

Distinct Melanocortin 2 Receptor Accessory Protein Domains Are Required for Melanocortin 2 Receptor Interaction and Promotion of Receptor Trafficking

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Melanocortin 2 receptor (MC2R) is the receptor for the pituitary hormone ACTH. When activated, MC2R stimulates cAMP production and adrenal steroidogenesis. The functional expression of the receptor requires melanocortin 2 receptor accessory protein (MRAP), a single-transmembrane domain protein involved in the trafficking of MC2R from the endoplasmic reticulum to the cell surface. Mutations in both MC2R and MRAP cause the inherited disease familial glucocorticoid deficiency. At present, little is known regarding the mechanism of MRAP in MC2R functional expression. Here we report the characterization of MRAP in the trafficking of MC2R to the cell surface and the formation of a functional receptor. We identify the transmembrane domain of MRAP as the MC2R interaction domain and a conserved N-terminal tyrosine-rich domain of MRAP that is required for trafficking MC2R to the cell surface. (*Endocrinology* 150: 720–726, 2009)

The melanocortin 2 receptor (MC2R) is the smallest member of the G protein-coupled receptor (GPCR) superfamily and belongs to the melanocortin subfamily of receptors (1, 2). The pituitary hormone ACTH acts through MC2R to induce intracellular production of cAMP and the stimulation of steroidogenesis in the adrenal cortex. Mutations in MC2R are responsible for around 25% of the cases of autosomal recessive ACTH insensitivity syndrome, familial glucocorticoid deficiency (3–5). Analysis of MC2R function, using heterologous expression in cultured cells, has been difficult because, unlike the other melanocortin receptors, it is retained in the endoplasmic reticulum and due to lack of cell surface expression is unable to form a functional ACTH-responsive receptor (6, 7). This characteristic of MC2R led to the hypothesis that it requires a specific accessory factor, present in adrenal cell types, to traffic efficiently to the cell surface.

In an effort to find causative genes mutated for familial glucocorticoid deficiency, a disease locus was mapped to chromosome 21, identifying mutations in a gene encoding a small single transmembrane domain protein that showed high adrenal expression, which was subsequently named MRAP (melanocortin 2 receptor accessory protein) (8). MRAP interacts with MC2R

and facilitates MC2R cell surface expression, thereby producing an ACTH-responsive receptor. In addition, knockdown of endogenous mouse MRAP in Y1 adrenocortical cells, which express a functional endogenous MC2R, leads to insensitivity to ACTH (9). Interestingly, recent work suggests that MRAP exists as a unique antiparallel homodimer in which one copy of MRAP has a C terminus outorientation and the other an N terminus outorientation (10). It also appears that this MRAP homodimer is in a stable complex with MC2R, although it remains unclear how this complex forms.

Despite the recent advances in the understanding of MRAP, little is known regarding the mechanism of how it traffics MC2R to the cell surface. Superficially, MRAP is similar to a number of other GPCR accessory proteins that have been identified in the past decade. Like MRAP, the receptor activity-modifying proteins (RAMPs), which are required for the expression of the calcitonin and calcitonin-like receptors (11–13), and the receptor trafficking proteins and related receptor expression-enhancing proteins (REEPs), which traffic a number of odorant receptors (14), are small single-transmembrane domain proteins. However, notwithstanding the similar basic structure and parallel roles of these GPCR accessory proteins, there is no sequence

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Abbreviations: GPCR, G protein-coupled receptor; HA, hemagglutinin-tagged human; MC2R, melanocortin 2 receptor; MRAP, melanocortin 2 receptor accessory protein; RAMP, receptor activity-modifying protein; REEP, receptor expression-enhancing protein; SDS, sodium dodecyl sulfate.

identity and no indication that they function by a common mechanism.

Here we report the identification of the transmembrane domain as the site for MRAP interaction with MC2R. In addition, we identify a requirement for a conserved tyrosine-rich region in the N terminus of MRAP for the cell surface expression of MC2R and the consequent formation of a functional ACTH-responsive receptor.

Materials and Methods

DNA constructs

MRAP-3XFLAG was constructed by directional cloning into the *Hind*III and *Eco*RI restriction sites of the p3xFLAG-CMV-14 expression vector (Sigma, Poole, UK) after PCR amplification of human MRAP α (forward primer, ACGCTGAAA GCTTAGTGCCACAGACATG, reverse primer, ACCAGTTGAATTCGCTATGGC CACGAT). Each of the MRAP deletion constructs was directionally cloned into p3xFLAG-CMV-14 using the *Hind*III and *Eco*RI restriction sites (NT2 forward, ATTGCCAAGCTTACCAACGC CATGGCCCCA, reverse, As MRAP α , NT3 forward, CTGGACAAGCTTGA CCTCATGCCCGTGGAC, reverse, As MRAP α , NT4, AAGCTGAAGCTTATGC ATTCCATCGCA; reverse, As MRAP α , CT2 forward, As MRAP α , reverse, CGGGGAG-GAATTCACGACATGTAGAGCAA, TMT forward, As NT4, reverse, As CT2). The cDNA clone for hemagglutinin-tagged human (HA) MC2R was obtained from the Missouri S&T cDNA Resource Center (www.cdna.org).

Cell culture and transfection

CHO cells were grown in DMEM:F12 (1:1) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Plasmids were transiently transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK). Plasmid DNA amounts were kept constant in transfection with pCDNA3.1.

Immunoprecipitation and Western blotting

Lysates were prepared 24 h after transfection. Cells were washed (2 \times PBS, 10 min) and lysates generated using lysis buffer (0.1% *n*-dodecyl- β -maltoide in PBS with protease inhibitors). Lysates were centrifuged, passed through a 0.22- μ m filter, and either sodium dodecyl sulfate (SDS) loading buffer added for Western blotting or immunoprecipitation performed. For immunoprecipitation, lysates were incubated for 2 h at 4 C with either anti-FLAG beads (Sigma) or anti-HA beads (Sigma). After incubation, beads were washed five times in lysis buffer, supernatant removed, and beads resuspended in SDS loading buffer. To detect the FLAG epitope, beads were heated for 5 min at 90 C to detect HA-MC2R, and beads were heated at 70 C for 2 min. Proteins were resolved by SDS-PAGE and Western blotting performed as described previously (8).

Immunofluorescence

Twenty-four hours after transfection, coverslips were washed (PBS 2 \times 5 min) and fixed in 4% formaldehyde (10 min), cells were permeabilized by washing with PBS-Tween 20 (0.1%; 3 \times 5 min). Cells were blocked for 1 h with blocking buffer (PBS, 1% BSA, 10% donkey serum), incubated with primary antibody (anti-FLAG; Sigma; at 1:200; or anti-HA; Sigma; 1:200) in blocking buffer, washed (PBS-Tween 20, 3 \times 10 min), incubated with secondary antibody (FITC or CY3; both Sigma; 1:200), and washed (PBS-Tween 20, 3 \times 10 min). Coverslips were mounted in fluorescent mounting medium (Dako, High Wycombe, UK). Cells were imaged using a laser-scanning confocal microscope (LSM 510; Zeiss, Jena, Germany).

Fluorescent cell surface assay

To measure cell surface expression of HA-MC2R, cells were grown in 24-well plates. Transfections were performed in triplicate in parallel on 2 \times 24-well plates. Twenty-four hours after transfection, one plate was used for the detection of cell surface HA-MC2R and the other used as a control for total cellular HA-MC2R. Cell surface HA-MC2R was detected by staining live cells with anti-HA antibody (1:500) for 20 min at 37 C in cell growth media. Cells were then washed in PBS (3 \times 5 min), fixed in 4% formaldehyde (10 min), washed in PBS (2 \times 5 min), and incubated with an infrared secondary antibody (goat antimouse, 1:1500, Licor, Lincoln, NE) in blocking buffer (Licor). After further washing (PBS, 3 \times 5 min), plates were scanned using an infrared scanner (Licor Odyssey). To control for total protein, cells were washed twice in PBS (5 min), fixed in 10% formaldehyde (10 min), washed (PBS 2 \times 5 min), permeabilized (0.025% Triton X-100, 10 min), washed (PBS 2 \times 5 min), blocked for 1 h (Licor blocking buffer), incubated with anti-HA (1 h), washed (PBS 3 \times 5 min), incubated with infrared secondary antibody (Licor), washed (3 \times 5 min PBS), and quantitated on the infrared plate reader (as above). Results are presented as the percentage of total protein detected at the cell surface.

cAMP reporter assay

cAMP production was assessed using a cAMP luciferase reporter construct and assayed using the Dual Glo luciferase reporter system (Promega, Southampton, UK), as reported previously (9). Briefly, 24 h after cotransfection of HA-MC2R and MRAP constructs with α -GSU-846 luciferase and pRL-CMV *Renilla* luciferase, cells were incubated with 10⁻⁶ M ACTH for 6 h. After stimulation cells were harvested and Dual Glo luciferase assay performed. Luciferase activity was measured using a multiplate reader (Wallac Victor 2; PerkinElmer, Norwalk, CT), and values normalized to the pRL-CMV *Renilla* luciferase activity.

Statistical analysis

Statistical analysis was performed using the *t* test. Results are expressed as mean \pm SEM.

Results

Human MRAP α interacts with MC2R and is required for receptor cell surface expression and ACTH responsiveness

We have previously shown that mouse MRAP is able to interact with MC2R and is required for the cell surface expression of MC2R (8). To investigate how MRAP directs the functional expression of MC2R, we first tested whether human MRAP α has the same effect as mouse MRAP. Human MC2R epitope tagged at the N terminus with HA-MC2R was expressed only at the cell surface of permeabilized CHO cells, as visualized by immunofluorescence when coexpressed with MRAP α (epitope tagged at the C terminus with FLAG, subsequently referred to as MRAP-FLAG) (Fig. 1A). Using a fluorescent cell surface assay, in which live cells are incubated with HA-antibody, and therefore, only cell surface HA epitopes are detected and normalized against total HA-MC2R in fixed permeabilized cells, and there was a significant increase in HA-MC2R at the cell surface in the presence of MRAP-FLAG (Fig. 1B). Furthermore, using a cAMP luciferase reporter assay, a cAMP response to ACTH stimulation was detected only when HA-MC2R was expressed in the presence of MRAP-FLAG, demonstrating that MRAP is required for functional MC2R cell surface expression (Fig. 1C). Coimmunoprecipitation confirmed that human MRAP-FLAG and HA-

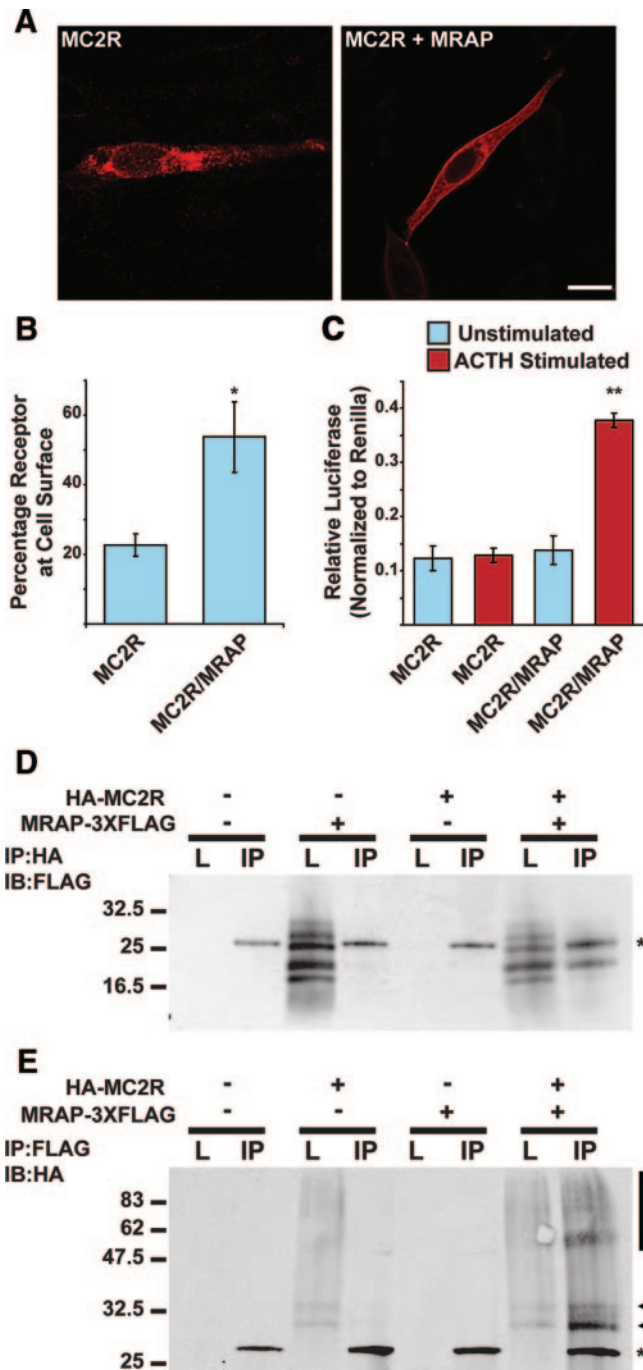


FIG. 1. Human MRAP α interacts with and promotes trafficking of MC2R to the cell surface producing an ACTH-responsive receptor. **A**, HA-MC2R was detected at the cell surface of transiently transfected CHO cells by immunofluorescent staining only when coexpressed with MRAP (bar, 10 μ m). **B**, Cell culture plate based fluorescent cell surface assay showing surface anti-HA staining of live cells as a percentage of total anti-HA staining in permeabilized cells. There was a significant increase in HA-MC2R detected at the cell surface when coexpressed with MRAP-FLAG. Mean \pm SEM; $n = 3$. *, $P < 0.05$. **C**, Luciferase assay to measure cAMP response after 6 h 10^{-6} M ACTH stimulation. There is a significant increase in cAMP after ACTH stimulation when HA-MC2R is coexpressed with MRAP-FLAG. Mean \pm SEM; $n = 3$. **, $P < 0.005$. **D** and **E**, Coimmunoprecipitation between HA-MC2R and MRAP-FLAG. L, Lysate; IB, immunoblotting; IP, immunoprecipitation. *, Light chain. **E**, Arrowheads and vertical bar represent MC2R.

MC2R interact (Fig. 1, D and E). Human MRAP-FLAG resolved as several bands between 16 and 32 kDa on SDS-PAGE (Fig. 1D). MRAP-FLAG has a predicted molecular mass of about 24 kDa,

and the major band may correspond to an unmodified MRAP. Like mouse MRAP, human MRAP contains a predicted N-glycosylation site, and glycosylated MRAP corresponds to the slowest migrating band detected (data not shown). The other bands may correspond to posttranslational modifications or, because some of the bands are smaller than the predicted molecular weight, cleavage products. Immunoprecipitation using HA-conjugated agarose beads and immunoblotting using FLAG antibody specifically detected MRAP-FLAG. HA-MC2R resolved as a series of bands, typical of the migration pattern of other GPCRs on SDS-PAGE. HA-MC2R bands were specifically detected after immunoprecipitation with FLAG-conjugated agarose beads and immunoblotting using an HA antibody (Fig. 1E). Therefore, human MRAP α interacts with MC2R and promotes its trafficking to the cell surface, in which it forms a functional receptor.

Identification of functional MRAP domains

In an effort to identify the MRAP domains involved in the interaction with and functional expression of MC2R, we produced a series of human MRAP truncation constructs that are FLAG tagged at their C terminus (Fig. 2A and supplemental Fig.

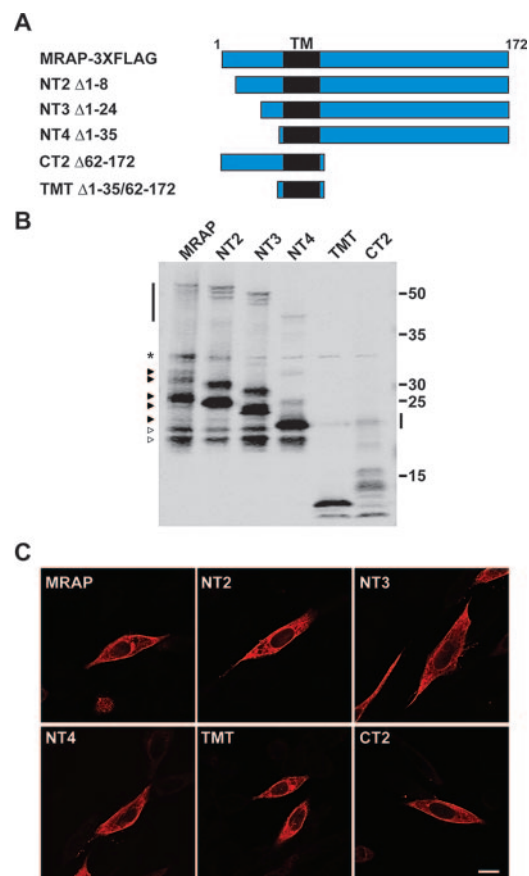


FIG. 2. Expression of MRAP truncation constructs. **A**, Schematic showing MRAP truncation constructs in relation to full-length MRAP α . TM, Transmembrane domain. Each construct is epitope tagged at the C terminus with FLAG. Numbers represent deleted residues, inclusive. **B**, Western blot showing the expression of each of the constructs when coexpressed with HA-MC2R. *, Nonspecific band. Arrowheads on left side represent wild-type MRAP bands. White arrowheads represent putative cleavage products. Vertical bars represent SDS-resistant homodimers. **C**, Each of the MRAP constructs can be seen at the cell surface of permeabilized CHO cells by immunofluorescent staining. Bar, 10 μ m.

1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Each of the constructs was detectable by Western blotting and immunofluorescence after transient transfection into CHO cells (Fig. 2, B and C). We also produced constructs encoding the MRAP N terminus and C terminus alone; however, these were not detected by either Western blot or immunofluorescence, suggesting that these constructs either do not express or that the proteins are rapidly degraded (data not shown). As with the full-length MRAP-FLAG construct, the three N-terminal truncation constructs, NT2, NT3, and NT4, were detected as multiple bands by Western blot, with the major band, corresponding to unmodified MRAP (Fig. 2B). Interestingly, as reported previously with mouse MRAP (9), specific immunoreactive species migrating at about 50 kDa were detected, suggesting the presence of SDS-resistant homodimers. Curiously, lower molecular weight bands observed with full-length MRAP-FLAG were also present at the same molecular weight with each of the N-terminal truncation constructs. This implies that MRAP undergoes degradation at specific sites, or it might be that the N terminus of MRAP undergoes specific processing events. A similar phenomenon was observed with the TMT and CT2 constructs by Western blot. The transmembrane domain construct was detected as two bands at about 10 kDa, and the same bands were also detected with the CT2 construct. As with the N-terminal truncations, a faint band was detected at about 20 kDa that might represent an SDS-resistant dimer.

Each of the MRAP truncation constructs could be detected at the cell surface by immunofluorescence using the FLAG antibody on permeabilized cells (Fig. 2C), signifying that the MRAP transmembrane domain alone is sufficient for the cell surface expression of MRAP.

The transmembrane domain of human MRAP is sufficient for MRAP/MC2R interaction

To identify the MRAP domain required for its interaction with MC2R, HA-MC2R was coexpressed with each of the MRAP truncation constructs and coimmunoprecipitated. Immunoblotting using FLAG identified each of the MRAP truncation proteins after immunoprecipitation with HA-conjugated beads (Fig. 3A). In the reverse experiment, immunoprecipitation using FLAG-conjugated agarose specifically precipitated HA-MC2R with each of the FLAG tagged MRAP truncation proteins (Fig. 3B). A concern with immunoprecipitation of membrane proteins is that if lysates are incompletely solubilized, two proteins that are not in physical contact may be present in a membrane fragment and produce spurious interaction data (15). These coimmunoprecipitations have therefore been performed using lysates that have been passed through a 0.22- μ m filter to ensure that membrane fragments have been removed. Using unfiltered lysates and different detergent conditions, the same results were obtained (data not shown). We therefore mapped the MRAP/MC2R interaction domain to a 27-amino acid region between residues 36 and 62 of MRAP that consists almost entirely of the transmembrane domain. It is thus likely that MC2R interacts with MRAP via one or more of its transmembrane domains.

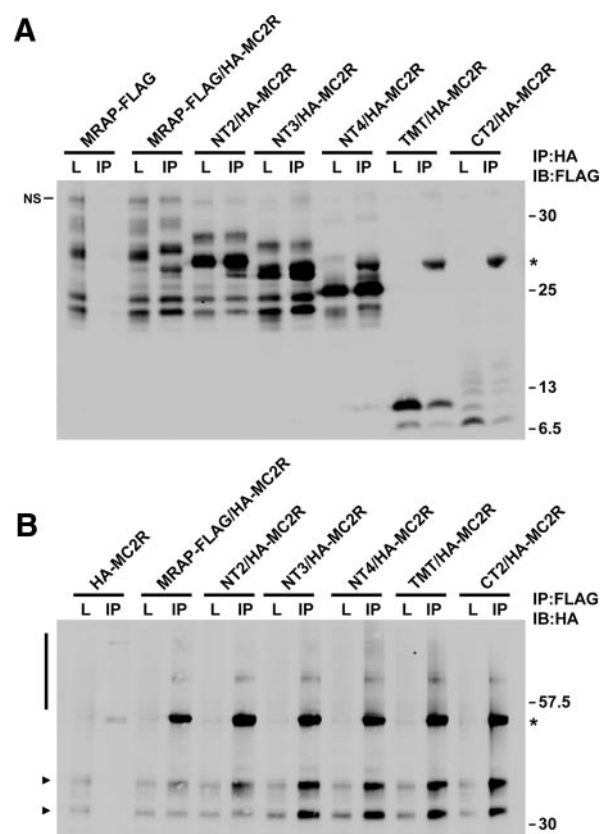


FIG. 3. The MRAP transmembrane domain is required for receptor interaction. A and B, Coimmunoprecipitation between HA-MC2R and each of the FLAG tagged MRAP constructs. Each of the MRAP constructs interact with HA-MC2R as seen by immunoblotting with FLAG after immunoprecipitation with HA and vice versa. L, Lysate, IP, Immunoprecipitation; IB, immunoblotting. *, Light chain (A) and heavy chain (B). NS, Nonspecific in A. Bar and arrows in B show HA-MC2R.

The N terminus of MRAP is required for MC2R cell surface expression and ACTH responsiveness

We then tested whether the interaction between MRAP and MC2R was sufficient to mediate the trafficking of MC2R to the cell surface. HA-MC2R was cotransfected with each of the MRAP truncation constructs and cell surface expression monitored using immunofluorescence and the fluorescent cell surface assay. HA-MC2R was detected at the cell surface of permeabilized cells in the presence of both the NT2 and CT2 constructs as detected by immunofluorescence staining (Fig. 4A). However, HA-MC2R remained intracellular when cotransfected with each of the NT3, NT4, and TMT constructs. As with full-length MRAP-FLAG, the amount of HA-MC2R detected at the cell surface was around 2-fold higher when coexpressed with the NT2 construct, indicating that the N-terminal eight amino acids of MRAP, which contain the glycosylation site, are not required for MRAP to traffic MC2R to the cell surface (Fig. 4B). In the presence of CT2, the amount of HA-MC2R at the cell surface was significantly higher compared with coexpression with either MRAP-FLAG or NT2. No difference in the total levels of HA-MC2R was seen (data not shown). The increase in HA-MC2R at the cell surface seen with the CT2 construct is in contrast to previously published data using a mouse MRAP construct with a C-terminal deletion (10). This mouse construct supported functional MC2R expression; however, this was not greater than that

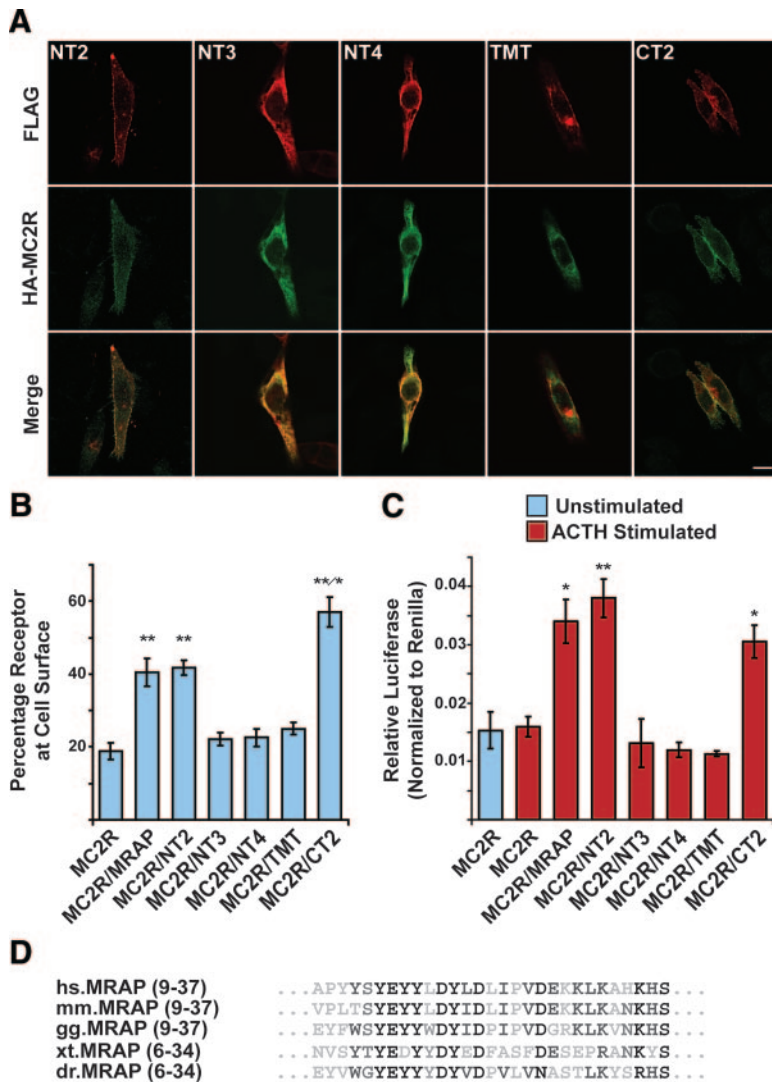


FIG. 4. MC2R cell surface trafficking requires the N terminus of MRAP and is regulated by the C terminus of MRAP. **A**, Immunofluorescent staining showing localization of HA-MC2R with each of the FLAG-tagged MRAP constructs in permeabilized transiently transfected CHO cells. *Top panels*, Anti-FLAG staining of MRAP constructs (red). *Middle panels*, Anti-HA staining of HA-MC2R (green). *Bottom panels*, Merge. HA-MC2R colocalizes with each of the MRAP constructs, and is detected at the cell surface in the presence of the NT2 and CT2 constructs. With NT3, NT4, and TMT, HA-MC2R is not seen at the cell surface. *Bar*, 10 μ m. **B**, Fluorescent cell surface assay showing the percentage of MC2R at the cell surface when cotransfected with each of the MRAP constructs. Significant cell surface expression is seen in the presence of MRAP, NT2, and CT2 (**, $P < 0.005$) compared with HA-MC2R alone. There is no increase seen with the remaining constructs. HA-MC2R cell surface expression is significantly higher when coexpressed with CT2 compared with MRAP or NT2 (*, $P < 0.05$). Mean \pm SEM; $n = 4$. **C**, Luciferase assay to measure cAMP response after 6 h 10^{-6} M ACTH stimulation. There is a significant increase in cAMP after ACTH stimulation when HA-MC2R is coexpressed with MRAP, NT2, and CT2. Mean \pm SEM; $n = 3$. *, $P < 0.05$; **, $P < 0.005$. **D**, Alignment of amino acids 9–37. Residues deleted in the NT3 construct are underlined. hs, *Homo sapiens*; mm, *Mus musculus*; gg, *Gallus gallus*; xt, *Xenopus tropicalis*; dr, *Danio rerio*.

seen with wild-type MRAP. This could be explained either by species differences or may be due to the presence of both N- and C-terminal epitope tags on the mouse MRAP fusion, which could impair or alter its function.

When cotransfected with NT3, NT4, or TMT, there was no significant increase in HA-MC2R detected at the cell surface compared with HA-MC2R alone using the fluorescent cell surface assay, demonstrating that an interaction between MC2R and MRAP alone is not sufficient for trafficking but that the

MRAP N-terminal domain between residues 9 and 24 is also required.

To investigate whether the MRAP truncation constructs affect the function of MRAP, we performed a cAMP luciferase reporter assay (Fig. 4C). In line with the cell surface assay, there was only a cAMP response when HA-MC2R was coexpressed with either full-length MRAP-FLAG, NT2, or CT2, although there was no increase in signaling in the presence of CT2 to match the increase in HA-MC2R cell surface expression. Therefore, the N terminus of MRAP is required for the cell surface expression of MC2R and the resultant formation of an ACTH-responsive receptor. The key region is a conserved tyrosine-rich region that begins between residues 9 and 24 (Fig. 4D) because the NT3 construct, which has the first 24 amino acids deleted, is unable to support MC2R cell surface expression.

Discussion

MRAP is a small single-transmembrane domain protein, which is required for the proper function of the ACTH receptor MC2R. It has previously been reported that MRAP interacts with MC2R and promotes its trafficking to the cell surface. However, the means of the MRAP/MC2R interaction and the role of MRAP in receptor trafficking have so far been elusive. Here we studied the role of MRAP and identified that the transmembrane domain of MRAP as the site of MC2R interaction, whereas a conserved 15-residue region in the N terminus of MRAP is required for trafficking. In addition MRAPs C terminus, which shows little sequence conservation between species, may have a role in the control of MC2R cell surface expression.

The identification of the MRAP transmembrane domain as the site of MRAP/MC2R interaction highlights differences between the mechanisms of MRAP and the RAMPs. For RAMP1 it is the large extracellular N-terminal domain that has been shown to be sufficient for its interaction with the calcitonin-like receptor (13, 16–18).

Although the MRAP transmembrane domain is sufficient for interaction with MC2R, it is not able to support MC2R trafficking. The key region controlling trafficking is a 15-amino acid tyrosine-rich region between residues 9 and 24 in the N terminus of MRAP because the NT3 MRAP construct is unable to promote MC2R trafficking. The role of this 15-amino acid domain in the cell surface expression of MC2R is not yet known. It is not required for the MRAP/MC2R interaction, and it does not appear to be needed for MRAP to reach the cell surface. It is possible that it plays a role in the stabilization of the MRAP/MC2R interaction during trafficking to the cell surface. Alternatively, it might interact with another protein that regulates MRAP/MC2R cell

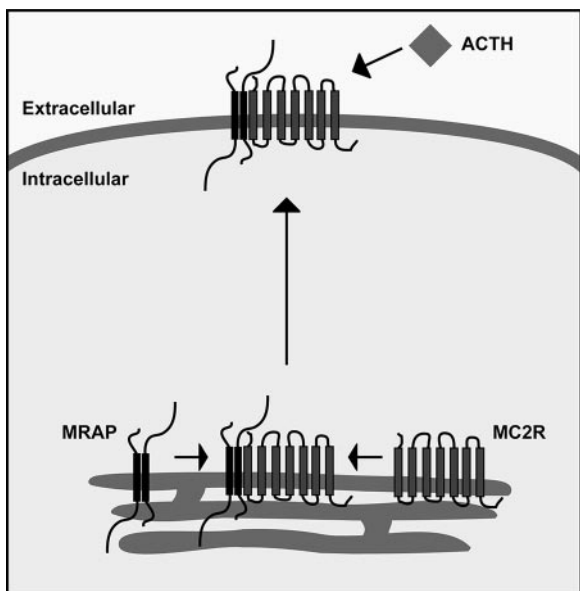


FIG. 5. Hypothetical model of MRAP/MC2R interaction and receptor trafficking. MRAP (black), which acts as an antiparallel homodimer (10), interacts with MC2R (gray) within the endoplasmic reticulum via its transmembrane domain. This heterotrimeric structure then traffics to the cell surface through a process dependent on a 15-amino acid tyrosine-rich region of the N terminus of MRAP, in which it is capable of recognizing and responding to the ACTH peptide.

surface expression. Recently the mouse MRAP protein has been shown to function as an antiparallel homodimer. The N terminus of MRAP could be involved in establishing this unique structural confirmation either intrinsically or by interacting with an unidentified partner; however, the presence of an apparent SDS-resistant dimer, as seen by Western blot when this region is deleted (Fig. 2B), suggests that the loss of MC2R trafficking is not through a failure of MRAP homodimerization. At present it is unclear whether this novel 15-amino acid tyrosine-rich domain is unique to MRAP or whether it is a general feature of proteins involved in the trafficking of GPCRs. Although sequence homology is not readily identifiable between this domain and other proteins, it does resemble a region within another family of GPCR trafficking proteins. REEP1 was first identified as a transmembrane protein that is able to promote the cell surface expression of odorant receptors (14). Like MRAP, the REEPs have a tyrosine-rich region in their N termini. It is therefore possible that a tyrosine-rich domain may be a more common feature of proteins involved in GPCR trafficking.

The C terminus of MRAP is not required for MRAP to interact with MC2R or for MRAP to traffic MC2R to the cell surface. Instead, the C terminus may play a role in the amount of MC2R that reaches the cell surface because deletion of MRAPs C terminus resulted in significantly higher MC2R cell surface expression compared with MC2R with wild-type MRAP. A possible role for the C terminus of MRAP in regulating MC2R cell surface expression is supported by the different effects the two human MRAP isoforms, which differ in their C terminus, have been shown to have on MC2R (19). When coexpressed with MRAP β , MC2R cell surface expression was significantly higher than when coexpressed with MRAP α .

In summary, MRAP is a small single-transmembrane domain protein that is required for the cell surface expression of the G protein-coupled MC2R (Fig. 5). We previously reported that MRAP interacts with MC2R and enhances its trafficking to the cell surface, producing a functional ACTH-responsive receptor. However, both the mechanism of the interaction and the means of trafficking have been elusive. The N terminus and transmembrane domain of MRAP are conserved throughout vertebrates, whereas its C terminus is highly divergent. In this study, we have shown that the C terminus of MRAP is not necessary for either MC2R interaction or trafficking but instead may have a regulatory role in MC2R cell surface expression. Furthermore, we have shown a requirement for a tyrosine-rich region of the N terminus of MRAP in trafficking MC2R to the cell surface and demonstrated that the MRAP transmembrane domain is sufficient for the MRAP/MC2R interaction.

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References

1. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD 1992 The cloning of a family of genes that encode the melanocortin receptors. *Science* 257:1248–1251
2. Cone RD, Mountjoy KG, Robbins LS, Nadeau JH, Johnson KR, Roselli-Rehffuss L, Mortrud MT 1993 Cloning and functional characterization of a family of receptors for the melanotropic peptides. *Ann NY Acad Sci* 680:342–363
3. Clark AJ, McLoughlin L, Grossman A 1993 Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor. *Lancet* 341:461–462
4. Weber A, Kapas S, Hinson J, Grant DB, Grossman A, Clark AJ 1993 Functional characterization of the cloned human ACTH receptor: impaired responsiveness of a mutant receptor in familial glucocorticoid deficiency. *Biochem Biophys Res Commun* 197:172–178
5. Clark AJ, Cammas FM, Watt A, Kapas S, Weber A 1997 Familial glucocorticoid deficiency: one syndrome, but more than one gene. *J Mol Med* 75:394–399
6. Noon LA, Franklin JM, King PJ, Goulding NJ, Hunyady L, Clark AJ 2002 Failed export of the adrenocorticotrophin receptor from the endoplasmic reticulum in non-adrenal cells: evidence in support of a requirement for a specific adrenal accessory factor. *J Endocrinol* 174:17–25
7. Rached M, El Mourabit H, Buronfosse A, Blondet A, Naville D, Begeot M, Penhoat A 2005 Expression of the human melanocortin-2 receptor in different eukaryotic cells. *Peptides* 26:1842–1847
8. Metherell LA, Chapple JP, Cooray S, David A, Becker C, Ruschendorf F, Naville D, Begeot M, Khoo B, Nurnberg P, Huebner A, Cheetham ME, Clark AJ 2005 Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. *Nat Genet* 37:166–170
9. Cooray SN, Almiro Do Vale I, Leung KY, Webb TR, Chapple JP, Egertova M, Cheetham ME, Elphick MR, Clark AJ 2008 The melanocortin 2 receptor accessory protein exists as a homodimer and is essential for the function of the melanocortin 2 receptor in the mouse Y1 cell line. *Endocrinology* 149:1935–1941

10. Sebag JA, Hinkle PM 2007 Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. *Proc Natl Acad Sci USA* 104:20244–20249
11. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM 1998 RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 393:333–339
12. Morfis M, Christopoulos A, Sexton PM 2003 RAMPs: 5 years on, where to now? *Trends Pharmacol Sci* 24:596–601
13. Hay DL, Poyner DR, Sexton PM 2006 GPCR modulation by RAMPs. *Pharmacol Ther* 109:173–197
14. Saito H, Kubota M, Roberts RW, Chi Q, Matsunami H 2004 RTP family members induce functional expression of mammalian odorant receptors. *Cell* 119:679–691
15. Milligan G, Bouvier M 2005 Methods to monitor the quaternary structure of G protein-coupled receptors. *FEBS J* 272:2914–2925
16. Steiner S, Muff R, Gujer R, Fischer JA, Born W 2002 The transmembrane domain of receptor-activity-modifying protein 1 is essential for the functional expression of a calcitonin gene-related peptide receptor. *Biochemistry* 41:11398–11404
17. Fitzsimmons TJ, Zhao X, Wank SA 2003 The extracellular domain of receptor activity-modifying protein 1 is sufficient for calcitonin receptor-like receptor function. *J Biol Chem* 278:14313–14320
18. Ittner LM, Luessi F, Koller D, Born W, Fischer JA, Muff R 2004 Aspartate(69) of the calcitonin-like receptor is required for its functional expression together with receptor-activity-modifying proteins 1 and -2. *Biochem Biophys Res Commun* 319:1203–1209
19. Roy S, Rached M, Gallo-Payet N 2007 Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms α and β in isogenic human embryonic kidney 293 cells. *Mol Endocrinol* 21:1656–1669