hMRAPa specifically alters hMC4R molecular mass and *N*-linked complex glycosylation in HEK293 cells

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Abstract

Human melanocortin 2 receptor accessory protein 1 (hMRAPa) is essential for human melanocortin 2 receptor (hMC2R)-regulated adrenal steroidogenesis. hMRAPa enhances hMC2R *N*-linked glycosylation and maturation, promotes hMC2R cell surface expression and enables ACTH to bind and activate the MC2R. However, hMRAPa is predicted to have functions beyond its critical role in hMC2R activity. It is more widely expressed than the hMC2R and it has been shown to co-immunoprecipitate with all other hMCR subtypes and other G-protein-coupled receptors, when these are co-expressed with each receptor in heterologous cells. The physiological relevance of hMRAPa interactions with these receptors is unknown. We hypothesised that hMRAPa could influence post-translational processing and maturation of these receptors, similar to its actions on the hMC2R. Here we used co-immunoprecipitation and western blotting techniques to characterise effects of hMRAPa-FLAG co-expression on the maturation of each HA-tagged hMCR subtype and the HA-tagged human calcitonin receptor-like receptor (hCL), co-expressed in HEK293 cells. While hMRAPa-FLAG interacted with all five HA-hMCR subtypes and the HA-hCL, it only altered HA-hMC4R molecular mass. This altered HA-hMC4R molecular mass was due to a change in endoglycosidase H-resistant complex *N*-linked glycosylation, which we observed for HA-hMC4R in both intracellular and cell surface fractions. This effect was specific to the HA-hMC4R as hMRAPa did not alter the molecular mass of any of the other receptors that we examined. In conclusion, the specific effects of hMRAPa on hMC4R molecular mass and complex *N*-linked glycosylation provide evidence in support of a role for MRAP α in hMC4R functions.

Key Words

- GPCR accessory protein
- melanocortin-2 receptor accessory protein
- melanocortin receptor
- glycosylation

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Introduction

The melanocortin system performs a wide variety of physiological roles in human health and disease (Getting 2006). The human melanocortin 2 receptor (hMC2R) is unique amongst the melanocortin receptors, because it does not signal in response to ACTH stimulation in the absence

of hMC2R accessory protein (*hMRAP1*). Human *MRAP1* (*MRAP*) was discovered as a gene mutated in a rare autosomal disorder of ACTH resistance, known as familial glucocorticoid deficiency (FGD) type 2 (Metherell *et al.* 2005). Mutations in *hMRAP1* underlie ~25% of FGD cases

(Metherell *et al.* 2006). *hMRAP1* is located on chromosome 21 and gives rise to two alternatively spliced hMRAP1 isoforms. The longer isoform, hMRAPa, is encoded by exons 1, 2, 3, 4 and 5 and the shorter isoform, hMRAPb, is encoded by exons 1, 2, 3, 4 and 6 (Metherell *et al.* 2005). *hMRAP2*, a paralog of *hMRAP1*, is located on chromosome 6 and its physiological function is unknown (Chan *et al.* 2009).

hMRAPa promotes hMC2R post-translational modification and maturation, cell surface expression and signalling in response to physiological ACTH concentrations (Roy *et al.* 2007, Sebag & Hinkle 2007, Webb *et al.* 2009). hMRAP2 promotes hMC2R cell surface expression, but, in contrast to hMRAPa, hMRAP2 only supports hMC2R signalling in response to supraphysiological ACTH concentrations (Sebag & Hinkle 2010). *hMRAPa* and *hMRAPb* mRNA have been shown to be expressed in the adrenal gland and other tissues including human brain, testis, breast and skin by RT-PCR (Gardiner *et al.* 2002, Metherell *et al.* 2005). *MRAPα* expression was also identified in specific mouse brain regions including hippocampus and hypothalamus, using *in situ* hybridisation (Gardiner *et al.* 2002). *hMRAP1* mRNA therefore has a broader tissue distribution than that of the *hMC2R*, which is primarily expressed in the adrenal and pituitary glands (Metherell *et al.* 2005, Cooray *et al.* 2008, Chan *et al.* 2009, Sebag & Hinkle 2009a,b). Hence, there has been intense interest in determining whether hMRAP1 proteins play roles in the functional expression of the other four widely expressed hMCR subtypes.

Both hMRAPa and hMRAP2 have been shown to co-immunoprecipitate with the hMC1R, hMC3R, hMC4R and hMC5R, but the effects of hMRAPs on the maturation and/or post-translational modification of these four hMCR subtypes were not examined (Chan *et al.* 2009). We hypothesise that hMRAPa may influence the maturation and/or post-translational modification of any of the hMCR subtypes in addition to the hMC2R. In this study, we have employed co-immunoprecipitation and western blotting techniques to examine the effects of hMRAPa on the molecular masses of all five hMCR subtypes, the human dopamine D2 receptor (hD2R) and human calcitonin receptor-like receptor (hCL), exogenously expressed in HEK293 cells.

Materials and methods

Construction of recombinant DNAs

N-terminal HA-tagged melanocortin receptor constructs and HA-tagged hD2R in pcDNA 3.1 were purchased

from the Missouri S&T cDNA Resource Centre (Rolla, MO, USA). N-terminal FLAG-tagged human receptor activity-modifying protein 1 (RAMP1) and HA-tagged hCL (McLatchie *et al.* 1998) both in pcDNA 3.1 were gifts.

hMRAPa constructs hMRAPa cDNA (Ultimate ORF IOH58589 from Invitrogen Corporation) was recombined with pcDNA 3.2 using Gateway LR Clonase II (Invitrogen Corporation) and according to the manufacturer's protocol. The hMRAPa insert was then transferred into pcDNA 3.1 to ensure that all of the hMRAPa constructs had the same expression backbone.

hMRAPa was amplified from hMRAPa in pcDNA 3.2 using the following primers: 5'-ATC GGA TCC ATG GCC AAC GGG ACC AAC-3' (sense) and 5'-ATC GAA TTC TCA GCT CTG CAA TTG AGA GGT-3' (antisense). To subclone hMRAPa-FLAG, human ovary total RNA (Ambion, Inc., Austin, TX, USA; 2 µg) was reverse transcribed to cDNA with Superscript III reverse transcriptase (Invitrogen Corporation) in a total volume of 20 µl and then 2 µl of cDNA were used to amplify hMRAPa-FLAG with the following primers: 5'-ATC GGA TCC ATG GCC AAC GGG ACC AAC-3' (sense) and 5'-ATC GAA TTC TCA CTT GTC ATC GTC GTC CTT GTA GTC GCT CTG CAA TTG AGA GGT-3' (antisense). RT-PCR for both hMRAPa and hMRAPa-FLAG was performed using iProof High Fidelity DNA polymerase (Bio-Rad Laboratories) and the following conditions: denature (98 °C, 1 min; 98 °C, 5 s), anneal (60 °C, 30 s), elongate (72 °C, 10 s) for 35 cycles and then one elongation at 72 °C, for 7 min. hMRAPa and hMRAPa-FLAG were subcloned into pcDNA 3.1 (Invitrogen Corporation) using BamHI and EcoRI sites and the recombinant DNA was verified by sequencing. The coding sequences are aligned with GenBank accession number NM_178817.3.

hMRAP2 Human ovary total RNA (Clontech; 2 µg) was reverse transcribed to cDNA with Superscript III reverse transcriptase in a total volume of 20 µl and then 2 µl of cDNA were used to amplify hMRAP2 by RT-PCR using the following primers: 5'-ATG TCC GCC CAG AGG TTA AT-3' (sense) and 5'-ATC GAA TTC TCA ATC CAG GTC TTT GTG TG-3' (antisense). PCR conditions were as described above for hMRAPa except that an annealing temperature of 62 °C was used. hMRAP2 was subcloned into pcDNA 3.1 using BamHI and EcoRI restriction sites and the recombinant DNA verified by sequencing. The coding sequence is aligned with GenBank accession number NM_206898.

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, 375 000 HEK293 cells/well were seeded into six-well plates. After 48 h when the cells were at 30–50% confluency, they were transfected with 1.5 µl of FuGENE 6 (Roche Applied Science) for every 0.5 µg of plasmid DNA, and then incubated for another 48 h at 37 °C under 5% CO₂.

Co-immunoprecipitation and western blotting

HEK293 cells were transfected with HA-tagged G-protein-coupled receptors (GPCRs) plus hMRAPa-FLAG, FLAG-hRAMP1 or pcDNA 3.1. Forty-eight hours post-transfection, the cells were lysed in PBS+1% *n*-dodecyl-β-D-maltoside (Sigma-Aldrich Ltd.) and Complete mini protease inhibitors (Roche Applied Science). Each cell lysate (500 µl) was pre-cleared with 25 µl of Protein G Sepharose Fast Flow beads (GE Healthcare Biosciences, Pittsburgh, PA, USA) for 1 h at 4 °C. The beads were then pelleted and each supernatant was incubated with 1 µg rabbit anti-FLAG polyclonal antibody (Sigma-Aldrich Ltd.) for 4 h at 4 °C. Immune complexes were precipitated with 50 µl of Protein G Sepharose beads (50% slurry in lysis buffer) for 1 h at 4 °C, washed four times with 1 ml of lysis buffer, once with 1 ml of 50 mM Tris-HCl, pH 8.0, and then incubated with 30 µl of 1× sample loading buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol) at 65 °C for 5 min, to elute bound proteins. The beads were then pelleted and the supernatants were loaded onto 10% Bis-acrylamide SDS-PAGE gels and electrophoresed at 150 V for 1–2 h. A fraction of each cell lysate (5–10 µl) used for co-immunoprecipitation was loaded in parallel with the co-immunoprecipitated samples and all samples were heated to 65 °C for 10 min prior to loading on SDS-PAGE. Precision plus Dual Colour pre-stained protein standard markers (Bio-Rad Laboratories) were used to estimate the molecular masses of specific bands. Proteins were electrophoretically transferred onto PVDF membranes and then incubated with blocking buffer (TBS+1% Tween 20 (TBS-T)+5% non-fat milk powder) for 45 min prior to incubation with mouse anti-HA.11 MAB (Covance Research Products, Princeton, NJ, USA) at 1:10 000 in TBS-T+1% non-fat milk powder, overnight at 4 °C. PVDF membranes were washed with TBS-T and incubated with HRP-conjugated anti-mouse

secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) at 1:10 000 in TBS-T+1% non-fat milk powder for 2 h at room temperature. The membranes were washed with TBS-T and then developed with ECL Plus (GE Healthcare Biosciences). Chemiluminescent signals were visualized on a Fuji LAS4000 Imager. Three independent experiments were performed for each GPCR.

For western blotting of HA-hMC4R with hMRAP2, HEK293 cells were transfected with HA-hMC4R with hMRAPa, hMRAP2 or pcDNA 3.1, and lysates were prepared as described above. Cell lysates (25 µg/lane) were diluted in 2× sample loading buffer, heated to 65 °C for 10 min before loading onto 10% Bis-acrylamide SDS-PAGE gels and electrophoresis was performed at 150 V for 1–2 h. Western blotting with anti-HA antibody (as described above) and anti-β-tubulin antibody was performed on SDS-PAGE gels run in parallel. To detect β-tubulin, the PVDF membrane was blocked with 5% BSA/TBS-T for 1 h and then incubated with rabbit anti-β-tubulin antibody (Cell Signalling Technology, Danvers, MA, USA), diluted 1/1000 in 5% BSA/TBS-T overnight at 4 °C. The membrane was then washed with TBS-T, incubated with HRP-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) at 1:10 000 in TBS-T+1% non-fat milk powder for 2 h at room temperature and washed again with TBS-T prior to development.

Analysis of N-linked glycosylation and sialylation

HEK293 cells were transfected with HA-hMC2R or HA-hMC4R with hMRAPa-FLAG or pcDNA 3.1. Total cell lysates were digested with either PNGase F (synthesised at the School of Biological Sciences, University of Auckland, Loo *et al.* (2002)), endoglycosidase H or neuraminidase (New England Biolabs, Ipswich, MA, USA). Lysates (100 µg) were digested with 2 µg of PNGase F in PBS+1% SDS for 4 h at 37 °C or with 500 U of endoglycosidase H or 50 U of neuraminidase according to the manufacturer's instructions for 16–18 h at 37 °C. Digests were diluted in 2× sample loading buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol)+50 mM dithiothreitol and western blotting with anti-HA antibody was performed as described above. Two independent experiments were performed for each receptor.

Cell surface protein fractionation

Biotinylation and fractionation of cell surface HA-hMC4R were performed using a Pierce Cell Surface Protein

Isolation Kit (Pierce Protein Research Products, Rockford, IL, USA). The manufacturer's protocol was followed but reagent volumes were reduced to biotinylate only one 10 cm plate of HEK293 cells transfected with HA-hMC4R and either hMRAPa-FLAG or pcDNA 3.1. Cell surface and intracellular fractions (20 μ l) were digested with PNGase F or endoglycosidase H as described above, but digests were incubated in 1 $\times$ sample loading buffer for 2 h at 37 $^{\circ}$ C. Half of each final reaction volume was analysed on SDS-PAGE and HA-hMC4R was detected by western blotting as described above. To assess sample loading, the western blot for the intracellular HA-hMC4R fraction was stripped with 2% SDS, 125 mM Tris-HCl pH 6.8, 100 mM β -mercaptoethanol for 50 min at 50 $^{\circ}$ C, washed thoroughly in Milli-Q water, and then blocked in 5% BSA/TBS-T. Detection of β -tubulin was performed as described above.

Results

Observed molecular masses for all five HA-hMCR subtypes differed from their predicted molecular masses

All five HA-hMCR subtypes were detected in cell lysates by western blotting, provided that lysates were not boiled prior to loading on SDS-PAGE (Fig. 1). The HA-hMC1R was detected as two bands of $\sim$ 30 and $\sim$ 34 kDa, which were less than its predicted molecular mass of $\sim$ 38 kDa (Fig. 1A, lane 3). The HA-hMC2R was detected as three bands of $\sim$ 38, $\sim$ 45 and $\sim$ 52 kDa, which were greater than its predicted molecular mass of 37 kDa (Fig. 1B, lane 3). The HA-hMC3R was detected as a single strong band at $\sim$ 44 kDa and a weak band at $\sim$ 33 kDa, which were greater and lesser respectively than its predicted molecular mass of $\sim$ 39 kDa (Fig. 1C, lane 3). The HA-hMC4R was detected as five bands of $\sim$ 44, $\sim$ 65, $\sim$ 70, $\sim$ 75 and $\sim$ 80 kDa, which were greater than its predicted molecular mass of $\sim$ 40 kDa (Fig. 1D, lane 3). The HA-hMC5R predominantly migrated as a single band at $\sim$ 60 kDa, which was greater than its predicted molecular mass of $\sim$ 40 kDa (Fig. 1E, lane 3). Weaker HA-hMC5R-specific bands were observed at $\sim$ 39, $\sim$ 52, $\sim$ 65 and $\sim$ 36 kDa (Fig. 1E, lane 3).

hMRAPa-FLAG interacted with all five HA-hMCR subtypes and this interaction specifically changed HA-hMC4R molecular mass

To investigate whether hMRAPa interacts with hMCR subtypes to alter their molecular mass, hMRAPa-FLAG and HA-hMCR subtypes were co-immunoprecipitated and each HA-hMCR subtype was detected by western blotting.

Figure 1

Interactions of the five different HA-hMCR subtypes with hMRAPa-FLAG. Lysates (L) of HEK293 cells transiently transfected with HA-hMCRs $\pm$ hMRAPa-FLAG were run in parallel with lysates that had been co-immunoprecipitated (IP) with anti-FLAG antibody. Western blotting was performed using anti-HA antibody. Predicted molecular masses are (A) HA-hMC1R, 37.9 kDa; (B) HA-hMC2R, 37 kDa; (C) HA-hMC3R, 39.2 kDa; (D) HA-hMC4R, 40.1 kDa and (E) HA-hMC5R, 39.8 kDa. The 3 $\times$ HA tag is $\sim$ 4.4 kDa. Predicted molecular masses were determined using the ExPASy Compute pI/MW tool: http://expasy.org/tools/pi_tool.html. Molecular masses (kDa) are indicated on the right-hand side of each blot. Bands of interest are indicated on the right-hand side of each lane with (closed left triangle). Asterisks indicate heavy and light chains of IgG. Closed diamonds indicate bands that cannot be distinguished as specific or non-specific. 1–6, Lane numbers.

Following co-immunoprecipitation of the HA-hMC1R and hMRAPa-FLAG, two bands at $\sim$ 30 and $\sim$ 33 kDa were observed (Fig. 1A, lane 2). The $\sim$ 30 kDa band was not

detected in hMC1R cell lysates (Fig. 1A, lanes 1 and 3). Bands of similar molecular mass were observed in cell lysates expressing HA-hMC1R alone or HA-hMC1R together with hMRAPa-FLAG (Fig. 1A, lane 1 vs lane 3).

Nine HA-hMC2R-specific bands of different intensities were observed following co-immunoprecipitation with hMRAPa-FLAG with the most intense bands observed at ~45 and ~52 kDa (Fig. 1B, lane 2). These bands were also observed in HA-hMC2R cell lysates and were more intense when the HA-hMC2R was expressed with hMRAPa-FLAG than when it was expressed alone (Fig. 1B, lane 1 vs lane 3). Not all of the HA-hMC2R-specific bands that were observed when the HA-hMC2R was co-immunoprecipitated with hMRAPa-FLAG were also observed in HA-hMC2R cell lysates. This may in part reflect lower protein concentrations in cell lysates compared with immunoprecipitates.

Following co-immunoprecipitation of the HA-hMC3R and hMRAPa-FLAG, intense HA-hMC3R-specific bands were observed at ~40 and ~80 kDa and a weaker band was observed at ~33 kDa (Fig. 1C, lane 2). The ~40 kDa-specific band was also observed in HA-hMC3R cell lysates both in the presence and the absence of hMRAPa-FLAG, while the ~33 kDa HA-hMC3R band was only faintly detected in the cell lysates for the HA-hMC3R expressed alone (Fig. 1C, lanes 1 and 3). The weaker signals and the absence of the ~80 kDa band in the cell lysates compared with the immunoprecipitates (Fig. 1C, lanes 1, 2 and 3) could reflect the lower protein concentrations in lysates compared with immunoprecipitates.

Following co-immunoprecipitation of the HA-hMC4R with MRAP α -FLAG, a strong smear at ~52 kDa and three bands at ~35, ~40 and ~45 kDa were observed (Fig. 1D, lane 2). The strong ~52 kDa HA-hMC4R-specific smear is probably three closely spaced bands at ~50, ~52 and ~60 kDa as observed in the cognate cell lysate (Fig. 1D, lane 1). The ~35, ~40 and ~45 kDa HA-hMC4R-specific bands observed following co-immunoprecipitation with hMRAPa-FLAG were not detected in HA-hMC4R cell lysates (Fig. 1D, lanes 1 and 3). Different HA-hMC4R-specific bands were also observed in cell lysates for the HA-hMC4R expressed with hMRAPa-FLAG compared with when it was expressed alone. In the presence of hMRAPa-FLAG, the ~80, ~70 and ~75 kDa HA-hMC4R bands observed when the HA-hMC4R was expressed alone were absent, the ~44 kDa band was present, the ~65 kDa band was shifted slightly downwards to ~60 kDa, and additional bands were observed at ~50 and ~52 kDa (Fig. 1D, lane 1 vs lane 3).

Following co-immunoprecipitation of HA-hMC5R with hMRAPa-FLAG, HA-hMC5R-specific bands were observed at ~36, ~39, ~44 and ~55 kDa (Fig. 1E, lane 2). Only the ~39 and ~36 kDa bands were also faintly observed in cell lysates when HA-hMC5R was expressed alone (Fig. 1E, lane 3). Specific bands in cell lysate for HA-hMC5R expressed with hMRAPa-FLAG were difficult to see, although a faint band was observed at ~55 kDa (Fig. 1E, lane 1). HA-hMC5R-specific bands may have been difficult to see because less protein was loaded in this lane compared with the immunoprecipitate lane. In further experiments with HA-hMC5R cell lysates, both when the HA-hMC5R was expressed alone and when it was expressed with hMRAPa-FLAG, it was observed as eight bands between ~40 and ~150 kDa ([Supplementary Figure 1](#), see section on supplementary data given at the end of this article, lanes 1 and 3).

hMRAPa-FLAG interacted with the HA-hCL and the HA-hD2R

To determine whether hMRAPa interacts with GPCRs other than the hMCR family, we performed co-immunoprecipitation of hMRAPa-FLAG with HA-hCL or the HA-hD2R. When the HA-hCL was expressed alone or co-expressed together with hMRAPa-FLAG, a single band at ~52 kDa was observed in cell lysates (Fig. 2, lanes 1 and 3). When the HA-hCL was co-expressed with FLAG-hRAMP1, the accessory protein that promotes hCL to function as a calcitonin gene-related peptide receptor, a single band was observed at ~60 kDa in cell lysate (Fig. 2, lane 5). Following co-immunoprecipitation with either hMRAPa-FLAG or FLAG-hRAMP1, the HA-hCL was observed as a single band at ~52 or ~60 kDa respectively (Fig. 2, lanes 2 and 6). Therefore the HA-hCL interacted with hMRAPa-FLAG indicating that hMRAPa may not be a specific accessory protein for the hMCR family. The HA-hD2R migrated as a single band at ~70 kDa in cell lysates ([Supplementary Figure 2](#), see section on supplementary data given at the end of this article, lanes 1 and 3). Following co-immunoprecipitation of HA-hD2R with hMRAPa-FLAG, the HA-hD2R was observed as a smear at ~60–80 kDa and two weak bands at ~50 and ~45 kDa ([Supplementary Figure 2](#), lane 2). The ~60–80 kDa smear was weakly observed when the HA-hD2R was co-immunoprecipitated from cells transfected with the HA-hD2R alone ([Supplementary Figure 2](#), lane 4) and we would not expect to see any specific bands in this lane. We cannot verify that the ~60–80 kDa smear represents a specific interaction of the HA-hD2R with hMRAPa-FLAG.

Figure 2

Interactions of HA-hCL with hMRAPa-FLAG or FLAG-hRAMP1. Lysates (L) of HEK293 cells transiently transfected with HA-hCL $\pm$ hMRAPa-FLAG or FLAG-hRAMP1 were run in parallel with lysates that had been co-immunoprecipitated (IP) with anti-FLAG antibody. Western blotting was performed

using anti-HA antibody. Molecular masses (kDa) are indicated on the right-hand side of each blot. Bands of interest are indicated on the right-hand side of each lane with (closed left triangle). 1–8, Lane numbers.

hMRAPa-FLAG enhanced *N*-linked glycosylation of HA-hMC2R and altered HA-hMC4R complex glycosylation

It was shown previously that hMRAPa promotes hMC2R *N*-linked glycosylation and therefore we hypothesised that the HA-hMC4R molecular mass changes induced by co-expression with hMRAPa-FLAG result from altered HA-hMC4R *N*-linked glycosylation. First, we verified that hMRAPa enhances hMC2R glycosylation in HEK293 cells. HEK293 cell lysates for the HA-hMC2R expressed alone or expressed with hMRAPa-FLAG were deglycosylated with PNGase F, endoglycosidase H or neuraminidase. HA-hMC2R-specific bands were observed at ~ 32 , ~ 35 , ~ 45 and ~ 52 kDa in the absence of treatment with endoglycosidases (Fig. 3A and C, lanes 1 and 3; B, lanes 3 and 5). The ~ 45 and ~ 52 kDa HA-hMC2R-specific bands, which were more intense when the HA-hMC2R was expressed with hMRAPa-FLAG compared with when the HA-hMC2R was expressed alone, were sensitive to PNGase F digestion (Fig. 3A, lanes 2 and 4) but not to endoglycosidase H digestion (Fig. 3B, lanes 4 and 6). The ~ 45 kDa HA-hMC2R-specific band was partly sensitive to neuraminidase digestion as it smeared downwards following neuraminidase treatment (Fig. 3C, lanes 2 and 4). The ~ 35 kDa HA-hMC2R-specific band was sensitive to both PNGase F (Fig. 3A, lanes 2 and 4) and endoglycosidase H (Fig. 3B, lanes 4 and 6), indicating that this band represents an intermediate in the HA-hMC2R glycosylation pathway. These results confirm that HA-hMC2R

has endoglycosidase H-resistant complex *N*-linked glycosylation when expressed in HEK293 cells and that hMRAPa-FLAG co-expression enhances HA-hMC2R complex *N*-linked glycosylation.

We went on to examine complex *N*-linked glycosylation of the HA-hMC4R expressed alone and together with hMRAPa-FLAG. In the absence of treatment with endoglycosidases, the HA-hMC4R expressed alone migrated as at least four bands between ~ 65 and ~ 80 kDa with a weaker band at ~ 44 kDa (Fig. 3D and F, lane 1; E, lane 3). When the HA-hMC4R was co-expressed with hMRAPa-FLAG, all HA-hMC4R-specific bands above ~ 65 kDa were absent and an additional band was observed at ~ 50 kDa (Fig. 3D and F, lane 3; E, lane 5). Following PNGase F digestion, the HA-hMC4R was observed as two bands at ~ 31 and ~ 33 kDa both when it was expressed alone and when it was co-expressed with hMRAPa-FLAG, which presumably represents native HA-hMC4R (Fig. 3D, lanes 2 and 4). Following endoglycosidase H digestion of HA-hMC4R expressed alone, the HA-hMC4R was observed as four bands between ~ 65 and ~ 80 kDa and a band at ~ 31 kDa (Fig. 3E, lane 4). Following endoglycosidase H digestion of HA-hMC4R co-expressed with hMRAPa-FLAG, the HA-hMC4R was observed as two smeary bands at ~ 50 and ~ 65 kDa and a single band at ~ 31 kDa (Fig. 3E, lane 6). All of the HA-hMC4R-specific bands above ~ 44 kDa were sensitive to PNGase F and resistant to endoglycosidase H, both when HA-hMC4R was expressed alone and expressed with

Figure 3

hMRAPa-FLAG influences both HA-hMC2R and HA-hMC4R *N*-linked glycosylation in HEK293 cells. Lysates of HEK293 cells transiently transfected with HA-hMC2R ± hMRAPa-FLAG were treated with either PNGase F (A), endoglycosidase H (B), or neuraminidase (C). Lysates of HEK293 cells transiently transfected with HA-hMC4R ± hMRAPa-FLAG were

treated with PNGase F (D), endoglycosidase H (E) or neuraminidase (F). Western blotting was performed using an anti-HA antibody. Molecular masses (kDa) are indicated on the left side of the western blots. Bands of interest are indicated with (closed left triangle). 1-6, lane.

hMRAPa-FLAG. Therefore, these bands represent HA-hMC4R with complex *N*-linked glycosylation. The ~44 kDa HA-hMC4R-specific band was sensitive to both PNGase F and endoglycosidase H digestion and therefore represents an intermediate glycosylated HA-hMC4R. Following neuraminidase digestion of HA-hMC4R expressed alone, the HA-hMC4R migrated as multiple bands from ~50 to 65 kDa and two weak bands at ~44 and ~70 kDa (Fig. 3F, lane 2). When HA-hMC4R was co-expressed together with hMRAPa-FLAG and the cell lysate was digested with neuraminidase, HA-hMC4R was observed as two strong bands at ~47 and ~50 kDa and a weaker band at ~44 kDa (Fig. 3F, lane 4). All of the HA-hMC4R-specific bands above ~44 kDa were partly sensitive to neuraminidase, except for the ~50 kDa band observed when the HA-hMC4R was co-expressed with hMRAPa-FLAG. Therefore, the HA-hMC4R contained Neu5Ac α 2-3R and Neu5Ac α 2-6R sialic acid residues, both when it was expressed alone and when it was expressed with hMRAPa-FLAG. The

neuraminidase-resistant ~50 kDa HA-hMC4R band observed in the presence of hMRAPa-FLAG may represent an alternative intermediate in the HA-hMC4R glycosylation pathway. Overall our data show that the HA-hMC4R has complex *N*-linked glycosylation, which varies following co-expression with hMRAPa-FLAG.

hMRAPa-FLAG changed the molecular mass of both cell surface and intracellular HA-hMC4R

To determine whether the HA-hMC4R with altered complex *N*-linked glycosylation could reach the cell surface, we performed a cell surface biotinylation assay to separate cell surface and intracellular HA-hMC4R fractions and then digested these fractions with endoglycosidases. HA-hMC4R was expressed on the cell surface and intracellularly both when the receptor was expressed alone and when it was expressed with hMRAPa-FLAG (Fig. 4). When the undigested HA-hMC4R was expressed alone, strong bands in the intracellular fraction were

Figure 4

HA-hMC4R molecular mass is changed at both the cell surface and intracellularly in the presence and the absence of hMRAPa-FLAG. HEK293 cells transiently transfected with HA-hMCRs $\pm$ hMRAPa-FLAG were cell surface labelled with sulfo-NHS-SS-biotin. Intracellular and cell surface fractions were separated following sorting of biotin-labelled from non-biotin-labelled fractions and then treated with PNGase F or endoglycosidase H. Western blotting was performed using anti-HA antibody. The western blot for the intracellular fractions (A) was stripped and reprobed for β -tubulin (55 kDa) as a loading control. The western blot for the cell surface fractions is shown with two different exposures (B and C). Molecular masses (kDa) are indicated on the left side of the western blots. Bands of interest are indicated on the right-hand side of each lane with closed left triangles. 1–7, Lane numbers.

observed at ~ 40 and ~ 60 kDa, with weaker bands observed at ~ 38 , ~ 44 , ~ 55 , ~ 70 , ~ 75 and ~ 80 kDa (Fig. 4A, lane 1). By contrast, in the presence of hMRAPa-FLAG the HA-hMC4R-specific bands above ~ 60 kDa were

absent and an additional band at ~ 50 kDa was observed in the undigested intracellular fraction (Fig. 4A, lane 4). Densitometric analysis of a β -tubulin loading control on the same membrane indicated that $\sim 33\%$ less sample was loaded in lane 4 compared with lane 1, which could account for the different HA-hMC4R signal intensities. Two different exposures of the membrane probed for the cell surface HA-hMC4R fractions are shown to permit more accurate estimation of HA-hMC4R band sizes (Fig. 4B and C). When the undigested HA-hMC4R was expressed alone, two bands were observed at ~ 40 and ~ 60 kDa, with a smear at ~ 70 – 80 kDa in the cell surface fraction (Fig. 4B, lane 1). Observation of this membrane at a shorter exposure time indicated that the ~ 70 – 80 kDa smear was comprised of three closely spaced bands and an additional band was present below at ~ 38 kDa (Fig. 4C, lane 1). When HA-hMC4R was co-expressed with hMRAPa-FLAG, the bands above ~ 60 kDa were absent, and additional bands at ~ 55 and ~ 50 kDa were observed in the undigested intracellular fraction (Fig. 4B and C, lane 4). hMRAPa therefore induces changes in HA-hMC4R molecular mass both intracellularly and at the cell surface.

All of the HA-hMC4R-specific bands above ~ 38 kDa were sensitive to PNGase F digestion both in the intracellular and cell surface fractions and both when HA-hMC4R was expressed alone or co-expressed with hMRAPa-FLAG. Additional HA-hMC4R-specific bands were also observed at ~ 31 and ~ 33 kDa, which presumably represent monomeric native HA-hMC4R (Fig. 4A, lanes 2 and 5; B, lanes 2 and 5). Additional weak bands were also observed at ~ 36 and ~ 38 kDa in the intracellular fraction for the HA-hMC4R expressed alone and digested with PNGase F (Fig. 4A, lane 2).

For the HA-hMC4R expressed alone, all of the HA-hMC4R-specific bands above ~ 50 kDa in both intracellular and cell surface fractions were resistant to endoglycosidase H digestion and represent HA-hMC4R with complex *N*-linked glycosylation (Fig. 4A and B, lane 3). However, the ~ 38 , ~ 40 and ~ 44 kDa bands were sensitive to endoglycosidase H and represent intermediates in the HA-hMC4R *N*-linked glycosylation pathway (Fig. 4A and B, lane 3). For the HA-hMC4R expressed with hMRAPa-FLAG in both the intracellular and cell surface fractions, all of the HA-hMC4R-specific bands above ~ 40 kDa were resistant to endoglycosidase H and represent HA-hMC4R with complex *N*-linked glycosylation (Fig. 4A and B, lane 6). Therefore, hMRAPa-FLAG co-expression altered HA-hMC4R complex *N*-linked glycosylation both intracellularly and at the cell surface.

Figure 5

hMRAP2 increases HA-hMC4R total protein expression. Western blotting with either anti-HA or anti-tubulin antibody was performed on lysates of HEK293 cells that were transiently transfected with HA-hMC4R + hMRAPa, hMRAP2 or pcDNA 3.1. Molecular masses (kDa) are indicated on the right-hand side of each blot. Bands of interest are indicated on the right-hand side of each lane with closed left triangles. 1–4, Lane numbers.

hMRAP2 increased HA-hMC4R total protein expression

To determine whether altered HA-hMC4R molecular mass is specific to co-expression with hMRAPa we performed western blotting of HA-hMC4R expressed alone or expressed together with either hMRAPa or hMRAP2. The HA-hMC4R was observed as nine bands of identical molecular masses when it was expressed together with hMRAP2, or when it was expressed alone (Fig. 5). This contrasts with Fig. 1D where five bands were observed for the HA-hMC4R expressed alone and four bands were observed for the HA-hMC4R expressed with hMRAPa-FLAG. Despite these differences, the higher molecular mass HA-hMC4R bands were altered following co-expression with hMRAPa (Fig. 5, lane 3) or with hMRAPa-FLAG (Fig. 1D) but not with hMRAP2 (Fig. 5, lane 4). However, the intensity of all of the HA-hMC4R-specific bands was increased following co-expression with hMRAP2, compared with HA-hMC4R expressed alone. An β -tubulin loading control verified that approximately the same amount of cell lysate was loaded in each lane.

Discussion

We show here that HA-hMC4R molecular mass is specifically altered following co-expression with hMRAPa-FLAG in HEK293 cells. hMRAPa-FLAG co-expression did not change the molecular mass of the other four

MCR subtypes or the hCL or hD2R. Furthermore, we show that the hMRAPa-induced changes in hMC4R complex N-linked glycosylation in a manner that differs from its previously reported effects on hMC2R glycosylation. We observed the expression of hMC4R with hMRAPa-induced changes in complex N-linked glycosylation at the cell surface as well as intracellularly, indicating that differential complex N-linked glycosylation could potentially alter hMC4R functional expression. Our data indicate that hMRAPa could be an accessory protein influencing functional expression of the hMC4R, provided these proteins are co-expressed *in vivo*.

The hMC2R is the only hMCR subtype for which immunoblotting following co-expression with hMRAPa has been performed and this revealed increased expression of higher molecular mass glycosylated hMC2R (Roy *et al.* 2007, 2010, Sebag & Hinkle 2007, 2009a). We confirmed that hMRAPa specifically increases the relative abundance of complex glycosylated (~ 45 and ~ 52 kDa) compared with lower molecular mass intermediate and monomeric (~ 32 and ~ 35 kDa) hMC2R forms. Additionally, we have shown for the first time that the HA-hMC2R is partly sensitive to neuraminidase digestion; therefore it contains some Neu5Ac α 2–3R and Neu5Ac α 2–6R sialic acid residues. We observed hMRAPa-FLAG-induced changes in HA-hMC4R molecular mass in cell lysates that were electrophoresed in parallel with co-immunoprecipitated samples. Others did not observe hMRAPa-induced changes in hMC4R molecular mass, because they immunoblotted for hMRAPa while we immunoblotted for each HA-hMCR subtype (Chan *et al.* 2009). The hMC4R has previously been shown to migrate at a molecular mass higher than its predicted molecular mass on SDS–PAGE (Ju *et al.* 2001, Peter *et al.* 2010). The hMC4R is predicted to be N-glycosylated at three N-terminal sites, Asn³, Asn¹⁷ and Asn²⁶, but it has never been determined if this modification occurs *in vitro* or *in vivo* (Schioth *et al.* 1997, Mountjoy 2000, Tao 2010). We have performed the first analysis of hMC4R N-linked glycosylation by western blotting and shown that the HA-hMC4R has extensive endoglycosidase H-resistant complex N-linked glycosylation when it is expressed in HEK293 cells. hMRAPa co-expression induces changes in the mass of the N-linked glycans attached to the HA-hMC4R. The HA-hMC4R was sensitive to neuraminidase digestion both when it was expressed alone and when it was expressed with hMRAPa-FLAG. Therefore hMRAPa-FLAG does not block the addition of sialic acid to the HA-hMC4R. hMRAPa-FLAG could influence oligosaccharide processing prior to the addition of terminal sialic acid as we observed a

neuraminidase-resistant HA-hMC4R-specific band when the HA-hMC4R was expressed with hMRAPa-FLAG. Alternatively, hMRAPa-FLAG may alter branching of complex oligosaccharide chains and/or hMRAPa may differentially influence hMC4R complex glycosylation at each of the three predicted *N*-linked glycosylation sites. Future studies are required to mutate each of the predicted hMC4R *N*-linked glycosylation sites individually and to express these mutant receptors with and without hMRAPa to determine whether hMRAPa influences hMC4R *N*-linked glycosylation at more than one site. Differential complex *N*-linked glycosylation at separate sites has been reported for the hMC1R (Herraiz *et al.* 2011). We attempted to purify the HA-hMC4R for proteomic analysis but were unsuccessful.

The effect on hMC4R molecular mass was specific to hMRAP1, since we observed this change following co-expression with either hMRAPa or hMRAPb but not following co-expression with hMRAP2 or FLAG-hRAMP1 (data not shown). hMRAP2 increased the intensity of all hMC4R molecular mass specific bands. This contrasts with the increased abundance of hMC2R high molecular mass bands that we and others have observed with hMRAPa co-expression. Other GPCR accessory proteins enhance the folding and stability of specific GPCRs (Ritter & Hall 2009). hMRAP2 could act to enhance the folding and/or the stability of the hMC4R, leading to increased hMC4R protein expression.

We observed no effect of hMRAPa-FLAG co-expression on the molecular masses of the hMC1R, hMC3R or hMC5R. However, the hMC3R and hMC5R both migrated at higher molecular masses than predicted, indicating that these receptors also undergo post-translational modification. The hMC5R was previously reported to have *N*-linked glycosylation (Sebag & Hinkle 2009a) and therefore HA-hMC5R-specific bands above ~40 kDa probably represent glycosylated HA-hMC5R. The high-molecular mass (~44 kDa) HA-hMC3R may be glycosylated hMC3R as the hMC3R has two predicted *N*-linked glycosylation sites (Schioth *et al.* 1997). The HA-hMC1R migrated at a lower molecular mass than predicted. Previously, FLAG-tagged hMC1R was observed to migrate at a lower molecular mass than untagged hMC1R (Sanchez Mas *et al.* 2002, Roberts *et al.* 2006, Sanchez-Laorden *et al.* 2006). Epitope tagging the hMC1R may alter hMC1R conformation resulting in greater mobility on SDS-PAGE. The hMC1R has also been reported to migrate as oligomers and/or *N*-linked glycoforms, but we did not observe any high-molecular mass hMC1R-specific bands (Sanchez Mas *et al.* 2002, Sanchez-Laorden *et al.* 2006).

In conclusion, we have shown that hMRAPa specifically alters hMC4R molecular mass and complex glycosylation. The functional significance of hMC4R complex *N*-linked glycosylation remains to be determined. *N*-linked glycosylation has been shown to modulate the cell surface expression, membrane dynamics, ligand binding and signalling of different GPCRs (Wheatley & Hawtin 1999, Murray *et al.* 2009). hMRAPa could influence any of these aspects of the hMC4R life cycle by altering hMC4R *N*-linked glycosylation. Regardless, our observation that hMC4R has differential complex *N*-linked glycosylation as a result of an interaction with hMRAPa indicates that hMRAPa may be an accessory protein modulating hMC4R functional expression.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-12-0220>.

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.

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