMC5R and zfMRAP1, immunofluorescence analyses indicated the probability of an interaction between rtMC5R and zfMRAP1 as evidenced by the appearance of co-localization in images taken of cells transfected with both cDNA constructs (Figure 5C). Interestingly, this potential interaction was observed in unpermeablized cells, indicating that rtMC5R was still being trafficked to the plasma membrane in the presence of MRAP1. Further imaging studies, including FRET, could provide more support for interactive behavior of rtMC5R with MRAP1.

Ligand selectivity studies on cells transfected only with rtMC5R indicated that α -MSH is a more potent ligand than ACTH(1-24). These results are in agreement with the ligand selectivity studies on mammalian MC5Rs (Haitina *et al.*, 2004). Hence, during activation of the HPC/M axis it is highly likely that target cells expressing rtMC5R receptor are activated.

In CHO cells transfected with rtMC5R and zfMRAP1, the presence of zfMRAP1 did not interfere with the functional activation of the transfected cells (Figure 8A and 8B). These data coincide with the immunofluorescence observations mentioned above, which showed rtMC5R on the plasma membrane, co-localized with mMRAP1. However, when the same analysis was done for CHO cells transfected with hMC5R and mMRAP1 (Beleckis, 2012), there was a significant drop in cAMP production following stimulation with either NDP- α -MSH or ACTH(1-24). These observations pointed to a clear difference between the interaction of rtMC5R/MRAP1 and hMC5R/MRAP1 which was further clarified by a cell surface ELISA analysis.

Sebag and Henkle (2009) used a cell surface ELISA analysis to show that co-expression of hMC5R and mMRAP1 resulted in a decrease in the expression of hMC5R on the plasma membrane. In these experiments some hMC5R/mRAP1 complexes appear to be retained in the ER. When the same paradigm was applied to CHO cells co-transfected with rtMC5R and zfMRAP1, there was no evidence for a decrease in rtMC5R surface expression (Figure 10). Again, this coincides with the results from immunofluorescence assays and cAMP reporter assays.

It is also conceivable that rtMC5R interaction with MRAP1 results in decreased internalization rates of MC5R from the plasma membrane, which in turn increases the duration of cell surface expression of rtMC5R and also explains the increased overall activation rates observed in the cAMP reporter assays. Yet another plausible explanation which was not explored is the possibility that MRAP1 causes a conformational change in rtMC5R which results in a change of ligand preference, causing MC5R to bind ACTH

with higher affinity. Such a phenomenon was suggested by Chan *et al.*, 2004. The latter hypothesis would be simple to test initially with a cyclic AMP activity assay similar to the one performed to determine the ligand preference of rtMC5R. Ultimately, this is an interaction that needs further investigation, preferably through the use of a rainbow trout MRAP1 ortholog.

Rainbow Trout MC5R expression in CHO cells: co-expression with rtMC2R

The following experiments were done to see if co-expression of rtMC5R and rtMC2R would alter either the trafficking of rtMC5R to the plasma membrane or affect the ligand selectivity of rtMC5R. Immunofluorescence analysis was suggestive of co-localization of rtMC5R with MC2R, as seen in Figure 5F. It was surprising that rtMC2R was trafficked to the plasma membrane in the absence of MRAP, as it has been well established that MC2R requires MRAP to be trafficked to the plasma membrane (Metherell *et al.*, 2006). It is possible that the presence of both receptors results in the formation of a heterodimer which allows MC2R to traffic to the plasma membrane, though it is likely that rtMC2R is not functional in this situation, as it has been demonstrated that the necessity of MRAP1 in MC2R function goes beyond initial trafficking to the plasma membrane (Metherell *et al.*, 2006).

Receptor functionality was also evaluated in CHO cells co-expressing rtMC5R and rtMC2R, and resulted in only a slight decrease in EC₅₀ values following stimulation with either NDP- α -MSH ($2.3 \pm 1.4 \times 10^{-10}$ M) or ACTH(1-24) ($1.6 \pm 0.52 \times 10^{-9}$ M), as compared to the same treatment of rtMC5R alone (NDP- α -MSH = $5.0 \pm 2.4 \times 10^{-10}$ M;

ACTH(1-24) = $63 \pm 25 \times 10^{-10}$ M) (figures 7C and 7D). These results give the impression that rtMC2R does not interfere greatly with the functionality of rtMC5R, however the inverse relationship may be harder to define. When cells co-expressing rtMC2R and MRAP1 are stimulated with ACTH(1-24) the EC₅₀ value ($1.8 \pm 1.0 \times 10^{-10}$ M) was significantly higher than cells co-expressing rtMC5R and rtMC2R (EC₅₀ = $3.6 \pm 1.0 \times 10^{-10}$ M) (p = 0.01) (Figure 9).

Due to the fact that both rtMC5R and rtMC2R can be functionally activated by ACTH, the cAMP levels that were measured when cells were transfected with both receptors and stimulated with ACTH(1-24) cannot be fully attributed to a single receptor or both receptors with absolute certainty (Figures 7A, 7D and 9). As a result of such uncertainty, any loss, decrease, or gain of function as a result of an interaction between the two receptors cannot be identified. The ambiguity of these results could potentially be alleviated by the use of rtMC5R and rtMC2R homologues that behave normally in all ways, but cannot be functionally activated. These receptors can be folded, glycosylated and trafficked to the plasma membrane as a normal, functional receptor, but lack the ability to be activated by ligand and subsequently produce cAMP, and would provide a way to determine which receptor is responsible for the cAMP production with some amount of certainty.

Cell surface assays offered little insight into what may be causing the differences in EC₅₀ values observed in the cAMP reporter assays. Simultaneous expression of rtMC5R with rtMC2R indicates a trend toward a significant (p = 0.053) drop in surface expression of rtMC5R (Figure 10). Additionally, measurement of rtMC2R expression

was not significantly different than when rtMC2R was expressed individually (Figure 11). Taken together, these data from cells co-expression rtMC5R with rtMC2R give the impression that rtMC5R can be slightly inhibited by rtMC2R, while rtMC2R may be enhanced by the presence of rtMC5R. Further functional characterization of the relationship between these two receptors will serve to clarify this matter.

Rainbow Trout MC5R expression in CHO cells: co-expression with rtMC2R and zfMRAP1

The immunofluorescence analysis indicated that co-transfection of all three cDNA constructs resulted in what appeared to be colocalization of rtMC5R with both MC2R (Figure 6C) and MRAP1 (Figure 6F). Again, these results indicate the possible interaction of rtMC5R with MC2R as well as MRAP1. Further imaging studies, including FRET techniques similar to those performed by Pisterzi *et al.* (2010) in which each protein would be tagged with a different fluorescent protein, could provide more support for interactive behavior of rtMC5R with MRAP1. In addition, simultaneous staining for all three proteins performed on cells that were transfected with rtMC5R, rtMC2R and zfMRAP1 would provide ideal evidence for potential colocalization and interaction.

Cyclic AMP reporter assays showed that the presence of all three proteins resulted in a significant decrease in EC_{50} values when cells were stimulated with NDP- α -MSH, as well as a drop in total saturation (Figure 8A). Additionally, identically transfected cells stimulated with ACTH(1-24) showed a right shift in EC_{50} and a drop in total saturation, though neither were significant relative to cells transfected with only rtMC2R and

zfMRAP1 (Figure 9). The seeming loss of function of rtMC5R in this case is concurrent with the initial hypothesis that MRAP1 would interfere with the functionality of the receptor. Furthermore, this change in rtMC5R function could be indicative of a regulatory mechanism as predicted. As stated previously, the activity of cells stimulated with ACTH(1-24) cannot be accurately interpreted due to the potential for dual activation of rtMC5R and rtMC2R.

The decrease in total activation of rtMC5R in cells transfected with all three cDNA constructs and stimulated with NDP- α -MSH coincides with a significant reduction in detectable expression of rtMC5R at the plasma membrane. These results strongly suggest a mechanism for the decrease of the trafficking of rtMC5R to the plasma membrane. As stated, such a mechanism could prevent HPA/I axis activation during times of HPC/M activity. Conversely, triple transfection was the only condition which resulted in a significant increase in detectable rtMC2R at the cell surface.

Conclusions

It has been shown that MRAP1 interacts and coimmunoprecipitates with all MCRs, including MC5R in mammals indicating the formation of heterodimers at the very least (Chan *et al.*, 2007). Additionally, larger heteromers have been demonstrated in other G-protein coupled receptors, including, but not limited to M₂ muscarinic receptors (Pisterzi *et al.*, 2010), D₂ Dopamine receptors (Guo *et al.*, 2008; Carriba *et al.*, 2008), α_{1b} -Adrenoceptors, cannabinoid CB₁, and adenosine A_{2A} receptors (Carriba *et al.*, 2008). Based on this information, we believe a decrease in surface expression of rtMC5R may

be due to formation of a large MC5R, MC2R, and MRAP1 heteromer within the cell which results in prevention of rtMC5R trafficking to the plasma membrane. In addition this same arrangement of proteins may affect the functionality of rtMC2R at the plasma membrane, resulting in the increased EC_{50} and decreased total saturation which are indicative of slightly decreased functionality.

After completion of this research, a rainbow trout MRAP1 homologue was discovered. Repetition of assays described in the present study using the rtMRAP1 homologue would provide more conclusive results regarding the authentic interaction of rtMC5R with MC2R and MRAP1. It is however unlikely that these results are completely inaccurate due to the use of zfMRAP1, as it has been shown that zfMRAP1 is capable of interacting with rtMC2R to produce a properly folded, functional receptor that can be trafficked to the plasma membrane and activated by ACTH (Liang *et al.*, 2011). It is still possible that the characterization of rtMC5R in this study is not physiologically accurate due to the use of heterologous MRAP1. As seen in figure 13, there is a great deal of inconsistency in MRAP1 sequences. The percent homology between zfMRAP1 and rtMRAP1 may determine the physiological accuracy of these results.

The *in vitro* studies presented in this thesis reveal three different ways in which rtMC5R can function in a target cell. When rtMC5R is either expressed alone in a target cell or co-expressed with MRAP1 there is no major shift in the activation of the receptor. Rainbow trout MC5R does not require MRAP1 for trafficking to the plasma membrane or for function activation. While it appears that rtMC5R may form a complex with MRAP1, this complex did not alter the functional expression of the receptor.

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*          [---Traff.to PM---]          [-----TM-----]
DaMRAP1 M-----KNSS-----EYVWGYEYDYDVPVLVNSTLKYRY----SIVLIFWMLAFIG 67
TrMRAP1 M-----ENST-----GTYEWEYDYIEPVIUDESKLYHKY----SVIIIFWPSLSGLVG
OnMRAP1 M---AGVENST-----SGIIEYDYDLPVIVDERTLKYKY----SVVIIWVWVITFV
GgMRAP1 M-----ANRT-----NSSEYFWSYDYIDPIVVDGRKLVNKY----SIVIAFWVGLAFAFM
MmMRAP1 M-----ANGT-----DASVPLTSYDYIDLPVDEKCLKANKH----SIVIALWLSLATFV
RnMRAP1 M-----ANRT-----DASVPFTSYDYIDLPVDEKCLKANKH----SIVIALWLSLATFV
MiMRAP1 M-----ANGT-----NASAPYYSYDYIDLPVDEKCLKANKH----SVVIAFWVSLAFAFM
MaMRAP1 M-----ANGT-----NASAPYYSYDYIDLPVDEKCLKANKH----SIVIAFWVSLAFAFM
HsMRAP1 M-----ANGT-----NASAPYYSYDYIDLPVDEKCLKANKH----SIVIAFWVSLAFAFM

CmMRAP2 MSENPFVVKNT-THHPGFNDYTWGYEY---DYGPVSEFGLKAHRCKLLLSIVIGFWVGLVVFV
DrMRAP2 M-PRFQLSNST---SVPNHNYEYSEY---DDEEPVSEFGLKAHRY----SIVIGFWVGLVVFV
TrMRAP2 MS---ARGNRS---QSSARRGDIYQYEY---DYDEPVSFGLRAHRY----SIVIGFWVGLVVFV
XtMRAP2 MSEQTQVTRNT-SHKQLNSDYTWYEY---EYAPVSEFGLKAHRY----SIVIGFWVGLVVFV
AcMRAP2 MAGPRFVSNRTSKHALLNSDYTWYEY---EYEPVSEFGLKAHRY----SIVIGFWVGLVVFV
GgMRAP2 MSALRLISNRT-SQQALSNSDYTWYEY---EYGPVSEFGLKAHRY----SIVIGFWVGLVVFV
MaMRAP2 MSAQRLISNRT-SQQSASNSDYTWYEY---EIGPVSEFGLKAHRY----SIVIGFWVGLVVFV
HsMRAP2 MSAQRLISNRT-SQQSASNSDYTWYEY---EIGPVSEFGLKAHRY----SIVIGFWVGLVVFV
----TM---]

DrMRAP1 FFFLILSLISHSGQLPRGPRVKKSGLPIMKGYASSQ 134
TrMRAP1 FLFLTLNLSHSGHIPGRS
OnMRAP1 CLFLTLNLSRSGNLPKHTSTNSLRTRPE
GgMRAP1 FLFLILLYMSRSGSNFVKQVVVRNVEESSNSEQPHGDNLSPPFPDPAVPGTSPCLPDHSGIHGSI
MmMRAP1 LLFLILLYMSWSGS-PQMRHSPQPCSWTHSFNPLCLRRASLQTTEPGRRAGTDQWLTQQSPS
RnMRAP1 LLFFILLYMSWSGS--QVRHSPQAYQVCSWTHGFHLPLCIQRCLRRSLQAPPSVEDPGRAGPDQW
MiMRAP1 LLFLILLYMSWSGS-PQMNSTQHHH-TCFWSHRLNPLHLQRHRAPPGLTQPSASLPQAEALDGGF
MaMRAP1 LLFLILLYMSWSGS-PQMRNSPKHHQTCFWSHSLSLQLCIQKCLPCHREPLGTS--WARSVEPGSRT
HsMRAP1 LLFLILLYMSWSAS-PQMRNSPKHHQTCFWSHGLNLHLCIQKCLPCHREPLATSQAQASVEPGSRT

CmMRAP2 FMFFVLTLLTKTGA-PHQENV--AKQHR--MNGFSVGYPMLQKPKDAFVHRVSEESRSLFH-CYVN
DrMRAP2 FMFFVLTLLTKTGA-PHPEAAEYKRMRLTSCADGLGQRETDGRTGLSRPLLEESRS-LFHCYIN
TrMRAP2 FMFFVLALLSKTRA-PRQENPESADKHHR-PDGYPVDIDSLQDEKAPSFTHPLLESRAYSHFQION
XtMRAP2 FMFFVLTLLAKTGA-PHQENVDSLEKQFRMDSFAPDFGRNTEADTDRIFSRNVTEESRSLFH-CYIN
AcMRAP2 FMFFVLTLLTKTGA-SHQENTDSAEKRLCMNMFVADFG--PLDSNRIFSQVAEESRSLFH-CYIN
GgMRAP2 FMFFVLTLLTKTGA-PHQENTDESERFRMNSFVADFG--PLESERVFSRQIAEESRSLFHFC-IN
MaMRAP2 FMFFVLTLLTKTGA-PHQDNAESSEKRFMNSFVSDFG--PLEPDKVFSRQNEESRSLFH-CYIN
HsMRAP2 FMFFVLTLLTKTGA-PHQDNAESSEKRFMNSFVSDFG--PLEPDKVFSRQNEESRSLFH-CYIN

GgMRAP1 SA 201
MmMRAP1 ASAPGPLALPG
RnMRAP1 PQQDSTTDEPKSRAGIDQWLQQQSPSASPAGPLALP
MiMRAP1 HVKDKPSPGDTQQLN
MaMRAP1 DPDQLLRQESSSTLPLGVFQIHPTLLWELTLNGGPLIRSKPSEPPPGDRTSQLQS
HsMRAP1 GPDQPLRQESSSTLPLGGFQTHPTLLWELTLNGGPLVRSKPSEPPPGDRTSQLQS

CmMRAP2 EVGHFERQQSGSRISGPKNEDLVTHEKEGEDIL-VFPWNCLAKEDI PNFVNSDQSSSLAEDDLIMC
DrMRAP2 EEE---REGGRAATDAGALTHGRSGIGNSRGQVRAEAREALLAHFNI PNFVNSSELNSALGEDDLLG
TrMRAP2 SQD---SGKKTSEDSRFEQNLAPQVSCCHLGGGSSPRRDTVEFNI PNFVNSLEQSSSELGEEELLYE
XtMRAP2 EVD----QPERIKNRNRAMONEII IQOTIRNSKVEDINMIAKFNI PNFVNTDQSSSIGDDDLLLY
AcMRAP2 ELE----GAKPCPKVPVTDNSIHFPQEVMTTRARLEELHCHTKENI PNFVNTDQ-SSLGEDDLLIS
GgMRAP2 EVEHLDKA--QQSQKGPDLLESNIHFQEVSRSSGTLLEDLNCLAKYNI PNFVNTQNSSLGGEDLLIS
MaMRAP2 EVERLDRAKTCHQTTALDSDVQL--QEAIRSNQGPPEELNRLMKEDI PNFVNTDQ-SFGEDDLLIS
HsMRAP2 EVERLDRAKACHQTTALDSDPVLINDGNIRSSGQPEELNRLMKEDI PNFVNTDQ-YFGEDDLLIS

CmMRAP2 DQIVLENKPISAHNIHQNSD 220
TrMRAP2 DPEIIMEEARPRCTH
DrMRAP2 PS-AVLERREAHCIDIHIIID
XtMRAP2 DPEMNLKAVHTSLCDFMN
AcMRAP2 EPEIILENKPIVHASHYIPD
GgMRAP2 QPFRVLESKMAHQSSHRILD
MaMRAP2 EPEIVLETKPLSQTSBKDLD
HsMRAP2 EPEIVLETKPLSQTSBKDLD

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Figure 13: Alignment of MRAP1 and MRAP2 amino acid sequences. The MRAP1 amino acid sequences for Homo sapiens (Hs), Macaca mulatta (Ma), Microcebes murinus (Mi), Rattus norvegicus (Rn), Mus musculus (Mm), Gallus gallus (Gg), Oreochromis niloticus (On), Takifugu rubriipes (Tr), and Danio rerio (Dr) were aligned with the MRAP2 amino acid sequences from Homo sapiens (Hs), Macaca mulatta (Ma), Gallus gallus (Gg), Anolis carolinensis (Ac), Xenopus tropicalis (Xt), Takifugu rubriipes (Tr), Danio rerio (Dr), and Callorhinchus milii (Cm). Shaded in gray are amino acid positions which display 70% or greater primary sequence identity in both MRAP paralogs. Shaded in yellow are amino acid positions in which 66% or greater primary sequence identity is shared in MRAP1 orthologs. In pink are positions in which amino acids share 80% or better primary sequence identity in mammalian MRAP1 orthologs only. Positions in which amino acids share 75% or greater primary sequence identity in only MRAP2 orthologs are shaded in green. The N-linked glycosylation site is indicated by an asterisk (*). The residues ¹⁸LDYI²¹, which ensure functional activation of MC2R are underlined. Figure 13 from Liang *et al.*, 2011.

The co-expression of rtMC5R and rtMC2R, a situation that could occur in some target cells, appears to result in the formation of heterodimers. However, at present it is not clear if this is a major or minor event. It did appear that the sensitivity of rtMC5R under these conditions had decreased, but this shift in EC50 value was not significant. Hence, it appears that in target cells expressing both receptors the presence of rtMC2R did not decrease functional activation. In addition, based on the immunofluorescence staining experiments it appears that at least some rtMC2R is expressed on the plasma membrane. Whether the rtMC2R on the plasma membrane can be functionally activated is not clear. Additional experiments using mutated forms of rtMC5R which lack the ability to be functionally activated, but not the ability to properly traffic to the plasma membrane would be required to resolve this issue.

Of the three *in vitro* conditions, the co-expression of rtMC5R, rtMC2R, and zfMRAP1 most closely approximated the distribution of melanocortin receptors and MRAP in interrenal cells. Under these conditions, it appears that the two receptors and MRAP1 are forming a multimeric complex. In addition, under these conditions, the functional activation of rtMC5R was significantly impaired because of the presence of MRAP1. Hence, it would be reasonable to predict that, in the rainbow trout interrenal cell, the presence of MC2R, MC5R, and MRAP1, the role of rtMC5R is probably not significant on the surface. As a result activation by α -MSH may not be an issue. If this conclusion is correct we predict that on the plasma membrane of interrenal cells there is a population of MC2R/MRAP1 complexes for functional activation of target cell by ACTH (i.e., HPA/I axis) and a diminished population of either MC5R, or MC5R/MC2R

heterodimers on the surface of these cells. Future experiments that quantify the proportion of these various receptor/accessory protein complexes are required to confirm this hypothesis.

To summarize, this research demonstrated that rtMC5R can be successfully expressed and functionally activated in CHO cells, and, similar to mammals, α -MSH is the preferred ligand for rtMC5R (Cone, 2006). The presence of MRAP1 does not seem to inhibit the expression or function of rtMC5R, rather it appears that it may increase expression and total activation levels. In contrast, the presence of MRAP1 in conjunction with MC2R reduces expression and total activation levels of rtMC5R. The cause of these contrasting effects should be a direction of future study, in addition to repeating the characterization of rtMC5R with the use of rtMRAP1 to ensure the accuracy of these findings. The interaction of rtMC5R with MRAP1 and MC2R is one that is complicated, and ultimately needs a great deal more research to fully characterize.

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