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Use of chimeric melanocortin-2 and -4 receptors to identify regions responsible for ligand specificity and dependence on melanocortin 2 receptor accessory protein

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Abstract

The melanocortin 2 (MC_2) receptor differs from other melanocortin family members in its pharmacological profile and reliance on an accessory protein, MC_2 receptor accessory protein (MRAP), for surface expression and signal transduction. To identify features of the MC_2 receptor responsible for these characteristics, we created chimeras between MC_2 and MC_4 receptors and expressed these in CHO cells, where MRAP is essential for trafficking and signaling by MC_2 but not MC_4 receptors. Replacing the first transmembrane segment of the MC_2 receptor with the corresponding region from the MC_4 receptor allowed some surface expression in the absence of an accessory protein, while ACTH-induced cAMP production remained entirely MRAP-dependent. On the other hand, replacing the last two transmembrane domains, third extracellular loop and C-terminal tail of the MC_4 receptor with the corresponding regions from the MC_2 receptor resulted in MRAP-dependent signaling. Surprisingly, replacing the second and third transmembrane domains and the intervening first extracellular loop of MC_2 receptors with MC_4 sequences generated a chimera (2C2) that responded to both adrenocorticotropic hormone (ACTH) and to the potent MSH analog 4-norleucine-7-D-phenylalanine-α-melanocyte stimulating hormone (NDP-α-MSH), which does not activate native MC_2 receptors. The 2C2 chimeric receptor was able to respond to NDP-α-MSH without MRAP, but MRAP shifted the EC_{50} value for NDP-α-MSH to the left and caused constitutive activity. These results identify the first transmembrane domain as important for surface expression and regions from the second through third transmembrane segments of the MC_2 receptor as important for MRAP dependent-signal transduction and ligand specificity.

Index words

Accessory protein; ACTH; G protein-coupled receptor; melanocortin receptor; MRAP; MSH

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1. Introduction

The melanocortin 2 (MC_2[1]) receptor is an essential G protein-coupled receptor that plays a crucial physiological role, mediating responses of the adrenal cortex to adrenocorticotropic hormone (ACTH). The MC_2 receptor differs from the four other members of the melanocortin receptor family in several respects(Cone, 2006). First, the MC_2 receptor is not functional unless expressed together with an accessory protein (melanocortin-2 receptor accessory protein, or MRAP)(Hinkle and Sebag, 2009;Metherell et al., 2005;Roy et al., 2007;Sebag and Hinkle, 2007;Webb and Clark, 2010). In humans, inactivating mutations in either the MC_2 receptor or MRAP cause severe ACTH resistance and a deficiency of adrenal glucocorticoids, which can be fatal if left untreated(Chan et al., 2008). In vitro, the MC_2 receptor cannot undergo maturation and trafficking to the cell surface or signal unless it is expressed in a cell that makes MRAP(Metherell et al., 2005;Sebag and Hinkle, 2007). Other melanocortin receptors do not require MRAP for surface localization or signaling(Chan et al., 2009;Sebag and Hinkle, 2007). The melanocortin-1, -3, -4 and -5 receptors respond to the endogenous melanocortin agonists α-, β-, and γ-melanocyte stimulating hormone (MSH) and ACTH, but the MC_2 receptor is alone in not responding to any of the MSH peptides and is only stimulated by ACTH(Cone, 2006).

The MRAP protein has a single transmembrane domain and forms a unique anti-parallel homodimer(Sebag and Hinkle; 2007; 2009a; b). The amino-terminal and transmembrane domains are highly conserved, whereas the carboxyl-termini differ among species, differ depending on splicing, and can be deleted without loss of function(Roy et al., 2007; Sebag and Hinkle, 2007; 2009b; Webb et al., 2009). MRAP has two distinct functions, one to promote MC_2 receptor folding, maturation and localization on the plasma membrane, and the other to promote MC_2 receptor-mediated cAMP signaling(Sebag and Hinkle, 2009b). MRAPs with mutations in a highly conserved, tyrosine-rich region of the amino-terminus support cell surface localization of the MC_2 receptor but not high affinity ACTH binding or signaling(Sebag and Hinkle, 2009b; Webb et al., 2009). The product of a second gene, MRAP2, also allows MC_2 receptor trafficking but not high affinity agonist binding or signal transduction(Roy et al., 2010; Sebag and Hinkle; 2009a). The MC_2 receptor and MRAP co-precipitate and have been shown to interact closely by bimolecular fluorescence complementation performed in living cells(Chan et al., 2009; Sebag and Hinkle; 2009a).

It is not known what features of the MC_2 receptor are responsible for its dependence on an accessory protein or its unique pharmacological profile. In this study, we created chimeras between MC_2 and MC_4 receptors and expressed these in CHO cells, where MRAP is essential for trafficking and signaling by MC_2 but not MC_4 receptors. We use these chimeric receptors to identify regions of the MC_2 receptor responsible for MRAP dependence and peptide ligand specificity for cAMP-dependent signaling.

2. Materials and methods

2.1 Plasmid construction

Mutations were introduced into plasmids encoding human MC_2 and MC_4 receptors by standard PCR methods to form unique restriction sites that did not result in changes in amino acid sequence whenever possible, as shown schematically in Supplemental Fig. 1. Restriction fragments from one receptor were generated by enzyme digestion, gel purified, and ligated into the corresponding digested plasmid containing the other receptor.

[1]Abbreviations: ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine 3',5'-monophosphate; CRE, cAMP response element; ECL, extracellular loop; ER, endoplasmic reticulum; ICL, intracellular loop; MSH, melanocyte stimulating hormone; NDP-α-MSH, 4-norleucine-7-D-phenylalanine-α-MSH; TM, transmembrane domain.

Quikchange mutagenesis (Stratagene) was used to introduce a double HA epitope tag following the initiator methionine. Stitching PCR and Quikchange mutagenesis were used to generate chimeras with smaller changes than those obtained by combining restriction fragments (2C1a,b,c and 2C2a,b,c). All plasmids were sequenced to verify that the constructs were free of undesired PCR-induced base pair changes. Several of the MC_2-like chimeric receptors contained an extra amino-terminal Met followed by a 2x-HA epitope, which did not alter expression or signaling patterns. Construction of a plasmid encoding mouse MRAP carrying a single N-terminal V5 tag and a triple C-terminal Flag epitope (referred to as MRAP) has been described(Sebag and Hinkle, 2007). MRAP constructs were in pCI-Neo and receptor and control constructs in pcDNA3.

2.2 Cell growth and transfection

CHO cells were grown in DMEM/F12 medium with 5% fetal calf serum and transfected 24 to 48 h before experiments with FugeneHD, which was used as recommended by the manufacturer. Cells were transfected with plasmids encoding receptors and either MRAP or RAMP3 or GFP, which served as controls and behaved equivalently in expression and cAMP studies; GFP was used as a control in luciferase reporter assays.

2.3 Characterization of receptor expression

Cells were plated in 24 well dishes and transfected the next day with 250 ng total DNA/well (100–200 ng of receptor DNA and 50–150 ng MRAP). The next day, the density of receptors on the plasma membrane was measured by an ELISA that has been described(Jones et al., 2007). In brief, live cells were incubated with mouse monoclonal anti-HA antibody diluted 1:5000 for 30–60 min and then washed extensively and fixed with 2% paraformaldehyde. Cells were then washed, incubated with horseradish peroxidase-labeled secondary antibody, washed again and incubated with TMB substrate. Background signal was determined in mock-transfected cells and subtracted.

For fluorescence microscopy, cells were grown on glass coverslips and transfected with receptor and GFP and with either MRAP or control plasmid. Live cells were incubated with 1:1000 anti-HA antibody in complete medium for 30 min at 37°C, washed, fixed in paraformaldehyde, and incubated with 1:250 rhodamine-labeled goat anti-mouse serum for 1 h at room temperature. Coverslips were washed and then mounted in Prolong Gold with DAPI to stain nuclei. GFP labeling allowed the identification of successfully transfected cells. Images were obtained with a 100×/1.3 MA objective using standard filter sets, captured with a CoolSnap ES camera and analyzed using Metamorph software from Universal Imaging. Exposure times and image handling were identical for images of surface receptor.

2.4 Characterization of receptor responses

To measure cAMP, cells were incubated for 30 min with 0.1 mM isobutylmethylxanthine and vehicle, 1 μM ACTH or NDP-α-MSH, as noted, in DMEM/F12 with 0.1% BSA. The concentration of cAMP was then quantified using the Lance time-resolved FRET assay from Perkin Elmer as recommended. In luciferase reporter assays, cells were plated on 96-well dishes and transfected the next day with 40 ng/well total DNA. Transfections typically were performed using 13.3 ng/well of a cAMP-dependent luciferase reporter containing multiple copies of the rat insulin promoter(Chepurny and Holz, 2007), generously provided by Dr. George Holz (Syracuse, NY), 21.3 ng/well receptor DNA and 5.3 ng/well MRAP or GFP DNA. After 24 h, medium was replaced with 40 μl fresh DMEM/F12 containing 0.1% BSA with vehicle, agonists or 20 μM forskolin. Incubation was continued for 5 h, when medium was aspirated and luciferase substrate, One Step Luciferase Assay Reagent (Nanolight Technologies), was added. Luminescence was quantified on either a Perkin Elmer Victor or

BioTek platereader. ACTH(1–24) and NDP-α-MSH were purchased from Phoenix Biochemicals.

2.5 Data analysis

Data points for receptor expression represent the mean and standard error of values obtained in multiple experiments in which each assay point was performed in duplicate or triplicate. Either MC_2 or MC_4 receptor was included in every experiment to provide reference values, and data are normalized to the values obtained with the parental receptors as described. Data points for receptor signaling represent the mean and range or standard error of values obtained in a representative of at least two experiments in which each assay point was performed in duplicate (Fig. 2B and 3B) or triplicate. EC_{50} values were determined using PRISM software. Differences in pairs of values (with or without MRAP) were analyzed by Student's two-tailed t-test. Differences in groups of three or more were evaluated by ANOVA with Tukey's analysis. Transmembrane segments of the MC_2 receptor were drawn based on predictions in TMHMM (Viklund and Elofsson, 2004) and alignment of MC_2 and MC_4 receptors as predicted by BLAST.

3. Results

3.1 Expression and signaling of chimeric receptors

We prepared chimeric receptors in which segments of the human MC_2 receptor, which relies on the accessory protein MRAP for trafficking and signaling, were replaced with corresponding sequences from the human MC_4 receptor, which is able to function without MRAP. We tagged all receptors with the HA epitope at the amino-terminus to facilitate quantification of receptor on the plasma membrane. Receptors were transiently expressed in CHO cells together with MRAP or either RAMP3 (an accessory protein for several class B receptors that does not affect melanocortin receptors)(Parameswaran and Spielman, 2006) or GFP as controls.

To measure receptors on the plasma membrane, we used a protocol that has been carefully validated previously(Jones et al., 2007; Sebag and Hinkle, 2007). Intact cells were incubated with monoclonal anti-HA antibodies, washed to remove free antibody, and then fixed and signal quantified by standard ELISA protocols. To measure receptor signaling, we either quantified cAMP directly or used a reporter assay. In the direct assay, cells were incubated with or without ACTH or NDP-α-MSH in the presence of a phosphodiesterase inhibitor for 30 min when the cAMP concentration was determined. In the reporter assay, cells were transfected with cDNA encoding luciferase driven by multiple copies of a cAMP-dependent cAMP response element (CRE) from the rat insulin promoter (CRE-luciferase) (Chepurny and Holz, 2007) together with receptors and MRAP, then incubated with or without agonist or forskolin for 5 h, when luciferase activity was measured. Forskolin stimulates adenylyl cyclase directly. The reporter assay is more sensitive and was useful in studying chimeric receptors with weak signaling. In cells expressing MC_2 receptor and MRAP, the EC_{50} for ACTH was approximately 10^{-10} M in the CRE-luciferase assay and over 20-fold higher, 2.4×10^{-9} M, in the direct cAMP assay, highlighting the difference in sensitivity.

The MC_2 receptor was expressed poorly on the plasma membrane in the absence of MRAP and strongly in the presence of the accessory protein (Fig. 1A). ACTH-dependent cAMP generation by the MC_2 receptor was highly dependent on MRAP (Fig. 1B). In contrast, MRAP decreased surface expression of the MC_4 receptor with little effect on cAMP response. The ability of MRAP to inhibit MC_4 receptor expression was also observed by Chan et al.(Chan et al., 2009). In our experiments, MRAP did not have a significant effect on MC_4 receptor signaling. To determine whether the same concentrations of MRAP were

required to affect MC_2 and MC_4 receptors, we transfected cells with different amounts of MRAP cDNA and measured receptor expression (Fig. 1C). MRAP increased MC_2 receptor on the cell membrane and inhibited MC_4 receptor trafficking at the same concentrations. Changing the ratio of MRAP to MC_2 receptor did not change the EC_{50} for ACTH response (data not shown).

Receptor constructs are shown schematically in the figures, and sequence details are provided in Supplemental Fig. 1. Signaling by chimeric receptors was tested in both direct cAMP assays (shown in Supplemental Fig. 2) and in CRE-luciferase reporter assays (shown in Figs. 2 and 3 for receptors that are primarily MC_2 or MC_4, respectively). To be sure that all chimeric receptors would be activated regardless of ligand specificity, we challenged cells with a combination of 1 μM ACTH and 1 μM NDP-α-MSH in the reporter assays.

In general, MRAP promoted the trafficking of the chimeras that are predominantly MC_2 and reduced cell surface expression of those that are predominantly MC_4. A few of the chimeric receptors were not expressed well in either case. The MC_2-like chimeric receptors either required MRAP for signaling or failed to respond to ACTH, with the exception of chimera 2C5 (Fig. 2). The MC_4-like receptors that were capable of signaling did not require MRAP, with the exception of the 4C4 receptor. MRAP enhanced signaling by the 4C4 chimera, which contains the last two transmembrane domains, third extracellular loop (ECL3) and C-tail of the MC_2 receptor. MRAP inhibited surface expression of the 4C4 chimera and the MC_4 receptor parent. The converse receptor, 2C4, with a C-terminus from the MC_4 receptor, did not signal. A number of the chimeric receptors were present at high levels on the plasma membrane but failed to give any cAMP response (most notably 2C1 and 4C2), whereas the 2C2 receptor expressed at low levels but signaled strongly.

One clear finding was the presence of significant levels of the 2C1 chimera on the cell surface in the absence of MRAP. The 2C1 receptor has the N-terminus, first transmembrane domain (TM1) and first intracellular loop (ICL1) from the MC_4 receptor. To verify the ELISA result, we expressed the parent MC_2 receptor and the 2C1 chimera with and without MRAP and stained for surface receptor using fluorescence microscopy. As shown in Fig. 4, the 2C1 receptor was localized on the plasma membrane with or without MRAP. In contrast, the MC_2 receptor was visible only in cells co-expressing MRAP. We narrowed the region responsible for MRAP-independent trafficking by generating mutants containing only TM1, TM1 and ICL-1, or only ICL-1 from the MC_4 receptor. Replacing TM1 of the MC_2 receptor with the corresponding transmembrane segment from MC_4 was sufficient to allow the receptor to undergo trafficking to the cell surface even in the absence of MRAP (Fig. 5A, 2C1b). Despite substantial surface expression, the 2C1 chimera gave essentially no cAMP or CRE-luciferase response with or without the accessory protein (Supplemental Fig. 2, Figs. 2B and 5B). Signaling results were remarkably different for the derivatives of 2C1. Simply replacing the extracellular N-terminal region of the 2C1 receptor with the native MC_2 receptor sequence (chimera 2C1a) restored strong signaling. The receptor containing only TM1 from the MC_4 receptor (chimera 2C1b) gave a very strong ACTH response that was highly dependent on MRAP, as did receptor 2C1c with only ICL1 from the MC_4 receptor (Fig. 5B).

These results raise the possibility that N-terminal regions of the MC_2 receptor are responsible for the requirement for MRAP for proper trafficking. We attempted to learn whether these domains of the MC_2 receptor were sufficient to confer MRAP dependence to the MC_4 receptor by studying the converse of the 2C1 receptor, 4C1 (Fig. 3). The 4C1 receptor was expressed very poorly on the plasma membrane and gave a small but significant response in the CRE-luciferase system that was the same with or without MRAP. Signaling by the 4C1 chimera was too weak to detect in cAMP assay (Supplemental Fig. 2).

3.2 Ligand specificity of chimeric receptors

The 2C2 chimera, which has TMs 2 and 3 and the connecting first extracellular loop (ECL-1) from the MC_4 receptor, showed low, MRAP-enhanced surface expression but striking agonist responses in both the CRE-luciferase (Fig. 2) and direct cAMP assays (Supplemental Fig. 2). The 2C2 receptor also gave a significant response in the sensitive reporter assay in the absence of MRAP (Fig. 2). We also tested the ability the chimeric receptors to respond to ACTH or NDP-α-MSH individually. Unexpectedly, we found that the 2C2 chimera responded to NDP-α-MSH as well as ACTH and displayed substantial constitutive activity in the CRE-luciferase assay (Fig. 6). These properties resemble those of the MC_4 receptor.

Complete concentration-response curves for ACTH and NDP-α-MSH are shown in Fig. 7. Cells transfected with the 2C2 chimera and MRAP responded to both ACTH (EC_{50} = 37 nM) and NDP-α-MSH (EC_{50} = 7.2 nM). The MC_4 receptor is much less sensitive to ACTH than the MC_2 receptor. Likewise, the 2C2 chimera was 380-fold less sensitive to ACTH than the MC_2 receptor (EC_{50} = 37 vs. 0.097 nM). The 2C2 receptor was not as sensitive to NDP-α-MSH as the MC_4 receptor, which has an EC_{50} of 0.011 nM for NDP-α-MSH and an EC_{50} of 1.3 nM for ACTH in the CRE-luciferase assay. The substantial constitutive activity of 2C2 was entirely MRAP-dependent.

When it was expressed without MRAP, the 2C2 receptor responded to NDP-α-MSH but the potency was relatively low (EC_{50} = 57 nM). The maximal responses of the 2C2 receptor to NDP-α-MSH were approximately the same with or without the accessory protein. On the other hand, ACTH elicited no significant response via the 2C2 receptor expressed without MRAP.

To dissect the basis for the unexpectedly strong signaling of the 2C2 chimera, we engineered receptors that contained either ECL1 from the MC_4 receptor (2C2a), ECL1 plus TM2 (2C2b), or ECL1 plus TM3 (2C2c). Two of these receptors trafficked well to the cell surface with MRAP but displayed very low signaling (Fig. 7), while the third did not express or signal.

The 2C3 chimeric receptor, which has TMs 4 and 5 and the connecting ICL-2 loop from the MC_4 receptor, also expressed and signaled well (Fig. 2 and Supplemental Fig. 2). The 2C3 receptor resembled the MC_2 receptor, requiring MRAP for trafficking and signaling and responding to low concentrations of ACTH (EC_{50} = 0.59 nM) but not to NDP-α-MSH (Fig. 7). The 2C3 receptor exhibited weak constitutive activity.

4. Discussion

It is clear that MRAP and MC_2 receptors interact closely from the time they are first synthesized in the endoplasmic reticulum (ER), but it is not known whether the proteins bind one another directly or whether MRAP makes contact with ACTH. A key question is why MRAP, which co-precipitates with all five melanocortin receptors in cell culture model systems, is only essential for trafficking and signaling by MC_2 receptors. Using chimeric receptors, we have identified some of the features of the MC_2 receptor important for MRAP-dependent trafficking and signaling.

4.1 MC_2 receptor trafficking

In the absence of MRAP, most MC_2 receptor is trapped in the ER. Several models for the role of MRAP can be envisioned. For example, MC_2 receptor may have an ER retention signal that is masked by MRAP. A number of motifs that cause ER retention or ER retrieval have been identified on the cytoplasmic side of membrane proteins(Ma et al., 2001;

Margeta-Mitrovic et al., 2000; Zerangue et al., 1999). The MC_2 receptor has one potential ER retention signal in its cytoplasmic tail, a Lys-Lys pair absent in other melanocortin receptors, but mutating the Lys-Lys to Ala-Ala does not alter the requirement for MRAP (data not shown). Another possibility is that MRAP exposes an ER export signal, but the motifs known to promote exit of membrane proteins from the ER (Ma et al., 2001; Margeta-Mitrovic et al., 2000; Zerangue et al., 1999) are absent from the MC_2 receptor. Perhaps the most likely explanation is that the MC_2 receptor cannot fold correctly unless it is bound to MRAP. Without MRAP, MC_2 receptors may be unable pass the rigorous quality control checkpoints found in the ER, resulting in their eventual degradation(Petaja-Repo et al., 2001).

Substituting TM1 from the MC_4 receptor into MC_2 resulted in some surface expression without MRAP, suggesting that the first membrane helix may be important for MRAP dependent trafficking; however, MRAP still increased surface expression and was essential for signal transduction. Sequence differences in the first transmembrane segments of MC_2 and MC_4 receptors occur in the outer half, a region that is not completely conserved even among mammalian MC_2 receptors. Replacing the N-terminus through ICL1 of MC_4 receptors with corresponding regions from MC_2 impaired surface localization, but MRAP did not rescue expression (Fig. 3). Chen et al. (Chen et al., 2007b) made cassette substitutions, introducing TMs 2, 3, 4, 5 and 6 of the MC_2 receptor into the MC_4 receptor. None of the individual cassette substitutions had a significant effect on surface expression of the MC_4 receptor, ruling out models in which a single helix, other than possibly TM1 or TM7, imposes MRAP-dependence. Fridmanis and coworkers characterized membrane localization, peptide binding and signaling by a large series of MC_2/MC_4 receptor chimeras but did not test the effects of MRAP (Fridmanis et al., 2010). They concluded that the extracellular N-terminus of MC_2 receptors is important for receptor trafficking and that TMs 3 and 4 of the MC_2 receptor comprise an ER retention signal that can be transferred to MC_4. We did not study exactly the same chimeras but did find that introducing TMs 2–3 or 4–5 of MC_2 receptor had little effect on MC_4 receptor surface expression.

4.2 Constitutive activity of melanocortin receptors

Constitutive activity of MC_4 receptors is readily detectable in vitro and important for maintaining normal energy balance in vivo, and certain mutations in the extracellular N-terminal domain of the MC_4 receptor cause a loss of constitutive activity and an obesity phenotype(Srinivasan et al., 2004). In contrast, MC_2 receptors do not display constitutive activity. Our finding that chimeras 2C2 and 2C3 are constitutively active, i.e. they stimulate cAMP-dependent pathways in the absence of agonist (Fig. 2), suggests a possible additional role of TMs 2 through 4 in the constitutive activity of melanocortin receptors.

4.3 MC_2 receptor signaling

The effects of MRAP on signaling by chimeric receptors are summarized in Fig. 8. Although only cAMP-dependent responses were monitored in these experiments, we have previously shown that MRAP controls the affinity of the MC_2 receptors for ACTH(Sebag and Hinkle, 2007), making it likely that all signal pathways would be affected similarly by domain swaps. The carboxyl-terminus of the MC_2 receptor (TM6 through the C-tail) may be important for MRAP-stimulated signaling, because splicing the C-terminus of the MC_2 receptor onto MC_4 receptors rendered the chimeras (4C4, 2C6) MRAP-dependent for signaling; the 2C5 chimera did not follow this pattern, however. Replacing TMs 2 through 3 of the MC_2 receptor with corresponding sequences from MC_4 allowed for some MRAP-independent signaling (2C2, 2C5), pointing to the importance of this region as well.

Fridmanis and colleagues have put forth a model in which two MRAP molecules interact with each molecule of MC_2 receptor(Fridmanis et al., 2010). In their scheme, one MRAP molecule contacts TMs 4 and 5, which are important for binding the four basic amino acids in ACTH. Our data do not support this model, because replacing TMs 4 and 5 of the MC_2 receptor with corresponding regions from MC_4 (chimera 2C3) did not prevent MRAP-dependence and replacing TMs 4 and 5 of the MC_4 receptor (chimera 4C3) did not impose an MRAP requirement for signaling.

4.4 Ligand specificity of MC_2 receptors

The MC_2 receptor is activated by subnanomolar concentrations of ACTH but not by even micromolar concentrations of MSH. ACTH and MSH share the first 13 amino acids, including the HisPheArgTrp core sequence found in natural melanocortin agonists. The LysLysArgArg sequence in positions 15–18 of ACTH is also important for activity. Three acidic residues at the top of TM3 of the MC_2 receptor are critical for binding, two conserved among melanocortin receptors (Asp103 and Asp107) and one unique to MC_2 (Asp104) (Chen et al., 2007a). The 2C2 chimera, which has TMs 2 and 3 and ECL1 from the MC_4 receptor, was able to respond not only to ACTH but also to NDP-α-MSH; MRAP was necessary for the ACTH response but 2C2 was able to respond to high concentrations of NDP-α-MSH without any accessory protein. The converse chimera, an MC_4 receptor with TMs 2 through 3 from MC_2, expressed well on the plasma membrane but did not signal with ACTH or MSH. The results are consistent with the idea that the outer region of TM3 dictates peptide specificity as well as ACTH affinity.

4.5 General observations and conclusions

Mutations in human MC_2 receptors are the most common cause of familial glucocorticoid deficiency, which is characterized by ACTH resistance, and most of the missense mutations that give rise to this disease interfere with receptor trafficking(Chung et al., 2008). Although any problem with protein folding could result in ER retention, these mutant receptors may fail to reach the plasma membrane or signal effectively because they cannot interact appropriately with MRAP. The disease-causing mutations in the human MC_2 receptor map to many different regions, but are particularly concentrated in the second intracellular loop. Because ICL2 is highly conserved, our chimera studies would not be expected to reveal the importance of this region. None of the known mutations is found in the TM1 region that appeared to be important for MRAP-dependent trafficking. Mutations in acidic residues at the top (outside) of TM3 prevent ACTH signaling and cause familial glucocorticoid deficiency (Chen et al., 2007a; Chung et al., 2008), and these acidic residues may be important for effects on ligand specificity and affinity observed in chimeras studied here.

It is a common practice to normalize signaling by transfected G protein-coupled receptors to surface expression, which is typically measured by FACS, miscroscopy or ELISA using an antibody to an N-terminal eptiope. Here we found a very poor correlation between surface receptor levels and agonist responses for several chimeras. In fact, some receptors (2C2 without MRAP, 2C5 and 4C4 with MRAP) gave barely detectable surface expression but robust signaling. This may reflect an unrecognized shortcoming in the surface receptor assay, a lack of desensitization that exaggerates responses in the luciferase reporter assay, or highly efficient signaling by the chimeric receptors. Regardless of the reason, these findings suggest that caution is warranted in normalizing signaling data to receptor levels.

An additional question is whether a chimeric receptor that does not localize to the cell surface or signal is useful in identifying critical regions or simply uninformative. We encountered one chimera (2C2) that signaled very well with substitution of two TMs and the intervening extracellular loop, whereas chimeras containing smaller segments from the same

region were completely inactive. Another chimera (2C1) did not signal at with substitutions of the N-terminus, TM1 and ICL1, whereas chimeras containing smaller fragments covering this entire region were fully active. Any conclusion about what receptor segments are important based on the lack of activity of the 2C1 chimera would be misleading. Our work results point out the difficulties in interpretating the failure of an inactive chimera to express or signal.

In summary, three laboratories have examined the expression and signal transduction of MC_2 and MC_4 receptor chimeras (Chen et al., 2007b; Fridmanis et al., 2010). This is the first study to include MRAP. The activity profiles obtained do not pinpoint specific sequences responsible for the dependence of MC_2 receptor function on MRAP. Instead, experiments with chimeric receptors support the concept that MRAP, either directly or indirectly, affects the conformation of multiple receptor regions in order to promote receptor movement from the ER to the plasma membrane and G protein-mediated signal transduction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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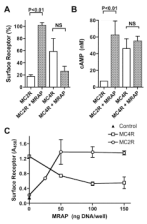

Fig. 1.

Effects of MRAP on MC_2 and MC_4 receptors. Cells were transfected with plasmids encoding MC_2 receptor (MC_2R), MC_4 receptor (MC_4R) and either RAMP3 (Control) or MRAP. (A and C) Surface expression of receptors was measured by ELISA as described under *Methods*. Results in (A) are expressed as a percent of the value with MC_2 receptor and MRAP. (B) Cells were incubated with 1 μM ACTH in buffer containing 0.1 mM isobutylmethylxanthine for 30 min when cAMP was measured. cAMP levels in mock transfected cells have been subtracted. (C) Cells were transfected with 100 ng/well of receptor DNA and 0 to 150 ng/well of MRAP or control cDNA. Points show average from (A) 7 or (B) 2 experiments, each performed in duplicate or triplicate. NS=non-significant.

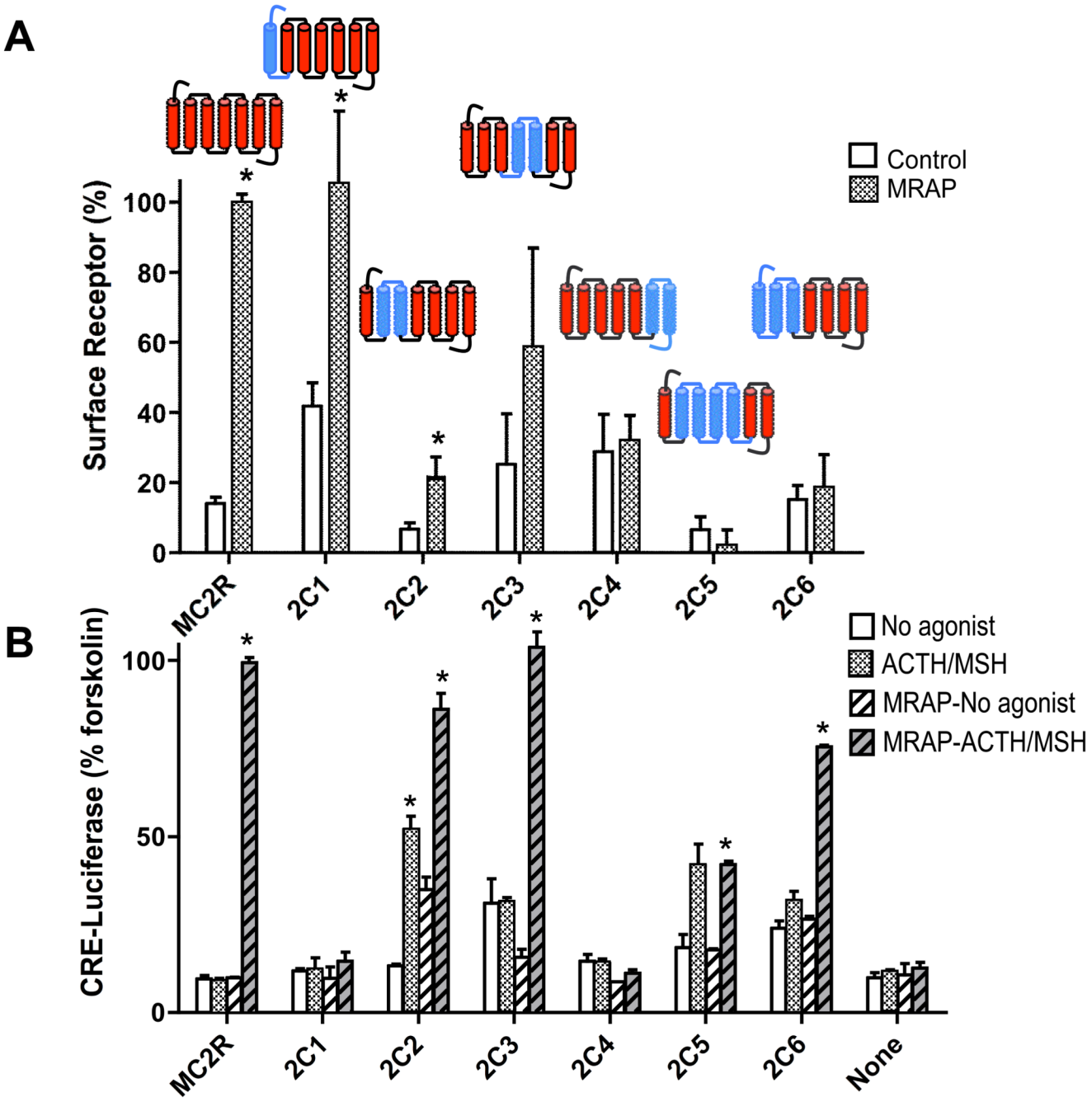

Fig. 2.

Effects of MRAP on MC_2-like receptor chimeras. (A) Cells were transfected with plasmids encoding receptors and either RAMP3 (Control) or MRAP. Surface receptor is expressed percent of the values obtained with MC_2 receptor and MRAP. Points are averaged from 3 to 23 experiments. (B) Cells were transfected with plasmids encoding CRE-luciferase, receptors and either control plasmid or MRAP. The next day cells were incubated with vehicle, 20 μM forskolin or 1 μM ACTH plus 1 μM NDP-α-MSH for 5 h when luciferase activity was measured. Data are expressed relative to the response to forskolin. *P<0.05 vs no MRAP.

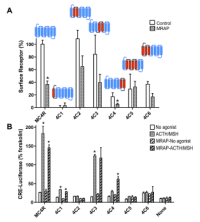

Fig. 3.

Effects of MRAP on MC_4-like receptor chimeras. (A) Cells were transfected with plasmids encoding receptors and either RAMP3 (Control) or MRAP. Surface receptor is expressed percent of the values obtained with MC_4 receptor without MRAP. Points are averaged from 3 to 16 experiments. (B) Cells were transfected with plasmids encoding CRE-luciferase, receptors and either control plasmid or MRAP. The next day cells were incubated with vehicle, 20 μM forskolin or 1 μM ACTH plus 1 μM NDP-α-MSH for 5 h when luciferase activity was measured. Data are expressed relative to the response to forskolin. *P<0.05 vs no MRAP.

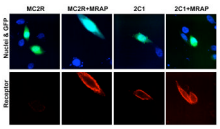

Fig. 4.

Surface expression of MC_2 and 2C1 receptors. Cells were transfected with plasmids encoding GFP and either MC_2 receptor or 2C1 chimeric receptor together with either control plasmid or MRAP. Live cells were incubated with monoclonal anti-HA antibody and then fixed and stained with rhodamine-labeled secondary antibody. Top panels show overlays of staining for nuclei in blue and GFP, marking successfully transfected cells, in green. Lower panels show HA-tagged surface receptors in red.

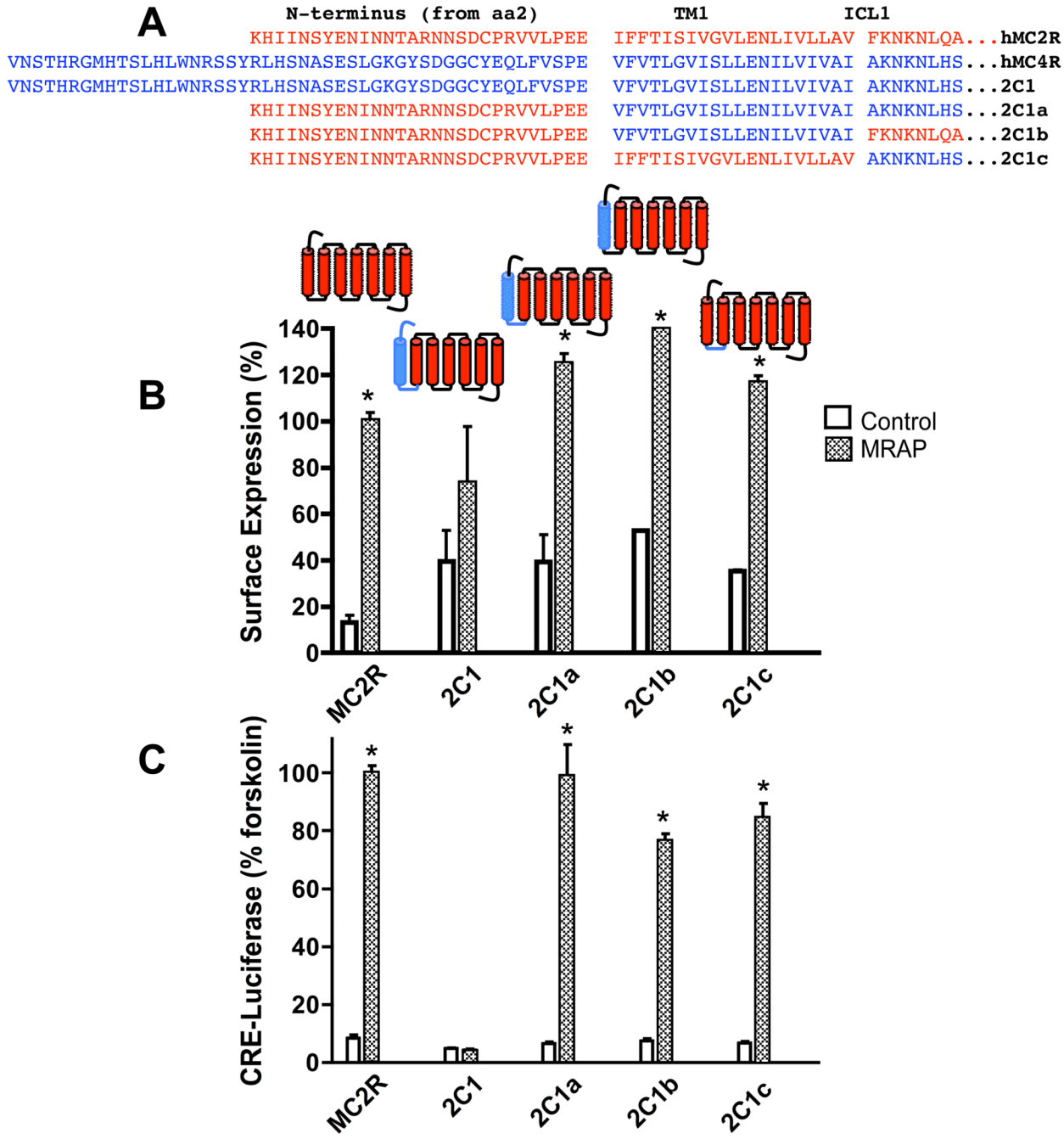

Fig. 5.

Importance of amino-terminal regions of MC_2 receptor. (A) Amino acid sequences of receptor constructs, beginning at amino acid 2 after the HA tag (MYPYDVPDYAYPYDVPDYA). MC_2 receptor sequences are shown in red, MC_4 in blue. (B) Cells were transfected with plasmids encoding receptors and either MRAP or control plasmid. Surface receptor expression is normalized to values obtained with MC_2 receptor plus MRAP. (C) Cells were transfected with CRE-luciferase, receptors and MRAP or control plasmid. The next day cells were incubated with 20 μM forskolin or 100 nM ACTH for 5 h when luciferase activity was measured. Data are expressed as a percent of the response to forskolin. *P < 0.01.

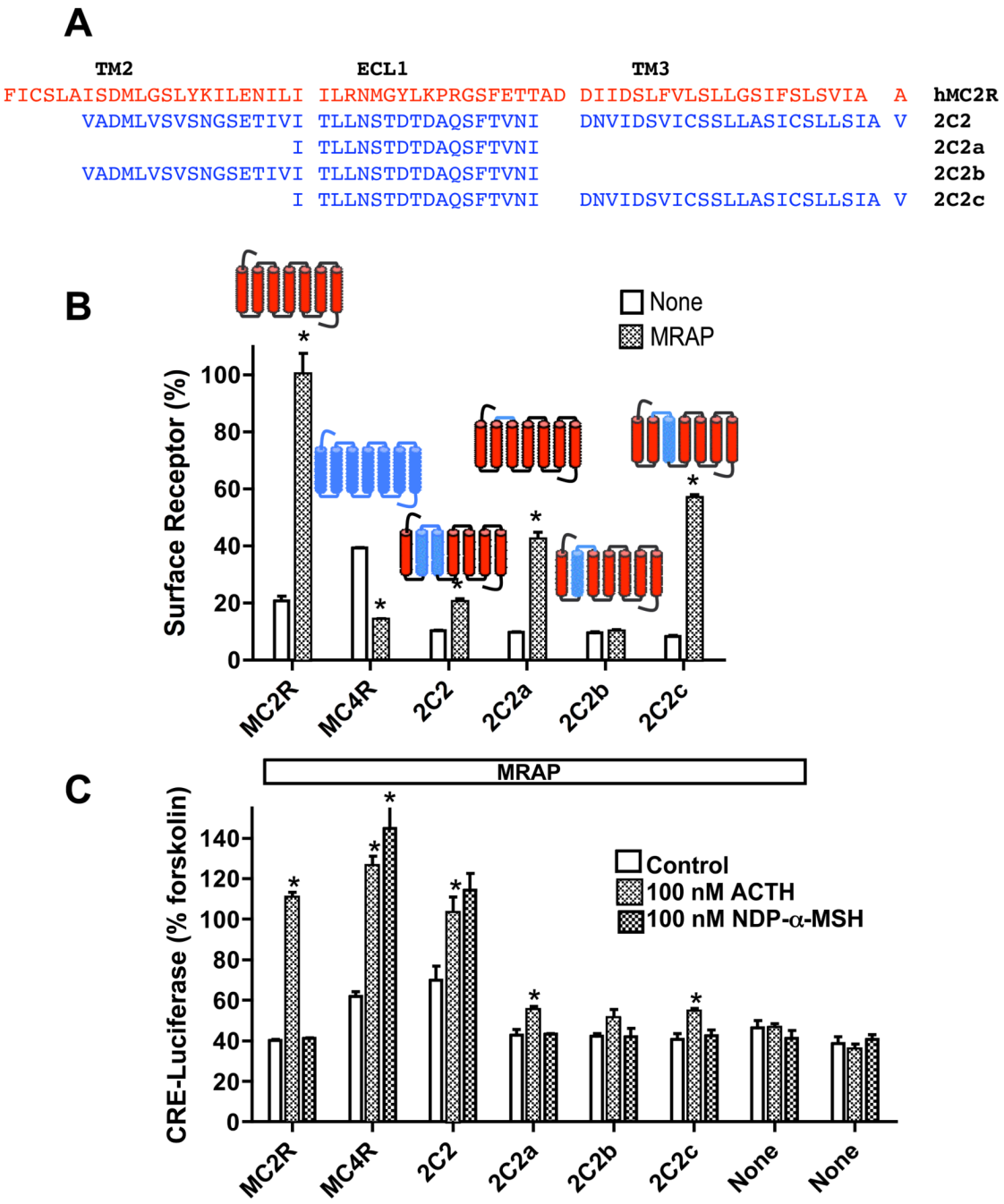

Fig. 6.

Importance of regions from TM2 through TM3 of the MC_2 receptor. (A) Amino acid sequences of receptor constructs. MC_2 receptor sequences are shown in red, MC_4 in blue. (B) Cells were transfected with plasmids encoding receptors and either control plasmid or MRAP. (C) Cells were transfected with CRE-luciferase, receptors and MRAP or control plasmid. The next day cells were incubated with vehicle, 20 μM forskolin, 100 nM ACTH or 100 nM NDP-α-MSH for 5 h when luciferase activity was measured. Surface receptor is expressed relative to the expression of MC_2 receptor with MRAP and CRE-luciferase relative to the response to forskolin. *$P < 0.05$.

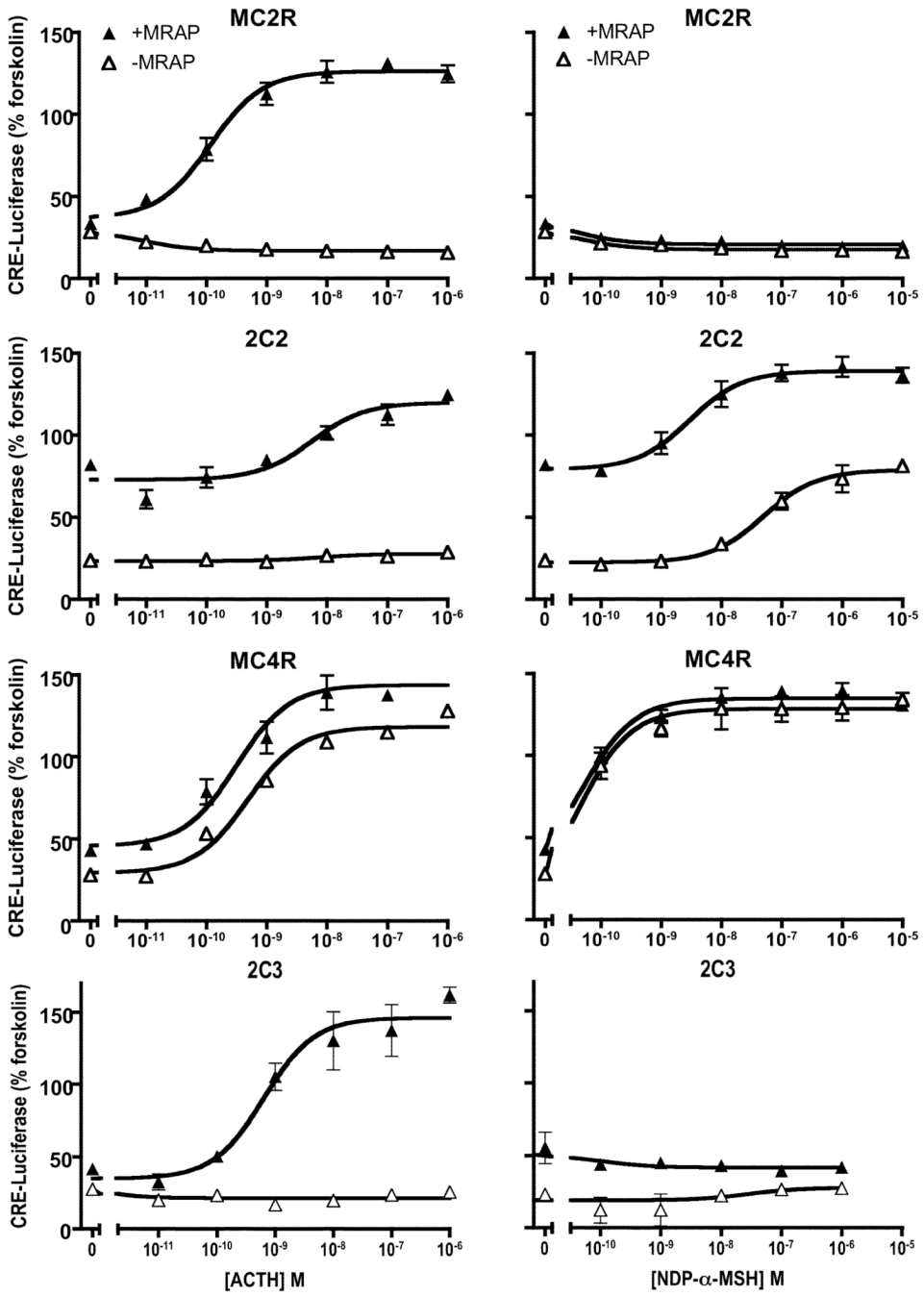

Fig. 7.

Ligand specificity of MC_2, MC_4, 2C2 and 2C3 receptors. Cells were transfected with CRE-luciferase, receptors and MRAP or control plasmid. The next day cells were incubated with vehicle, 20 μM forskolin or different concentrations of ACTH or NDP-α-MSH for 5 h when luciferase activity was measured. Data are expressed relative to the response to forskolin. Shown are the mean and standard error from representative experiments performed in triplicate.

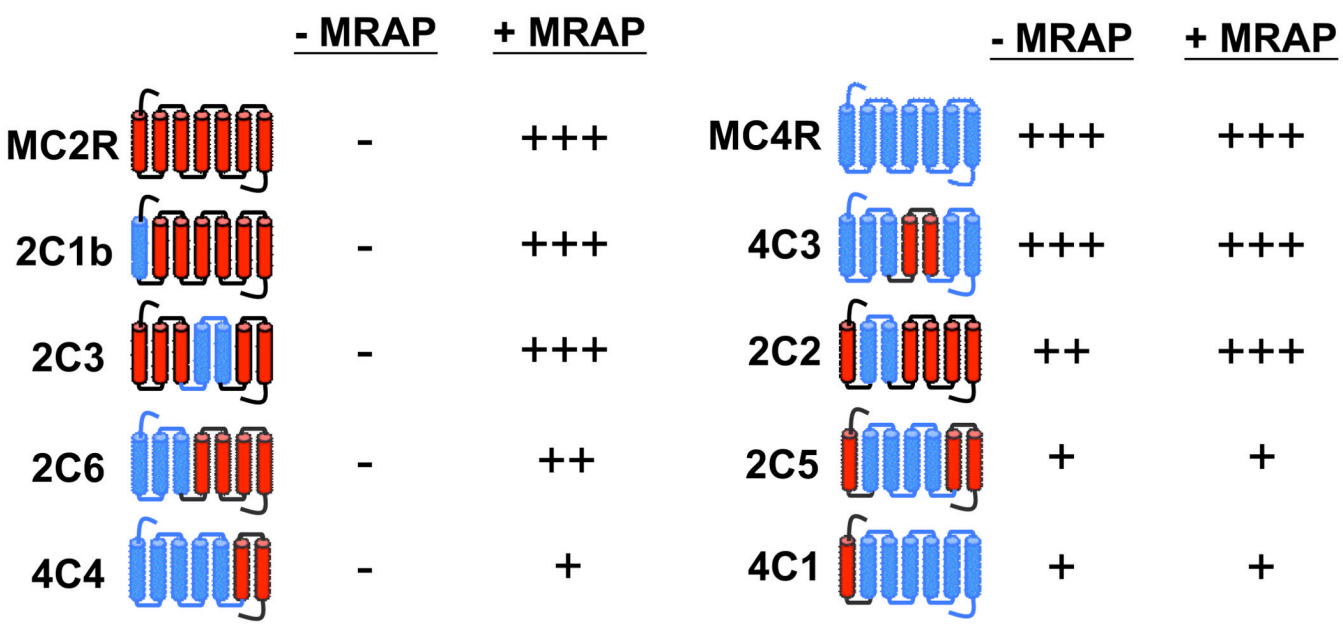

Fig. 8.

Summary of MRAP dependence of signal transduction by chimeric receptors. Receptors that failed to signal are not shown. Responses are based on results from 3 or more experiments like those shown in Figs. 2 and 3 and are denoted: (−) not significant, (+) <33%, (++) 33–66%, or (+++) >66% the activity of the parent receptor.