Localisation of the melanocortin-2-receptor and its accessory proteins in the developing and adult adrenal gland

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Abstract

The melanocortin-2-receptor (MC2R)/MC2R accessory protein (MRAP) complex is critical to the production of glucocorticoids from the adrenal cortex. Inactivating mutations in either MC2R or MRAP result in the clinical condition familial glucocorticoid deficiency. The localisation of MC2R together with MRAP within the adrenal gland has not previously been reported. Furthermore, MRAP2, a paralogue of MRAP, has been shown in vitro to have a similar function to MRAP, facilitating MC2R trafficking and responsiveness to ACTH. Despite similar MC2R accessory functions, in vivo, patients with inactivating mutations of MRAP fail to be rescued by a functioning MRAP2 gene, suggesting differences in adrenal expression, localisation and/or function between the two MRAPs. In this study on the rat adrenal gland, we demonstrate that while MRAP and MRAP2 are expressed throughout the adrenal cortex in low quantities. In the developing adrenal gland, both MRAP and MRAP2 are equally well expressed. The MC2R/MRAP2 complex requires much higher concentrations of ACTH to activate compared with the MC2R/MRAP complex. Interestingly, expression of MC2R and MRAP in the undifferentiated zone would support the notion that ACTH may play an important role in adrenal cell differentiation and maintenance.

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Introduction

Glucocorticoids are produced by the zona fasciculata (ZF) of the adrenal cortex in response to ACTH stimulation of the melanocortin-2-receptor (MC2R), also known as the ACTH receptor. Ligand binding and activation of this G protein-coupled receptor principally results in stimulation of adenylyl cyclase, which catalyses the conversion of ATP to cAMP. Subsequent activation of protein kinase A leads to phosphorylation of cAMP response element protein, which in turn activates the transcription of genes involved in steroidogenesis (Lefkowitz et al. 1970, Lehoux et al. 1998).

It has emerged over recent years that the MC2R is dependent on the co-expression of an accessory protein, the MC2R accessory protein (MRAP; Metherell et al. 2005). The MC2R only responds to ACTH in heterologous cells when co-expressed with MRAP (Metherell et al. 2005, Roy et al. 2007, Sebag & Hinkle 2007), and knockdown of MRAP in mouse adrenal Y1 cells (Cooray et al. 2008) and inactivating mutations in patients with familial glucocorticoid deficiency type 2 (FGD type 2; OMIM #607398) (Metherell et al. 2005) lead to loss of ACTH responsiveness. It is also clear that MRAP mRNA is expressed in the adrenal gland (Metherell et al. 2005). It is generally assumed that the glucocorticoid-producing fasciculata cell expresses both MC2R and MRAP, although this has not been formally established. Furthermore, MRAP has a single paralogue in the mammalian genome – MRAP2. MRAP and MRAP2 share a 39% amino acid identity in the N-terminus and transmembrane domain (Chan et al. 2009). The MRAP2 gene is expressed in the brain and the adrenal gland. In vitro MRAP2 appears to have a qualitatively similar ability to MRAP, enabling MC2R trafficking from the endoplasmic reticulum to the cell surface and the formation of a functional ACTH receptor (Chan et al. 2009). However, studies with mouse or zebrafish MRAP2 did not show this effect when a lower concentration of ACTH was used (Sebag & Hinkle 2009, Agulleiro et al. 2010). Since the presence of MRAP2 in the adrenal gland does not protect patients with MRAP mutations from developing glucocorticoid deficiency, the question arises as to whether MRAP and MRAP2 are expressed at different levels, in different cell types or whether they differ functionally to lower doses of ACTH. In this study, we analysed the quantitative expression, anatomical localisation and functional properties of these two MRAPs.
**Materials and methods**

**Plasmid and probes**

Vectors used in the cAMP reporter assays, MRAP-flag and MRAP2-flag were cloned, as previously described (Chan et al. 2009). HA-MC2R was purchased from the Missouri S&T cDNA Resource Center (University of Missouri at Rolla, Rolla, MO, USA). Templates for creating riboprobes for in situ hybridisation (ISH) were produced by inserting full-length M*rap* (forward primer 5'-CTGTCGCTCTACACCTGAT-3', reverse primer 5'-TAACCATGTGATTCAGAGG-3'), M*rap2* (forward primer 5'-CTGTCGAAGGCTCCTGACCC-3', reverse primer 5'-GAACACATCCCTGGAAGGC-3'), or M*cr* (forward primer 5'-ATCTGCAGTTTGGCCATTTC-3', reverse primer 5'-ATGCCCATTGACCTGGAAGG-3') cDNA produced from rat adrenal RNA into pGEMT-Easy vector (Promega). All constructs were confirmed by sequencing.

**Nonradioactive-ISH and immunohistochemistry**

Adrenal glands from adult Wistar male rats were snap frozen, or fixed in 4% paraformaldehyde and embedded in paraffin. Wistar rat embryos (embryonic day 17-18) were collected in ice-cold PBS (Sigma), fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded in OTC compound (Fisher Scientific, Loughborough, UK). Digoxigenin (DIG)-labelled sense and antisense cRNA riboprobes were produced from rat adrenal RNA into pGEMT-Easy vector (Promega). All constructs were confirmed by sequencing.

**Real-time quantitative RT-PCR**

cDNA was synthesised from rat total adrenal RNA (extracted from the adrenal glands of two adult male Wistar rats) by reverse transcription using M-MLV Reverse Transcriptase (Promega), following the manufacturer's guidelines. Gene expression levels were analysed by SYBR Green assays, using KAPA SYBR Fast Universal qPCR kit (Anachem, Luton, UK) and a Stratagene thermocycler. Thermal profile used: 1 × 95 °C for 3 min, 40 × (95 °C for 3 s, 55 °C for 20 s and 72 °C for 1 s), 1 × (95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s). Primers used for rat cDNA were *Mrap* (forward, 5'-ATTCTGCTCTACATGTCTTG-3', reverse, 5'-TACCTCTGCTGTACTG-3') and *Mrap2* (forward, 5'-CAGAGGCCTGACCTTAAAGC-3', reverse, 5'-CAGAAATCAATCAGAGG-3'). Gene expression levels were normalised using the housekeeping gene *Gapdh* (Promega). All constructs were confirmed by sequencing.

**Cell culture, transfection and cAMP reporter assay**

Human embryonic kidney (HEK293) cells were grown in DMEM (Sigma) containing 10% foetal bovine serum (Sigma) and penicillin/streptomycin (Invitrogen). Cells were grown to around 70–80% confluency in six-well plates and transfected with HA-MC2R, plus either MRAP-flag, MRAP2-flag, MRAP-flag and MRAP2-flag or pcDNA 3.1 (+) empty vector, along with pGL4.18 luciferase and pRL-CMV Renilla luciferase plasmid constructs, using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. Twenty-four to forty-eight hours after transfection, cells were collected and assayed using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured using a plate reader (LUMistar Omega; BMG Labtech, Aylesbury, UK) and normalised to the pRL-CMV Renilla luciferase activity.

**Statistical analysis**

The data represent mean ± s.e.m. of at least three independent experiments performed in duplicate. Prism software (La Jolla, CA, USA) was used to evaluate EC50 values. Statistical analysis was performed using Student’s t-test.

**Results**

**Mrap, Mrap2 and Mcr2 localisation in the adult and foetal rat adrenal gland**

ISH was performed to visualise the localisation of *Mrap*, *Mrap2* and *Mcr2* mRNA expression within the...
adrenal gland (Fig. 1). Mrap and Mrap2 were readily detectable specifically in the adrenal gland during development (Fig. 1A–F). Mrap and Mrap2 NR-ISH was performed in combination with CYP11B1 IHC, which demonstrated that cortical, but not medullary cells, expressed Mc2r accessory proteins (Fig. 1G–G″).

In the adult adrenal gland, Mrap and Mc2r were expressed throughout the cortex (Fig. 1H–K), although their expression was weaker in the zona glomerulosa (ZG) compared with the ZF. In the ZF, a gradient was observed with higher levels of expression seen at the periphery decreasing towards the cortico-medullary boundary. When combined Mc2r NR-ISH/CYP11B1 IHC was performed, the inner layer of cells within the undifferentiated zone (ZU), also known as the zona intermedia, which was negative for CYP11B1, was strongly positive for Mc2r expression (Fig. 1L–L″). Combined Mrap NR-ISH/CYP11B1 IHC revealed the same pattern of expression (data not shown). In contrast, Mrap2 was detected at low levels throughout all zones of the adrenal cortex (Fig. 1M).

Figure 1  In situ hybridisation showing localisation of Mrap, Mrap2 and Mc2r in foetal and adult Wistar rat adrenal glands. (A–G) Foetal Wistar rat adrenal glands (embryonic age 17–5 days); (H–N) adult male Wistar rat adrenal glands. (A and B) Low-magnification images of foetal rat adrenal glands incubated with Mrap antisense and sense (S) riboprobes. Mrap is present throughout the foetal rat adrenal gland. The sense probe resulted in no specific staining. (C and D) 10× magnified images of foetal rat adrenal glands. No Mrap staining in the adrenal capsule (C) or subcapsular zone (indicated by T). (E and F) Foetal rat adrenal glands incubated with Mrap2 antisense and sense (S) riboprobes. Mrap2 is present throughout the foetal rat adrenal gland. The sense probe resulted in no specific staining. (G) Combined Mrap ISH and CYP11B1 immunohistochemistry. Nuclei are stained with DAPI. Cells positive for Mrap also express CYP11B1, suggesting these cells are capable of glucocorticoid synthesis and are destined to localise to the zona fasciculata postnatally. (H–K) Mc2r and Mrap are present in the zona fasciculata (ZF), with relative sparing of the capsule, zona glomerulosa (ZG) and medulla (M). Expression was strongest at the periphery, with a gradient reduction towards the medulla. The sense probes resulted in no specific staining (h,j). (L) Combined Mc2r ISH and CYP11B1 immunohistochemistry. Mc2r is expressed in the ZF, in cells co-expressing CYP11B1. Mc2r is also expressed highly in the undifferentiated zone, in cells which lack CYP11B1 expression. (M and N) Mrap2 is expressed at low levels throughout the adult rat adrenal gland. The sense probe resulted in no specific staining (N).
Quantification of Mrap and Mrap2 in rat adrenal gland

Quantitative PCR was performed on rat total adrenal gland cDNA for Mrap and Mrap2 expression. Results were expressed relative to Gapdh or copy numbers per reaction. Mrap was expressed at significantly higher levels compared with Mrap2 (18× greater than Mrap2 when expressed relative to Gapdh, \( P<0.0001 \), and 21× greater when expressed as copy number per reaction, \( P=0.0015 \); Fig. 2).

Functional MC2R differences between MRAP and MRAP2

HEK293 cells, which lack endogenous melanocortin receptors, were transfected with MC2R and either MRAP, MRAP2 or both. Stimulation with ACTH generated a dose-dependent increase in cAMP production, as assessed by the luciferase activity produced from the cAMP-responsive αGSU-846 luciferase reporter construct. Cells transfected with MC2R and MRAP2 were able to produce cAMP; however they required significantly higher concentrations of ACTH to achieve this (EC50 1×10^{-9} for MRAP and 9.3×10^{-7} for MRAP2; \( P<0.0001 \)). Cells expressing both MRAP and MRAP2 did not exhibit a reduction or augmentation of the MRAP/MC2R cAMP response to ACTH stimulation (Fig. 3).

Discussion

The importance of MRAP in MC2R function is exemplified by the clinical condition of FGD, in which disabling mutations of either gene results in life-threatening isolated glucocorticoid deficiency (Metherell et al. 2005). Although the expression of MRAP has been demonstrated in the adrenal gland by RT-PCR, little is known about the localisation of MRAP mRNA within the different zones of the adrenal gland (Metherell et al. 2005). In addition, the presence of MRAP2 in the adrenal gland could indicate the potential for redundancy (Chan et al. 2009), although this is unlikely as patients with MRAP mutations are not rescued by an intact MRAP2 gene.

Our ISH studies have shown that Mrap and Mc2r are highly expressed in the rat adrenal ZF in cells expressing CYP11B1 and thus being capable of glucocorticoid synthesis. The level of expression appears to be highest at the periphery, with a gradient reduction towards the medulla. Interestingly, the highest level of Mrap and Mc2r expression is in the inner part of ZU, directly adjacent to the ZF. The ZU lies between the ZF and ZG in the adult rat adrenal and has been postulated to contain stem cells that contribute to steroidogenic cell maintenance (Wright & Voncina 1977, Mitani et al. 2003). Expression of Mrap and Mc2r in the ZU in relatively undifferentiated, CYP11B1-negative cells is interesting and suggests that ACTH may have a role in adrenal cell differentiation and maintenance. The concept that ACTH plays an...
An important role on adrenal growth and adrenal zonal maintenance has been proposed for some time (reviewed in Karpac et al. 2007 and Chan et al. 2011). Hypophysectomised animal models have adrenocortical atrophy (Robinson et al. 1983) and similarly atrophied adrenal glands were noted in POMC-knockout mice (Coll et al. 2004, Karpac et al. 2005). The administration of ACTH alone appeared to restore adrenal weight, morphology and function to normal in these models (Robinson et al. 1983, Coll et al. 2004). The adrenal histology of FGD would support the suggestion that MC2R and/or MRAP may be important for adrenal zonal development as the glands from deceased patients demonstrate disorganisation of glomerulosa cells with almost complete absence of fasciculata and reticularis cells (Clark & Weber 1998).

In comparison to the high expression levels seen with Mrap and Mc2r, ISH studies for Mrap2 showed very low levels of expression in adult rat adrenal but high expression in the developing adrenal gland. Real-time RT-PCR quantification of Mrap and Mrap2 expression in the adult rat adrenal gland demonstrated that Mrap expression is significantly greater than Mrap2 expression, supporting the low expression levels detected by ISH.

We previously showed that MRAP2 was able to rescue the expression and function of the MC2R in non-adrenal cells (Chan et al. 2009). Later studies, however, suggested that although MRAP2 was capable of assisting MC2R to the cell surface, the receptor was unresponsive to ACTH (Sebag & Hinkle 2009, Agulleiro et al. 2010). Here, we demonstrate that MRAP2 does indeed enable functioning of the MC2R in response to ACTH stimulation in vitro, however, much higher concentrations of ACTH are required to generate the same amount of cAMP in the presence of MRAP2 compared with MRAP. The 1000-fold difference in $EC_{50}$ provides an explanation and hence agreement for the different results obtained by other groups who utilised different ACTH doses for stimulation (Chan et al. 2009, Sebag & Hinkle 2009, Agulleiro et al. 2010). It is likely that even in FGD type 2, where ACTH concentrations are often $>1000$ pg/ml (Clark & Weber 1998), this is still inadequate to stimulate MC2R function. This suggests that although MRAP2 can enable MC2R function, this is not its role in vivo.

Studies have shown that MRAP2 can heterodimerise with MRAP in vitro (Chan et al. 2009) and such heterodimers adopt an antiparallel conformation (Chan et al. 2009, Sebag & Hinkle 2010). Western blotting of tissues has revealed band sizes of which are suggestive of homodimers rather than heterodimers (Cooray et al. 2008, Chan et al. 2009). Nevertheless, experiments on Y1 and CHO cells suggest that MRAP2 has an inhibitory role and competes with MRAP to bind the MC2R, reducing cAMP production in response to ACTH stimulation (Sebag & Hinkle 2010) in vitro. In this study, we did not show any reduction in cAMP generation of the MRAP/MC2R complex with the addition of MRAP2. Likewise, another study on zebrafish MRAP2 did not report an inhibitory effect, but found that MRAP2 had additive effects on the MRAP/MC2R cAMP response to ACTH (Agulleiro et al. 2010). This may, however, represent the different cell lines used in the studies and hence different levels of expression achieved (Chan et al. 2009, Agulleiro et al. 2010, Sebag & Hinkle 2010). While the hypothesis of a dominant-negative action of MRAP2 is an attractive one, it is difficult to envisage such a mechanism in view of the low levels of MRAP2 expression in adrenal tissue, the diffuse zonal localisation in the adrenal gland and the amount of MRAP2 expression that may be required to significantly shift the $EC_{50}$ for this to be relevant. Whether MRAP2 expression in the foetal adrenal gland determines the tone of the hypothalamic–pituitary–adrenal axis in adult life has yet to be studied and can only be answered with the generation of knockout mice.

In conclusion, we have demonstrated that Mc2r, Mrap and CYP11B1 are co-expressed in fasciculata cells in the rat adrenal gland, consistent with their role in ACTH stimulation of steroidogenesis. Furthermore, Mrap and Mc2r are expressed in some cells of the rat adrenal ZU, suggesting that ACTH may be important in adrenal cell differentiation and maintenance. The role of MRAP2 in the adrenal gland is unclear. Although MRAP2 is capable of facilitating MC2R function in response to ACTH stimulation, the concentration of ACTH required is much higher than that likely to be generated in vivo. However, the apparent high expression of MRAP2 in the developing adrenal gland may suggest a role in early development.
Availability of global or tissue-specific Mrap2-knockout mice would certainly help clarify the role of this protein during development and in adult homeostasis.

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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