Amino Acid Residues Implicated in the Interaction of Melanocortin Ligands and Their Receptors: A Study of MC2R Selectivity

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ABSTRACT

Melanocortin receptor ligand selectivity has been a question not easily answered. The inability to functionally express melanocortin 2 receptor (MC2R) has inhibited the study of why MC2R is only stimulated by ACTH, a melanocortin hormone. With the recent discovery of the MC2R accessory protein (MRAP), creating a heterologous system is now feasible. Using a general cell line like CHO-K1 cells, which do not express endogenous MCRs, we were able to create a heterologous expression system and test the selectivity of MC2R using analog variants of ACTH(1-24). Our results indicate an amino acid requirement in the C-terminal portion of ACTH(1-24) for activation, which supports the 2-step method of activation hypothesized for MC2R. This site, the tetra basic cleavage site, when altered does not stimulate cAMP production and does not compete with ACTH(1-24) for binding. We also demonstrate the potential for a non-mammalian MC2R system in cloning full length Silurana tropicalis MC2R and completed localization studies with this system with MRAP using CHO-K1 cells.
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INTRODUCTION

Overview:

Ligand selectivity exhibited by receptors in the melanocortin receptor family is a question that has been hard to answer until recently. Without the discovery of the MC2R accessory protein (MRAP), obtaining functional MC2R in a heterologous system was nearly impossible. The focus of this thesis is three-fold: first, using endogenous expression systems to analyze a representative from each MCR class that will allow for functional studies in native surroundings. The second goal was creation a heterologous system for functional expression system of MC2R that can be easily cultured. The final goal was to look at the interaction and the ligand sequence requirement for activation of MCRs will be investigated using analog variants of ACTH hormone.

Melanocortin Receptor Family:

Melanocortin receptors (MCRs) are a family of G-protein coupled receptors (GPCR) activated by melanocortin peptides (Cone, 2006). The G protein-coupled receptor superfamily is divided into five different classes (Class A to Class E/F; Fredriksson et al., 2003). Class A is the rhodopsin or rhodopsin-like family, which includes the melanocortin receptors. Other families of GPCRs are adhesion, secretin, glutamate, and frizzled/TAS2. A unifying feature of the GPCRs is that each receptor has seven membrane spanning domains that are connected by internal and external polypeptide loop domains (Gantz and Fong, 2003). While the 3-dimensional structure of
melanocortin receptors has not been resolved, the structures of other GPCRs have been determined, such as the bovine rhodopsin and the human β-adrenergic receptor (Cone, 2006).

GPCRs typically activate second messenger systems in order to relay messages the receptors receive. Melanocortin receptors are no different in that they relay messages using the adenylate cyclase/cAMP messenger pathway to propagate a biological response (Gantz and Fong, 2003). Another interesting feature about melanocortin receptor stimulation is the association of melanocortin signaling to intracellular systems that increase intracellular Ca\(^{2+}\) via activation of IP\(_3\) (Konda et al., 1994), and induce either janus kinase/signal transducer and activator of transcription (Buggy, 1998), the influx of extracellular Ca\(^{2+}\) (Kojima et al., 1985), as well as activation of MAP kinase (Englaro et al., 1995), and other PKCs (Kapas et al., 1995). The activation of MCRs is derived from proopiomelanocortin, or POMC, which is the common precursor for different stimulatory ligands that activate melanocortin receptors.

POMC:

The melanocortin peptides come from a large preprohormone complex called proopiomelanocortin (or POMC) that is cleaved into many hormone products that may or may not undergo further processing (Pritchard et al., 2002; Fig. 1). Processing of the POMC gene product is tissue specific in that main cleavage products are made via processing by prohormone convertase 1/3 (PC1/3) in the anterior lobe of the pituitary and a distinct set of end-products can be generated from this precursor in the intermediate pituitary and hypothalamus through the action of prohormone convertases 2 {PC2}
Figure 1: Processing of the preprohormone POMC
POMC is processed into several different melanocortin ligands depending on the tissue. Primary cleavage takes place in the anterior pituitary where ACTH and β-LPH are major cleavage products via PC1/3. Further processing of ACTH and β-LPH takes place in the intermediate pituitary where ACTH is cleaved into α-MSH and CLIP and β-LPH is cleaved into γ-LPH and β-endorphin. γ-MSH is formed from cleavage of the N-terminal fragment of POMC while β-MSH is a C-terminal cleavage product of β-LPH. Hormones are then circulated in the bloodstream until they get to their target tissues and cause biological responses. ACTH and the MSH family are primary melanocortin receptor agonists with differing affinities across the MCR family.
(Pritchard *et al.*, 2002). Products generated by PC1/3 include adrenocorticotropic hormone, ACTH(1-39), β-LPH, and the N-terminal region of the prohormone complex (16K Fragment). ACTH(1-39) is the primary hormone end product from the corticotropic cells of the anterior pituitary and does not undergo any additional posttranslational processing in that tissue. This hormone is now in its biologically active form and is capable of activating its target tissue following exocytosis.

Once ACTH(1-39) is secreted, it travels through the blood stream to the adrenal cortex and binds to the ACTH receptor known as melanocortin 2 receptor, MC2R. The activation of the adrenal cortex then leads to a production of cortisol. Cortisol is also involved in a negative feedback loop in the Hypothalamus/Pituitary/Adrenal Cortex axis (HPA) to stop the production and release of ACTH from the pituitary by way of inhibiting the production of corticotropin releasing hormone (CRH) from the hypothalamus. Though ACTH is biologically active, ACTH is capable of being cleaved further to yield other active albeit smaller peptides. In the intermediate lobe of the pituitary and the arcuate nucleus of the hypothalamus, the N-terminal region of POMC is cleaved into γ-Melanocyte stimulating hormone (γ-MSH) while ACTH(1-39) is cleaved into 2 peptide products: α-MSH and CLIP (Eipper and Mains, 1980) The β-LPH peptide product is further cleaved to yield γ-LPH as well as β-endorphin, another of the well characterized major hormone products from POMC. γ-LPH can be further cleaved to yield δ-MSH as an end product (in sharks only) and β-MSH (Dores and Lecaude, 2005). All of the melanocortin ligands share a common linear four amino acid motif, HFRW, which is required for biological activity (Dores and Lecaude 2005). POMC, in addition to being found primarily in the CNS, can also be found in regions such as genitourinary
tract, GI tract, adrenal, lung, spleen, and thyroid and immune system cells in humans (Cone, 2006). POMC has been characterized from every major class of vertebrates (Dores and Lecaude, 2005).

Cloning the first MCRs

The first melanocortin receptor was cloned from a cDNA library from human melanoma cells since finding the α-MSH receptor was very desirable for therapeutic reasons (Cone, 2006). Competing groups cloned the first melanocortin receptor using similar PCR methods to obtain whole length sequence from human melanoma (Chhajlani and Wikberg, 1992 and Mountjoy et al., 1992). This first melanocortin receptor was designated MSH-R, or MC1-R, and the next goal was to clone the ACTH receptor. The ACTH hormone was compared to α-MSH and showed a high level of sequence identity at the amino acid level and it was assumed that there must be similarities in the respective receptors. Based on work done previously, it was known that ACTH could stimulate glucocorticoid production from cells in the adrenal cortex. From the same study, it was also known that α-MSH could not cause the same effect (Schwyzer, 1977). As stated before, high degrees of sequence identity shared between α-MSH and the ACTH ligands would aid in finding the ACTH receptor using a low-stringency PCR method and degenerate primers (Mountjoy et al., 1992). Using the degenerate PCR method, it was shown that there was low levels of ACTH-R in the CNS however there was high amounts of receptor mRNA in the adrenal cortex. This tissue became the target tissue for cloning full-length receptor and shortly after identifying this tissue as a ready-pool of receptor, the full ACTH-R, or MC2R, was cloned (Mountjoy et al., 1992). One caveat to the low
ACTH-R mRNA in the CNS was that there was another MCR showing up in very high amounts that was not MSH-R. Following the same cloning strategy used previously for MC1R and MC2R, the final three receptors were found; MC3R, MC4R, and MC5R.

MCR locations and functions:

MC1R is found in melanocytes and is thought of as the classical α-MSH receptor. (Gantz and Fong, 2003) It functions to determine skin and hair pigmentation though the receptor is not limited to melanocytes. MC1R is also found in fibroblasts, endothelial cells, keratinocytes, as well as antigen expressing cells. In addition to its pigmentation properties, when MC1R is expressed in leukocytes, it adopts an anti-inflammatory role and immunomodulatory properties (Luger et al., 1999). MC3R and MC4R are both found in the CNS and share functions in energy homeostasis. (Gantz and Fong, 2003). MC5R is more of an enigma and is found in numerous tissues, including adrenal gland, adipocytes, leukocytes, and others. MC5R is also found, though in limited amounts, in the CNS (Gantz and Fong, 2003). The function of MC5R, though still vague, is narrowed to exocrine function. MC2R is the classical ACTH receptor and is found in the adrenal cortex in the zona reticularis and the zona fasiculata where it mediates the effects of ACTH on steroid secretion (Bentley et al., 2003). What separates MC2R from the other four receptors is its ligand affinity; MC2R can only be activated by ACTH whereas the other MCRs can be activated by any of the MSH species (α, β, γ) or ACTH. More about MC2R will be discussed later.
Figure 2: Organizational Scheme of MCRs
The MCR family shares a high degree of sequence identity due to the transmembrane spanning regions and ligand binding domains. These regions (indicated by TM) are highly hydrophobic and do not differ significantly from MCR to MCR. MCR genes are mostly intron-less and all MCRs share a common ligand binding region. Differences begin to appear in the N-termini and C-termini of the MCR gene products.
MCR gene evolution:

The five melanocortin receptors found in mammals appear to have evolved from a common ancestral gene. It has been shown that all melanocortin receptors share a common amino acid binding motif (Baron et al., 2009). The family of melanocortin receptors also shares a high degree of sequence identity at the nucleotide level. Recall that GPCRs are defined as having seven transmembrane spanning regions that form the receptor on the plasma membrane. These membrane-spanning regions are hydrophobic in nature and are quite conserved between all the receptors (Fig. 2). In addition to physical similarities the MCRs also share affinities to melanocortin ligands, with the exception of MC2R (Fig. 3). It has been previously hypothesized that the five-melanocortin receptors were a product of two distinct genome duplication events (Holland et al., 1994). This hypothesis is referred to as the 2R hypothesis and the idea behind it describes an initial genome duplication event that yielded the first two paralogous genes and then another duplication that yielded four paralogous genes. Nearly 40 years ago, there was a study that analyzed the genome of several chordates and the evidence for two genome duplication events presented itself (Ohno et al., 1970). A specific gene family, HOX genes, is examined to shed light on the idea of genome duplication and gene redundancy. In mammals, there are four “clusters” of HOX genes that regulate body plan development (Schughart et al., 1988 and Schughart et al., 1989). In an organism such as the protochordate amphioxus, there is only a single HOX gene cluster. Since protochordates appear in the fossil record before vertebrates, the working assumption is that the amphioxus is the ancestral prototype for the HOX gene cluster progression.
Figure 3: MCR ligand binding affinity

MCRs are capable of being activated by a range of melanocortin ligands with the exception of MC2R. Depending on the MCR, there is a preference of ligands that activate the receptor with all Class A MCRs being activated preferentially by α-MSH. Only the Class B MCR, MC2R, is selective to a single melanocortin, ACTH. All MCRs with the exception of MC5R share Agouti as a common antagonist, with MC3R and MC4R also sharing a common antagonist in AGRP, Agouti related protein. This scheme helps to show the MCR relatedness as well as differences that may have come from genome duplication events.

*Adapted from Gantz and Fong, 2003
Subsequent analysis and sequencing of the HOX cluster from amphioxus shows conservation in sequence identity and furthers the evidence for genome duplication events. Figure 4 shows the hypothesized genome duplication event that results in the four HOX clusters seen in mammals today. The first duplication event would have appeared at the time of the jawless vertebrates diverging from protochordates around 500 million years ago resulting in two HOX clusters. The next genome duplication would have occurred 450 million years ago when the jawed vertebrates diverged from the jawless vertebrates. This would give mammals the four HOX clusters we see today and some parallels can start to be drawn between the HOX cluster duplications and the melanocortin receptor gene family. Melanocortin receptor gene family duplications follow the same progression of the HOX clusters (Fig. 5) with one exception, the MC2R/MC2R event in which a single gene duplication resulted in the creation of MC5R from MC2R. Evidence comes by the way of studies on chromosome MCR loci with relation to each other. Human and mouse (*Mus musculus*) MC2R and MC5R are located on the same chromosome, Chromosome 18, with short distances separating the two gene loci, 40 kilobases in humans and 67 kilobases in mice (Logan *et al.*, 2003). Though MC4R is also located on Chromosome 18, it is not thought to be a gene duplication product as the distance between MC2R/MC5R and MC4R is tremendous. In chickens (*Gallus gallus*), though on a different chromosome than human and mouse, MC2R and MC5R are located in close proximity to each other on Chromosome 2 (Schioith *et al.*, 2003) along with MC4R much further away as with human and mouse MC2R and MC4R. It is important to note the similarities of chicken chromosome 2 and human and
Figure 4: Illustrating the 2R Hypothesis in HOX genes. Using the HOX gene family, a hypothesis was formed about the numbers of protein coding genes and why their functions are not as redundant or overlapping as expected.
Figure 5: Hypothesis of radiation of MCR gene family. Using the 2R Hypothesis that models the HOX gene family radiation, applying the same hypothesis to the MCR gene family reinforces the idea of several genome duplication events leading to multiple copies of similar genes that function independent of each other and in a widely various manner. Note the single gene duplication event of MC2R that leads to the creation of a distinct family member, MC5R.

Figure 6: Schematic of the melanocortin receptor duplication events, as described by the 2R hypothesis.
mouse chromosome 18 as many of the same genes located on human or mouse chromosome 18 are located on chicken chromosome 2 (Schioth et al., 2003). The location of chicken, human, and mouse MC2R and MC5R seem to point to gene duplication event early in vertebrate evolution (Schioth et al., 2003). Even non-mammalian species demonstrate MC2R/MC5R chromosome linkage such as the case with zebrafish (Danio rerio) and pufferfish (Takifugu rubripes). In zebrafish, MC2R and MC5Rα are only separated by 750 kilobases on chromosome 16 (Logan et al., 2003). Fugu is even closer with distances of only 2.6 kilobases separating the last exon of MC2R with the first exon of MC5R (Logan et al., 2003).

MCR Classification:

Since the last genome duplication event, the melanocortin receptors have adapted different functions in different tissues. In addition to different tissues and functions, it is possible still to develop ligand selectivity such as with the case of MC2R. Melanocortin receptors are grouped into two different classes. Class A includes MC1R, MC3R, MC4R, and MC5R and Class B, which only include MC2R. Class A melanocortin receptors are capable of binding all the major melanocortin ligands such as ACTH, α-MSH, β-MSH, and γ-MSH with differing affinities. MC1R, MC3R, and MC4R all share common antagonist, Agouti, while MC3R and MC4R share another antagonist, AGRP (Fig. 3). The Class A MCRs can be easily expressed in cultured cell lines that normally do not express MCRs such as HEK cells or CHO cells (Adan et al., 1994 and Desarnaud et al., 1994 respectively).
Meanwhile the lone Class B melanocortin receptor, MC2R, is only capable of binding ACTH. MC2R has only one antagonist, Agouti, and MC2R is not easily functionally expressed in cell culture (Noon et al., 2002). Studies on functional expression initially showed that MC2R expression was possible in some cell lines that cell lines that expressed MCRs other than MC2R (Rached et al., 2004). This lead to difficulty in quantifying results as the cell line described in Rached et al. studies, Cloudman M3, had endogenous MC1R being produced (Cooray et al., 2008). Contaminating MC1R expression would skew results showing activation of MC2R because MC1R is also highly responsive to ACTH in addition to α-MSH (Mountjoy et al., 1992). Other cell lines would need to be utilized for functional expression but it was still very difficult to express MC2R in cells that do not express MCRs. It has been recently discovered that the reason for the poor functional expression is the absence of an accessory protein that assists in the translocation and activation of MC2R (Noon et al., 2002). This accessory protein, called melanocortin receptor accessory protein or MRAP, is essential for functional studies of MC2R (Sebag and Hinkle, 2007). Later, functional studies, i.e. cAMP production, would show that fluorescent fusion proteins and other bulky C-terminal attachments would hinder functional activation. Smaller N-terminal epitope tags would be the way to track cell localization of the MC2R protein as well as study functional activation (Roy et al., 2007).

MRAP:

The MRAP protein is a small transmembrane protein with only one membrane-spanning region that contains numerous hydrophobic residues (Metherell et al., 2005).
Human MRAP has been the most studied since it was first cloned from human cDNA and has been further characterized showing structure and function. Human MRAP mRNA products are alternatively spliced producing 2 variants of the protein. The $\alpha$-form is 172 amino acids long while the $\beta$-form is 102 amino acids long (Sebag and Hinkle, 2007). Interestingly, MRAP forms antiparallel homodimers that when paired with MC2R form stable complexes in the plasma membrane. It is worth noting that MC2R will associate with either form of MRAP, either the $N_{in}$-$C_{out}$ homodimer or the opposite $N_{out}$-$C_{in}$ form (Sebag and Hinkle, 2007). While other groups have reported MC2R trafficking to the plasma membrane, it is likely due to endogenous MRAP from the cell line being used as numerous papers have shown that MC2R is neither functional nor adequately trafficked to the plasma membrane (Metherell et al., 2005, Noon et al., 2002, and Cooray et al., 2008).

MC2R Selectivity Issues:

Reasons for MC2R ligand selectivity have been elusive mostly due to the inability to properly express functional protein. Having a viable expression solution for MC2R was half the fight and based on the previous studies, the MRAP finding was in no small part key to evaluating the important questions relevant to the only Class B MCR. As stated before, all MCRs contain a common binding site motif (Baron et al., 2009) and *Silurana tropicalis* is no different when compared to other MCR sequences. So, why then, if the core sequence of the melanocortin receptors is identical should there arise a selective activation specificity for only one receptor? It is important to note that studies done almost 40 years ago established the necessary functioning region of ACTH(1-39)
via deletion analogs (Schwyzer, 1977). ACTH(1-39) deletions of the C-terminal region still produced reactive species that would stimulate glucocorticoids up to the ACTH(1-24) analog. After the proline residue at position 24, deletion analogs would result in non-functional species of ACTH.

MC2R activation hypothesis:

One hypothesis is the binding area for ACTH in the MC2R protein has developed an extended binding pocket apart from the other four MCRs to include amino acids from the remaining 15 through 24 residues of the fully active ACTH(1-24) peptide (Baron et al., 2009). Another possible explanation is that when ACTH(1-24) first comes into contact with an inactive MC2R, there is a conformational change in the MC2R that only after the initial contact does the HFRW pocket open (Baron et al., 2009). Studies conducted by Mosberg and colleagues (Pogozheva et al., 2005) begin to shed light on potential residues important in ligand binding in MC4R. By using the crystal structure of rhodopsin and calculating the distance constraints, a model of the active form of mu opioid receptor, a member of the rhodopsin family, was developed (Fowler et al., 2004). Based on the assumption that melanocortins as well as other Rhodopsin-related GPCRs share common activation schemes, the Mosberg group used the mu opioid receptor model to model the MC4R active site. Using alanine-scanning mutations in residues predicted to bind α-MSH, several residues were found to be of vital importance based on cAMP production assays after stimulation with α-MSH (Pogozheva et al., 2005; Figure 6). Of the 11 residues found to be important in binding, 10 of them are conserved in human MCRs, 9 are conserved in frog MCRs (S. tropicalis), fugu MCRs (Takifugu rubripes),
<table>
<thead>
<tr>
<th>Amino Acid Position in Human MC4R</th>
<th>Region within Human MC4R</th>
<th>Amino Acid Position in α-MSH</th>
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<tr>
<td>H^{264}</td>
<td>TM6</td>
<td>E^5, W^9</td>
</tr>
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<td>TM2</td>
<td>W^9</td>
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<tr>
<td>D^{122}, D^{126}</td>
<td>TM3</td>
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<td>D^{189}</td>
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<tr>
<td>F^{261}</td>
<td>TM6</td>
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</tr>
<tr>
<td>N^{285}, L^{288}</td>
<td>TM7</td>
<td>F^7</td>
</tr>
</tbody>
</table>

Figure 6: The HFRW binding site in MC4R
Previous work done shows the HFRW binding site in hMC4R using Rhodopsin as a model. The residues shown correlate highly with residues in MCRs in several organisms including humans, mouse, chicken, and bony fish.

*Adapted from Pogozheva, 2005
and lamprey MCRs (*Lampetra fluviatilis*), 8 are conserved in the known MCRs of horn shark (*Heterodontus francisci*) and dogfish shark MCRs (*Squalus acanthias*) (Baron *et al.*, 2009). In the sites where the amino acids do not match, there are neutral amino acid substitutions, and thus, if over 450 million years of evolution the majority of the residues are the same it is a safe assumption that all vertebrates share the same HFRW binding site (Baron *et al.*, 2009)

If the first hypothesis is to be considered, there is data that has been gathered as far back as 1981 that too must be considered. In the extended binding site hypothesis, it is assumed that the HFRW site is initially available and no conformational change to expose that site is necessary. ACTH(1-24) would then bind the HFRW pocket in MC2R and cause the downstream production of cAMP. If this were the case then the site would also be open for α-MSH to bind and activate. This is not the case however as shown by Mountjoy *et al.* in 1992 that cAMP production is not observed when MC2R expressing cells are treated with α-MSH. The notion of α-MSH out-competing ACTH(1-24) is quickly dismissed from the work done by Buckley *et al.* in 1981. Finally, it has also been shown that truncated forms of ACTH(1-24) that lack the C-terminal portion of the peptide cannot activate MC2R either (Schwyzer, 1977). These observations point to an activation scheme that requires several steps to change the inactive form of MC2R into an active form that produces cAMP.

MC2R Activation Scheme Requirements:

There are several assumptions made about the multi-step activation of MC2R by ACTH(1-24) that should be noted. First is that the HFRW site is closed in the inactive
form of MC2R. Second, when ACTH(1-24) dissociates from MC2R, the receptor reverts to a closed conformation thus again hiding the HFRW binding site. The multi-step method is as follows; 1) the C-terminal region of ACTH(1-24) makes initial contact with MC2R outside of the HFRW binding site as it is still closed, 2) secondary interaction of ACTH(1-24) with MC2R would open the HFRW site, and 3) ACTH(1-24) binds to the HFRW site now exposed on the MC2R protein (Baron et al., 2009). This hypothesis would take into account the observations make by previous labs that the C-terminal region of the ACTH(1-24) peptide is of significant importance in activation. α-MSH is potentially too short to induce the conformational change in MC2R as it is lacking the amino acids after position 16 that are present in ACTH(1-24). ACTH(1-24) C-terminal residues include the tetra basic cleavage sites that if further processed would result in the α-MSH peptide. These tetra basic residues located at positions 15-18 (lysine and arginine), along with the proline residue at position 19, could contribute to ACTH(1-24) ability to activate MC2R fully. For the purpose of this thesis, the working hypothesis is that changing the basic residues at positions 15-18 along with the proline at position 19 would inhibit the activating nature of ACTH on MC2R.

As has been predicted and shown that the residues in figure 6 play a vital role in the binding of ligands to MCRs then changing residues in ACTH, specifically the conserved regions such as HFRW or the tetra basic cleavage site, would reveal exactly which residues are needed for ligand binding (Pogozheva et al., 2005). The goals of this thesis are as follows. Changing the basic residues at positions 15-18, along with the proline at position 19, and testing for functional activation of endogenous MC2R will
<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>ACTH(1-24)</td>
<td>SYSMEHFRWGVGKKRRPVKVYP</td>
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</table>

Figure 7: ACTH(1-24) analogs used for this study
Shown are the different variations of ACTH(1-24) used for this study. Underlined are the residues that have been identified as required for all MCR activation (HFRW motif) and the tetra basic region hypothesized to be important in the activation of MC2R (KKRRP motif).
shed light on whether or not these residues are important for activation (Fig. 7). In addition, will these analogs of ACTH(1-24) out compete the wild-type version of ACTH?

Since it has been shown in previous work by Buckley et al. that α-MSH is incapable of displacing ACTH(1-39) and α-MSH is also not capable of outcompeting ACTH(1-39), being able to find an analog of ACTH(1-39) that competes for MC2R binding would uncover the mechanism of activation and amino acid residues that are key for proper activation. In addition, testing analogs that have replaced the HFRW moiety in ACTH(1-24) with alanine residues as well as replace the basic arginine residue in ACTH(1-24) at position 8 with an alanine residue tests the hypothesis that the basic residue in the HFRW motif of ACTH(1-24) peptide is key in binding (Fig. 7). Finally, testing a non-mammalian MC2R (S. tropicalis) using the same analogs in cell lines that do not produce MCRs will further support for the hypothesis that the HFRW binding site and, by relation, the ligand binding site on MC2R, has been evolutionarily conserved in chordates for at least 450 million years.
MATERIALS AND METHODS

Cloning MC2R:

For this study, it was necessary to obtain multiple tissue samples from *S. tropicalis* to perform various experiments and cDNA/gDNA extractions. Mature adult female *S. tropicalis* (Nasco, Fort Atkinson, WI) were defined as older than six months, capable of reproducing, and were chosen due to the fact that since the females are larger than the males the dissection would be easier. An animal care plan for the sacrifice of the frogs was arranged by Dr. Robert Dores and approved by the University of Denver IAUCUC. Animals were subjected to an overdose (>500mg/mL) of MS-222 (Sigma, St. Louis, MO) for 10-15 minutes before sacrifice by decapitation. Whole brains were obtained as a single sample by cutting the top of the skull off and separating the brain from the spinal cord. Dissecting the brain away from the sella turcica and removing the whole pituitary from the posterior side of the brain yielded whole pituitary samples. Adrenal tissue was obtained along with the kidney tissues, as there were no distinguishable islets that could be separated from the kidney tissue. Muscle tissue was also obtained from the thoracic region of all the animals. Similar tissues from each of the six animals were pooled into one 1.5mL Eppendorf tube and flash frozen using liquid nitrogen. Samples were then stored at -70°C until further use.
Generation of gDNA:

Genomic DNA was obtained using a tissue homogenization protocol adapted from the Promega Wizard Genomic DNA Purification kit (Promega, Madison, WI). 10-20mg of adrenal/kidney tissue was used in the preparation as well as 1mL of 1.0mm glass beads (BioSpec Products, Bartlesville, OK). Tissue was homogenized using a Fastprep FP120 (Thermo Savant, Waltham, MA) on settings as follows, 45 seconds @ 5.5 speed. There were a total of 3 cycles of homogenization with periods of ice incubation in between each cycle for 30-60 seconds. A phenol/chloroform extraction was done on genomic samples to further clean the sample. An equal volume of phenol:chloroform was added to the sample and the aqueous layer was extracted. A chloroform extraction was applied to the aqueous layer from the phenol/chloroform step and the sample was then spun at 16.1 RCF at room temperature for 5 minutes. The aqueous layer was again taken and then subjected to an ethanol precipitation. 3M NaOAc was added to a final concentration of 0.3M as well as three volumes of ice cold 100% ethanol. The sample was then spun at 16 RCF at 4°C for 15 minutes. The supernatant was removed and the pellet was washed with ice-cold 70% ethanol and spun for 5 minutes at room temperature at 16.1 RCF. The pellet was allowed to air dry for 10 minutes where afterwards DNA rehydration solution (10mM Tris and 1mM EDTA) was added and allowed to rehydrate overnight at 4°C. DNA obtained by this procedure was tested for purity and yield via 1% agarose gel electrophoresis and spectrophotometry reading at 260/280 OD. Once purity and concentration were determined, aliquots of diluted stocks were made for future applications and again were tested for concentration. All stocks were stored at -20°C.
Human MC2R:

Human wild type MC2R was obtained from Missouri S&T (www.cdna.org). The complete coding sequence was available in a standard vector from Invitrogen without epitope tags or other fusion proteins (pcDNA3.1+). When the vector arrived, it was subcloned it into Z-competent cells (Zymo, Orange, CA). For stocking purposes, mini prep reactions were performed and DNA to use for downstream applications was frozen at -20°C. Transformed colonies were stocked in 15% glycerol and stored at –80°C.

PCR:

PCR primer design was based on *Silurana tropicalis* sequence obtained from the genome consortium site (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). Full-length sequences for MC2R was not fully established by the consortium however, review of the raw sequence revealed an open reading frame that would correspond to human MC2R. This sequence was then BLAST analyzed to ensure homology with other known full-length MC2R sequences that has been properly submitted to NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez/). BLAST analysis indicated that the region was indeed the MC2R region and shared varying degrees of homology with several organisms. All primers were ordered from IDT online (www.idtdna.com) for both human and frog versions. One forward primer and 2 reverse primers were ordered for both organisms. One reverse primer would include the stop codon and the other would replace the stop codon for future experiments. hMC2R (5’-CACCATGAAGCACATTATCAACTCGTAT) and fMC2R (5’-CACCATGATGAAGATGGATATAGCAAATGAA) forward primers have extra 4 base
sequences at the beginning to aid in the cloning reaction downstream. The reverse primers that include the stop codon for 5’ epitope tagging for each are as follows: fMC2R STOP REV (5’-CTACATTGTCATCCAAACAGCATAACATTTTTTTGAA) hMC2R STOP REV (5’-CTACCAGTACCTGCTGCAGAAGAT). The second set of primers omits the stop codon to future downstream experiments can include 3’epitope tagging; fMC2R MUT REV (5’-CATTGTCATCCAAACAGCATAACATTTTTTTGAA), hMC2R MUT REV (5’-CTTCCAGTACCTGCTGCAGA).

PCR analysis was done using a proofreading polymerase to ensure no mutations were introduced and that there were no extra bases added to the sequence. Taq polymerase is known to add adenine overhangs to the ends of PCR products and for our study it was necessary for strict amplification of error-free product (Lawyer et al., 1993). Pfx50 polymerase was obtained from Invitrogen for use during PCR. Reaction mix was as follows; 5uL Pfx50 pol. PCR mix, 1.5uL 10mM NTPs, 1.5uL 10nM Forward primer, 1.5uL 10nM Reverse primer, 5uL template DNA, 1uL Pfx50 polymerase, 36uL dH2O. Reaction volume total for each reaction was 50uL typically with 3 reactions done at a time. PCR reactions were carried out on a MJ Research PTC-200 thermocycler using the following conditions; 94°C for 2 minutes for initial denature followed by 94°C for 15 seconds, annealing temperature for 45 seconds, 68°C for 1 minute then looping back to the second denature step for 35 cycles total, a final extension of 68°C for 5 minutes and finally a 4°C hold indefinitely, Annealing temps for frog and human MC2R varied (XtMC2R 66.2°C and HsMC2R 62.8°C) and were run separately. PCR products were run on 1.5% TAE agarose gels with 500 ng/mL ethidium bromide for visualization. 1ul of 6X loading dye with visual markers to aid in migration visualization was added to each
sample. Electrophoresis was preformed 1X TAE buffer at 115V for 12-17 minutes or until the smallest visual migration marker was near the bottom of the gel. Gels were then visualized under UV light using a GelDoc Transilluminator System (Bio-Rad, Hercules, CA) and Quantity One imaging software.

Purification:

Once positive reactions were identified, the rest of the PCR reaction was put through a Qiaquick PCR purification kit to remove unused nucleotides and primers (Qiagen Valencia, CA). Once purified, samples were then quantified on a Nanodrop ND-1000 spectrophotometer using ND-1000 V3.5.2 software. For cloning into the TOPO Entry vector, 5ng of the PCR product for both frog and human MC2R was used for the reaction combining 20ng/uL vector, 1uL of the supplied salt solution (1.2M NaCl, 0.06M MgCl₂) (Invitrogen, Carlsbad CA). The mixture was then allowed to sit at room temperature for 5 minutes before transforming into bacteria. TOP10 OneShot cells from Invitrogen were used for the transformation where 2uL of the TOPO reaction was added to one vial (50uL) of the cells and allowed to incubate on ice for 5 minutes. The cell mixture was then heat shocked at 42°C for 30 seconds and put back on ice immediately. 250uL of S.O.C. media was added to the cell mixture and was incubated at 37°C for 1 hour with shaking of 200rpm. After the outgrowth period, the mixture was plated at 50uL per plate. Antibiotic resistance of kanamycin at 100ug/mL on LB agar plates was used for selection. Plates were incubated overnight at 37°C and the following day, colonies were picked for stock plates using LB plates with 100ug/mL kanamycin for selection. These stock plates were allowed to grow overnight at 37°C and the resulting stocks were then
transferred to liquid LB media containing 100ug/mL kanamycin and grown overnight at 37°C with 100rpm shaking for miniprep. Overnight growth was done using a SI-300 incubating shaker from JEIO Tech (Korea).

Overnight samples ready for miniprep were assayed using a Zyppy Plasmid Miniprep Kit. (Zymo Research Orange, CA) Once minprepped, samples were then sequenced for gene insert verification. Using the nanodrop spectrophotometer, DNA concentration was obtained for miniprep samples for optimization of the sequencing reaction. Sequencing primers were supplied with the TOPO reaction kit and were used for sequence verification of the gene insert. M13 forward and M13 reverse primers were supplied at 0.1ug/uL in 8.0pH TE buffer. Following the Beckman-Coulter protocol for DCTS quickstart sequencing kit, reaction mixtures were set up as follows; 4uL of the quickstart mixture, 1uL of either the M13 forward primer or the M13 reverse primer (to ensure both 5’ and 3’ sequence integrity), and 5uL of the miniprep DNA. Once the samples were mixed, they were loaded into the thermocycler mentioned before using the following thermal profile; 30 cycles of 94°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes. Once the reaction was done, samples were loaded into 96-well plates and analyzed on a Beckman-Coulter CEQ 2000 autosequencer. Data was then visualized using 4peaks chromatogram software (mekandtoj.com) and BLAST analyzed using the BLAST tool at http://ncbi.nlm.nih.gov.

Expression vector generation:

Positive clones in the ENTRY vector were then set up for clonase reactions for movement of the MC2R gene into the mammalian expression vector. A vector for both 5’
and 3’ tagging were used for frog and human genes for use in downstream applications. The 5’ tagged vector utilized a V5 epitope tag that would allow for immunostaining while the 3’ tagged construct utilized a GFP fluorescent marker that would not require staining at all. Following the protocol provided with the destination vectors, both frog and human MC2R entry clones were set up for 5’ and 3’ tagging. Entry clone concentration was determined using the nanodrop spectrophotometer as mentioned before for optimization of the clonase reaction. pcDNA-DEST47 vector (Invitrogen Carlsbad CA) and pcDNA3.1/nV5-DEST (Invitrogen Carlsbad CA) were the vectors used, the DEST47 being the GFP-3’ version and the nV5 being the 5’V5 epitope version. Reactions were set up as follows; 50-150ng of ENTRY clone mixed with 150ng of the Destination vector and TE buffer pH8. To this, 2uL of LR Clonase II enzyme was added and incubated at 25°C for 1 hour. After the incubation, 1uL of Proteinase K was added to the samples and incubated at 37°C for 10 minutes. These samples were then transformed into Z-competent DH5α bacteria (Zymo Research Orange, CA) following manufacturer protocol. Both of the destination vectors had ampicillin resistance markers so the transformation reactions were plated on LB agar plates with 100ug/mL ampicillin for selection purposes. Plates were allowed to grow overnight at 37°C and colonies were picked using sterile toothpicks and stock plates were made as previously described. To confirm the correct orientation of the insert, sequencing was preformed as previously described but primer used was targeted to the T7 promoter site (5’-TAATACGACTCACTATAGGG-3’). Once positive clones were identified, overnight samples were made for maxi prep using 100mL LB media and 100ug/mL ampicillin for
resistance selection. These samples were grown at 37°C with 200rpm shaking for 16 hours before assaying.

Maxiprep was accomplished using an Endo-free Maxi Prep Kit (Qiagen Valencia, CA) in order to use the DNA for (delete: in) transfections of mammalian cell lines. Endotoxins produced by bacteria can inhibit transfection or cause the mammalian cells to change physiology and die so it was important to ensure purity of DNA. Once DNA was collected, it was analyzed using the nanodrop spectrophotometer as described before to determine purity and yield. DNA was then split into two aliquots, one for +4°C storage for quick use while the other aliquot went to the -20°C freezer for stock and future use.

Cell Culture:

Experiments showing MC2R expression were conducted using the CHO mammalian cell line. Chinese hamster ovary cells, or CHO, were ordered from ATCC (www.atcc.org) and grown according to the ATCC standards. Complete media is defined as follows for CHO cell line; Kaighn’s Modification of F12 media (F12K) (www.atcc.org) which includes 2mM L-glutamine, 1.5g/L sodium bicarbonate, 10% v/v Fetal Bovine Serum (FBS) (Atlanta Biological Atlanta, GA), 100U penicillin and 100ug streptomycin antibiotics (Gibco Carlsbad CA), and 50mg Normocin (Invivogene CA). Cells were grown in 37°C incubators with 5% CO₂ and between 70-80% humidity. CHO cells were propagated until 75% confluent and then split using 0.05% trypsin/0.53mM EDTA (Invitrogen Carlsbad, CA). Once cells were dense enough for transfection, they were plated on two-chamber slides previously treated with 100ug/mL poly-L-lysine for attachment at a density of 1x10⁶ per reaction. 1ug of construct DNA was used per
reaction along with 2uL Lipofectamine 2000 (Invitrogen Carlsbad, CA) in a total volume of 200uL OptiMEM media (CellGro). Cells were allowed to grow 24 hours before fixing and mounting. Cells were fixed to the slides using 4% paraformaldehyde (EMS Hatfield, PA) for 15 minutes at room temperature. Slides were then washed three times in 1x PBS solution and treated with VECTA-SHIELD with DAPI (Vector Laboratories Burlingame, CA) and sealed with a coverslip. Visualization took place on a Zeiss Axioplan 2 microscope using SlideBook software version 3.

MC2R and MRAP co-localization studies:

After acquiring the FLAG-MRAP and V5-MRAP constructs (Dr. Patricia Hinkle, U of Rochester) co-localization studies were necessary to ensure MC2R and MRAP were being translocated properly to move forward with functional studies of MC2R. CHO cells were grown and prepared for transfection as described above and transfected with either the frog V5-MC2R construct or the human V5-MC2R construct and the MRAP-FLAG construct using Lipofectamine as previously described. 24 hours post-transfection, the cells were fixed to the chamber slides as described before using 4% paraformaldehyde and washing with 1xPBS three times. For cells lacking the MRAP construct, MC2R was not expressed on the cell surface so permeabilization studies were necessary. After fixing with cells, they were treated with 0.3% Triton-X100 (Sigma) for 10 minutes at room temperature. Cells were then washed three times in 1xPBS. After washing, cells were incubated with primary antibody for the respective tag as follows. All antibodies (1° and 2°) were diluted in a 1xPBS+1%BSA mixture. Primary antibodies were diluted 1:100 and a volume of 200uL was used per transfection well. Incubations took place in 37°C
incubator without CO\textsubscript{2} for 1 hour. Wells were rinsed three times with 1xPBS and secondary antibody was added at 200uL per well. Secondary antibody was diluted 1:800 in the 1xPBS+1\%BSA solution. Depending on the primary antibody, either a donkey-\(\alpha\)-mouse Alexa488 fluorophore (anti-V5) or a donkey-\(\alpha\)-rabbit Cy3 fluorophore (anti-FLAG) was used. Wells were incubated for 30 minutes at 37\textdegree C without CO\textsubscript{2} and then washed with 1xPBS three times. Cells were then stained with VECTA-Shield was previously described and visualized using Zeiss Axioplan 2 microscope using SlideBook software version 3.

Endogenous Expressing Cell Lines:

GT1-7 (GnRH neurons) cells were used for this study as GT1-7 line expresses an MC4R when properly induced (Sebag and Hinkle, 2006). The GT1-7 media is as follows: DMEM which includes 4.6g/L glucose (CellGro), 10mL of 100mM sodium pyruvate per liter (CellGro), 100U penicillin and 100ug streptomycin (Gibco), 3.7g/L sodium bicarbonate (Sigma), 10\% FBS (Atlanta Biological), and 50ug Normocin (Invivogen). Cell lines were grown in 37\textdegree C incubators with 5\% CO\textsubscript{2} and between 70-80\% humidity until density reached 75-80\% confluency. Cells were then treated with trypsin-EDTA as previously described. Cells were plated at a density of 5 or 6 x10\textsuperscript{5} into 6-well plates (Costar) that were tissue culture treated for adherency along with 2mL of media. GT1-7 cells were allowed to re-attach for 24 hours and then induced for MC4R expression using 100nM dexamethazone (Sigma) for 30 hours prior to cAMP assay.

INS-1 (rat insulinoma cell line) cell line used for this study was a gift from Dr. Joe Angleson (U of Denver). INS-1 has been previously shown to endogenously express
MC2R (Al-Majed et al., 2004 and Angleson, unpublished). INS-1 cell media includes the following: RPMI media (CellGro), 2g/L Sodium Bicarbonate (Sigma), 2.38g/L HEPES (Sigma), 10mL of 100mM Sodium Pyruvate per liter (CellGro), 100U penicillin and 100ug streptomycin antibiotics (Gibco), 10% FBS (Atlanta Biologicals), and 50ug Normocin (Invivogen). Cell lines were grown in 37°C incubators with 5% CO$_2$ and between 70-80% humidity until density reached 75-80% confluency. Cells were then treated with trypsin-EDTA as previously described. Cells were plated at a density of 5 or 6 x10$^5$ into 6-well plates (Costar) that were tissue culture treated for adherency along with 2mL of media.

Murine M3 cell line studies:

Cloudman M3 cell line (mouse melanocyte cell line) were obtained from ATCC (www.atcc.org) and used initially for MC2R expression studies but later discarded for use of CHO cell line. Cells were grown in the following media preparation: Ham’s F12K modified to contain 1.5g/L sodium bicarbonate, 2mM L-glutamate, 100U penicillin and 100ug streptomycin antibiotics (Gibco), 15% horse serum (Atlanta Biological), 2.5% FBS (Atlanta Biological), and 50ug Normocin (Invivogen). Cell lines were grown in 37°C incubators with 5% CO$_2$ and between 70-80% humidity until density reached 75-80% confluency. Cells were then treated with trypsin-EDTA as previously described.

Mammalian Cell Nucleofection:

To create a large enough population of positively transfected CHO cells to perform functional assay, the AMAXA Nucleofector II system (Lonza Walkersville Inc.)
was utilized to obtain a high percentage of transfected cells. According to the manufacturer protocol and verified protocol in the Lonza database, CHO cells were grown to 80% confluency before transfection. Cells were trypsinized as previously described and counted using a hemocytometer. Cells were concentrated to $1 \times 10^6$/mL for each transfection reaction. Cells were then spun using a swinging bucket centrifuge at a setting of 4 for 10 minutes. Media was removed and cells were resuspended in 100uL type “T” solution per transfection reaction from AMAXA transfection kit “T”. 1ug of template DNA was added for each reaction and mixed by inversion. Mixture was aliquoted 100uL per cuvette and shocked in the Nucleofector II under the pre-existing conditions of program U-023. Mixture was then removed from cuvette by adding warm CHO cell media and plating in a 6-well tissue culture treated plate. Cells were then incubated for 30-48 hours (roughly 85-90% confluency) before assaying for cAMP production.

cAMP EIA assay:

All means of functional assaying were done as follows including CHO cell lines transfected with StMC2R, HsMC2R, and the GT1-7 cell line. Ligands were diluted as needed on assay days otherwise stored at -20°C. Peptides (New England Peptide) were resuspended in physiological media that contained 1mM MgCl$_2$, 10mM HEPES, 5mM CaCl$_2$, 140mM NaCl, 2mM KCl, 1% BSA, and 10mM D-glucose for working concentrations. To prevent degradation of intracellular cAMP a phophodiesterase inhibitor, IBMX, was used at a concentration of 1mM diluted in the physiological media described before and added to all wells. Cells were then incubated at 37°C without CO$_2$
for 15 minutes with 1mL total volume containing ligand and IBMX. Ligand mixture was removed and wells were incubated with 1mL 0.1M HCl at room temperature along with well scrapping using sterile scrappers (BioxBrand) to ensure total cell lysis for 10 minutes. Liquid was collected and spun at 4.5 RCF for 5 minutes to pellet cellular debris and supernatant was collected and assayed directly. Lysate was assayed using a direct cAMP EIA kit (Assay Designs Ann Arbor, MI) following manufacturer protocols for reagent addition and incubation times. The plates were then read using a Synergy HT Plate Reader (BioTek) using the absorbance setting of 405nm and manually corrected for blank controls.

Data interpretation:

All data from cAMP EIA experiments were normalized to maximal response as to compare experiments from separate days and transfections. Maximal response for each experiment is defined as maximal signal for saturating ligand doses for each experiment. Activation values were found and all other data points were divided by maximal value. Data shown in graphs are representative of percent activation based on maximal activation. Error calculated for each data set was accomplished by dividing each data point individually by the value for maximal activation and then averaged together and standard error calculated.
RESULTS

Studies using an endogenous MCR system: GT1-7 cell line and MC4R:

In order to test activity of analogs of ACTH(1-24) on MC2R it was first necessary to identify a positive control MCR cell line that could be used to demonstrate the efficiency of the cAMP assay when cells were challenged with the wild-type form of ACTH(1-24). There are cell lines that express endogenous MCRs and can be used for functional assays. One caveat is that the cell line must only express one MCRs in order that functional studies would not be hindered by signal from other expressed MCRs. Endogenous MCR expressing cell lines expressing only one of the melanocortin receptor were chosen to reduce the possibility of background interference in downstream experiments. Use of GT1-7 line (rat GnRH neurons) that express endogenous MC4R, were 3-fold: one was to establish a dose response curve (DRC) to ensure EC₅₀ value that was in keeping with reported values, discover working parameters for the cAMP EIA assay, and finally using endogenous cell lines would circumvent the problem of not having a satisfactory expression system in non-MCR expressing cell lines. As previously shown, MC4R is responsive to all melanocortin ligands and both ACTH(1-24) and a form of MSH were tested on this cell line (Cone, 2006). Measurements taken for cAMP production were normalized to maximal activation and figure 8 shows typical standard curves for the cAMP EIA assay when using GT1-7 cells and stimulating with ACTH(1-24) or NDP-MSH. Figure 9 shows another DRC using the KKRRP analog and comparing
GT1-7 cells were induced to express MC4R and stimulated with either NDP-MSH or ACTH(1-24) and assayed for cAMP production. Doses of ligand range from 1uM ($10^{-6}$) to 10pM ($10^{-11}$).

Figure 8: GT1-7 DRC for NDP-MSH and ACTH(1-24)

GT1-7 cells were induced to express MC4R and stimulated with either NDP-MSH or ACTH(1-24) and assayed for cAMP production. Doses of ligand range from 1uM ($10^{-6}$) to 10pM ($10^{-11}$).
Figure 9: GT1-7 DRC with ACTH(1-24) and KKRRP analog
GT1-7 cells were induced to express MC4R and stimulated with either
ACTH(1-24) or KKRRP analog and assayed for cAMP production.
Doses of ligand range from 1uM (10^{-6}) to 10pM (10^{-11})
it to ACTH(1-24). EC\textsubscript{50} values obtained for NDP-MSH, an analog of α-MSH, ACTH(1-24), and KKRRP analog are 3nM, 5nM, and 50nM respectively. Establishing the sensitivity of the GT1-7 cell line to ACTH(1-24) would allow for future experiments using analogs of that peptide to test hypotheses about the method of activation by melanocortin receptors. For the GT1-7 study, there were four analog variants of the ACTH(1-24) peptide that would be used (Fig. 7). With the HFRW-motif analogs (HFRW, HFAW, HARW), the critical binding site and subsequent activation would be tested with the single amino acid substitutions while the C-terminal analog (KKRRP) would test the requirement of the amino acids in the basic cleavage position. cAMP was measured as a function of receptor being stimulated by the ligand using a cAMP EIA kit. Analogs were initially tested individually to check for activation before testing for antagonist activity. All ligands were tested at a saturating dose 1\mu M and roughly EC\textsubscript{50} equivalent dose of 1nM.

It is known that for MCR activation, there must be an intact HFRW-motif on the melanocortin ligand (Schwyzer, 1977). By changing residues within that motif, we confirm that our endogenous system follows this rule. By testing the KKRRP analog, which changes residues at the C-terminal end of the peptide, checking to see if the HFRW-site on the ligand is sufficient for activation in this assay. Single analog activation assays done using GT1-7 cells are shown in figure 10. Results shown are normalized to maximal activity and are done in triplicate. ACTH(1-24) shown at doses of 1\mu M and 1nM confirm that the cells are responsive to positive stimulus as well as show in a dose dependant manner the activation of the MC4R. Analogs tested include the N-terminal variants that have modified HFRW-motifs as well as the C-terminal analog that has a
GT1-7 cells were assayed after incubation with ligands individually to test for cAMP production. Differences between ACTH(1-24) and KKRRP analog are not significantly different at either dose (1uM or 1nM) suggesting KKRRP analog can activate MC4R with the HFRW-motif intact but without the basic cleavage site downstream.
Figure 11: GT1-7 Competition Assay
N-terminal analogs were assayed with ACTH(1-24) to check for antagonistic characteristics. ACTH(1-24) was used at doses of $10^{-9}$ M and $10^{-10}$ M while all analogs were tested at $10^{-6}$ M. Asterisks represent significance to the $P<0.05$ level. $N=3$ for all experiments.
modified dibasic cleavage site for processing, KKRRP. Analogs were also tested at 1uM and 1nM for activation. None of the HFRW-motif modified analogs were capable of stimulating cAMP production above background activity at either high dose or low dose. This is in keeping with reports about HFRW-motif requirement. Activation is seen for the KKRRP analog at both doses that are roughly equivalent to the values obtained for the ACTH(1-24) dose points. This suggests that MC4R, when activated by ACTH(1-24), does not need the C-terminal region of the peptide, only the N-terminal portion that contains the α-MSH region.

Competition assays using GT1-7 cells were grown as described in the materials section and induced with dexamethazone to stimulate the expression of MC4R. By co-incubating a natural agonist (ACTH(1-24) with a ligand shown to not have any stimulatory effect (HFRW-motif analogs Figure 10), we could discover if these analogs would compete with ACTH(1-24) for binding site and thus activation. Choosing dose points for the ACTH(1-24) component of the competition assay reflects the desire to check whether a 1000-fold higher dose of analog (1uM) could compete with a roughly EC₅₀ dose range (1nM and 100pM) of ACTH(1-24). Ligands were co-incubated with cells and cells were then prepared as described to assay the supernatant directly for cAMP using the cAMP EIA kit (Assay Designs, MI). ACTH(1-24) was run alone at doses 1nM and 100pM for control reasons. There is significant (P<0.05) competition between the HFRW-motif analogs and ACTH(1-24). Both dose points for ACTH(1-24) show significantly diminished cAMP production when challenged with saturating levels of the analogs (Fig.11). This shows the ability of the HFRW analogs to block the HFRW
binding site on MC4R but the inability of the analog to properly activate the receptor into producing cAMP.

Initial MC2R endogenous system and heterologous expression system.

INS-1 cell line was used initially as an endogenous MC2R system for this study of MC2R binding selectivity. INS-1 did not require induction of expression as with GT1-7 cell line and has been reported to functionally express MC2R (Al-Majed et al., 2004 and Angleson, unpublished) however, inconsistency with results lead us to develop our own functional system for MC2R expression. Before moving on to other cell lines, preliminary experiments were conducted on INS-1 cells as shown in figure 12. An ACTH(1-24) dose response curve was generated using INS-1 cells that were plated and assayed using the cAMP EIA kit as described before. EC$_{50}$ for INS-1 cells was found to be 0.5nM. The ability to express MC2R in a true heterologous system lead us away from the INS-1 experiments as troubleshooting cell culture conditions would have taken longer than creation of a heterologous system.

As reported in Rached et al.’s studies, Cloudman M3 cell lines could be used as a viable cell line for functional expression of the MC2R. After successfully cloning the S. tropicalis MC2R and the human MC2R into C-terminal GFP constructs, M3 cell line was used for localization studies. However, as shown in figure 13a, human MC2R-GFP is seen in an expression pattern that does not resemble that of surface staining.

Upon further literature review, it was revealed that C-terminal GFP fusion proteins using MC2R inhibited trafficking to the plasma membrane as well as functional activation (Roy et al., 2007). Expression constructs were changed in favor of an N-
Figure 12: INS-1 DRC
INS-1 cells were assayed for cAMP production after stimulation with ACTH(1-24). N=2 for all dose points. Error bars were not created as there were not enough experiments completed before problems arose.
Figure 13: Cloudman M3 Cell Line Experiments with MC2R
Panel A shows Cloudman M3 cells transfected with hMC2R-GFP. Panel B shows permeabliized M3 cells with V5-hMC2R epitope tagged construct while Panel C shows permeablized V5-fMC2R transfected cells. None show appropriate cell surface localization.
terminal V5 epitope tag and M3 cells were again used for visualization. Again, figure 13b-c shows a staining pattern not consistent with surface staining for both human MC2R as well as frog MC2R. Two problems came to light after further review of the M3 cell line. One is the evidence of multiple MCRs being expressed in the M3 cells. This would complicate functional studies attempted on any expressed MC2R. The second problem and answer comes by way of the discovery of a unique accessory protein whose function is aiding the MC2R protein in translocation to the plasma membrane and aiding in functional activation (Sebag and Hinkle, 2009). These discoveries lead us to move to a heterologous expression system using CHO cell line and obtaining the accessory protein, MRAP.

Human and Frog MC2R localization with MRAP in CHO cells:

Subcloning the frog and human MC2R genes into the final destination vector is discussed in the materials section. PCR amplification and ligation into the entry vector allowed for restriction digest analysis to ensure appropriate size of the inserts (fig 14). Vectors containing the appropriate band size were then sequence verified for both human and frog MC2R. Genes were then moved to the V5-tagged construct for immunofluorescence. CHO cells were transfected using the lipid-based system and grown on poly-lysine treated glass slips. Figure 16-top panel shows permeabilized human V5-MC2R transfected CHO cells and figure 17-top panel shows permeabilized frog V5-MC2R transfected CHO cells. Note the apparent lack of cell surface localization and the general internalization of both the receptors due to lack of MRAP.
Figure 14: fMC2R and hMC2R ENTR vector confirmation
After ligation into the TOPO-ENTR vector, a digest confirmation was done using NotI and AscI restriction enzymes. Bands in lanes 2 and 3 are fMC2R and bands in lanes 4 and 5 are hMC2R.
After obtaining an MRAP construct from the Hinkle lab, localization and functional studies could be performed in CHO cells. FLAG-MRAP construct was transfected into CHO cells and plasma membrane localization of MRAP is clearly seen (Fig. 15). After obtaining this verification of the construct, double transfections were performed using both human and frog MC2R constructs with MRAP to ensure MC2R was being trafficked properly. Figure 16-b and figure 17-b show human and frog MC2R localizing to the cell membrane respectively. Functional assays would not be accomplished without this surface staining data.

Activation Assays using CHO and HsMC2R+MRAP:

Initial functional studies were done on human MC2R to show that the CHO system would give results in keeping with published data. The first experiment was a dose response curve (DRC) using ACTH(1-24) as the stimulatory ligand. Knowing that MC2R is not responsive to other melanocortin ligands (Gantz and Fong, 2003), only three high doses of NDP-MSH were used as a negative control. Figure 18 shows the DRC for CHO cells transfected with HsMC2R construct and MRAP construct that has been normalized to maximal activation. All dose points are experiments done in triplicate ± standard error. EC$_{50}$ value recorded for HsMC2R in the CHO line was 5nM, which is mildly shifted when compared to other reported values (Roy et al., 2007).

These next experiments involve the N-terminal analogs as well as the C-terminal analogs and whether or not they could activate HsMC2R. These analogs are variants of ACTH(1-24) with certain residues or motifs replaced with alanine residues to test the necessity of that residue or region with respect to functional activation. Like with the
Figure 15: CHO cells with FLAG-MRAP
CHO cells were transfected with FLAG-MRAP construct to verify MRAP expression and cell surface localization. FLAG-MRAP construct a gift from Dr. Patricia Hinckle of U of Rochester.
Figure 16: CHO cells with hMC2R and hMC2R+MRAP
Top panel shows a permeablized CHO cell with V5-hMC2R construct (green) without MRAP. Bottom panel shows a non-permeablized CHO cell with both V5-hMC2R and FLAG-MRAP (red). Compared to Figure 15, surface localization of both MRAP and hMC2R is apparent.
Figure 17: CHO cells with V5-fMC2R and V5-fMC2R+MRAP
CHO cells were transfected with V5-fMC2R only (top panel) and then with V5-fMC2R+FLAG-MRAP (bottom panel). Surface localization is observed in non-permeablized cells with MC2R (green) & MRAP (red) while the top panel is permeablized and shows no plasma membrane localization.
Figure 18: CHO cell line DRC with hMC2R & MRAP
CHO cells were transfected with hMC2R construct and stimulated with ACTH(1-24) and NDP-MSH and assayed for cAMP production. Graph shows results normalized for maximal activation.
Figure 19: CHO cell/hMC2R/MRAP system N-terminal analog activation assay
CHO cells assayed for analog activation via cAMP production. Doses points for assay were 1uM and 1nM for all ligands. No significant activation occurred for any of the analog peptides at either dose. N=3 for all dose points.
experiments using the HFRW-motif analogs and the MC4R system, activation is not anticipated for single analog activation scenarios. As for the C-terminal analog, KKRRP, referring back to the 2-step activation scheme, KKRRP should not be able to activate MC2R. As can be seen in figures 19 and 20, the range of ACTH(1-24) analogs used did not provide any significant activation of the CHO/HsMC2R system. ACTH(1-24) activation at doses of 1uM and 1nM show that the system is sensitive to stimulus and not inactive. The N-terminal analogs not being able to activate the MC2R system is in keeping with the data obtained for the GT1-7/MC4R system discussed earlier. One exciting exception is the KKRRP, which had been shown to be able to activate the GT1-7 cell line, but was not able to activate the CHO/hMC2R system. This observation points to a C-terminal sequence requirement for binding and, in particular, the tetra basic region.

CHO/hMC2R+MRAP Ligand Competition assays:

Finally, since the analogs do not activate the MC2R system, competitive assays were set up to test the potential antagonist activity of the analogs, particularly the C-terminal KKRRP analog. The first group of analogs tested was the N-terminal HFRW-motif analogs (Fig 21). At the ACTH(1-24) 1nM dose, none of the analogs proved to be significantly antagonistic which contrasts very differently with the significant competition observed with the GT1-7 competition experiments. Though, looking at the ACTH(1-24) 100pM dose, both the HFRW and HARW analogs significantly (P<.05) out-competed the ACTH(1-24) where in the GT1-7 experiments, all three N-terminal analogs showed competition. The second experiment using the C-terminal KKRRP analog showed no antagonistic properties at either ACTH(1-24) dose of 1nM or 100pM.
Figure 20: CHO cell/hMC2R/MRAP system C-terminal analog activation assay
CHO cells assayed for analog activation via cAMP production. Dose points were 1uM & 1nM for all ligands. No significant activation occurred for any of the analog peptides. N=3 for all dose points.
Figure 21: CHO cell with hMC2R+MRAP ligand competition
ACTH(1-24) was co-incubated with analog peptides and cAMP was measured to determine antagonist activity. Values in dose point of 1nM are not significantly different while HFRW and HARW analogs at the ACTH(1-24) dose point of 100pM appear to have strong antagonist characteristics.
Figure 22: CHO cells with hMC2R+MRAP analog competition
KKRRP was tested with ACTH(1-24) for antagonistic characteristics.
KKRRP analog, while stimulatory in GT1-7 cells due to MC4R activation
scheme, is not stimulatory in CHO cells transfected with hMC2R and
MRAP nor can compete for the active binding site possibly pointing to a
requirement of that motif in binding and activation of MC2R/ACTH.
(Fig 22). This result further points to a region in the C-terminus that is required for binding and activation.
DISCUSSION

Melanocortin receptors are a family of G-protein coupled receptors in the same superfamily as Rhodopsin (Cone, 2006). These melanocortin receptors (MCRs) are stimulated by a group of ligands known as melanocortins that are derived from a common preprohormone, POMC (Pritchard et al., 2002). Once activated, these MCRs signal via adenylate cyclase pathway resulting in the second messenger cAMP being produced. MCRs have a differing affinity for these ligands (Fig. 3) and are further classified based on binding, Class A (MC1R, MC3-5R) and Class B whose only member is MC2R. MC2R is unique by the fact that it only binds the POMC product, ACTH, unlike the Class A MCRs, which bind the entire range of POMC melanocortin cleavage products (Gantz and Fong, 2003). Since all of the melanocortin ligands share a unique amino acid motif (HFRW) that is fundamental in binding to the MCR receptors and activating them, it is curious then why the MC2R is only capable of being activated by ACTH and not by the other melanocortin ligands.

Melanocortin receptors arose likely due to genome duplication events throughout the evolution of the Chordates. For example, the evolution of the HOX genes is believed to follow duplication events called the 2R hypothesis where 2 independent genome duplication events resulted in 4 HOX gene clusters (Ohno et al., 1970). Relating this to melanocortin receptors shows a similar pattern of gene evolution with the slight exception of MC2R and MC5R, which are thought to be a product of a single gene
duplication event (Schioth et al., 2003). Why then does the Class A receptors bind the whole range of melanocortin ligands and why MC2R does not has been an elusive question. Functional studies of MC2R have been hindered due to poor activation and trafficking of the mature receptor to the plasma membrane (Roy et al., 2007). This issue was circumvented as a result of the discovery of an accessory protein dubbed melanocortin 2 receptor accessory protein or MRAP (Metherell et al., 2005). The introduction of MRAP to certain cell lines made it feasible to establish heterologous MC2R systems a realized system.

For this thesis, we asked the question about what is important and necessary for the activation of MC2R. We know from previous studies that the HFRW amino acid motif is required for activation of MCRs (Schwyzer, 1977). Knowing then that the only difference separating ACTH(1-24) and α-MSH is the C-terminal portion of the ACTH peptide, it seemed appropriate to design analogs in which the amino acids in the C-terminal of ACTH(1-24) underwent alanine substitution to address the question of which amino acid positions in the C-terminal of ACTH were required for activation of MC2R. We accomplished this by using both homologous and heterologous expression systems for MC2R and a homologous expression system for MC4R.

GT1-7 ACTH(1-24) and NDP-MSH Dose Response Curves:

One of the model systems we used was GT1-7 cell line. GT1-7 cells can be induced to express MC4R following dexamethazone stimulation (Sebag and Hinkle, 2006). Being able to use a cell line that already produces an MCR of choice without expressing others is an ideal system to work in for functional studies on the activation
properties of various melanocortin ligands. Beginning with dose response curves for both
ACTH(1-24) and NDP-MSH did several things. The first was to establish that in our
hands using the GT1-7 cell line would be a functional system to measure a Class A
melanocortin response to stimulus. Since MC4R is responsive to both ligands [i.e. α-
MSH and ACTH(1-24)], we wanted to establish a dose response curve for ACTH(1-24).
We would then have a Class A MCR dose response curve for ACTH(1-24) that we could
compare to the ACTH(1-24) dose response curves for MC2R. Looking at the DRC for
ACTH(1-24) and α-MSH in figure 8 shows, after normalization, similar activation
patterns for the ligands. EC$_{50}$ values observed for NDP-MSH and ACTH(1-24) are
roughly 1nM and 5nM respectively.

GT1-7 Single Analog Experiments:

Showing that the cells are responsive allowed us to move to the analog
experiments for Class A MCRs for future comparison to MC2R systems we would test.
The first of those experiments is shown in figure 10 where we looked at analogs of
ACTH(1-24). Testing whether these HFRW-motif analogs would activate MC4R is a
control for MCR stimulation because it is known that the HFRW motif in melanocortin
ligands is key for activation of all MCRs (Schwyzer, 1977). The experiments show that
indeed, either substituting alanines for the entire motif, or doing individual alanine
substitutions for F$^7$ or R$^8$ resulted in HFRW-motif analogs that were incapable of
stimulating cAMP production.

The inability of the HFRW-motif analogs to stimulate cAMP production in the
MC4R system can most likely be attributed to the method of activation. Because Class A
MCRs can be activated by a host of melanocortins, they only require the HFRW binding site to be activated. Other regions of the peptide do not seem to be needed to activate the receptor as evidenced by the KKRRP analog stimulation resulting in cAMP message (Figure 10). The level of activation between ACTH(1-24) and KKRRP is not significantly different at either 1uM or 1nM doses. Since the KKRRP analog was capable of stimulating MC4R to roughly the same degree as ACTH(1-24) a dose response curve was generated using KKRRP analog and GT1-7 cells (figure 9). The dose curve shown is slightly shifted when compared to ACTH(1-24) but at the dose points used for the activation assay, there is not significant difference between observed cAMP values. EC$_{50}$ values observed for ACTH(1-24) and KKRRP are roughly 5nM and 50nM respectively.

GT1-7 Competition Assays:

GT1-7 cells were set up to determine if any of the N-terminal analogs could compete with ACTH(1-24) for binding and activation. ACTH concentrations used were 1nM and 100pM to simulate doses near EC$_{50}$ values observed. Analog ligands were held constant at 1uM doses for the purpose of testing greater than 1000-fold higher saturation. Looking at figure 11, there is significant competition at both doses of ACTH(1-24) when challenged with 1uM analogs. The out-competition of ACTH(1-24) by the ligands points to the analog ligands being able to bind the site on the receptor but not confer the appropriate conformational change to cause a cAMP message to be generated.
INS-1 Cell and Cloudman M3 Experiments:

To complement the GT1-7 experiments, a homologous MC2R expression system was attempted using INS-1 cells. These cells have been shown to express MC2R endogenously and could have been a potential answer to the problem of MC2R expression systems and functional assays (Al-Majed et al., 2004 and Angleson, unpublished). Figure 12 shows the initial dose response curve for ACTH(1-24) and α-MSH using the INS-1 system. The EC$_{50}$ value observed for this system was roughly 800pM and there was no response for α-MSH at saturating doses. Issues with cell culture producing functional cells prevented further studies on this system so one alternative was the possibility of a heterologous expression system. Groups had previously shown that it was possible to express hMC2R in a murine cell line called Cloudman M3 (Rached et al., 2005).

A model endocrine system organism, *Silurana tropicalis*, was chosen to potentially represent a non-mammalian example of MCR study and MC2R was cloned from adrenocortical tissue of this frog. Human MC2R was obtained and both MC2Rs were individually transfected into the M3 cells. Figure 13 shows initial expression studies using the M3 system. Initial constructs had both human and frog receptors attached to a GFP tag on the C-terminus. Since a subsequent review of the literature indicated that other groups had shown that attaching GFP to the C-terminus of a MC2R inhibits the function of the receptor, the MC2R/GFP fusion protein was discarded in favor of a smaller N-terminal tag epitope tag on the MC2Rs. Panels B and C of Figure 13 show human and frog V5 tagged constructs transfected into M3 cells. The lack of cell surface organization raised questions as to whether the receptor was being properly trafficked to
the membrane surface. The apparent problems with the M3 cell line in our hands were circumvented by the recent discovery of an accessory protein, MRAP, which solved the plasma membrane localization problem (Metherell et al., 2005).

CHO Heterologous System Localization Experiments:

The discovery of the MRAP protein made functional studies of MC2R much more reasonable. Being able to use cell lines, like CHO cells, that do not express MCRs endogenously is a great boon to understanding ligand selectivity of MC2R. The first experiment in this thesis regarding a heterologous MC2R expression system was to verify surface transport of MC2R when MRAP is present. Figure 15 shows CHO cells transfected with MRAP-FLAG construct. The MRAP protein itself localizes to the plasma membrane in a very succinct manner. The top panel of figure 16 shows CHO cells transfected with hMC2R construct with the N-terminal V5 tag. When comparing this panel with previous experiments in M3 cells, distribution of MC2R is very similar. M3 cells were purported to have MRAP-like activity and the ability to functionally express MC2R but staining patterns of M3 cells with hMC2R (figure 13 panel A) and the top panel of figure 16 (hMC2R) and the top panel of figure 17 (StMC2R) shows a consistent pattern of cytosolic, possibly ER-bound MC2R that does not transport properly. While it is reasonable to believe a minority of the receptor being produced can make it to the cell surface, this would not be enough to test for functional activation and it has also been shown that MRAP is involved with MC2R activation (Sebag and Hinkle, 2007).
CHO Heterologous System Dose Response Experiments:

Cell surface localization of MC2R is the first step in being able to test functionality. Bottom panels of figures 16 and 17 show human and frog MC2R/MRAP co-localization respectively. By demonstrating that the MC2R could be translocated to the cell surface, we could then focus on testing if the receptor was viable. We show a dose response curve for CHO cells with hMC2R and MRAP that has been stimulated with ACTH(1-24) and α-MSH (Figure 18). α-MSH had no effect at saturating doses of 10nM up to 1μM, which is to be expected. ACTH(1-24) stimulation yielded an EC_{50} value of about 2nM. This value is close to other group’s reported values (Roy et al., 2007) but there are slight differences. Roy et al. used human embryonic kidney cells (HEK293) while we used CHO-K1 line so the possibility of subtle differences in cell response could affect the values we observed. Another point of difference is the manner in which cAMP was detected. For our study, we used a cAMP EIA kit that used antibodies to detect and bind cAMP that is produced after cell stimulation. Roy et al. used a radiolabeling assay and separation of labeled ATP from labeled cAMP using chromatography. The potential for differing results is more than likely due to assay quality and sensitivity. Regardless, the CHO/MC2R+MRAP system is the best way to test receptor functionality and ultimately MC2R ligand selectivity.

CHO Heterologous Single Analog Experiments:

As stated before, all MCRs require the HFRW-motif in the ligands to be properly activated. Using the GT1-7 cell line, we showed that MC4R is incapable of being activated by analogs where the HFRW motif is changed, even by a single amino acid.
When testing MC2R in the CHO/MRAP system, these analogs were tested as well but the emphasis of the analog activation experiments was the C-terminal ACTH(1-24) analog, KKRRP (Figure 7). Using the GT1-7 system, it was shown that KKRRP was capable of activating MC4R. In this thesis, we propose that there is a multi-step activation procedure for MC2R that is not shared with the Class A MCRs. MSH-like peptides and ACTH share the core motif required for activation (HFRW) but the remainder of the sequence is varied. The activation for Class A MCRs is solely dependent on an intact HFRW site and amino acid residues at either the N-terminal or C-terminal regions are not important for Class A MCR activation. We hypothesize that there is some requirement in the C-terminal region of ACTH(1-24) that is necessary for primary activation of MC2R. It has been hypothesized that in the C-terminus of ACTH(1-24), there is a core sequence that docks with the MC2R first that allows for a conformational change that possibly exposes the HFRW-binding site (Baron et al., 2009). Following the initial binding event it has been further proposed that the ACTH peptide then is fully nested in the receptor and full activation is achieved. By replacing the tetra basic cleavage site positions 16-20 in ACTH(1-24) with alanines (along with the proline residue at position 20), we can test if that motif is involved in receptor activation. The cleavage site is where PC2 will cleave ACTH resulting in α-MSH and CLIP. This C-terminal region is the only difference between ACTH and α-MSH so activation of MC2R must be dependent on a set of residues in this region.

Single analog tests were performed on the CHO system as shown in figures 19 and 20. As expected, HFRW-motif analogs were incapable of activating MC2R. Again the absence or alteration of this key site is the cause for the inactivity of these analogs. In
figure 20, multiple C-terminal analogs were tested for activation and none showed activation. Included in this set of analogs was the KKRRP analog and though it activated the MC4R to nearly the same degree as ACTH(1-24), it could not activated MC2R. This begins to possibly explain the exciting possibility of MC2R selectivity. As discussed in the introduction, the multi-step activation scheme depends on an initial binding event that confers a conformational change in the MC2R that would allow for the HFRW-binding site to be available for binding with the ligand. Failure to cause this conformational change would presumably prevent peptides with the intact HFRW-motif from binding and ultimately activating the biomessenger pathway. The KKRRP peptide does in fact have a functional HFRW site based on the GT1-7 activation data leading one to believe there is a requirement for initial docking of the ACTH(1-24) peptide in the C-terminal region, more specifically the tetra basic region.

Going along with the KKRRP analog activation assay were several analogs that are truncated versions of ACTH(1-24) that only contain C-terminal residues after the cleavage site. These analogs do not contain HFRW motifs and cannot activate the receptor. Data shown confirms this however future experiments using these truncated analogs and ACTH(1-24) and possibly KKRRP in competition assays could be useful in further proving the C-terminal requirement for activation.

CHO Heterologous System Competition Assays:

To understand which regions of ACTH that could competitively inhibit the action of wild-type ACTH, competition assays using the analog peptides and ACTH(1-24) were performed. Figure 21 shows assays using the N-terminal analogs with the altered HFRW-
motif. At ACTH(1-24) dose of 1nM, there is no significant competition between the analogs and ACTH. There is, however, competition between ACTH and the HFRW and HARW analogs strongly reduces cAMP production at the ACTH(1-24) dose of 100pM. This is in keeping with data observed for GT1-7 cells, however, there was a greater competition in GT1-7 cells at the 1nM dose of ACTH(1-24) that was not observed for the CHO/hMC2R experiment. The HFAW analog did not show any kind of competition in either dose of ACTH(1-24), which contrasts with the data observed for GT1-7 cells.

Figure 22 shows competition data for ACTH(1-24) and KKRRP analog. There is not significant decrease in cAMP production even when KKRRP is at a very large molar excess. This is the final piece in the activation puzzle. KKRRP’s inability to even slightly compete with ACTH(1-24) points to the basic residues being necessary for the initial binding of ACTH to the MC2R and releasing the HFRW site for active binding. Future experiments will help validate this claim of the basic residues being necessary.

Future Directions:

MC2R functional studies have been a source of frustration for years until the recent discovery of MRAP. Now we can create heterologous expression systems to measure MC2R functionality and begin to explore its selective nature. In this thesis, we have shown the GT1-7 cell system to be a valid Class A MCR system to study functional activation. Having a cell system that endogenously expresses MCRs of interest will be useful in the future when delving deeper into physiological function of MCRs. The INS-1 MC2R endogenous system is also a valid system to study when the cell culture issues are resolved. Closer examination of INS-1 cells is necessary to understand physiological
expression conditions and requirements and will aid in long-term understanding of MC2R. Together, comparing the expression and function of these two endogenous systems will lead to new realizations of Class A and Class B MCRs physiological role in cells and ultimately in tissues.

Most work done in MCR research is accomplished using mammalian genes. We have successfully cloned and expressed a non-mammalian MC2R that can be applied to future studies on MC2R. *Silurana tropicalis* is a model system for development as well as a benchmark system for endocrinology studies. More in depth studies of this receptor will shed light on whether or not this system can be used for MC2R studies for both pharmacological and physiological purposes.

Probably the most exciting aspect of this thesis is the CHO cell heterologous system we have created. By using the CHO cell line and human MC2R along with MRAP, we have demonstrated a fully functional MC2R system capable of shedding light on the selective binding caveat. While not the first to show this CHO system is functional, we are the first to begin exploring the activating properties of human MC2R using analog peptides of ACTH(1-24). While the multi-step method of MC2R activation is not a new concept, we have shown initial steps in isolating the key region for this hypothesized mechanism. The tetra basic region in ACTH is an area for PC2 to cleave ACTH into α-MSH and CLIP. Realizing that the only difference between the peptides that bind Class A MCRs and MC2R is the cleavage region leads one to suspect that areas importance in activation. By making alanine substitutions in that region, we were able to test this hypothesis and have shown that these residues are indeed important in the
functional activation of MC2R lending credence to the multi-step activation hypothesis of MC2R.

Future experiments will include re-examining INS-1 cell culture conditions. Cell culture conditions will be evaluated and tested so in later experiments, analog assays such as analog activation and competition can be completed. This will be important for the endogenous expression systems future as being able to compare two endogenously expressing cell lines will put all data on an even keel so to speak. Another future direction will be getting the non-mammalian MC2R heterologous system up and running. Being able to show localization with MRAP is the first major hurdle to pass in validating this system. Another hurdle is functional activation. Collaboration with Dr. Patricia Hinkle of the University of Rochester has given us the initial push for these activation studies. Figure 23 is an ACTH dose response curve that was performed by Dr. Hinkle and Dr. Sebag using our fMC2R construct and MRAP. The resulting DRC shows activation of the receptor albeit to a shifted degree. Our task is to now work together with that lab to confirm those readings for future analog experiments. The CHO/hMC2R system has proven to be the most informative set of experiments for this thesis and for the future several more analogs will be tested for competition. Additionally, competitive binding studies will need to be done in support of the analog activation studies.
Figure 23: Frog MC2R DRC
ACTH DRC performed by Drs Hinkle and Sebag at the University of Rochester using frog MC2R construct. Data shows a shifted EC$_{50}$ value when compared to observed hMC2R value.
BIBLIOGRAPHY


