

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

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The ACTH receptor [melanocortin 2 receptor (MC2R)] gene produces a functional receptor only when transfected into cells of adrenocortical origin, implying that it may require an adrenal-specific accessory factor. Recently we showed that the MC2R accessory protein (MRAP) is essential for the cell surface expression of the MC2R in such models. Using RNA interference (RNAi) technology, we have further explored the action of MRAP in the functioning of the MC2R in Y1 mouse adrenocortical cells that endogenously express MRAP and MC2R. We created stable cell lines expressing mouse MRAP short hairpin RNA (shRNAs) by transfecting cells with an expression vector containing the MRAP small interfering

RNA sequence. The knockdown of MRAP resulted in a reduction in MC2R signaling. The overexpression of a mouse MRAP-Flag construct did not restore the expression of MRAP due to its degradation by the mouse shRNAs. The introduction of human MRAP that is resistant to silencing by mouse MRAP shRNAs resulted in the rescue of the MC2R signaling. MRAP migrates on SDS-PAGE with markedly lower mobility than predicted for a 14.1-kDa protein. Coimmunoprecipitation and mass spectroscopy suggests that MRAP exists as a homodimer that is resistant to dissociation by sodium dodecyl sulfate and reducing agents. (*Endocrinology* 149: 1935–1941, 2008)

THE ACTION OF ACTH on the adrenal cortex to stimulate steroidogenesis is a unique and vital component of the pituitary-adrenal axis. Failure of this action is incompatible with normal life. It is perhaps surprising therefore that this action is mediated through a single G protein-coupled receptor (GPCR), the ACTH receptor or melanocortin 2 receptor (MC2R) (1). The functional characterization of the MC2R in heterologous cells has been extremely challenging because it is expressed at the cell surface very inefficiently in comparison with other GPCRs. For this reason there had been some controversy initially as to whether the MC2R was indeed the cognate receptor for ACTH. The finding that mutations in the human MC2R were associated with ACTH insensitivity provided some reassurance of this (2). Several groups have attempted to characterize the transfected MC2R in a number of cells including HEK293, NIH3T3, CHO-K1, and other cell lines without success (3, 4). Others also transfected COS 7 cells (5) and the Cloudman M3 melanoma cell line (6) with the MC2R and demonstrated expression of the

receptor, although this was confounded by the endogenous melanocortin receptor present in these cell lines. However, the Y6 and OS3 cell lines, derived as sister lines to the mouse Y1 cell line (7, 8) are the only cell lines to date that have provided reasonable expression of the MC2R with no other interfering melanocortin receptors (9–11). These cell lines do not express endogenous MC2R despite retaining appropriate signaling pathways and have enabled the majority of the characterization of the transfected receptor to date.

Because MC2R is efficiently translated in nonadrenal cell lines but is still nonfunctional, we have proposed that the receptor is dependent on an accessory factor for cell surface trafficking and function (3). We recently identified a novel single transmembrane domain protein that we named MC2R accessory protein (MRAP) as such a factor (12). We showed that MRAP and MC2R colocalized in the cell and coimmunoprecipitated and that cotransfection resulted in the functional expression of the MC2R in CHO-K1 and SKN-SH cells. Furthermore, a variety of mutations in MRAP were shown to be associated with familial glucocorticoid deficiency type 2, an autosomal recessive ACTH insensitivity syndrome (12).

The concept of accessory factors for GPCR expression is not new. The *Drosophila* cyclophilin gene *Nina A* (neither inactivation nor after potential A) and its mammalian homolog, Ran binding protein 2, have been identified as being essential for the folding and trafficking of R1–6 rhodopsin and red/green opsin to the cell surface (13–15). A protein named ODR4 was found to be necessary for the efficient targeting of odorant receptors to olfactory cilia in *Caenorhab-*

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Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; α -GSU, glycoprotein hormone α -subunit; HA, hemagglutinin; MC2R, melanocortin 2 receptor; MRAP, MC2R accessory protein; NP-40, Nonidet P-40; RAMP, receptor activity modifying protein; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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ditis elegans (16). Dopamine receptor interacting protein (DRiP78) is required for the trafficking of the D1 dopamine receptor (17), and the small single-transmembrane domain proteins receptor activity modifying proteins (RAMPs) are required for trafficking and ligand specificity of the calcitonin receptor and the calcitonin-like receptor (18, 19). More recently receptor transporter proteins 1 and 2 and the distantly related receptor expression-enhancing proteins have been shown to promote functional cell surface expression of some of the odorant receptors (20). The RAMPs, receptor transporter proteins, and receptor expression-enhancing proteins are all relatively small single transmembrane domain containing proteins that otherwise have no sequence homology to MRAP or each other. Despite the observation that mutations in MRAP cause ACTH insensitivity, the physiological relevance of functional observations made from protein overexpression in transfected cell lines was not fully clear. Furthermore, our previous studies had not clarified the specificity of the action of MRAP.

In the work reported here, we therefore used the RNA interference approach to knock down the expression of MRAP in Y1 mouse adrenocortical cells and investigated its effect on MC2R signaling. Furthermore, because MRAP is detected on immunoblotting at a molecular mass of 32 kDa in Y1 cells that is significantly higher than the predicted size of 14.1 kDa, we explored the possibility that it may exist as a dimeric structure.

Materials and Methods

All chemicals, tissue culture reagents, and antibodies stated in this paper were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. ACTH (1–39) was purchased from Bachem/Peninsula Laboratories (St. Helen's, Merseyside, UK). MRAP small interfering RNA (siRNA) duplexes were purchased from Dharmacon (Erembodegem, Belgium).

Cell culture

Y1 mouse adrenocortical cells were grown on collagen-coated tissue culture plates in the presence of DMEM/Ham's F10 (1:1) media (Life Technologies, Inc., Paisley, UK) supplemented with 12.5% horse serum, 2.5% fetal calf serum, and 1% penicillin/streptomycin. SKN-SH and CHO cells were grown in the presence of DMEM/Ham's F12 (1:1) media with 10% fetal calf serum and 1% penicillin/streptomycin.

MRAP siRNA duplexes and plasmid constructs

Four 21-nucleotide siRNA duplexes targeting the first coding exon of the mouse MRAP sequence were synthesized. The sequences of the four MRAP siRNA duplexes were: duplex 2, AGTATTACCTGGACTA-CATTT; duplex 4, ATGAGTATTACCTGGACTATT; duplex 5, GCT-GAAAGCCAAACAAGCATT; duplex 6, TCACCAGCTATGAGTAT-TATT. The optimum target sequence for MRAP was determined by RT-PCR analysis of MRAP gene expression in Y1 cells transfected with 25 nM each of these four MRAP siRNA duplexes. The target sequence of the MRAP siRNA duplex 6 was then used to design two complementary 55-mer siRNA template oligonucleotides encoding MRAP short hairpin RNAs (shRNAs) with *Bam*H1 and *Hind*III overhangs. The two oligonucleotide used were; 5'-GATCCTCACCAGCTATGAGTAT-TATTC AAGAGATAATACTCATAGCTGGTGACGA-3' and 5'-AGCT-TCCTCACCAGCTATGAGTA TTATCTCTTGAATAATACTCATAGC-TGGT GAG-3'. The oligonucleotides were then annealed and cloned into the *Bam*H1- and *Hind*III-digested pSilencer 4.1 CMV-Neo expression vector (Ambion, Cambridgeshire, UK). pRL-CMV luciferase (Promega, Southampton, UK) control plasmid was used to correct for transfection efficiency in luciferase assays. The cAMP luciferase reporter

construct, glycoprotein hormone α -subunit (α -GSU)-846 (containing 846 bp of the 5' flanking sequence and 44 bp of exon 1 of the human α -GSU gene, cloned in frame with the luciferase gene in the plasmid pA3Luc) was kindly donated by Professor J. Burrin (21). The mouse MRAP-Flag construct was a kind gift from Professor G. Cooper (22). The mouse MRAP-HA construct was prepared using 5'-ATCGGGATCCATG-GCCA ACGGGACC-3' as the sense primer and 5'-GGTAGTCTGG-GACGTCGTATGGGTAGGGGAGAGCCA-3' as the antisense primer. Human MRAP was amplified using 5'-ATGGCCAACGGACCAACG-3' as the sense primer and 5'-TCAGCTCTGCAATTGAGA-3' as the antisense primer and cloned into pcDNA3.1.

Stable transfections of shRNA expression plasmids

For the creation of stable cell lines expressing MRAP shRNA, the pSilencer 4.1 CMV-Neo-MRAP shRNA plasmid was linearized using *Eco*R1 and gel purified before transfecting into Y1 cells at 50% confluency. As a control the pSilencer 4.1CMV Neo plasmid (Ambion) encoding a hairpin siRNA, which shows no homology to any known gene, was also transfected into cells. Cells were selected using 500 μ g/ml G418 in growth media for 20 d. Surviving clones were transferred onto a 96-well plate and when confluent were transferred to 24-, 12-, and six-well plates.

RT-PCRs and Western blotting

To detect the expression of MRAP and MC2R in Y1 cells, total RNA was extracted from cells and cDNA was synthesized and used for PCR with the MC2R and MRAP primers. MRAP primers used were 5'-ACTGTTCATGGCCAACGG-3' as the sense primer and 5'-AGTGT-GAGG CCAGCTGAT-3' as the antisense primer. Primers used to amplify MC2R were 5'-ATGAAGCATATTATCAATTTCG-3' as the sense and 5'-CTAATACCGGTTGCAGAA-3' as the antisense primers. To detect the knockdown of MRAP expression in cells transiently transfected with MRAP siRNAs, cells were collected 24 or 48 h after transfection, and total RNA was extracted from the cell lines and used for cDNA synthesis. RT-PCRs were carried out using the MRAP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer pairs. For Western blotting, cells were scraped into lysis buffer containing PBS+1% dodecyl maltoside+protease inhibitor cocktail, and 20 μ g of protein samples, as determined by protein assay (Bio-Rad, Hertfordshire, UK), were separated by SDS-PAGE. Western blotting was performed using monoclonal anti-hemagglutinin (HA), clone HA-7 antibody, anti-Flag antibody M2, or an affinity-purified rabbit polyclonal MRAP antibody –7819 (produced to order by Severn Biotech, Worcestershire, UK) raised against an N-terminal peptide DLIPVDEKLLKA. This peptide was synthesized with an additional cysteine at both ends to provide two reactive sites for the coupling to the carrier protein keyhole limpet hemocyanin via sulfo-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate chemistry. The serum was affinity purified using a Thio-Link Gel gel column (Severn Biotech), following the manufacturer's instructions. We characterized the antibody as being specific for MRAP at 1:20 dilution or Flag antibody at 1:1000 dilution. Horseradish peroxidase-conjugated goat antimouse/rabbit secondary antibodies (Upstate Biotechnology, Lake Placid, NY) were used at 1:5000 dilution.

Coimmunoprecipitations

To detect the dimerization of MRAP, CHO cells were transfected with MRAP-Flag and MRAP-HA constructs. Twenty-four hours after transfection, cells were scraped into Nonidet P-40 (NP-40) lysis buffer [150 mM NaCl, 1.0% (vol/vol) NP-40, 50 mM Tris (pH 8.0)]+protease inhibitor cocktail. For coimmunoprecipitations 150 μ g of the supernatants were incubated with either 5 μ g of HA or Flag antibody and left at 4 C overnight. Protein Sepharose beads (GE Healthcare, Buckinghamshire, UK) were added and incubated for another 2 h at room temperature. One hundred microliters of sodium dodecyl sulfate sample buffer were then added, and the samples were subjected to Western blotting using HA or Flag antibody at a dilution of 1:1000.

cAMP assay

Cells were covered with 10^{-5} M 3-isobutyl-1-methyl-xanthine in serum-free medium for 30 min at 37 C. Cells were then stimulated with

10^{-6} M ACTH for 30 min to generate cAMP. Cells were placed on ice, scraped into the media, and boiled for 5 min. They were then centrifuged and the supernatant was used in the cAMP competitive protein-binding assay as described (23).

Dual-Glo luciferase assay

For the siRNA experiment, 25 nM concentration of the MRAP siRNA duplex 6 or 25 nM of the negative control cyclophilin siRNA was transfected into Y1 adrenocortical cells along with α -GSU-846 luciferase and pRL-CMV *Renilla* luciferase plasmid constructs. Twenty-four or 48 h after transfection, cells were stimulated with 10^{-6} M ACTH for 6 h. For the rescue of MC2R signaling in the clonal cell line 26, cells were transiently transfected with α -GSU-846 luciferase, pRL-CMV *Renilla* luciferase and human MRAP plasmid constructs. After 48 h they were stimulated for 6 h with ACTH (10^{-6} M). Cell lysates were then harvested and assayed using the Dual Glo luciferase reporter assay system (Promega). Luciferase activity was measured using a multiplate reader (Wallac Victor 2 PerkinElmer, Bar Hill, UK), and values were normalized to the pRL-CMV *Renilla* luciferase activity.

Mass spectrometry analysis

CHO cells were seeded into four 25-cm² cell flasks and transfected transiently with an MRAP-HA construct. Twenty-four hours after transfection, cells were lysed with 250 μ l NP-40 lysis buffer containing the proteinase inhibitor cocktail. The total protein concentration was measured by Bradford assay. MRAP-HA was purified by immunoprecipitation of 150 μ g of protein with 60 μ l of anti HA conjugated beads, washed with the NP-40 lysis buffer, and resolved by SDS-PAGE. Proteins were analyzed using Western blotting with the anti-HA antibody or by colloidal Coomassie-blue staining.

Bands unique to MRAP-HA-transfected cells were excised, subjected to in-gel digestion with trypsin, and analyzed by a liquid chromatography-tandem mass spectrometer (QToF-micro; Waters Corp., Milford, MA). The mass spectral data were processed into peak lists (tandem mass spectrometer data) and searched against the Swiss Prot database using the MASCOT search algorithm (www.matrixscience.com). One missed cleavage per peptide was allowed. For identified MRAP peptides to be considered significant, the peptide score was typically greater than 40 ($P < 0.05$), and manual interpretation confirmed agreement between spectra and peptide sequence. In addition, MASCOT searches of all

spectra were performed against a randomized version of the National Center for Biotechnology Information (Bethesda, MD) database using the same parameters as in the main search. In no case did this search retrieve more than a single peptide, and in all instances the peptide score was below the $P = 0.05$ significance level.

Statistical analysis

The data reported in this paper are the mean \pm SEM of at least three independent experiments, each performed at least in duplicate. Statistical analysis of cAMP and luciferase assays was performed using ANOVA.

Results and Discussion

Knockdown of MRAP and effect on MC2R signaling

We used mouse Y1 adrenocortical cells as a model to investigate the role of MRAP in the expression of the MC2R because this cell line expresses both MC2R and MRAP endogenously (Fig. 1A). We knocked down the expression of MRAP in cells by the RNA interference technique using synthetic siRNA duplexes and creating stable cell lines expressing MRAP shRNAs.

Four MRAP siRNA duplexes targeting the first coding exon of the mouse MRAP sequence were transfected into Y1 adrenocortical cells to determine the most potent sequence for MRAP knockdown (Fig. 1B). We tested four siRNA duplexes because siRNA duplexes designed against different targets of the same gene can show noticeable differences in silencing efficiency. They showed variable levels of MRAP gene knockdown. In particular duplexes 2 and 6 showed a significant reduction in MRAP expression, compared with the wild-type Y1 cells. Duplex 6 appeared to be the most potent siRNA duplex sequence to knock down the expression of MRAP. Twenty-five nanomolar concentration of duplex 6 was then used to transfect Y1 cells to measure its effect on MC2R signaling. Using the Dual Glo luciferase assay

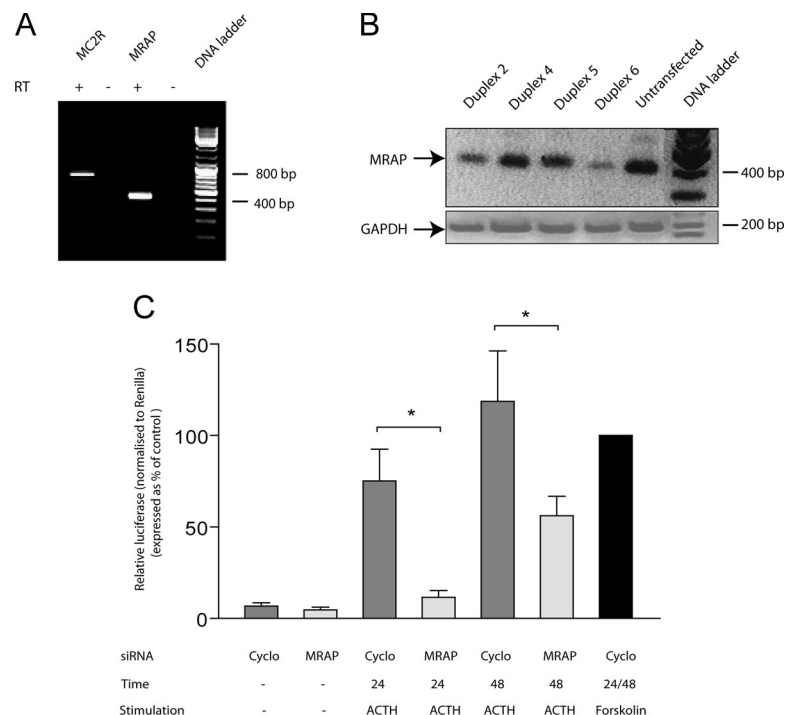


FIG. 1. Expression of MRAP siRNA and MC2R signaling. A, Y1 cells express endogenous MRAP (384 bp) and MC2R (891 bp) as determined by RT-PCR analysis. Lanes 2 and 4 represent no-RT controls. B, RT-PCR analysis to show the knockdown of MRAP expression in Y1 cells transfected with 25 nM each of the four MRAP siRNA duplexes. GAPDH was used as an internal control. C, Luciferase assay to measure MC2R signaling in Y1 cells expressing 25 nM MRAP siRNAs. Cyclophilin (Cyclo) was used as a control. Stimulations were carried out 24 and 48 h after transfection with 10^{-6} M ACTH. Data expressed as a percentage of the Cyclo-transfected and forskolin-stimulated control (100%) (n = 4). *, $P < 0.05$.

system (Promega), we found a significant reduction in the MC2R signaling in cells transfected with MRAP siRNA, compared with control cells transfected with cyclophilin siRNA, at 24 and 48 h after transfection (Fig. 1C). This provided additional support that MRAP may be involved in the functional expression of MC2R, consistent with our previous findings in a transfection-based model.

These experiments were carried out using transient transfection of synthetic MRAP siRNA duplexes, and therefore, to ensure that all cells expressed MRAP siRNA and also to obtain long-term knockdown of MRAP, we explored a plasmid-based approach to create stable cell lines. Duplex 6 tar-

geting the nucleotide sequence 5'-TCACCAGCTATGAG-TATTA-3' located at nucleotides 31–50 in the N-terminal coding region of MRAP was used to design oligonucleotides encoding MRAP shRNAs. The sense and antisense template oligonucleotides were designed to encode a hairpin structure with a 19-mer stem and a 2-nt overhang derived from the 21-nt mRNA target site. They were cloned into the pSilencer 4.1 CMV-Neo expression vector and transfected into Y1 cells to select stable cell lines using G418. RT-PCR analysis showed different levels of MRAP knockdown in several clonal cell lines, with clone 26 showing the most efficient knockdown of MRAP expression (Fig. 2A).

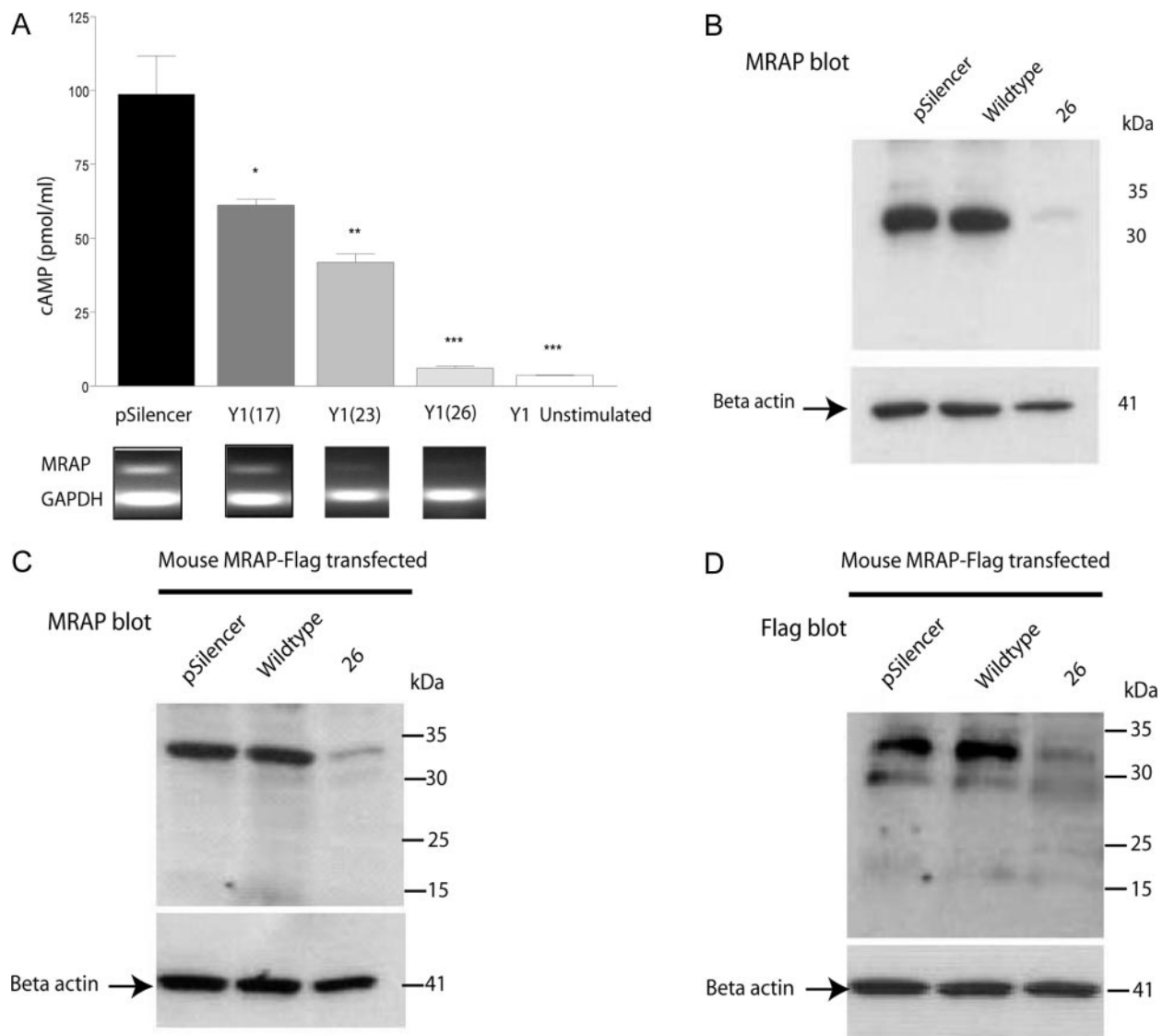


FIG. 2. Stable knockdown of MRAP using MRAP shRNA. A, cAMP response in clonal cell lines expressing MRAP shRNA. Stimulations were carried out in the presence of 3-isobutyl-1-methyl-xanthine (10^{-5} M) with 10^{-6} M ACTH for 30 min. Y1 wild-type unstimulated cells were used as a control. Results are the mean of three individual experiments each performed in duplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with pSilencer control). Shown in the panel below is the expression of MRAP in each of the clonal cell lines using RT-PCR. GAPDH was used as an internal control. B, Immunoblot to demonstrate the knockdown of MRAP protein expression. Whole-cell lysates from wild-type Y1 cells, Y1 cells transfected with the pSilencer 4.1 CMV-Neo vector, and Y1 knockdown clone 26 cells were blotted with anti-MRAP antibody. β -Actin was used as a loading control. C, Overexpression of mouse MRAP-Flag into cells expressing mouse MRAP shRNAs. Immunoblotting was carried out using the MRAP antibody (B) and the Flag antibody (D). Wild-type Y1 cells and a clonal cell line expressing pSilencer 4.1 CMV-Neo with a nonspecific shRNA were used as controls.

We therefore used this cell line to perform Western blotting to demonstrate the knockdown of MRAP protein expression. Lysates from cells transfected with pSilencer 4.1CMV-Neo control and wild-type Y1 cells were subjected to SDS-PAGE along with lysates from clone 26. Western blotting using the anti-MRAP polyclonal antibody (7819) (Fig. 2B) showed a predominant protein band of approximately 32 kDa in the pSilencer and wild-type Y1 cells. This 32-kDa band appeared to be markedly reduced in the lane containing clone 26.

The effect of MRAP silencing on MC2R signaling was determined by measuring cAMP production in response to ACTH (10^{-6} M). Three of the cell lines expressing MRAP shRNAs (clones 17, 23, and 26) were studied. The stably selected cell line transfected with pSilencer 4.1 CMV-Neo-negative control, which contains a siRNA sequence that shows no homology to any gene in the databases, was used as a control (Fig. 2A). The three clonal cell lines expressing MRAP shRNAs, which showed variable degrees of MRAP knockdown, showed a significant reduction of cAMP production in response to ACTH. Clone 26, which showed the most effective knockdown of MRAP expression, showed cAMP levels that did not increase significantly above basal levels in response to ACTH, confirming that MRAP needs to be expressed in cells for the MC2R to become functional.

Rescue of MRAP function

Because the possibility remained that the MRAP shRNA was having a nonspecific effect on ACTH signaling, an attempt was made to rescue the knockdown by transfection of MRAP. mMRAP-Flag was transfected into clone 26 cells. Immunoblotting using either anti-MRAP 7819 or anti-Flag antibody did not show that this maneuver had overcome the knockdown of MRAP protein expression (Fig. 2C). This was not unexpected because the mouse MRAP shRNAs expressed in this clonal cell line is able to degrade the introduced mouse MRAP transcript in a sequence-specific manner, leading to the failure of the MRAP protein to be expressed. Immunoblotting performed using the Flag antibody confirmed the failure of the MRAP protein to be expressed in the clonal cell line (Fig. 2D).

To determine whether the loss of MC2R signaling could be restored by the reintroduction of MRAP, the clonal cell line 26 was transfected with hMRAP-pcDNA3.1 that is resistant to silencing by the mouse MRAP shRNA sequence. It is well documented that the target gene recognition is highly sequence specific because even 1- or 2-bp mismatches between the siRNA and the target gene could greatly reduce or even abolish the silencing effect (24). The human MRAP sequence shows 10 of 19 mismatches to the mouse MRAP shRNA sequence and therefore would escape the knockdown by the mouse MRAP shRNA. This construct was transfected into cells along with the pRL-CMV *Renilla* luciferase plasmid, and α -GSU-846 luciferase plasmids. Forty-eight hours after transfection, cells were stimulated with 10^{-6} M ACTH for 6 h before harvesting for luciferase assays. Figure 3 shows that cells rescued with the hMRAP regain significant cAMP signaling ability. The failure to completely restore the signal to

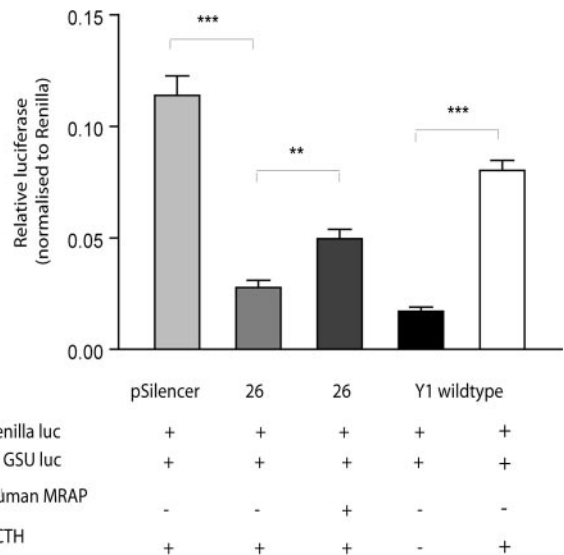


FIG. 3. Luciferase assay to assess the effect of MRAP expression on MC2R signaling in response to ACTH (10^{-6} M) in the clonal cell line 26 transfected with the human MRAP sequence ($n = 3$). **, $P < 0.01$; ***, $P < 0.001$.

wild-type levels may reflect the sequence divergence between hMRAP and the endogenous mouse MRAP.

Dimerization of MRAP

An interesting observation made with regard to the MRAP protein in these experiments was that the predominant immunoreactive species migrated at approximately 32 kDa on Western blots using Y1 adrenocortical cells after transfection of hMRAP. The monomeric structure of mouse MRAP with a predicted molecular mass of 14.1 kDa was occasionally observed faintly on Western blots (*e.g.* Fig. 2, C and D). However, the 32-kDa band, which is constantly detected in Y1 cells and extracts from adrenal glands (data not shown), was suspected to be due to the dimerization of MRAP in a manner that is resistant to denaturing and reducing conditions.

We have previously shown that the overexpression of MRAP into nonadrenal cells such as SKN-SH and CHO cells that lack endogenous MRAP results in the detection of a band that corresponds to the size of the predicted MRAP monomer as well as a significantly higher molecular weight band (12). To probe the nature of the higher molecular weight band, we transfected CHO cells with the MRAP-HA construct and immunoprecipitated cell lysates using anti-HA conjugated beads. This was analyzed by immunoblotting or colloidal Coomassie-blue staining (Fig. 4A). The additional protein bands that appeared in the cells transfected with MRAP-HA were characterized by mass spectrometry. Two MRAP peptides were identified in both gel slices (corresponding to the monomer and dimer of MRAP) (Fig. 4A) having Mascot scores greater than 40 ($P < 0.05$). These data support our hypothesis that MRAP may exist as a dimer.

We also cotransfected CHO cells with MRAP-Flag, MRAP-HA, or MRAP-Flag and MRAP-HA constructs. Immunoprecipitation of MRAP was carried out using anti-HA conjugated beads, and MRAP was detected by immunoblotting

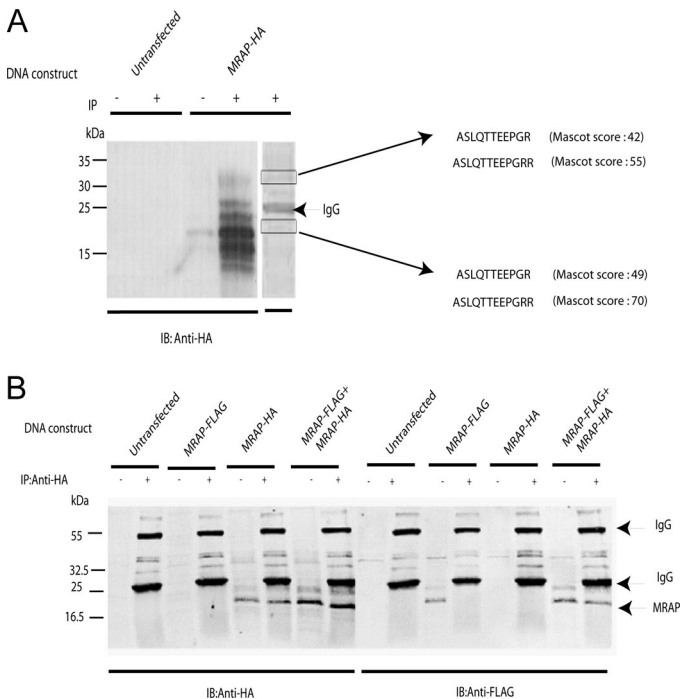


FIG. 4. Dimerization of MRAP. **A**, Comparison of protein profile between nontransfected and MRAP-HA-transfected CHO cells. Proteins were immunoprecipitated using the anti-HA conjugated IgG beads, and the protein profiles were obtained by immunoblot analysis using the HA antibody or by colloidal Coomassie blue staining. The boxed bands were excised and subjected to in-gel digestion with trypsin and mass spectrometry analysis. The identified ionic peptides are listed together with their Mascot scores. **B**, Interaction between MRAP-Flag and MRAP-HA constructs by coimmunoprecipitation. Cells were transfected with MRAP-Flag, MRAP-HA, or MRAP-Flag and MRAP-HA constructs. Immunoprecipitations were performed using the anti-HA-conjugated beads and after separation by SDS-PAGE were immunoblotted with anti-Flag or anti-HA antibodies, as indicated. The lanes without the immunoprecipitations are cell lysates. The arrowheads depict the MRAP protein and the light (~25 kDa) and heavy IgG (~55 kDa) bands.

with the Flag antibody. In cells cotransfected with the two proteins immunoprecipitation with the anti-HA conjugated beads coprecipitated MRAP-Flag, showing that the MRAP-Flag and MRAP-HA tagged proteins were able to interact with each other and potentially form a dimeric structure (Fig. 4B). The same observation was made when immunoprecipitations were performed using the anti-Flag antibody and immunoblotted with the anti-HA antibody on lysates from cells cotransfected with the MRAP-Flag and MRAP-HA constructs (data not shown). Therefore, we hypothesize that MRAP is able to exist as a dimer in CHO cells when exogenously expressed and that the endogenous 32-kDa protein that was mainly detected in the Y1 adrenocortical cell line is likely to be due to the dimerization of MRAP. It is not clear why the majority of the protein (endogenous or transfected) runs at the higher molecular weight in Y1 cells and at the lower size in nonadrenal cells. It is tempting to speculate that some further posttranslational modification unique to the adrenal cell line promotes formation of the stable dimeric structure despite strong denaturing and reducing conditions.

There are some interesting, if superficial, parallels here with the RAMP proteins that associate with the calcitonin

receptor and calcitonin-like receptor to create calcitonin gene-related peptide, adrenomedullin, calcitonin, or amylin receptors (18, 19, 25). RAMP1 has been shown to exist as a disulfide-linked homodimer in the absence of receptor and to reside in the endoplasmic reticulum. Only in the presence of receptor does this homodimer dissociate to form RAMP1-receptor heterodimers, which translocate to the cell surface (26). The MRAP data are distinct in that MRAP can translocate to the plasma membrane without receptor (12) and that the MRAP homodimer persists in the presence of the MC2R, despite reducing conditions. It is not yet clear whether MRAP remains in a dimeric form when complexed with the MC2R in adrenal cells.

In summary, we have shown that MRAP plays an essential role in the function of MC2R. This role requires the direct protein-protein interaction between MRAP and the receptor. Whereas MRAP demonstrates several functional similarities to other receptor accessory proteins, its distinct features suggest a number of unique aspects. It remains to be seen whether other melanocortin receptors have similar requirements for an accessory protein.

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