

hMRAPa increases α MSH-induced hMC1R and hMC3R functional coupling and hMC4R constitutive activity

Emma I Kay¹, Rikus Botha¹, Johanna M Montgomery¹ and Kathleen G Mountjoy^{1,2}

Departments of ¹Physiology ²Molecular Medicine and Pathology, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

Correspondence
should be addressed to
K G Mountjoy
Email
k.mountjoy@auckland.ac.nz

Abstract

Human melanocortin 2 receptor accessory protein (hMRAPa) is hypothesised to have functions beyond promoting human melanocortin 2 receptor (hMC2R) functional expression. To understand these potential functions, we exogenously co-expressed hMRAPa-FLAG with each of the five hMCR subtypes in HEK293 cells and assessed hMCR subtype coupling to adenylyl cyclase. We also co-expressed each HA-hMCR subtype with hMRAPa-FLAG to investigate their subcellular localisation. hMRAPa-FLAG enhanced α -melanocyte stimulating hormone (α -MSH)-stimulated hMC1R and hMC3R but reduced NDP- α -MSH-stimulated hMC5R, maximum coupling to adenylyl cyclase. hMRAPa-FLAG specifically increased hMC4R constitutive coupling to adenylyl cyclase despite not co-localising with the HA-hMC4R in the cell membrane. hMRAPa-FLAG co-localised with HA-hMC1R or HA-hMC3R in the perinuclear region, in cytoplasmic vesicles and at the plasma membrane, while it co-localised with HA-hMC2R, HA-hMC4R and HA-hMC5R predominantly in cytoplasmic vesicles. These diverse effects of hMRAPa indicate that hMRAPa could be an important modulator of the central and peripheral melanocortin systems if hMRAPa and any hMCR subtype co-express in the same cell.

Key Words

- ▶ GPCR accessory protein
- ▶ constitutive activity
- ▶ subcellular localisation
- ▶ melanocortin 4 receptor

*Journal of Molecular
Endocrinology*
(2013) 50, 203–215

Introduction

The five human melanocortin receptor (hMCR) subtypes are implicated in a wide range of physiological processes (Getting 2006). Only the hMC2R is dependent on an accessory protein for its functional expression both in cultured cells and *in vivo*. Human melanocortin receptor accessory protein (hMRAP1) promotes hMC2R post-translational modification and maturation, receptor cell surface expression and hMC2R signalling in response to physiological concentrations of ACTH, resulting in glucocorticoid production (Roy *et al.* 2007, Sebag & Hinkle

2007, Webb *et al.* 2009). Three hMRAP proteins have been identified to date. The *hMRAP1* gene is located on chromosome 21 and gives rise to two alternatively spliced hMRAP1 isoforms known as hMRAPa and hMRAPb, both of which promote hMC2R functional expression (Metherell *et al.* 2005, Roy *et al.* 2007). *hMRAP2*, a paralog of *hMRAP1*, is located on chromosome 6 but the physiological function of hMRAP2 is unknown (Chan *et al.* 2009). hMRAP2 promotes hMC2R cell surface expression but hMRAP2 only promotes hMC2R signalling

in response to supraphysiological ACTH concentrations (Sebag & Hinkle 2010).

hMRAPa is hypothesised to have additional functions to promoting hMC2R functional expression. *hMRAP1* mRNA is widely expressed compared with *hMC2R* mRNA, which is restricted to the adrenal and pituitary glands (Metherell *et al.* 2005, Cooray *et al.* 2008, Chan *et al.* 2009, Sebag & Hinkle 2009a,b). hMRAPa interacts with all five hMCR subtypes when it is co-expressed with these receptors in heterologous cells and it specifically alters hMC4R molecular mass and complex N-linked glycosylation (Chan *et al.* 2009, Kay *et al.* 2013). Furthermore, hMRAPa expression in Chinese hamster ovary (CHO) cells influenced hMC4R and hMC5R but not hMC1R and hMC3R functional expression, in response to a single nanomolar concentration of the superpotent synthetic melanocortin peptide [Nle4, D-Phe7]- α -melanocyte stimulating hormone (NDP- α -MSH) (Chan *et al.* 2009). However, Hinkle *et al.* (2011) performed full NDP- α -MSH concentration–response curves and observed no effect of hMRAPa on hMC4R coupling to adenylyl cyclase when hMC4R and hMRAPa were transiently co-expressed in CHO cells. Hence, there is conflicting data on the effects of hMRAPa on NDP- α -MSH-induced hMC4R coupling to adenylyl cyclase and the effects of hMRAPa on endogenous melanocortin peptide stimulated coupling of the hMC1R, hMC3R and hMC4R to adenylyl cyclase have not been examined. Therefore, the effects of hMRAPa on functional expression of hMCR subtypes other than the hMC2R warrant further investigation, especially given the physiological significance of the hMCR family.

In this study, we investigated the effects of hMRAPa co-expression on hMC1R, hMC3R or hMC4R coupling to adenylyl cyclase in HEK293 cells, in response to a range of concentrations of the endogenous melanocortin peptide, α -MSH. NDP- α -MSH was used to stimulate hMC5R coupling to adenylyl cyclase. We previously demonstrated that hMRAPa interacts with the calcitonin receptor-like receptor (hCL) *in vitro*, and therefore, we also investigated potential hMRAPa effects on calcitonin gene-related peptide (α -CGRP)-stimulated coupling of the hCL to adenylyl cyclase (Kay *et al.* 2013). We then characterised the effects of hMRAPa on the subcellular localisation of hMC1R, hMC3R, hMC4R and hMC5R. We here show that hMRAPa co-expression with each hMCR subtype differentially influences functional expression of these receptors in ways that have not previously been described but has no effect on hCL functional expression.

Materials and methods

Peptides

ACTH_{1–24}, ACTH_{1–39}, α -MSH and α -CGRP were purchased from Bachem (Bubendorf, Switzerland).

Construction of recombinant DNAs

N-terminal HA-tagged melanocortin receptor constructs were purchased from the Missouri S&T cDNA Resource Centre (Rolla, MO, USA). N-terminal FLAG tagged receptor activity modifying protein 1 (RAMP1) in pcDNA 3.1 was a gift. hMC1R, hMC2R, hMC3R, hMC4R and hMC5R in pcDNA Neo, hMRAPa-FLAG in pcDNA 3.1, mMRAPa-FLAG in pcDNA 3.1 and HA-tagged hCL in pcDNA 3.1 have previously been described (Mountjoy *et al.* 1992, 1994, McLatchie *et al.* 1998, Wong *et al.* 2002, Xu *et al.* 2002, Kay *et al.* 2013).

RT-PCR analysis of hMRAP subtype and hMC1–5R mRNA expression

Human total RNA for brain, adipose tissue, adrenal gland, ovary and testes was purchased from Clontech Laboratories. Human heart total RNA was purchased from Ambion (Austin, TX, USA). Total RNA was extracted from HEK293 cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Each RNA was reverse transcribed to produce cDNA with Superscript III reverse transcriptase (Invitrogen Corporation) in a total volume of 20 μ l. For each hMCR subtype RNA sample, a transcription reaction was also prepared without reverse transcriptase (RT-controls). Primer sequences for RT-PCR for hMC1–5R, hMRAPa, hMRAPb and hMRAP2 are described in [Supplementary Table S1](#), see section on [supplementary data](#) given at the end of this article. RT-PCR for hMC1–5R was performed on 2 μ l human cDNA using FastStart Taq Polymerase (Roche Applied Science) and the following conditions: denature (98 °C, 5 min; then 95 °C, 30 s), anneal (62 °C, MC1R; 53 °C, MC2R; 56 °C, MC3R and MC4R; 65 °C, MC5R; 30 s), elongate (72 °C, 70 s) for 34 cycles and then a final elongation at 72 °C, for 7 min. hMC1–5R PCR was performed on the RT-cDNA for each tissue to check for genomic DNA contamination as the coding hMCR subtypes are intronless genes. RT-PCR for hMRAPa, hMRAPb and hMRAP2 was performed on 2 μ l HEK293 cDNA using iProof High Fidelity DNA polymerase and the following conditions: denature (98 °C, 5 s), anneal (65 °C,

30 s), elongate (72 °C, 10 s) for 35 cycles and then one elongation at 72 °C, for 7 min. RT-control PCRs were not performed for hMRAPs as the primers were designed to target intron–exon boundaries in the *hMRAP* and *hMRAP2* genes. The products of the PCRs were analysed by electrophoresis on 1.2% agarose/TAE gels.

Cell culture and transfection

HEK293 human embryonic kidney cells were grown in DMEM supplemented with 10% (v/v) newborn calf serum and 1% (v/v) penicillin and streptomycin (Invitrogen Corporation) at 37 °C under 5% CO₂. For transient transfections, 75 000 HEK293 cells/well were seeded into 24-well plates. After 48 h when the cells were at 30–50% confluency, they were transfected with 1.5 µl FuGENE 6 (Roche Applied Science) for every 0.5 µg plasmid DNA. Transient transfections were incubated for 48 h at 37 °C under 5% CO₂. To generate stable cell lines, two million HEK293 cells were seeded per 10 cm dish and 24 h later when the cells were 30–50% confluent they were transfected with 30 µl FuGENE 6 (Roche Applied Science) and 9 µg plasmid DNA. Transfected cells were maintained for 48 h at 37 °C under 5% CO₂ before commencing the antibiotic selection with 1 mg/ml G418 (Invitrogen Corporation). Transfected cells were selected with G418 for 2 weeks until stable colonies had formed. Stable colonies were trypsinised and the cells were then maintained as monolayers of cultured cells in the presence of 500 µg/ml G418 for subsequent experiments.

Measurement of adenylyl cyclase activity

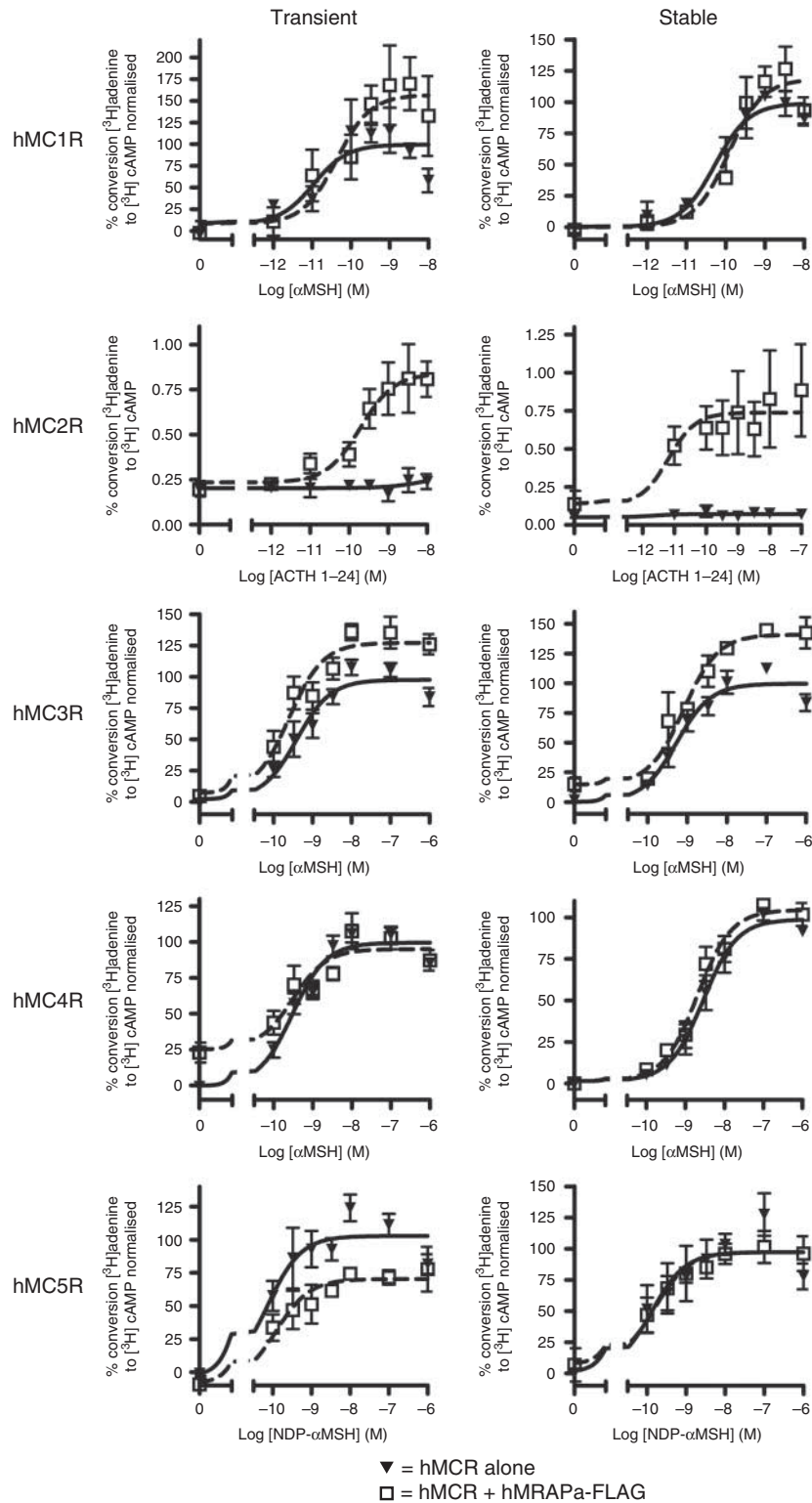
Different methods of transfection were used to assess hMCR subtype or HA-hCL coupling to adenylyl cyclase in the presence or absence of hMRAPa-FLAG. HA-hMC1R, HA-hMC3R, HA-hMC4R and HA-hMC5R functions were assessed by transiently transfecting each subtype in parallel with untagged hMCR subtypes in HEK293 cells. HA-hMC2R function was assessed by transiently transfecting HA-hMC2R or hMC2R in parallel into HEK293 cells stably expressing hMRAPa-FLAG. Neither the HA-tag on any of the hMCR subtypes nor the FLAG tag on hMRAPa altered protein function when compared with untagged hMCRs or hMRAPa respectively (Supplementary Figure S1 and Supplementary Table S2, see section on supplementary data given at the end of this article). For the HA-hMC1R comparison with untagged hMC1R, both HA-hMC1R and untagged hMC1R were constitutively

active (Supplementary Figure S1A) and 0.5 µg DNA of these constructs were used for these transfections. Others have observed constitutive activity of hMC1R in HEK293T cells and this constitutive activity increased with increasing amounts of transfected hMC1R plasmid DNA (Sanchez-Mas *et al.* 2004). Untagged hMCR subtypes were used to characterise the effects of hMRAPa-FLAG on hMCR subtype coupling to adenylyl cyclase and 0.25 µg of each DNA was used for transfections. Untagged hMCR subtypes were transiently transfected together with hMRAPa-FLAG or with the empty vector, pcDNA 3.1, into HEK293 cells. hMCR stably transfected HEK293 cells were transiently transfected with hMRAPa-FLAG or pcDNA 3.1. HA-hCL was transiently co-transfected with hMRAPa-FLAG, FLAG-hRAMP1 or pcDNA 3.1 into HEK293 cells.

Transfected cells in 24-well plates were equilibrated with [³H]adenine (2.5 µCi/ml) for 2 h at 37 °C under 5% CO₂. Cells transfected with hMCRs or HA-hCL were washed once with PBS and then incubated for 1 h with increasing concentrations of either ACTH_{1–24}, ACTH_{1–39}, α-MSH or α-CGRP at 37 °C under 5% CO₂. NDP-α-MSH was used to stimulate the hMC5R as the physiological agonist for this receptor has not been characterised (Bednarek *et al.* 2007). HA-hD2R transfected cells were washed once with PBS and incubated in DMEM without serum for 20 min and then incubated with 10 µM forskolin (Sigma–Aldrich) and increasing concentrations of quinpirole at 37 °C under 5% CO₂. All peptides were diluted in DMEM +0.5 mM isobutylmethylxanthine +0.1% BSA. Adenylyl cyclase activity was measured as accumulation of [³H]cAMP as described previously (Mountjoy *et al.* 1999).

Immunocytochemistry and microscopy

HEK293 cells were seeded onto glass coverslips in 24-well plates which had been soaked overnight in 75% acetic acid, washed thoroughly with milli Q water and then coated with poly-L-lysine (0.2 mg/ml in PBS) overnight at 37 °C. After 24–48 h when the cells were 30–50% confluent, they were transiently transfected with HA-tagged MCR subtypes with hMRAPa-FLAG or pcDNA 3.1. Forty-eight hours later, the cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and then were washed twice with PBS. The cells transfected with HA-hMC3R and not permeabilised were fixed with 2% paraformaldehyde in PBS. For permeabilisation, cells were washed with PBS +0.25% Triton X-100, washed with PBS and then blocked with PBS +0.1% BSA for 20 min at room temperature. The



cells were incubated with mouse anti-HA monoclonal (1:500 in PBS) and rabbit anti-FLAG polyclonal (1:10 000 in PBS) antibodies overnight at 4 °C. The cells were then washed with PBS three times for 5-min intervals. Fluorescent secondary antibody detection was performed using goat anti-mouse FITC (1:1000 in PBS +0.1% BSA) and goat anti-rabbit Alexa 568 (1:1000 in PBS +0.1% BSA) secondary antibodies (Invitrogen Corporation) for 2 h at room temperature. Cells were washed with PBS three times for 5-min intervals and then each coverslip was mounted onto a clean glass slide with Citifluor AF1 anti-fade reagent (Citifluor, Leicester, UK). Slides were left to cure overnight at 4 °C in the dark before microscopy.

Confocal microscopy was performed to determine HA-hMCR subtype and hMRAPa-FLAG subcellular localisation. Z-stack images at 1 µm intervals were acquired using a 60× oil immersion lens on an Olympus TE1000 confocal microscope using 3× optical zoom and Olympus Fluoview 1.7 acquisition software. A minimum of three Z-stacks of images were acquired for each transfection. Maximal intensity z-projections were processed in Image J (Rasband 1997–2011, In <http://imagej.nih.gov/ij/>).

Widefield fluorescence images for counting cells and nuclei were acquired with a Zeiss Axioplan 2 upright microscope and a 60× oil immersion lens using Metamorph software. Fluorescent cells were counted to determine the percentage of cells expressing HA-hMC4R, hMRAPa-FLAG or HA-hMC4R and hMRAPa-FLAG under different transfection conditions. Nuclei were counter-stained with 20 µM Hoechst 33258 (Sigma–Aldrich) in PBS to quantify the total cell number. For each transfection, a minimum of six fields of view were acquired and exposure times were kept constant. Counts of nuclei in each field of view, excluding those on the edges of the image, were performed using Image J to determine total cell number. Cells expressing both HA-hMC4R and hMRAPa-FLAG, or either of these proteins, were counted and the number was expressed as a percentage of the total cell number. Three independent experiments were performed.

Statistical analyses

GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to prepare graphs and to perform statistical analysis. Concentration–response curves were fitted to raw adenylyl cyclase data (not shown except for hMC2R) to determine maximum and minimum response values for each hMCR subtype expressed alone. For each independent experiment, adenylyl cyclase activity for the hMCR+hMRAPa-FLAG co-transfection was normalised to the minimum and maximum response best-fit values for the same hMCR subtype expressed alone. Normalised data from at least three independent experiments were pooled and sigmoidal concentration–response curves were fitted using GraphPad Prism 5.0. These pooled, normalised curves were used to calculate minimum and maximum responses and EC₅₀ values for hMCR subtype coupling to adenylyl cyclase, all of which were used for statistical comparisons. Statistical significance was determined using the non-parametric sum of squares *f* test. A *P* value of <0.05 was considered significant.

Results

hMC2R did not functionally express in HEK293 cells in the absence of co-transfected hMRAPa

To validate HEK293 cells as a suitable model for the investigation of MRAP interactions with the melanocortin receptors, we examined the presence of *MRAP* mRNA expression in our HEK293 cell line. Using RT-PCR, we readily detected *hMRAP2* but only weakly detected *hMRAPa* and did not detect any *hMRAPb* mRNA (Supplementary Figure S2, see section on supplementary data given at the end of this article). If endogenous MRAP proteins were expressed in our HEK293 cells, then they were not present at sufficient levels to promote hMC2R functional expression (Fig. 1). HEK293 cells were therefore used as a model to study effects of transfected hMRAPa on transfected hMCR subtypes.

Figure 1

Effects of hMRAPa on hMCR subtype coupling to adenylyl cyclase. Each untagged hMCR subtype was expressed transiently with transient hMRAPa-FLAG or stably with transient hMRAPa-FLAG. hMCR coupling to adenylyl cyclase was stimulated with increasing concentrations of α-MSH (hMC1R, hMC3R and hMC4R), ACTH (hMC2R) or NDP-α-MSH (hMC5R) for 1 h and adenylyl cyclase activity measured. For hMC1R, hMC3R, hMC4R and

hMC5R, normalised data from at least three independent experiments performed in duplicate were pooled and plotted as mean ± s.e.m. Best-fit values for baseline and maximum responses and EC₅₀ for these curves are shown in Table 1. For the hMC2R, raw data from three independent experiments performed in duplicate were pooled and plotted as mean ± s.e.m.

hMRAPa-FLAG had differential effects on hMCR subtype coupling to adenylyl cyclase

To characterise the effects of hMRAPa on hMCR signalling, we performed both transient and stable transfections of each untagged hMCR subtype with or without transiently transfected hMRAPa-FLAG and measured adenylyl cyclase activity. hMRAPa-FLAG significantly increased α -MSH-induced hMC1R maximum coupling to adenylyl cyclase for both the transiently transfected hMC1R (~57% increase, $P < 0.0001$) and the stably transfected hMC1R (~18% increase, $P < 0.04$), compared with the hMC1R expressed alone (Fig. 1 and Table 1). ACTH₁₋₂₄ stimulated transiently or stably transfected hMC2R coupling to adenylyl cyclase when hMRAPa-FLAG was co-expressed with the hMC2R (Fig. 1). hMRAPa-FLAG significantly increased α -MSH-induced hMC3R maximum coupling to adenylyl cyclase for both transiently transfected hMC3R (~30% increase, $P < 0.0003$) and stably transfected hMC3R (~40% increase, $P < 0.0001$), compared with hMC3R expressed alone (Fig. 1 and Table 1). hMRAPa-FLAG significantly increased baseline coupling of the transiently transfected hMC4R to adenylyl cyclase (~25% increase, $P < 0.03$) compared with transient

expression of hMC4R alone (Fig. 1 and Table 1). Increased baseline coupling of hMC4R to adenylyl cyclase was not observed when the hMC4R was stably expressed with transiently transfected hMRAPa-FLAG (Fig. 1 and Table 1). hMRAPa-FLAG significantly decreased NDP- α -MSH-stimulated hMC5R maximum coupling to adenylyl cyclase (~30% decrease, $P < 0.0004$) compared with hMC5R expressed alone (Fig. 1 and Table 1). There was no effect on the hMC5R maximum response when hMC5R was stably transfected together with transient hMRAPa-FLAG (Fig. 1 and Table 1).

hMRAPa-FLAG did not promote α CGRP-induced HA-hCL activity in HEK293 cells

We previously identified that in addition to interacting with hMCR subtypes, hMRAPa-FLAG interacts with HA-hCL (Kay *et al.* 2013). We therefore examined effects of hMRAPa-FLAG on the adenylyl cyclase coupling of this receptor to determine whether hMRAPa can influence functional expression of G-protein-coupled receptors (GPCRs) other than the MCRs. FLAG-hRAMP1 but not hMRAPa-FLAG co-expression with the HA-hCL promoted HA-hCL coupling to adenylyl cyclase in response

Table 1 Statistical analysis of effects of hMRAPa-FLAG on hMCR coupling to adenylyl cyclase in HEK293 cells. Mean \pm s.e.m. values are shown for baseline and maximum responses and EC₅₀ values are shown with 95% CIs in brackets

Transfection	Baseline response	Maximum response	EC ₅₀ (nM)
Transient hMC1R + pcDNA 3.1	5.06 \pm 16.5	99.5 \pm 9.70	0.010 (0.001, 0.074)
hMRAPa-FLAG	10.9 \pm 20.5	158 \pm 17.9 [†]	0.048 (0.007, 0.342)
Stable hMC1R + pcDNA 3.1	0.08 \pm 4.68	99.0 \pm 4.19	0.053 (0.027, 0.104)
hMRAPa-FLAG	-0.23 \pm 7.94	118 \pm 8.68*	0.118 (0.047, 0.296)
Transient hMC3R + pcDNA 3.1	2.19 \pm 7.70	97.6 \pm 4.74	0.393 (0.192, 0.806)
hMRAPa-FLAG	7.33 \pm 10.1	127 \pm 5.63 [†]	0.253 (0.125, 0.514)
Stable hMC3R + pcDNA 3.1	0.23 \pm 6.70	99.7 \pm 4.19	0.533 (0.282, 1.009)
hMRAPa-FLAG	14.8 \pm 8.92	141 \pm 6.68 [†]	0.811 (0.396, 1.661)
Transient hMC4R + pcDNA 3.1	-0.04 \pm 6.42	99.6 \pm 3.72	0.304 (0.17, 0.534)
hMRAPa-FLAG	25.2 \pm 8.89*	95.1 \pm 5.18	0.309 (0.102, 0.940)
Stable hMC4R + pcDNA 3.1	0.60 \pm 3.84	98.8 \pm 4.51	2.780 (1.695, 4.559)
hMRAPa-FLAG	2.13 \pm 4.61	105 \pm 4.57	2.084 (1.226, 3.541)
Transient hMC5R + pcDNA 3.1	-2.31 \pm 13.1	103 \pm 6.05	0.074 (0.025, 0.218)
hMRAPa-FLAG	-8.02 \pm 10.1	70.4 \pm 4.95 [†]	0.123 (0.042, 0.356)
Stable hMC5R + pcDNA 3.1	2.10 \pm 14.1	99.4 \pm 7.05	0.144 (0.044, 0.474)
hMRAPa-FLAG	7.19 \pm 13.1	95.2 \pm 6.53	0.143 (0.042, 0.485)

Statistically significant differences for each hMCR + hMRAPa-FLAG compared to the same hMCR + pcDNA 3.1 were * $P < 0.04$; [†] $P < 0.0004$; [‡] $P < 0.00001$.

to α CGRP stimulation (Supplementary Figure S3, see section on supplementary data given at the end of this article).

Different percentages of cells expressed only HA-hMC4R when the HA-hMC4R was stably or transiently co-expressed with transiently transfected hMRAPa-FLAG

To determine whether the different responses observed between transiently and stably transfected cells could be explained by varying proportions of cells expressing each recombinant DNA construct, we used fluorescence microscopy to determine the percentage of cells expressing HA-hMC4R alone, hMRAPa-FLAG alone or both HA-hMC4R and hMRAPa-FLAG. In cells stably transfected with HA-hMC4R alone, more cells expressed HA-hMC4R compared to when HA-hMC4R was transiently transfected alone. The low apparent transfection efficiencies can be explained by under detection of HA-hMC4R by our anti-HA antibody. When the HA-hMC4R was stably transfected with transient hMRAPa-FLAG, a higher percentage of the cells expressed HA-hMC4R alone compared to when HA-hMC4R was transiently co-transfected with hMRAPa-FLAG. Similar percentages of cells expressed both HA-hMC4R and hMRAPa-FLAG when HA-hMC4R was stably or transiently co-transfected with hMRAPa-FLAG (Supplementary Table S3, see section on supplementary data given at the end of this article).

HA-hMCR subtypes co-localised with hMRAPa-FLAG in different intracellular compartments

The intracellular localisation of each HA-hMCR subtype expressed alone and expressed with hMRAPa-FLAG was characterised in permeabilised HEK293 cells using confocal microscopy. The HA-hMC1R (Fig. 2A), HA-hMC2R (Fig. 2F), HA-hMC3R (Fig. 2K), HA-hMC4R (Fig. 2P) and HA-hMC5R (Fig. 2U) were each observed in the perinuclear region and in cytoplasmic vesicles when they were expressed alone. Prominent perinuclear clusters were observed for the HA-hMC1R, HA-hMC3R and HA-hMC5R, but these were less prominent for the HA-hMC2R and HA-hMC4R. When the HA-hMC1R (Fig. 2B, C, D and E) or HA-hMC3R (Fig. 2L, M, N and O) was co-expressed with hMRAPa-FLAG, they co-localised with hMRAPa-FLAG in large clusters associated with the nucleus and in cytoplasmic vesicles. Only the HA-hMC3R co-localised with hMRAPa-FLAG in the immediate perinuclear region (Fig. 2N). When the HA-hMC2R (Fig. 2G, H, I and J), HA-hMC4R (Fig. 2Q, R, S and T) or HA-hMC5R (Fig. 2V, W,

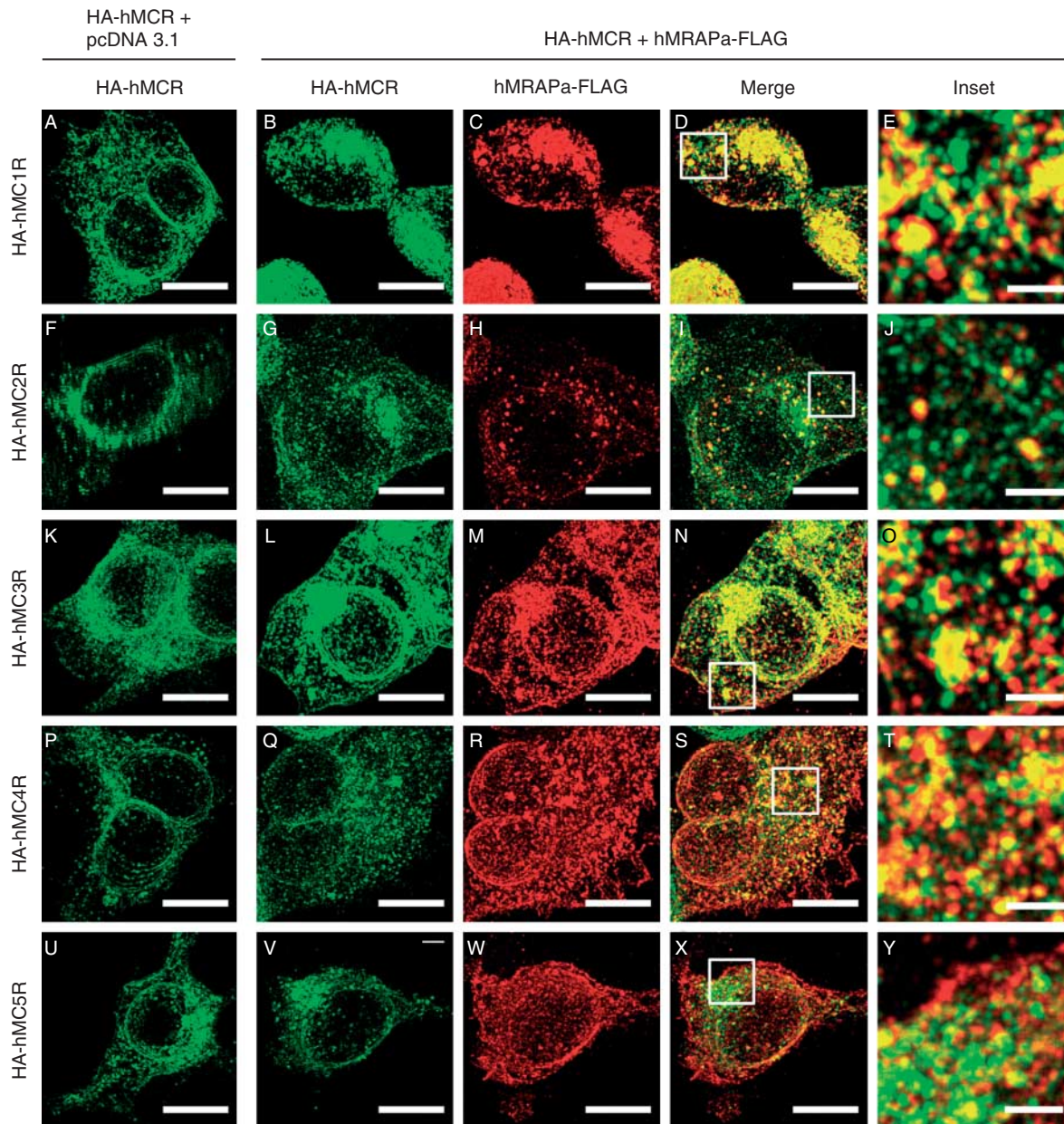
X and Y) was co-expressed with hMRAPa-FLAG, each receptor subtype co-localised with hMRAPa-FLAG in cytoplasmic vesicles.

hMRAPa-FLAG co-localised with HA-hMC1R, HA-hMC2R and HA-hMC3R but not with HA-hMC4R on the cell surface of HEK293 cells, while hMRAPa-FLAG blocked HEK293 cell surface expression of HA-hMC5R

Cell surface expression of HA-hMCRs and hMRAPa-FLAG was characterised in non-permeabilised HEK293 cells by confocal microscopy and under these conditions HA-hMC1R (Fig. 3A), HA-hMC2R (Fig. 3F), HA-hMC4R (Fig. 3P), HA-hMC5R (Fig. 3U) were detected on the surface of cells fixed with 4% paraformaldehyde when the receptors were expressed alone. The HA-hMC3R was detected on the surface of cells fixed with 2% paraformaldehyde when it was expressed alone (Fig. 3K). The HA-hMC1R (Fig. 3B), HA-hMC2R (Fig. 3G), HA-hMC3R (Fig. 3L), HA-hMC4R (Fig. 3Q) and hMRAPa-FLAG (Fig. 3C, H, M, R and W) were still expressed on the cell surface following transient co-expression of each HA-hMCR subtype with hMRAPa-FLAG. HA-hMC1R co-localised with hMRAPa-FLAG in vesicles on or near to the plasma membrane (Fig. 3B, C, D and E). HA-hMC2R either co-localised with hMRAPa-FLAG at the cell surface or it was expressed in close proximity to hMRAPa-FLAG at the cell surface (Fig. 3G, H, I and J). HA-hMC3R co-localised with hMRAPa-FLAG in vesicles on or close to the plasma membrane (Fig. 3L, M, N and O). HA-hMC4R and hMRAPa-FLAG did not co-localise at the cell surface apart from in occasional small clusters (Fig. 3Q, R, S and T). HA-hMC5R cell surface expression was almost undetectable when HA-hMC5R was co-expressed with hMRAPa-FLAG (Fig. 3V) compared with HA-hMC5R expressed alone (Fig. 3U). The HA-hMC5R that did express at the cell surface did not co-localise with hMRAPa-FLAG (Fig. 3X and Y).

hMRAPa and hMC1–5R subtype mRNA are expressed in a range of human tissues

To identify tissues in which hMRAPa and hMC1–5R could be co-expressed, RT-PCR was performed on a range of human cDNAs. *hMRAPa* mRNA expression was detected in brain, adrenal gland, adipose tissue, testes, ovary, heart and placenta (Fig. 4A). *hMC1R* mRNA expression was detected in testes and ovary; *hMC2R* mRNA was detected in adrenal gland, testes and ovary; *hMC3R* mRNA was detected in brain, adrenal gland and testes; *hMC4R* mRNA

**Figure 2**

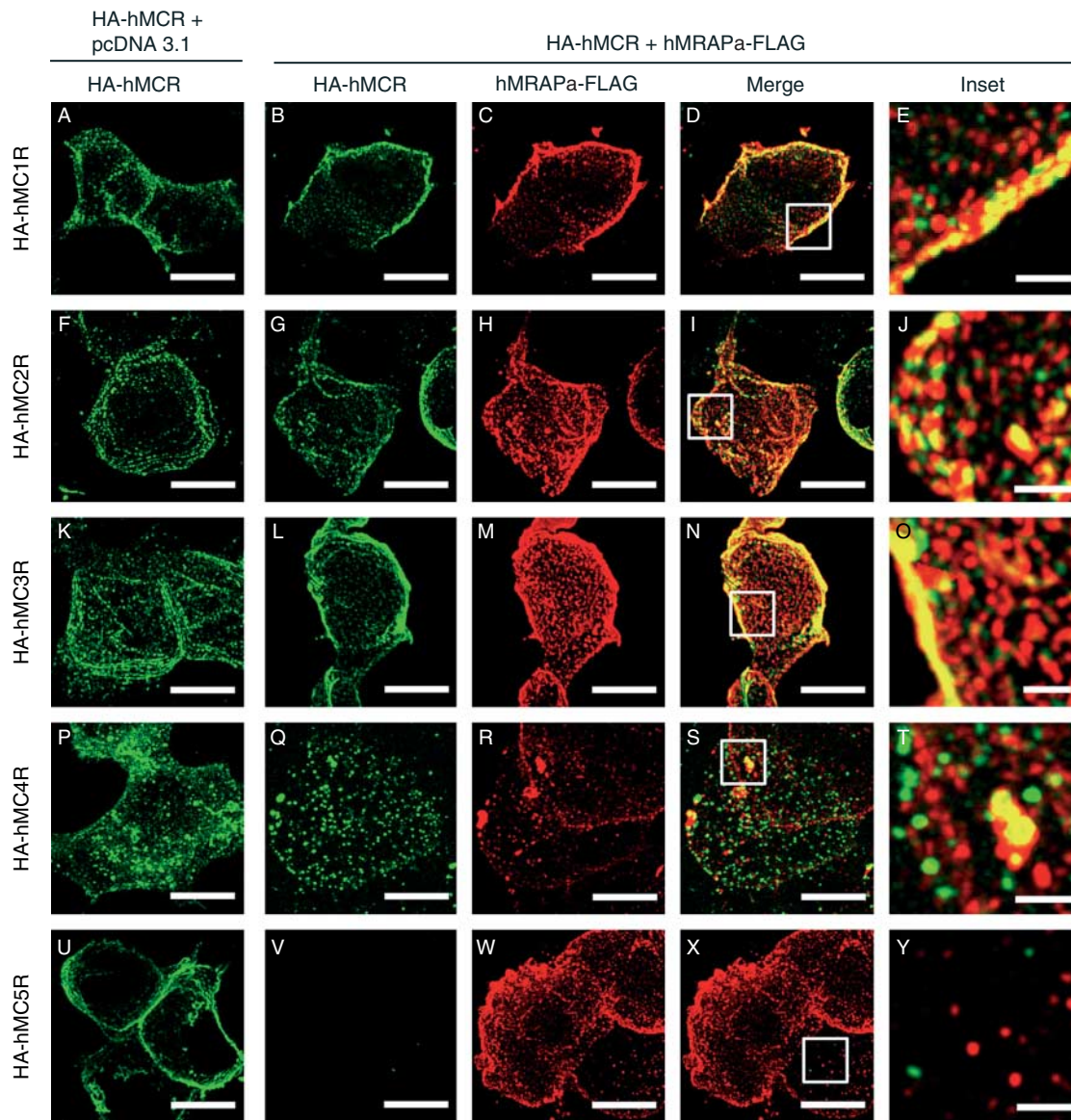
Different patterns of intracellular expression of HA-hMCR subtypes transiently co-transfected with pcDNA 3.1 or hMRAPa-FLAG. HA-hMCR1R + hMRAPa-FLAG (A, B, C, D and E), HA-hMCR2R + hMRAPa-FLAG (F, G, H, I and J), HA-hMCR3R + hMRAPa-FLAG (K, L, M, N and O), HA-hMCR4R + hMRAPa-FLAG

(P, Q, R, S and T) and HA-hMCR5R + hMRAPa-FLAG (U, V, W, X and Y) in permeabilised (intracellular) HEK293 cells using confocal microscopy. Scale bars = 10 and 2 μ m for insets.

was detected in brain, adrenal gland, adipose tissue, testes, ovary and heart; and *hMC5R* mRNA was detected in brain, adrenal gland, adipose tissue and testes (Fig. 4B). *hMC1–5R* mRNA expression was not detected in reverse transcriptase negative (RT⁻) cDNA samples confirming that *hMC1–5R* were amplified from cDNA and not genomic DNA (Fig. 4B).

Discussion

Our study shows that hMRAPa differentially alters the function of all five melanocortin receptor subtypes, in ways that have not previously been described. We show that hMRAPa enhances hMC1R and hMC3R coupling to adenylyl cyclase in response to stimulation with α -MSH.

**Figure 3**

Different patterns of cell surface expression of HA-hMCR subtypes transiently co-transfected with pcDNA 3.1 or hMRAPa-FLAG. HA-hMCR1R + hMRAPa-FLAG (A, B, C, D, and E), HA-hMCR2R + hMRAPa-FLAG (F, G, H, I and J), HA-hMCR3R + hMRAPa-FLAG (K, L, M, N and O), HA-hMCR4R + hMRAPa-FLAG

(P, Q, R, S and T) and HA-hMCR5R + hMRAPa-FLAG (U, V, W and Y) in non-permeabilised (cell surface) HEK293 cells using confocal microscopy. Scale bars = 10 and 2 µm for insets.

Furthermore, we show that hMRAPa co-expression with the hMC4R specifically increases hMC4R constitutive activity despite not co-localising with the hMC4R in the cell membrane. In contrast to the hMC4R, we show that hMRAPa co-localises with hMC1R and hMC3R in the perinuclear region, in the cytoplasm as well as at the cell surface of HEK293 cells. Previously, hMRAPa was shown to be essential for hMC2R functional expression (Roy *et al.* 2007, Sebag & Hinkle 2007, Webb *et al.* 2009). hMRAPa

also blocks hMC5R dimerisation leading to hMC5R retention in the ER and reduced hMC5R cell surface expression (Sebag & Hinkle 2009a). Our data using HEK293 cells as an expression system confirms these findings, but we also show that hMRAPa-induced blockade of hMC5R cell surface trafficking is not absolute, as we observed some limited NDP- α -MSH-stimulated coupling of the hMC5R to adenylyl cyclase when the hMC5R was co-expressed with hMRAPa. These diverse

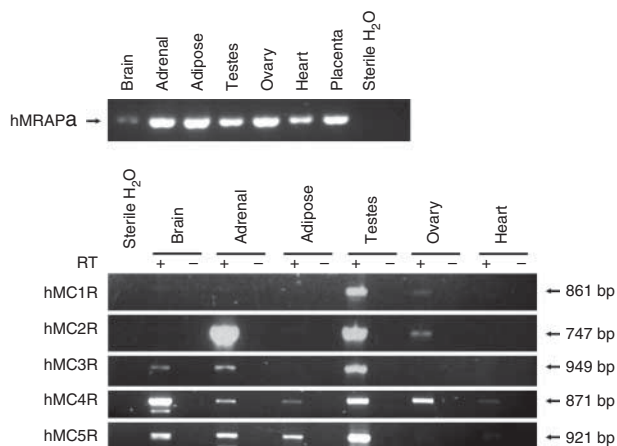


Figure 4

Expression of *hMRAPa* and *hMC1–5R* mRNA in human tissues. To amplify *hMRAPa* from human cDNA, RT-PCR was performed using primers targeting *hMRAP* intron–exon boundaries (A). To amplify *hMC1–5R* from human cDNA, RT-PCR was performed using specific primers targeting full-length coding *hMC1–5R*. RT-PCR using *hMC1–5R* primers was performed in parallel on an RT-control for each human RNA to check for genomic DNA contamination (B).

effects of *hMRAPa* indicate that *hMRAPa* could be an important modulator of the central and peripheral melanocortin systems.

It is intriguing that transient co-expression of *hMRAPa*-FLAG with *hMC4R* increases *hMC4R* constitutive activity. This effect is not peculiar to the epitope tag on *hMRAPa* as we demonstrated that untagged *hMRAPa* also increases *hMC4R* constitutive activity (data not shown). Furthermore, this effect is specific to *hMRAPa* as co-transfection of *hMRAP2* with the *hMC4R* in HEK293 cells does not increase *hMC4R* constitutive activity (data not shown). When *hMC4R* was stably expressed with transient *hMRAPa*-FLAG, we did not observe increased *hMC4R* constitutive activity. Under these transfection conditions, a higher percentage of cells expressed *hMC4R* alone compared to when the *hMC4R* and *hMRAPa*-FLAG were transiently co-expressed, potentially masking the effect of *hMRAPa*-FLAG on *hMC4R* constitutive activity.

hMRAPa-induced increase in *hMC4R* constitutive activity may be associated with differential *hMC4R* complex N-linked glycosylation as we previously demonstrated that *hMRAPa* co-expression specifically alters *hMC4R* complex N-linked glycosylation (Kay *et al.* 2013). The first 24 amino acids of the *hMC4R* N-terminus have been shown to act as a tethered intramolecular ligand and to be responsible for ~80% of *hMC4R* constitutive activity (Srinivasan *et al.* 2004, Ersoy *et al.* 2012). Predicted *hMC4R* N-linked glycosylation sites at Asn³ and Asn¹⁷ fall within

this region and are adjacent to residues that are susceptible to point mutations associated with human obesity (Arg⁷ and Arg¹⁸). R7H and R18C *hMC4R* mutants were shown to have reduced constitutive activity but similar cell surface expression when compared with wild-type *hMC4R* (Srinivasan *et al.* 2004). Therefore, differential *hMC4R* N-linked glycosylation could alter *hMC4R* constitutive activity and *hMRAPa* co-expression could modulate these effects on the *hMC4R* (Ersoy *et al.* 2012). *hMRAPa* most likely induces changes in *hMC4R* complex N-linked glycosylation as this receptor transits through the secretory pathway as *hMC4R* and *hMRAPa* co-localise in intracellular vesicles.

We observed varying effects of *hMRAPa*-FLAG co-expression on agonist-stimulated coupling of *hMC1R*, *hMC2R*, *hMC3R* and *hMC5R* to adenylyl cyclase. *hMRAPa*-FLAG co-expression enhanced maximum coupling of both the *hMC1R* and *hMC3R* to adenylyl cyclase. This conflicts with earlier work showing that *hMRAPa* had no effect on *hMC1R* or *hMC3R* coupling to adenylyl cyclase in CHO cells stimulated with 1 nM NDP- α -MSH (Chan *et al.* 2009). We generated full concentration–response curves using the physiological agonist α -MSH to stimulate each of these receptors expressed in HEK293 cells. Our data may conflict with those of Chan *et al.* because we used a different agonist and a different cell line. *hMRAPa*-FLAG co-expression reduced NDP- α -MSH-stimulated coupling of the *hMC5R* to adenylyl cyclase provided that HA-*hMC5R* and *hMRAPa*-FLAG were transiently co-expressed. In the *hMC5R* stable cell population, the effect of *hMRAPa*-FLAG on *hMC5R* signalling was probably masked by a high ratio of cells expressing *hMC5R* alone. Sebag and Hinkle showed that *hMRAPa* expression resulted in *hMC5R* intracellular retention but they did not study *hMC5R* functional coupling to adenylyl cyclase (Sebag & Hinkle 2009a). Interestingly, we showed that despite *hMRAPa*-FLAG-induced *hMC5R* intracellular retention, NDP- α -MSH-stimulated *hMC5R* coupling to adenylyl cyclase was only reduced and not completely abolished. The few *hMC5R* expressed at the cell surface were clearly sufficient to maintain *hMC5R* coupling to adenylyl cyclase.

We are the first to investigate *hMRAPa* effects on both *hMCR* subcellular localisation and functional expression in parallel. The HA-*hMC1R* and HA-*hMC3R* each co-localise with *hMRAPa*-FLAG intracellularly and at the cell surface indicating that these proteins associate in the secretory pathway and traffic to the cell membrane together. It is unknown how these interactions lead to increased *hMC1R* and *hMC3R* coupling to adenylyl

cyclase. We did not observe any obvious differences in the level of HA-hMC1R or HA-hMC3R cell surface expression in the presence or absence of hMRAPa-FLAG co-expression. hMRAPa-FLAG could stabilise HA-hMC1R and HA-hMC3R expression at the cell surface without increasing their overall abundance. Alternatively, hMRAPa-FLAG may associate with these receptors in a post-signalling intracellular compartment to decrease receptor degradation and increase receptor recycling. Our confocal data for the HA-hMC2R expressed with and without hMRAPa-FLAG reflects previous data obtained by Roy *et al.* (2007, 2010, 2012) for Myc-tagged hMC2R with and without hMRAPa co-expression in HEK293 cells. Our confocal data for the HA-hMC4R indicates that the HA-hMC4R and hMRAPa-FLAG associate in an intracellular compartment, most likely the Golgi apparatus, where hMRAP α -FLAG alters HA-hMC4R complex N-linked glycosylation, and then they traffic to the plasma membrane independently. The hMRAPa effect on hMC4R constitutive activity is probably not due to a cell surface interaction of hMRAPa with hMC4R. We observed almost total intracellular retention of HA-hMC5R in the presence of hMRAPa-FLAG, which concurs with the findings of Sebag & Hinkle (2009a).

We observed expression of hMC2R in the plasma membrane in the absence of exogenous hMRAPa, which could be explained by endogenous MRAP2 expression in our HEK293 cells. In contrast to our work and that performed by Roy *et al.*, others have shown that in non-adrenal cell types, e.g. SK-N-SH and CHO cells, hMC2R cell surface expression depends on co-expression with an MRAP protein (Metherell *et al.* 2005, Sebag & Hinkle 2007, 2009a,b, Chan *et al.* 2009). We have identified both hMRAPa and hMRAP2 mRNA expression in HEK293 cells and Roy *et al.* (2010) identified hMRAP2 expression in HEK293/FRT cells. However, no one has examined whether CHO cells express MRAPa or MRAP2 mRNA. hMC2R cell surface expression in the absence of co-transfected hMRAPa may depend on the cell line (e.g. CHO vs HEK293 or HEK293/FRT) used for protein expression, especially if CHO cells do not express these proteins. If the endogenous functional hMRAP proteins are expressed in HEK293 cells, they might be present at sufficient levels to traffic the hMC2R to the cell surface in the absence of transfected hMRAPa, but not sufficient to allow the hMC2R to couple to adenylyl cyclase. Therefore, we do not expect any endogenous MRAPa and MRAP2 protein expression in HEK293 cells to influence our data for the other hMCR subtypes. Further evidence in support of this prediction comes from our observation that hMRAP2

co-expression does not influence coupling of any of the hMCR subtypes to adenylyl cyclase in HEK293 cells apart from weakly activating the hMC2R at a supraphysiological concentration of ACTH (unpublished data). Our data for hMRAP2 effects on the hMC2R confirms previous findings (Roy *et al.* 2010, Sebag & Hinkle 2010, Gorrigan *et al.* 2011). All the effects of hMRAPa that we observed on hMCR functional expression were over and above any effects resulting from endogenous MRAP protein expression in our cells as we compared cells co-transfected with hMCRs and hMRAPa-FLAG with cells co-transfected with hMCRs and pcDNA 3.1.

The physiological significance of the differential effects of hMRAPa on hMC1R, hMC3R, hMC4R and hMC5R function will only be realised if hMRAPa expression co-localises with these receptors *in vivo*. We used RT-PCR to show hMC1–5R and hMRAPa mRNA expression in a range of human tissues. MRAPa but not MC2R was expressed in brain, adipose tissue and heart. However, one or more of the other MCR subtypes were expressed in these tissues and therefore MRAPa could potentially interact with these. Both hMC4R mRNA (Chagnon *et al.* 1997) and hMRAPa mRNA (Gardiner *et al.* 2002) were previously identified in brain, adrenal gland and heart. The hMC4R has diverse functions in the brain including maintenance of energy homeostasis, cachexia, neuroprotection, addictive behaviours and pain perception (Tao 2010). Therefore, hMRAPa regulation of hMC4R constitutive activity could influence many physiological functions. Furthermore, hMC4R constitutive activity has been associated with both the development of and protection from obesity (Xiang *et al.* 2006, Stutzmann *et al.* 2007). If hMRAPa increases hMC4R constitutive activity *in vivo*, this could influence body weight. The Mc3R is also expressed in rodent brain regions involved in body weight regulation (Roselli-Rhefuss *et al.* 1993). Rare mutations in the hMC3R that are associated with obesity had reduced maximum coupling to adenylyl cyclase when recombinant mutant hMC3R were compared with native hMC3R *in vitro* (Mencarelli *et al.* 2008). Hence, if hMRAPa increased hMC3R maximum coupling to adenylyl cyclase *in vivo*, this could also influence body weight and metabolism.

In conclusion, we have identified differential effects of hMRAPa on hMC1R, hMC2R, hMC3R, hMC4R and hMC5R coupling to adenylyl cyclase and subcellular localisation. Although only hMC2R functional expression critically depends on hMRAPa co-expression, hMRAPa could be a modulator of all the five hMCR subtypes if hMRAPa co-expresses with these receptors *in vivo*. We

believe that hMRAP α is the first naturally occurring molecule shown to increase hMC4R constitutive activity and only the second intracellular protein shown to increase the constitutive activity of a GPCR. Homer1a enhances the constitutive activity of the metabotropic glutamate receptors mGluR1a and mGluR5 in neurons (Ango *et al.* 2001). hMRAP α may be a melanocortin system-specific accessory protein as we and others have shown that while hMRAP α immunoprecipitates with other GPCRs *in vitro*, it does not alter their function (Chan *et al.* 2009, Kay *et al.* 2013).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-12-0221>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Funding for this work was provided by the International Investment Opportunities Fund (NZ) and the Health Research Council of New Zealand IIOF-UOA-016 grant (K G M). E I K was supported by PhD scholarships from the Neurological Foundation of New Zealand and the University of Auckland.

Acknowledgements

The authors thank Jacqui Ross (University of Auckland) for assistance with fluorescence and confocal microscopy; Dr Deborah Hay (University of Auckland) for gifts of HA-hCL, FLAG-hRAMP1 (gift to Deborah Hay from Denise L Wootten, Monash University, Melbourne, Australia) and hCGRP α ; Dr Aimin Xu (University of Hong Kong) for mMRAP α -FLAG construct; and Dr Shaun Lott (University of Auckland) for PNGase F enzyme.

References

- Ango F, Prezeau L, Muller T, Tu JC, Xiao B, Worley PF, Pin JP, Bockaert J & Fagni L 2001 Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. *Nature* **411** 962–965. (doi:10.1038/35082096)
- Bednarek MA, MacNeil T, Tang R, Fong TM, Cabello MA, Maroto M & Teran A 2007 Potent and selective agonists of α -melanotropin (α MSH) action at human melanocortin receptor 5; linear analogs of α -melanotropin. *Peptides* **28** 1020–1028. (doi:10.1016/j.peptides.2007.02.011)
- Chagnon YC, Chen WJ, Perusse L, Chagnon M, Nadeau A, Wilkison WO & Bouchard C 1997 Linkage and association studies between the melanocortin receptors 4 and 5 genes and obesity-related phenotypes in the Quebec family study. *Molecular Medicine* **3** 663–673.
- Chan LF, Webb TR, Chung TT, Meimaridou E, Cooray SN, Guasti L, Chapple JP, Egertova M, Elphick MR, Cheetham ME *et al.* 2009 MRAP and MRAP2 are bidirectional regulators of the melanocortin receptor family. *PNAS* **106** 6146–6151. (doi:10.1073/pnas.0809918106)
- Cooray SN, Almiro Do Vale I, Leung K-Y, Webb TR, Chapple JP, Egertova M, Cheetham ME, Elphick MR & Clark AJL 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. (doi:10.1210/en.2007-1463)
- Ersoy BA, Pardo L, Zhang S, Thompson DA, Millhauseer G, Govaerts C & Vaisse C 2012 Mechanisms of N-terminal modulation of activity at the melanocortin-4 receptor GPCR. *Nature Chemical Biology* **8** 725–730. (doi:10.1038/nchembio.1008)
- Gardiner K, Slavov D, Bechtel L & Davison M 2002 Annotation of human chromosome 21 for relevance to Down syndrome: gene structure and expression analysis. *Genomics* **79** 833–843. (doi:10.1006/geno.2002.6782)
- Getting SJ 2006 Targeting melanocortin receptors as potential novel therapeutics. *Pharmacology and Therapeutics* **111** 1–15. (doi:10.1016/j.pharmthera.2005.06.022)
- Gorrigan RJ, Guasti L, King P, Clark AJ & Chan LF 2011 Localisation of the melanocortin-2-receptor and its accessory proteins in the developing and adult adrenal gland. *Journal of Molecular Endocrinology* **46** 227–232. (doi:10.1530/JME-11-0011)
- Hinkle PM, Serasinghe MN, Jakabowski A, Sebag JA, Wilson KR & Haskell-Luevano C 2011 Use of chimeric melanocortin-2 and -4 receptors to identify regions responsible for ligand specificity and dependence on melanocortin 2 receptor accessory protein. *European Journal of Pharmacology* **660** 94–102. (doi:10.1016/j.ejphar.2010.10.113)
- Kay EI, Botha R, Montgomery JM & Mountjoy KG 2013 hMRAP α specifically alters hMC4R molecular mass and N-linked complex glycosylation in HEK293 cells. *Journal of Molecular Endocrinology* **50**. In press. (doi:10.1530/JME-12-0220)
- 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. (doi:10.1038/30666)
- Mencarelli M, Walker GE, Maestrini S, Alberti L, Verti B, Brunani A, Petroni ML, Tagliaferri M, Liuzzi A & Di Blasio AM 2008 Sporadic mutations in melanocortin receptor 3 in morbid obese individuals. *European Journal of Human Genetics* **16** 581–586. (doi:10.1038/sj.ejhg.5202005)
- Metherell LA, Chapple JP, Cooray S, David A, Becker C, Ruschendorf F, Naville D, Begeot M, Khoo B, Nurnberg P *et al.* 2005 Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. *Nature Genetics* **37** 166–170. (doi:10.1038/ng1501)
- 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. (doi:10.1126/science.1325670)
- Mountjoy KG, Mortrud MT, Low MJ, Simerly RB & Cone RD 1994 Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Molecular Endocrinology* **8** 1298–1308. (doi:10.1210/me.8.10.1298)
- Mountjoy KG, Willard DH & Wilkison WO 1999 Agouti antagonism of melanocortin-4 receptor: greater effect with desacetyl- α -melanocyte-stimulating hormone (MSH) than with α -MSH. *Endocrinology* **140** 2167–2172. (doi:10.1210/en.140.5.2167)
- Roselli-Rehffuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, Entwistle ML, Simerly RB & Cone RD 1993 Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *PNAS* **90** 8856–8860. (doi:10.1073/pnas.90.19.8856)
- 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. *Molecular Endocrinology* **21** 1656–1669. (doi:10.1210/me.2007-0041)
- Roy S, Perron B & Gallo-Payet N 2010 Role of asparagine-linked glycosylation in cell surface expression and function of the human

- adrenocorticotropin receptor (melanocortin 2 receptor) in 293/FRT cells. *Endocrinology* **151** 660–670. (doi:10.1210/en.2009-0826)
- Roy S, Roy SJ, Pinard S, Agulleiro MJ, Cerda-Reverter JM, Parent JL & Gallo-Payet N 2012 The C-terminal domains of melanocortin-2 receptor (MC2R) accessory proteins (MRAP1) influence their localization and ACTH-induced cAMP production. *General and Comparative Endocrinology* **176** 265–274. (doi:10.1016/j.ygcen.2012.02.003)
- Sanchez-Mas J, Hahmann C, Gerritsen I, Garcia-Borron JC & Jimenez-Cervantes C 2004 Agonist-independent, high constitutive activity of the human melanocortin 1 receptor. *Pigment Cell Research* **17** 386–395. (doi:10.1111/j.1600-0749.2004.00160.x)
- Sebag JA & Hinkle PM 2007 Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. *PNAS* **104** 20244–20249. (doi:10.1073/pnas.0708916105)
- Sebag JA & Hinkle PM 2009a Opposite effects of the melanocortin-2 (MC2) receptor accessory protein MRAP on MC2 and MC5 receptor dimerization and trafficking. *Journal of Biological Chemistry* **284** 22641–22648. (doi:10.1074/jbc.M109.022400)
- Sebag JA & Hinkle PM 2009b Regions of melanocortin 2 (MC2) receptor accessory protein necessary for dual topology and MC2 receptor trafficking and signaling. *Journal of Biological Chemistry* **284** 610–618. (doi:10.1074/jbc.M804413200)
- Sebag JA & Hinkle PM 2010 Regulation of G protein-coupled receptor signaling: specific dominant-negative effects of melanocortin 2 receptor accessory protein 2. *Science Signaling* **3** ra28. (doi:10.1126/scisignal.2000593)
- Srinivasan S, Lubrano-Berthelier C, Govaerts C, Picard F, Santiago P, Conklin BR & Vaisse C 2004 Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans. *Journal of Clinical Investigation* **114** 1158–1164. (doi:10.1172/JCI200421927)
- Stutzmann F, Vatin V, Cauchi S, Morandi A, Jouret B, Landt O, Tounian P, Levy-Marchal C, Buzzetti R, Pinelli L *et al.* 2007 Non-synonymous polymorphisms in melanocortin-4 receptor protect against obesity: the two facets of a Janus obesity gene. *Human Molecular Genetics* **16** 1837–1844. (doi:10.1093/hmg/ddm132)
- Tao YX 2010 The melanocortin-4 receptor: physiology, pharmacology, and pathophysiology. *Endocrine Reviews* **31** 506–543. (doi:10.1210/er.2009-0037)
- Webb TR, Chan L, Cooray SN, Cheetham ME, Chapple JP & Clark AJ 2009 Distinct melanocortin 2 receptor accessory protein domains are required for melanocortin 2 receptor interaction and promotion of receptor trafficking. *Endocrinology* **150** 720–726. (doi:10.1210/en.2008-0941)
- Wong J, Love DR, Kyle C, Daniels A, White M, Stewart AW, Schnell AH, Elston RC, Holdaway IM & Mountjoy KG 2002 Melanocortin-3 receptor gene variants in a Maori kindred with obesity and early onset type 2 diabetes. *Diabetes Research and Clinical Practice* **58** 61–71. (doi:10.1016/S0168-8227(02)00126-2)
- Xiang Z, Litherland SA, Sorensen NB, Proneth B, Wood MS, Shaw AM, Millard WJ & Haskell-Luevano C 2006 Pharmacological characterization of 40 human melanocortin-4 receptor polymorphisms with the endogenous proopiomelanocortin-derived agonists and the agouti-related protein (AGRP) antagonist. *Biochemistry* **45** 7277–7288. (doi:10.1021/bi0600300)
- Xu A, Choi KL, Wang Y, Permana PA, Xu LY, Bogardus C & Cooper GJ 2002 Identification of novel putative membrane proteins selectively expressed during adipose conversion of 3T3-L1 cells. *Biochemical and Biophysical Research Communications* **293** 1161–1167. (doi:10.1016/S0006-291X(02)00354-6)

Received in final form 6 December 2012

Accepted 7 January 2013

Accepted Preprint published online 7 January 2013