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Analyzing the Effect on Ligand Sensitivity for Xenopus Tropicalis MC1R, MC3R, MC4R, and MC5R as a Result of Coexpression with Gallus Gallus MRAP1 in Chinese Hamster Ovary Cells

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Abstract

Some melanocortin receptors and the accessory protein MRAP1 have been found to interact in novel ways when co-expressed in the same cells following stimulation with either ACTH (1-24) or αMSH. These interactions have been seen for mammalian, bird, and some bony fish melanocortin receptors. Such analysis has not been done yet on the amphibian melanocortin receptors, MC1R, MC3R, MC4R, and MC5R. To this end, this study was done on the effects regarding ligand sensitivity for ACTH (1-24) and αMSH, when the MC1R, MC3R, MC4R, and MC5R paralogs of the amphibian, *Xenopus tropicalis* were expressed in Chinese Hamster Ovary cells either in the presence of absence of *Gallus gallus* (c) MRAP1. Based on previous studies on human MC1R, MC3R, MC4R, and MC5R, the expectation was that the sensitivity to stimulation by αMSH would be lower for all the *X. tropicalis* receptors. However, for every *X.* tropicalis receptor tested, co-expression with cMRAP1 had no negative or positive effect on sensitivity to stimulation by xtαMSH. In a similar manner co-expression of xtMC1R, MC3R, or MC5R with cMRAP1 had no effect, positive or negative, on stimulation with xtACTH(1-24). However, co-expression of xtMC4R with cMRAP1 did lower sensitivity to stimulation by xtACTH (1-24) in a statistically significant manner. To date, this type of negative interaction between an MC4R ortholog and an MRAP1 ortholog has not been reported for other vertebrates.

Analyzing the effect on ligand sensitivity for *Xenopus tropicalis* MC1R, MC3R, MC4R, and MC5R as a result of coexpression with *Gallus gallus* MRAP1 in Chinese Hamster ovary cells

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Abstract

Some melanocortin receptors and the accessory protein MRAP1 have been found to interact in novel ways when co-expressed in the same cells following stimulation with either ACTH (1-24) or αMSH. These interactions have been seen for mammalian, bird, and some bony fish melanocortin receptors. Such analysis has not been done yet on the amphibian melanocortin receptors, MC1R, MC3R, MC4R, and MC5R. To this end, this study was done on the effects regarding ligand sensitivity for ACTH (1-24) and αMSH, when the MC1R, MC3R, MC4R, and MC5R paralogs of the amphibian, *Xenopus tropicalis* were expressed in Chinese Hamster Ovary cells either in the presence of absence of *Gallus gallus* (c) MRAP1. Based on previous studies on human MC1R, MC3R, MC4R, and MC5R, the expectation was that the sensitivity to stimulation by αMSH would be lower for all the X. tropicalis receptors. However, for every *X. tropicalis* receptor tested, co-expression with cMRAP1 had no negative or positive effect on sensitivity to stimulation by xtαMSH. In a similar manner co-expression of xtMC1R, MC3R, or MC5R with cMRAP1 had no effect, positive or negative, on stimulation with xtACTH(1-24). However, co-expression of xtMC4R with cMRAP1 did lower sensitivity to stimulation by xtACTH (1-24) in a statistically significant manner. To date, this type of negative interaction between an MC4R ortholog and an MRAP1 ortholog has not been reported for other vertebrates.

1 INTRODUCTION

Melanocortin receptors are a family of G-protein coupled receptors (GPCR) that are only present in chordates. There are 5 receptors: melanocortin-1 receptor (MC1R), melanocortin-2 receptor (MC2R), melanocortin-3 receptor (MC3R), melanocortin-4 receptor (MC4R), and melanocortin-5 receptor (MC5R)[1]. Melanocortin receptors are activated by melanocortin related peptides such as adrenocorticotropin (ACTH), alpha-melanocyte stimulating hormone (αMSH), beta-melanocyte stimulating hormone (βMSH), and gamma-melanocyte stimulating hormone (γMSH). These peptides are derived from the precursor, proopiomelanocortin (POMC) that is made in the pituitary[2]. In mammals, the POMC gene is expressed in cells of the anterior pituitary. ACTH is a major melanocortin end product for these cells. However, the pituitary anterior lobe corticotrophs and the intermediate lobe melanotrophs that express POMC have αMSH, βMSH, and γMSH as major melanocortin end products.

In mammals, MC1R is involved in influencing pigmentation. MC2R is involved in the production of glucocorticoids from cells of the adrenal cortex. The MC3R and MC4R influence metabolic rate and feeding behaviors in the central nervous system. MC5R regulates secretions by the exocrine glands. Melanocortin receptors have also been identified with similar functions in the major groups of jawed vertebrates (i.e. cartilaginous fishes, bony fishes, amphibians, reptiles, birds, and mammals)[1]. This study focuses on the melanocortin receptors found in the genome of the amphibian *Xenopus tropicalis*. An analysis of the genome database of the *X. tropicalis* reveals all 5 melanocortin receptors are present in *X. tropicalis* genome[3].

Previous studies on the *X. tropicalis* MC2R indicate that it is comparable with the mammalian ortholog MC2R. MC2R requires co-expression with melanocortin receptor accessory protein 1 (MRAP1) for functional expression[4]. Like the mammalian MC2R ortholog, *X. tropicalis* MC2R can be activated with ACTH, but not by an MSH-sized melanocortin peptide[4]. However, in order for functional expression of *X. tropicalis* MC2R in

Chinese Hamster cells (CHO), the receptor must be co-expressed with mammalian MRAP1[4]or an MRAP ortholog from chicken (*Gallus gallus*)[5]. To date, an MRAP1 ortholog has not been detected in the genome database of *X. tropicalis*[3].

The absence of an MRAP1 ortholog can be explained by an incomplete genome project or the use of another accessory protein[6]. There are very few studies on vertebrates. Our understanding of the absence of an MRAP1 ortholog can be furthered by examining the effects of MRAP1 on *X. tropicalis* melanocortin receptors. The purpose of this study is to observe how the co-expression with a tetrapod MRAP1 affects the ligand sensitivity of *X. tropicalis* MC1R, MC3R, MC4R, MC5R. xtMC2R was excluded since its activation has been shown to be dependent on the expression of MRAP1[7]. An earlier study done on human MC1R, MC3R, MC4R, and MC5R co-expression with MRAP1[8] found that co-expression with MRAP1 lowered sensitivity to stimulation with [Nle4-D-Phe7]-alpha-melanocyte stimulating hormone, the synthetic analog for αMSH, for all 4 human receptors. However, this research did not examine the impacts of co-expression with ACTH. The operating assumptions of the research were that *X. tropicalis* MC1R, MC3R, MC4R, and MC5R (xtMC1R, xtMC3R, xtMC4R, and xtMC5R) when stimulated with a form of *X. tropicalis* αMSH, xtαMSH, in the presence of cMRAP1 would respond in a manner similar to what was seen in the human melanocortin study. In addition, it was assumed that co-expression with cMRAP1 would not affect receptors with stimulated with *X. tropicalis* ACTH (1-24).

2 METHODS

2.1 DNA Constructs

The cDNA sequences of xtMC1R, xtMC3R, xtMC4R, xtMC5R were obtained from *X. tropicalis* genome database[3]. The cDNA sequence of cMRAP1 was obtained from the *G. gallus* genome database[3]. Each cDNA was synthesized by GenScript (Piscataway, NJ) and inserted into a pcDNA3.1+ vector. The cAMP reporter gene construct CRE-Luciferase[9]was provided by Dr. Patricia Hinkle (University of Rochester, NY).

2.2 Tissue Culture

Chinese Hamster Ovary (CHO) cells (ATCC, Manassas, VA) were grown in Kaighn's Modification of Ham's F12K media (ATCC) supplemented with 10% fetal bovine serum, 100unit/ml penicillin, 100μg/ml streptomycin, 100μg/ml normocin, and maintained in a humidified incubator with 95% air and 5% CO^2 at 37°C. When CHO cells reached 80% confluence, the cells were split into subcultures using 0.05% trypsin/0.53mM EDTA.

2.3 ACTH Analog Peptides

X. tropicalis (xt) ACTH (1-24) and *X. tropicalis* αMSH (α-melanocyte-stimulating hormone) were purchased from New England Peptides (Gardiner, MA). For the cAMP-reporter gene assay, xtACTH (1-24) was used to stimulate transfected cells at concentrations ranging from 10^{-12} M to 10^{-6} M. xtαMSH was used at concentrations ranging from 10^{-12}M to 10^{-6}M.

2.4 cAMP-Reporter Gene Assay

For the cAMP-reporter gene assay[4], 3.0×106 CHO cells were transiently co-transfected with a xtMCR cDNA construct either alone or with *G. gallus* (c)MRAP1 cDNA construct, and the CRE-Luciferase cDNA construct (2μg each) using the Amaxa Cell Line Nucleofector II system (Lonza, Portsmouth, NH) utilizing the Solution T transfection kit and program U-23 as recommended by the company. After a 10-minute post-transfection recovery period, cells were then seeded in a white 96-well plate at a final density of 1×105 cells/well. 48 hours after transfection, cells were stimulated with concentrations of xtACTH (1-24) or xtαMSH diluted in serum-free CHO media for 4 hours at 37°C. Following the incubation period, the stimulating media was removed, and the luciferase substrate reagent, Bright GLO (Promega, Madison, WI), was applied to the wells for a 5-minute incubation period at room temperature. Luminescence was immediately measured using a Bio-Tek Synergy HTX plate reader (Winooski, VT). To determine the background levels of cAMP production, a set of transfected CHO cells were stimulated with serum free CHO media for the 4-hour incubation period, and the average background luminescence reading for these control wells was subtracted from the ligand-stimulated luminescence readings. The dose response curves for the stimulated cells were analyzed using the Michaelis-Menton equation to obtain EC_{50} values. All assays were done in triplicate. The data were plotted using the Kaleidograph software (www.synergy.com).

2.5 Statistical Analysis

Data points are expressed as the mean + standard error of the mean (n = 3). Statistical differences between the EC_{50} value of the xtMCR positive control and the xtMCR co-expressed with cMRAP1 were evaluated using one-way ANOVA followed by Tukey's multi-comparison test using GraphPad Prism 2 (GraphPad Software Inc, La Jolla, CA, USA) for equal variance. Significance was set at $P \leq 0.05$.

3 RESULTS

The results from the expression of *X. tropicalis* MC1R, MC3R, MC4R, and MC5R are depicted in Figure 1. The receptors were expressed in either the presence or absence of cMRAP1. The cells were then stimulated with xtACTH (1-24) or xtαMSH to determine if cMRAP1 influences sensitivity of the melanocortin receptors for stimulation by these hormones. All EC_{50} values can also be found in Table 1, and all P Values from One-Way ANOVA Analysis can be found in 2.

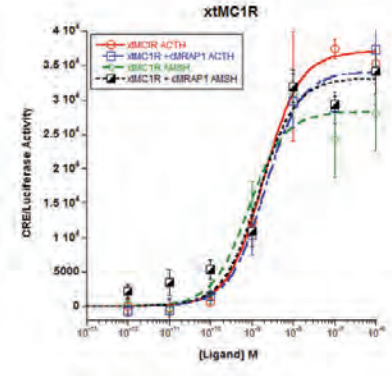

Figure 1. Expression of xtMC1R in the presence or absence of cMRAP1. As described in Methods, xtMC1R was expressed in CHO cells either in the presence or absence of cMRAP1, and transfected cells were stimulated in either xtACTH(1-24) or xtαMSH as described in METHODS. EC_{50} values appear in (Table 1), and the results of statistical analysis appear in (Table 2).

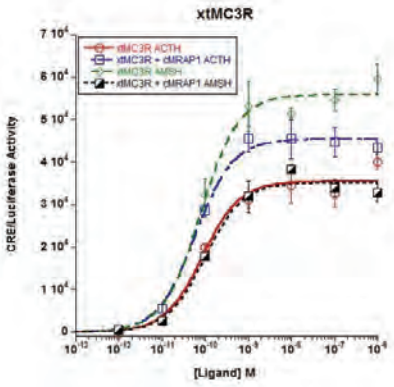

Figure 2. Expression of xtMC3R in the presence or absence of cMRAP1. As described in Methods, xtMC1R was expressed in CHO cells either in the presence or absence of cMRAP1, and transfected cells were stimulated in either xtACTH(1-24) or xtαMSH as described in METHODS. EC_{50} values appear in (Table 1), and the results of statistical analysis appear in (Table 2).

3.1 Coexpression of xtMC3R with cMRAP1

The cells expressed xtMC3R with or without cMRAP1 and were stimulated by xtACTH or xtαMSH (Figure 2). The EC_{50} values can be found in (Table 1). A One-Way ANOVA Analysis of variance did not find the results statistically significant for either hormone (Table 2).

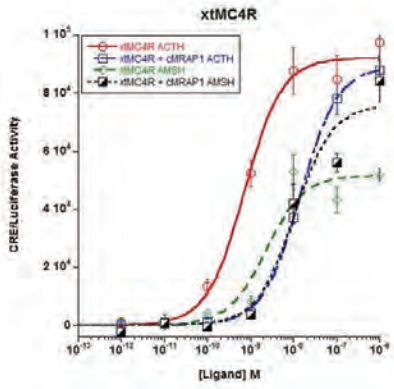

Figure 3. Expression of xtMC3R in the presence or absence of cMRAP1. As described in Methods, xtMC1R was expressed in CHO cells either in the presence or absence of cMRAP1, and transfected cells were stimulated in either xtACTH(1-24) or xtαMSH as described in METHODS. EC_{50} values appear in Table 1, and the results of statistical analysis appear in Table 2.

3.2 Coexpression of xtMC5R with cMRAP1

In Figure 4, the cells expressed xtMC5R with or without cMRAP1 and were stimulated by xtACTH or xtαMSH. The EC_{50} values can be found in Table 1. A One-Way ANOVA Analysis of variance did not find the results statistically significant for either hormone (Table 2).

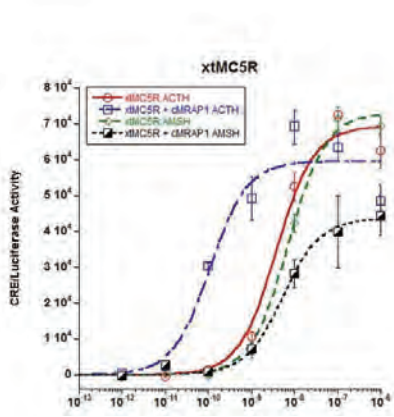

Figure 4. Expression of xtMC3R in the presence or absence of cMRAP1. As described in Methods, xtMC1R was expressed in CHO cells either in the presence or absence of cMRAP1, and transfected cells were stimulated in either xtACTH(1-24) or xtαMSH as described in METHODS. EC_{50} values appear in Table 1, and the results of statistical analysis appear in Table 2.

Table 1 EC_{50} values for xtMC1R, xtMC3R, xtMC4R, and xtMC5R with or without cMRAP1 and xtACTH or xtαMSH

Receptor	xtACTH or xtαMSH	Receptor with or without cMRAP1	EC_{50}Value (M)
xtMC1R	xtACTH	xtMC1R	$1.9x10^{-9}\pm2.6x10^{-10}$
		xtMC1R+cMRAP1	$2.1x10^{-9}\pm7.0x10^{-10}$
	xtαMSH	xtMC1R	$8.2x10^{-10}\pm3.3x10^{-10}$
		xtMC1R+cMRAP1	$1.5x10^{-9}\pm6.7x10^{-10}$
xtMC3R	xtACTH	xtMC1R	$8.6x10^{-11}\pm2.5x10^{-11}$
		xtMC1R+cMRAP1	$5.7x10^{-11}\pm8.4x10^{-12}$
	xtαMSH	xtMC1R	$7.4x10^{-11}\pm1.7x10^{-11}$
		xtMC1R+cMRAP1	$9.8x10^{-11}\pm2.2x10^{-11}$
xtMC4R	xtACTH	xtMC1R	$7.2x10^{-10}\pm1.3x10^{-10}$
		xtMC1R+cMRAP1	$1.3x10^{-8}\pm5.9x10^{-10}$
	xtαMSH	xtMC1R	$2.3x10^{-9}\pm4.4x10^{-9}$
		xtMC1R+cMRAP1	$1.0x10^{-8}\pm4.4x10^{-9}$
xtMC5R	xtACTH	xtMC1R	$3.6x10^{-10}\pm2.5x10^{-10}$
		xtMC1R+cMRAP1	$9.6x10^{-12}\pm8.8x10^{-12}$
	xtαMSH	xtMC1R	$2.8x10^{-9}\pm2.8x10^{-9}$
		xtMC1R+cMRAP1	$4.0x10^{-9}\pm3.3x10^{-9}$

Table 2 P Values from a One-Way ANOVA Analysis.

Receptor	Comparison	P Value
xtMC1R	MC1R vs MC1R+cMRAP1 stimulated with xtACTH(1-24)	0.85
	MC1R vs MC1R+cMRAP1 stimulated with xtαMSH	0.69
	MC1R alone stimulated with xtACTH(1-24) vs xtαMSH	0.99
xtMC3R	MC3R vs MC3R+cMRAP1 stimulated with xtαACTH (1-24)	0.76
	MC3R vs MC3R+cMRAP1 stimulated with xtαMSH	0.47
	MC3R alone stimulated with xtACTH(1-24) vs xtαMSH	0.99
xtMC4R	MC4R va MC4R+cMRAP1 stimulated with xtACTH(1-24)	0.006
	MC4R vs MC4R++cMRAP1 stimulated with xtαMSH	0.09
	MC4R alone stimulated with xtACTH(1-24) vs xtαMSH	0.94
xtMC5R	MC5R vs MC5R+cMRAP1 stimulated with xtACTH(1-24)	0.49
	MC5R vs MC5R+cMRAP1 stimulated with xtαMSH	0.99
	MC5R alone stimulated with xtACTH(1-24) vs xtαMSH	0.51

4 DISCUSSION

The purpose of this study was to see how co-expression with a tetrapod MRAP1 affects the ligand sensitivity of *X. tropicalis* MC1R, MC3R, MC4R, MC5R. An earlier study conducted on human MC1R, MC3R, MC4R, and MC5R co-expression with MRAP1[8] demonstrated that co-expression with MRAP1 lowered sensitivity to stimulation with NDM-MSH for all 4 human receptors. However, this research did not examine the effect of co-expression following stimulation with ACTH. It was hypothesized that stimulation of *X. tropicalis* MC1R, MC3R, MC4R, and MC5R with a form of xtαMSH in the presence of cMRAP1 would result in a response similar to that of human melanocortin study. We further hypothesized that co-expression with cMRAP1 would not affect receptors with xtACTH (1-24).

When mouse MRAP1 is co-expressed with human MC5R in CHO cells, the receptor is glycosylated but remains concentrated at the endoplasmic reticulum in the cell[7]. This research was corroborated by several other studies and demonstrated that MC2R activation is dependent on the co-expression of MRAP1. When MRAP1 is not co-expressed, MC2R remains trapped in the endoplasmic reticulum[7;10]. For this reason, MC2R was excluded from this study. However, unlike human MC5R, the activation of xtMC5R by ACTH (1-24) is not affected by co-expression with cMRAP1, and presumably there was no negative effect on the trafficking of xtMC5R to the plasma membrane(Figure 3).

In a different study, chicken melanocortin receptors (cMC2R, cMC3R, cMC4R, cMC5R) were transfected into CHO cells with chicken MRAP1 or MRAP2. αMSH was not used for stimulation due to the fact that avian pituitaries only have corticotropic cells and lack an intermediate lobe[11]. cMC2R again required the co-expression of cMRAP1[11]. cMC3R had no statistical change in sensitivity, while both MC4R and MC5R experienced an increase in sensitivity to ACTH with the co-expression of cMRAP1[11]. Studies on the co-expression of MRAP1 with melanocortin receptors have been primarily limited to humans, chickens, and mice. Since other studies on vertebrates have been limited, this study was done on the amphibian, *X. tropicalis* as a MRAP1 ortholog has not been found in its genome.

Our research showed no statistically significant shift for MC1R, MC3R, or MC5R sensitivity to ACTH. However, a one-way analysis of variance found that the comparison of MC4R vs. MC4R + cMRAP1 stimulated with xtACTH (1-24) to be statistically significant with a P Value of 0.006. Previous research is limited to studies on the interaction between human or mouse MC4R and human or mouse MRAP2. In a study on the interaction between human MC4R and human MRAP2, it was found that human MRAP2 negatively affected trafficking of the receptor to the plasma membrane and its activation with NDP-MSP[8]. Studies on mouse MC4R found that coexpression with MRAP2 increased sensitivity to mouse αMSH stimulation[12]. Hence, it would appear that co-expression of xtMC4R and MRAP1 may be an interaction that is unique to amphibians.

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6 EDITOR'S NOTES

This work was adapted from a senior thesis and has been condensed for publication. Contact DUURJ staff for the full publication.

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