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Review

Effects of melanocortins on adrenal gland physiology

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ABSTRACT

The melanocortin-2-receptor (MC_2 receptor), also known as the ACTH receptor, is a critical component of the hypothalamic–pituitary–adrenal axis. The importance of MC_2 receptor in adrenal physiology is exemplified by the condition familial glucocorticoid deficiency, a potentially fatal disease characterised by isolated cortisol deficiency. *MC_2 receptor* mutations cause ~25% of cases. The discovery of a MC_2 receptor accessory protein MRAP, mutations of which account for ~15%–20% of familial glucocorticoid deficiency, has provided insight into MC_2 receptor trafficking and signalling. MRAP is essential for the functional expression of MC_2 receptor. MRAP2, a novel homolog of MRAP, can also facilitate MC_2 receptor cell surface expression and function. Like MRAP, MRAP2 is a small transmembrane domain glycoprotein capable of homodimerising. In addition, MRAP/MRAP2 can heterodimerise. The presence of MRAP2 adrenal expression suggests a possible role for MRAP2 in adrenal physiology, which has yet to be elucidated. Importantly, new data shows that the MRAPs can interact with all the other melanocortin receptors ($MC_{1,3,4,5}$ receptor). In contrast to MC_2 receptor, this interaction results in reduced melanocortin receptor surface expression and signalling. MRAP2 is predominantly expressed in brain. Hypothalamic expression has been demonstrated for both MRAP and MRAP2. The ability of MRAPs to modulate different members of the melanocortin receptor family in a bidirectional manner is intriguing. Furthermore, central nervous system expression of MRAPs points to a role beyond MC_2 receptor mediated adrenal steroidogenesis.

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1. Introduction

The hypothalamo-pituitary-adrenal axis is responsible for the tight control of the diurnal production of cortisol at basal conditions and essential for the rapid increases in cortisol necessary at times of stress and illness. External stimuli such as physical and emotional stress trigger the synthesis and release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the medial parvocellular neurons of the paraventricular nucleus of the hypothalamus (Sawchenko and Swanson, 1985). CRH and AVP secreted into the hypophyseal portal circulation acts synergistically on corticotroph cells in the anterior pituitary gland to produce and release adrenocorticotropic hormone (ACTH), a melanocortin peptide derived from cleavage of the preprohormone precursor proopiomelanocortin (POMC), into the circulation (Plotsky, 1988). ACTH is released in sufficient concentrations to act on peripheral sites, principally the adrenal glands, to stimulate the production of glucocorticoids. Glucocorticoids can then negatively feed-back at the level of the hypothalamus, pituitary and additionally higher brain centers such as the hippocampus. The rapidity of the glucocorticoid feed-back (seconds to minutes) together with the stepwise intensification of the hormone signal at various levels of the axis provides amplification of the downstream cortisol signal which can be rapidly switched off. The control of this system is essential and perturbations of the system have been implicated in a wide range of physiological and disease states including immune modulation, psychiatric disorders, neonatal programming, metabolic disease, in addition to classical deficient and excess states such as adrenal insufficiency and Cushing's syndrome respectively.

2. ACTH actions on the adrenal gland

2.1. ACTH and adrenal steroidogenesis

ACTH is a 39 amino-acid peptide derived from the cleavage of the POMC protein by the action of prohormone convertase (PC) enzyme. In the anterior pituitary, PC1/3 cleavage sites are used to produce N-terminal proopiocortin, joining peptide, ACTH and β-lipotropin. In pituitary corticotrophs that lack PC2, these peptides are not cleaved further. Similar to the other endogenous melanocortin peptides derived from POMC namely α-, β-, γ-melanocyte-stimulating hormone (MSH), ACTH contains the amino acid sequence HFRW that is essential for biological activity (Schioth et al., 1997). Although ACTH can activate the other melanocortin receptors, its principal role is to regulate adrenal function through binding to its receptor the ACTH receptor (also known as the melanocortin-2-receptor), on the surface of adrenocortical cells.

The adrenal gland consists of an outer cortex and an inner medulla. The adrenal medulla, secretes catecholamines under the control of the sympathetic nervous system. The adrenal cortex is further divided into three distinct zones, the outer zona glomerulosa that secretes aldosterone, the middle zona fasciculata that secretes glucocorticoids (cortisol in humans and primates and corticosterone in rodents), and the inner zona reticularis that secretes adrenal androgens. ACTH predominantly acts on the zona fasciculata to produce glucocorticoids, a process which is essential for life, but can also act on the other zones all of which express the MC_2 receptor.

The action of ACTH on the zona reticularis results in the production of adrenal androgens dehydroepiandrosterone (DHEA), DHEA-sulfate, and androstenedione. ACTH dependent stimulation of the zona reticularis is seen naturally in normal puberty and is termed 'adrenarche'. Excessive ACTH drive on the zona reticularis also accounts for excessive androgen production in conditions such as Cushing's disease and congenital adrenal hyperplasia. Conversely patients with loss-of-function MC_2 receptor mutations have absent adrenarche (Weber et al., 1997). Premature adrenarche in childhood has also been associated to *MC_2 receptor* promoter polymorphisms (Lappalainen et al., 2008).

In comparison, the physiological role of ACTH on aldosterone production is unclear. Aldosterone synthesis from zona glomerulosa is principally controlled by angiotensin II acting on the angiotensin receptor. There is some evidence suggesting that ACTH acting through MC_2 receptor has a direct role in aldosterone production (Chan et al., 2009a; Chida et al., 2007; Lin et al., 2004).

2.2. Effects of ACTH on adrenal growth and maintenance

The role of ACTH in adrenal growth and maintenance is debatable. It has long been known that hypophysectomy of experimental animals results in adrenocortical atrophy (Robinson et al., 1983), whilst excess ACTH states such as Cushing's disease cause adrenocortical hyperplasia. It is uncertain if this is a direct effect of ACTH or due to co-secretion of another adrenal mitogen. One candidate is the N-terminal peptide from POMC, N-POMC[1–28] which has been shown to stimulate adrenal growth (Estivariz et al., 1982; Estivariz et al., 1988; Lowry et al., 1983). It is postulated that N-terminal POMC peptides with mitogenic activity are generated from the cleavage of circulating pro-γMSH (N-POMC[1–76]) by an adrenal specific protease (Bicknell et al., 2001). Studies on the POMC knock-out mice support the role of POMC derived peptides in adrenal growth and development. In POMC knock-out mice, the description of the adrenal glands varied from complete absence to small atrophied glands with disrupted architecture (Coll et al., 2004; Smart and Low, 2003; Yaswen et al., 1999). In one study the administration of ACTH alone but not N-POMC[1–28] sufficiently restored adrenal weight, cortical morphology, and plasma corticosterone concentrations in POMC knock-out mice (Coll et al., 2004, 2006). However, this is in contrast to some findings that ACTH replacement does not restore adrenal size in hypophysectomised rats (Payet and Lehoux, 1980). Similarly, cell based models have not supported a mitogenic role for ACTH. In fact ACTH has been shown to have antimitogenic activity on primary adrenal cells and mouse Y1 adrenocortical cells (Masui and Garren, 1971; Ramachandran and Suyama, 1975).

2.3. Extra-adrenal effects of ACTH

In addition to the adrenal gland, MC_2 receptor is also expressed in rodent adipose tissue (Wikberg, 1999). ACTH has an effect on adipocyte lipolysis and has been shown to cause insulin induced glucose uptake in white adipose tissue (Iwen et al., 2008). *MC_2 receptor* expression in a number of other tissues and cell-types is shown including gonads, skin, developing lung, pituitary and blood monocytes (Boston and Cone, 1996; Nimura et al., 2006). The precise role of ACTH in these tissues remains unknown.

2.4. Adrenal expression of other melanocortin receptors

The role of melanocortin receptors other than the MC_2 receptor in adrenal physiology is uncertain. MC_5 receptor is expressed in the adrenal cortex (Gantz et al., 1994; Griffon et al., 1994; van der Kraan

et al., 1998). The ability of α-MSH to stimulate aldosterone secretion (Vinson et al., 1980) together with abundant *MC_5 receptor* expression in the zona glomerulosa prompted suggestions that the MC_5 receptor maybe involved in melanocortin stimulated-aldosterone production (Griffon et al., 1994; van der Kraan et al., 1998). However, the lack of an adrenal phenotype in MC_5 receptor knock-out mice is not in keeping with this notion (Chen et al., 1997). Adrenal *MC_3 receptor* expression has also been demonstrated by some groups (Dhillo et al., 2003). The relevance, if any, is unclear and not supported by knock-out animal data (Chen et al., 2000).

3. MC_2 receptor in human adrenocortical pathology

The MC_2 receptor is the second of the five melanocortin receptors to be cloned. It is 297 amino acids in length and the smallest member of the G protein-coupled receptor (GPCR) family. The human *MC_2 receptor* gene maps to 18p11.2, and contains two exons, the second of which is the coding exon. Whilst the other melanocortin receptors (MC_1, MC_3, MC_4 and MC_5 receptors) are capable of binding melanocortin peptides α-, β- γ-MSH and ACTH, the MC_2 receptor is unique and binds specifically to ACTH alone. The MC_2 receptor, like the other melanocortin receptors, acts via the stimulatory G protein (Gs), which in turn activates adenylate cyclase, and hence cAMP generation. Intracellular cAMP activates protein kinase A which stimulates mitochondrial cholesterol import and gene expression of steroidogenic enzymes, ultimately resulting in steroidogenesis. A long standing question is the apparent difference in the ACTH dose required for steroidogenesis compared to cAMP production, demonstrated by 100 fold increase in the EC_{50} for cAMP generation. One explanation is that most cAMP assays fail to detect the small transient increases in cAMP that adequately stimulate steroidogenesis. An alternative explanation is the stimulation of an alternative pathway. Other secondary signal transduction pathways have been implicated in ACTH signalling involving, influx of extracellular calcium (Kojima et al., 1985), and MAP kinase (Le and Schimmer, 2001).

An interesting observation is that *MC_2 receptor* expression appears to be positively regulated by its own ligand. *In vitro*, exposing NCI-H295 human adrenocortical carcinoma cells to ACTH or forskolin for 24 h resulted in a 2 to 4 fold increase in *MC_2 receptor* expression (Mountjoy et al., 1994). In primary cell-lines a 21 fold increase in gene expression with a 4 fold increase in receptor number was demonstrated (Lebrethon et al., 1994).

Several groups hypothesised the involvement of MC_2 receptor in adrenal tumuorigenesis. Some evidence in support of this is the increased MC_2 receptor mRNA expression in aldosterone and cortisol secreting tumours (Arnaldi et al., 1998; Reincke et al., 1998). Loss of heterozygosity of the MC_2 receptor locus has also been described in some adrenal carcinomas (Reincke et al., 1997). Activating mutations of the MC_2 receptor are not a major cause of adrenocortical tumours (Latronico et al., 1995; Light et al., 1995). To date only one homozygous germ-line activating MC_2 receptor mutation has been described in a patient with cyclical Cushing's syndrome (Swords et al., 2002).

3.1. Familial glucocorticoid deficiency

Much of our current understanding about the action of ACTH on the adrenal gland comes from studying the condition familial glucocorticoid deficiency (OMIM 202200), otherwise known as hereditary unresponsiveness to ACTH. This rare autosomal recessive disease, first described by Shepard et al. in 1959 (Shepard et al., 1959), is clinically characterised by glucocorticoid deficiency in the presence of normal plasma renin and aldosterone levels. Typically ACTH levels at presentation are extremely high, >1000 pg/ml (normal range <80 pg/ml by RIA; <50 pg/ml by IRMA) (Clark and Weber, 1998). Patients present with hypoglycaemic seizures, transient hepatitis, hyperpigmentation, recurrent infections, failure to thrive, collapse, and coma in the neonatal period or early childhood. In familial glucocorticoid deficiency the adrenal glands are usually small in size. Post mortem adrenal histology from affected individuals reveals the absence of fasciculata or reticularis cells and disorganisation of glomerulosa cells (Clark and Weber, 1998).

Mild derangements of the renin–angiotensin–aldosterone system at the time of diagnosis are sometimes seen with this condition (Clark and Weber, 1998). The explanation of this observation is unclear but there is evidence that ACTH directly activates the production of aldosterone in the zona glomerulosa. *MC_2 receptor* mRNA localises to the zona glomerulosa and administration of synthetic ACTH to normal human subjects results in a rise in plasma aldosterone levels. Also, during periods of illness and acute stress, changes in the renin–angiotensin–aldosterone axis are seen (Clark and Weber, 1998). Furthermore, the MC_2 receptor knock-out mice have low serum aldosterone levels leading to hyperkalaemia. The renin–angiotensin–aldosterone system appears to attempt to compensate in the complete absence of MC_2 receptor, increasing angiotensin type 1b receptor expression in the zona glomerulosa, whilst CYP11B2 (aldosterone synthase) expression remains low (Chida et al., 2007). Finally, some familial glucocorticoid deficiency patients with 'severe' MC_2 receptor mutations have high renin levels suggestive of a compensated deficient mineralocorticoid state (Chan et al., 2009a; Lin et al., 2007). True mineralocorticoid deficiency and long-term fludrocortisone treatment is generally not seen (Dias et al., 2010).

Several features have been reported in familial glucocorticoid deficiency. For example, tall stature is seen in some patients with MC_2 receptor mutations. Absent adrenarche, presenting as delayed or absent pubic hair, is another feature and represents ACTH dependent production of adrenal androgens by the zona reticularis during normal puberty in childhood (Weber et al., 1997). A further finding is the highly elevated plasma ACTH seen in most patients which is often difficult to suppress despite treatment with high doses of hydrocortisone. The reason for this apparent lack of suppression is unknown. There is some suggestion of the existence of a short ACTH negative feedback loop at the level of the pituitary/hypothalamus. Hence, patients with mutations which render the MC_2 receptor inactive would lack negative inhibition of ACTH release at this level (Boscaro et al., 1988; Suda et al., 1987, 1986). Hyperpigmentation is almost always observed in familial glucocorticoid deficiency and is due to high ACTH levels which act on the MC_1 receptor in melanocytes.

3.2. Mutations in MC_2 receptor

The cloning of the MC_2 receptor by Mountjoy et al. in 1992 enabled researchers to identify point mutations in the MC_2 receptor in patients with familial glucocorticoid deficiency (Clark et al., 1993; Mountjoy et al., 1992; Tsigos et al., 1993). More than 30 mutations have been described in the MC_2 receptor, the majority of which are homozygous missense or compound heterozygous mutations (Fig. 1). The functional consequence of various MC_2 receptor mutations has been demonstrated by a number of groups (Elias et al., 1999; Fluck et al., 2002; Naville et al., 1997, 1996, 1999; Weber et al., 1995). The majority of MC_2 receptor mutations result in defective trafficking to the cell surface (Chung et al., 2008). To date there is no strong evidence to suggest that heterozygous carriers, i.e. parents or siblings of familial glucocorticoid deficiency patients, have abnormal cortisol secretion or response. However, a case report described a familial glucocorticoid deficiency patient harbouring a frameshift mutation (G217fs) on one allele and a T to C substitution in the −2 position of the MC_2 receptor promoter on the other allele (Tsiotra et al., 2006). Functional analysis of the promoter variant resulted in a reduction of MC_2 receptor function by 15% and the combination with a nonsense mutation is thought to lead to familial glucocorticoid deficiency (Tsiotra et al., 2006). This promoter variant is found in 10% of the population and has

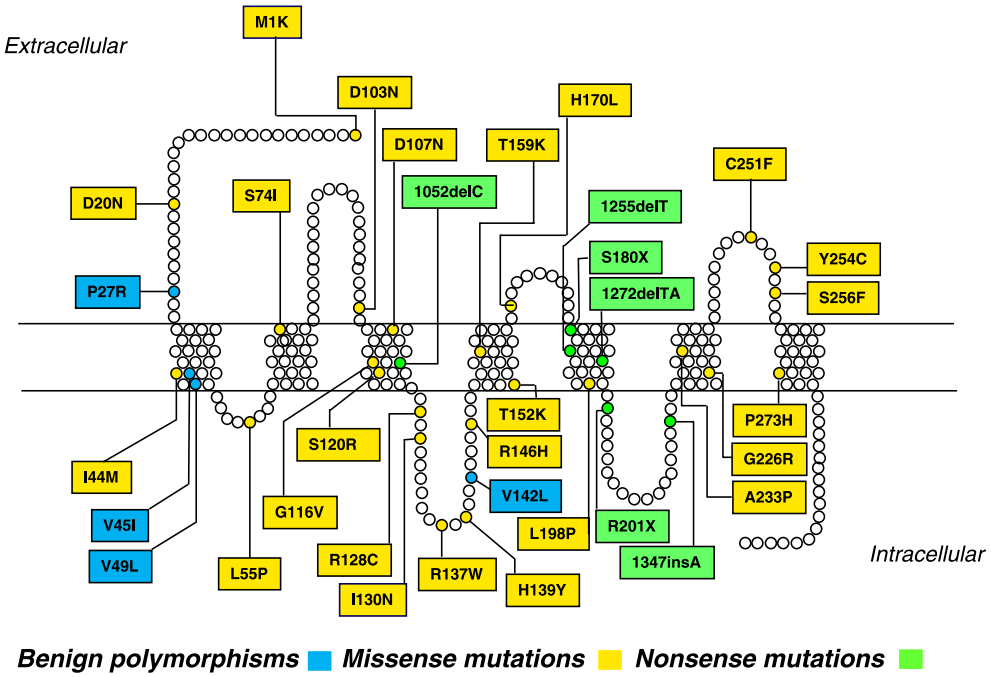

Fig. 1. Illustration of the human MC_2 receptor showing the locations of the loss-of-function mutations resulting in familial glucocorticoid deficiency.

been proposed to influence pituitary–adrenal axis activity in normal adults and children (Reisch et al., 2005; Slawik et al., 2004).

As well as the tall stature observed in some familial glucocorticoid deficiency patients with MC_2 receptor mutations (Elias et al., 2000; Imamine et al., 2005), a proportion of these patients have also been shown to have advanced or dissociated bone age, i.e. advanced bone maturation in the radius and phalanges with delay seen in the carpal bones. This excessive growth is more noticeable prior to the initiation of treatment and although hydrocortisone replacement appears to bring the height back towards the mid-parental target, children remain tall as adults (Clark and Weber, 1998). The rather limited data available suggests that the Insulin-like growth factor I-Growth Hormone axis is normal in these patients (Elias et al., 2000). It has been proposed that the excessive growth is due to high plasma ACTH levels (Imamine et al., 2005). The presence of all five melanocortin receptors in bone and the ability of ACTH to stimulate cAMP production and gene expression in bone cells provide a plausible mechanism (Elias et al., 2000; Mountjoy et al., 2003; Zhong et al., 2005). Not all familial glucocorticoid deficiency patients with inactivating mutations of the MC_2 receptor are tall, short stature has also been described (Selva et al., 2004). Around 25% of patients have mutations in the MC_2 receptor implying that other genetic causes can lead to the same clinical phenotype.

The search for additional causative genes linked familial glucocorticoid deficiency to a locus on 8q (Genin et al., 2002) in some patients. This locus was reanalysed by Metherell et al. revealing a larger region of homozygosity than previously described, now encompassing the locus for the steroidogenic acute regulatory gene (*STAR*). Mutational analysis of some patients revealed homozygous STAR mutations. Although mutations in *STAR* cause lipoid congenital adrenal hyperplasia, characterised by adrenal insufficiency together with gonadal deficiency, patients described by Metherell et al. habouring *STAR* mutations R192C and R188C were clinically indistinguishable from familial glucocorticoid deficiency (Metherell et al., 2009).

A proportion of patients did not show linkage to locus 8q or have a mutation in the MC_2 receptor, suggesting involvement of other genes. In keeping with this notion, distinct phenotypic variation has been described in a subset of Irish Travellers (an endogamous normadic group) with familial glucocorticoid deficiency. These patients present with later onset cortisol deficiency, associated with low birth weight, dysmorphic features and short stature. The majority of affected children are diagnosed over the age of 4 years, after an initial period of documented normal adrenal function in keeping with a possible degenerative process (O'Riordan et al., 2008).

4. MRAP

4.1. Discovery of MRAP as the cause of familial glucocorticoid deficiency type 2

In 2005, Metherell et al. performed a whole genome scan using single nucleotide polymorphism (SNP) array genotyping in one highly consanguineous familial glucocorticoid deficiency family, with three affected individuals all with normal MC_2 receptor. A new locus was mapped to chromosome 21q22.1 (Metherell et al., 2005). Examination of the tissue distribution of the genes expressed within the critical interval revealed a single candidate gene *C21orf61* (subsequently renamed melanocortin-2-receptor accessory protein — MRAP) that was highly expressed in the adrenal gland. mRNA expression was also detected in other tissues including testis, breast, brain, thyroid, lymph node, ovary and in fat (Metherell et al., 2005). The human *MRAP* gene consists of six exons. Exons 5 and 6 are alternatively spliced resulting in two transcripts termed MRAPα (exons1–5) and MRAPβ (exons 1–4 and 6). DNA sequencing of MRAP in this and other familial glucocorticoid deficiency families led to the identification of a number of mutations (Fig. 2).

When MRAP was discovered as the second genetic cause of familial glucocorticoid deficiency, little was known about the MRAP protein. An earlier study described it as a small protein upregulated in a differentiating mouse adiposite derived cell-line (3T3-L1). Xu et al. had called this protein of uncertain function *FALP* (Fat tissue-specific low molecular weight protein) (Xu et al., 2002). However, after the description of MRAP mutations in familial glucocorticoid deficiency patients, functional analysis revealed that MRAP was an essential MC_2 receptor accessory protein. This led to the protein and gene being renamed MRAP. MRAP is conserved between species in the N-terminus and transmembrane domain. The two protein isoforms

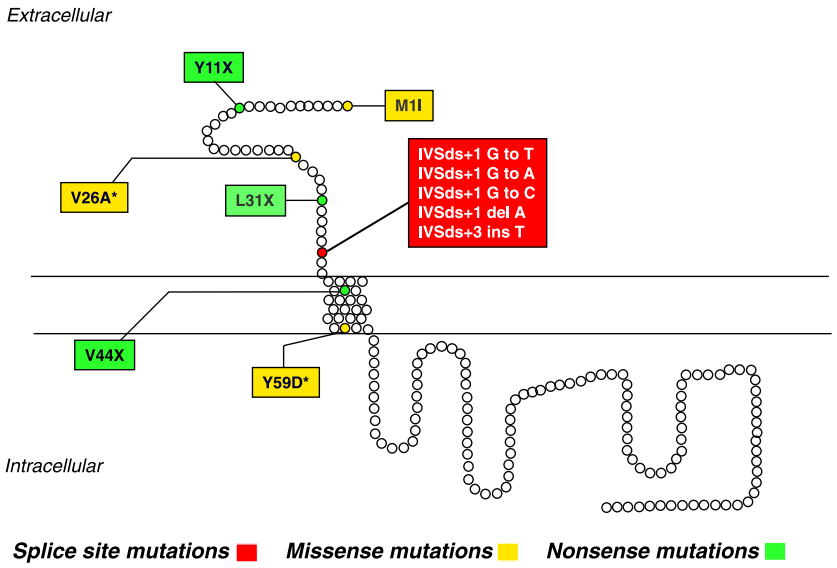

Fig. 2. Schematic representation of the human MRAPα protein and the mutations identified in familial glucocorticoid deficiency. The majority of the mutations result in complete absence or severe truncation of MRAP. *Two missense mutations (Y59D and V26A) are exceptions, associated with late onset disease.

MRAPα and MRAPβ differ only in the C-terminus and in humans are 19 kDa and 11.5 kDa in size respectively.

The majority of MRAP mutations described are homozygous mutations (nonsense and splice-site mutations) that would result in the complete absence or severe truncation of the protein (Metherell et al., 2005; Modan-Moses et al., 2006). In contrast, the majority of MC_2 receptor mutations are missense mutations. Familial glucocorticoid deficiency patients with *MRAP* mutations generally present very early in life relative to those with *MC_2 receptor* mutations (Chung et al., 2010), which may be a reflection of the mutations, i.e. the complete abolition of the MRAP protein compared to some residual function of the MC_2 receptor protein. Only two full length homozygous missense MRAP mutations have been identified: V26A and Y59D. Functional analysis demonstrates impaired, rather than absent, MC_2 receptor function. This correlated with a much later age of onset of disease with one patient presenting at 18 years of age (Hughes et al., 2009).

4.2. Structure and function of MRAP

In the past, studies using the MC_2 receptor were hindered by the inability to express the receptor at the cell surface and consequently the failure to generate a downstream cAMP response to ACTH. Functional expression of MC_2 receptor in the absence of other melanocortin receptors was only possible in a limited number of cell-types, such as the Y6 and OS3 cell-lines that were derived from mouse adrenocortical tumours. In most cell-lines, transiently transfected MC_2 receptor is retained in the endoplasmic reticulum (Noon et al., 2002), whereas in Y6 or OS3 cells MC_2 receptor localises to the cell surface where it forms a functional ACTH responsive receptor (Elias et al., 1999). The inability of MC_2 receptor to reach the cell surface in non-adrenal cell-types led to the belief that an adrenal-specific accessory factor was required to facilitate MC_2 receptor trafficking (Noon et al., 2002). Therefore, the identification of MRAP as one of the genes underlying familial glucocorticoid deficiency, led to the hypothesis that MRAP was an MC_2 receptor trafficking factor (Metherell et al., 2005).

MRAP is a small transmembrane protein, as demonstrated by immunocytochemistry localised to the cell surface (Metherell et al., 2005). To test the hypothesis that MRAP was an MC_2 receptor accessory protein, reciprocal co-immunoprecipitation was undertaken which demonstrated that MRAP and MC_2 receptor were able to form a complex. Furthermore, co-expression of MRAP and MC_2 receptor in non-adrenal cells enabled the MC_2 receptor to localise to the cell surface. MRAP and MC_2 receptor not only co-localised at the cell surface of heterologous cells, but the presence of MRAP enabled MC_2 receptor to generate cAMP in response to ACTH (Metherell et al., 2005). Mouse Y1 adrenocortical cells that endogenously express both MC_2 receptor and MRAP show a cAMP response to ACTH stimulation. siRNA knockdown of endogenous mouse MRAP in Y1 cells resulted in a loss of responsiveness to ACTH. MC_2 receptor function was rescued with transient expression of human MRAP, which is resistant to silencing by mouse siRNA (Cooray et al., 2008). These results together with studies by other groups confirmed that MRAP was an essential factor for MC_2 receptor trafficking and subsequent function (Roy et al., 2007; Sebag and Hinkle, 2007).

Although MRAP shares some functional similarity with the other accessory proteins (RAMPs, RTPs and REEPs), MRAP is in many ways unique (McLatchie et al., 1998; Saito et al., 2004). Firstly, MRAP shares no sequence homology with the other accessory proteins and does not possess an N-terminal signal peptide (Metherell et al., 2005). Secondly, unlike the RAMPs (McLatchie et al., 1998), there is little evidence to date that MRAP can affect ligand selectivity of the MC_2 receptor. The presence of MRAP did not allow MC_2 receptor to respond to NDP-MSH (Sebag and Hinkle, 2009b). Finally, MRAP has been shown to exist as a unique dual topology homodimer (Cooray et al., 2008; Sebag and Hinkle, 2007). Differentially tagged MRAP proteins were able to interact and co-immunoprecipitate, demonstrating the ability to form homodimers (Cooray et al., 2008; Sebag and Hinkle, 2007). Sebag and Hinkle showed that the MRAP homodimers adopted a dual antiparallel conformation (Sebag and Hinkle, 2007). It also appears that this dual topology homodimer is formed in the endoplasmic reticulum and in a stable complex with MC_2 receptor, as suggested by co-immunoprecipitation studies (Sebag and Hinkle, 2009b). The mouse MRAPα protein has a predicted molecular weight of 14.1 kDa. However, western blot analysis using an MRAP antibody to detect endogenous protein revealed a band over 30 kDa size. Mass spectroscopy identified this band as MRAP which suggested that endogenous MRAP exists as a homodimer resistant to the actions of sodium dodecyl sulfate (Cooray et al., 2008).

4.3. MRAP functional domains

Studies on mouse and human MRAPs have identified a number of MRAP functional domains. Webb et al. created a series of human

MRAP truncation constructs FLAG tagged at their C-terminus (Webb et al., 2009). Co-immunoprecipitation studies using these FLAG tagged MRAP truncation proteins and HA-MC_2 receptor mapped the MRAP/MC_2 receptor interaction domain to a 27 amino acid region between residues 36 and 62 of MRAP that consists almost entirely of the transmembrane domain (Webb et al., 2009). It is likely that MRAP interacts with MC_2 receptor via hydrophobic interactions with one or more of its transmembrane domains.

The key MC_2 receptor trafficking domain localised to a 15 amino acid tyrosine rich region between residues 9 and 24 of human MRAP (Webb et al., 2009). Like MRAP, REEP1 also has an N-terminal tyrosine rich region (Saito et al., 2004). It is therefore possible that a tyrosine rich domain may be a feature of some proteins involved in GPCR trafficking.

MRAP deletion constructs also identified mouse MRAP amino acids 31–37 (LKANKHS), just proximal to the transmembrane domain, to be responsible for dual topology (Sebag and Hinkle, 2009b). Deletion of these residues resulted in a single orientation of MRAP that failed to traffic the MC_2 receptor.

Sebag and Hinkle performed alanine-substitution of mouse MRAP amino acids 18–21 (LDYI) and showed that in mutating these four residues, MRAP maintained the ability to traffic MC_2 receptor to the cell surface but the MC_2 receptor failed to signal and generate cAMP. This suggests that residues 18–21 of MRAP are important for ACTH binding and subsequent signalling (Sebag and Hinkle, 2009b). The separation of functions within accessory proteins is seen with other accessory proteins. For example in RAMPs, where deletion of amino acids 91–103 of RAMP1, 86–92 of RAMP2 and 59–65 of RAMP3 results in an accessory protein that enables trafficking of CLR to the cell surface but not ligand binding or signalling (Kuwasako et al., 2003; Parameswaran and Spielman, 2006).

MRAP constructs, in which the entire C-terminus was deleted, appeared to function normally. The C-terminus is therefore not required for MRAP to interact with MC_2 receptor or for MRAP to traffic MC_2 receptor to the cell surface. Instead, the MRAP C-terminal deletion construct resulted in significantly higher MC_2 receptor cell surface expression than wild-type MRAP. The C-terminus may have a regulatory effect on the amount of MC_2 receptor that reaches the cell surface (Webb et al., 2009). This is supported by observations made by Roy et al. which showed that MRAPβ resulted in higher MC_2 receptor surface expression compared to MRAPα, which differed only in the C-terminus (Roy et al., 2007). The *in vivo* relevance of these observations is currently uncertain as all known human MRAP mutations would effectively disable both isoforms to a similar extent. Although, MRAPα and MRAPβ are expressed in the human adrenal gland at similar levels (Metherell et al., 2005), little is known about changes in expression with aging or in different physiological or disease states. MRAPβ is thought to only exist in primates as lower mammals appear to have a single isoform homologous to MRAPα (Webb and Clark, 2010).

5. MRAP2

5.1. Identification of MRAP2

Sequence analysis of the human genome showed that MRAP was similar to another putative protein encoded by gene C6orf117, located on chromosome 6q14.3, now named MRAP2 (melanocortin-2-receptor accessory protein 2) (Chan et al., 2009b). The human *MRAP2* gene consists of four exons (three are coding) and its protein product comprises of 205 amino acid residues, with a predicted molecular weight of 23.5 kDa (Fig. 3A). MRAP2 is a small single pass transmembrane domain protein homologous to MRAP, with 39% amino-acid identity to MRAP in the N-terminal and transmembrane domains (Fig. 3B). Like MRAP, MRAP2 has no predicted signal sequence and a putative N-linked glycosylation site with the consensus sequence NRTS at amino acid positions 9–12 (Fig. 3A).

MRAP2 is highly conserved through vertebrates (Fig. 3C). MRAP2 protein conservation between species is much greater than MRAP with 87% sequence identity between human and mouse compared to 63% sequence identity between human MRAPα and mouse MRAP. Thus, MRAP2 is thought to represent the ancestral gene (Webb and Clark, 2010).

5.2. MRAP2 structure and function

RT-PCR of human *MRAP2* using a panel of cDNA together with immunoblotting of rodent tissues demonstrated MRAP2 expression in the brain and adrenal gland (Chan et al., 2009b). Furthermore, in-situ hybridisation studies localise MRAP2 mRNA to the paraventricular nucleus (PVN) of the hypothalamus (Lein et al., 2007). However, in a recent study on zebrafish MRAP2, a more ubiquitous expression pattern was described (Agulleiro et al., 2010).

Studies in heterologous cell lines expressing MRAP2 demonstrated that MRAP2 like MRAP was a single-pass transmembrane domain protein, localised to the cell surface and glycosylated at the N-terminus (Chan et al., 2009b). MRAP2 dimerisation properties seem to mirror that of MRAP. In endogenously expressing tissue, western blotting using an antibody to the C-terminal region of MRAP2 revealed a ~47.5 kDa band. Mouse MRAP2 has a predicted molecular weight of 23.3 kDa, hence the immunoreactive band visualised could be consistent with an MRAP2 dimeric structure *in vivo* that is resistant to the actions of SDS, reducing agents and heat. Like MRAP, human MRAP2-FLAG runs as monomeric isoforms. In the case of MRAP2-FLAG two molecular species, ~27 and 29 kDa in size, were detected representing the deglycosylated and glycosylated protein respectively. The ability of MRAP2 to homodimerise and also heterodimerise with MRAP was demonstrated by co-immunoprecipitation of differentially tagged MRAP2 and MRAP proteins. N-terminal glycosylation and a C-terminal out orientation, as shown by deglycosylation studies and immunofluorescent staining of live cells respectively, suggest that MRAP2 also adopts a dual topology.

5.2.1. MRAP2 and MC_2 receptor

In view of similarities between MRAP2 and MRAP together with MRAP2 adrenal expression, we studied the ability of MRAP2 to assist MC_2 receptor trafficking and function. Co-immunprecipitation studies revealed that MRAP2 and MC_2 receptor could form a complex, whilst confocal microscopy and an immunofluorescent cell surface assay showed that MRAP2 was capable of facilitating the MC_2 receptor to the cell surface in heterologous cells expressing the receptor. In addition, the presence of MRAP2 enabled the receptor to respond to ACTH.

An interesting finding was the uncoupling of MRAP2 receptor trafficking and receptor signalling accessory roles. An MRAP2 mutant with an asparagine to glutamine substitution at amino acid position 9 (disabling N-linked protein glycosylation) was able to traffic the MC_2 receptor to the cell surface but failed to generate cAMP in response to ACTH. Hence suggesting that MRAP2 has two separate MC_2 receptor accessory roles: firstly in trafficking of the receptor, and secondly in enabling receptor responsiveness to ACTH. MRAP2 glycosylation status is clearly vital to MC_2 receptor function and could represent a disruption of protein dual topology. In contrast, MRAP glycosylation status is probably unimportant as an MRAP deletion construct lacking the N-glycosylation signal was capable of supporting the expression of an ACTH responsive receptor (Webb et al., 2009).

The finding that MRAP2 could enable a functional MC_2 receptor contradicted results from two subsequent studies which found that although mouse and zebrafish MRAP2 facilitated trafficking of MC_2 receptor to the surface of cells, the receptor failed to respond to ACTH (Agulleiro et al., 2010; Sebag and Hinkle, 2009b). This discrepancy can be explained by the different doses of ACTH used in the studies. A

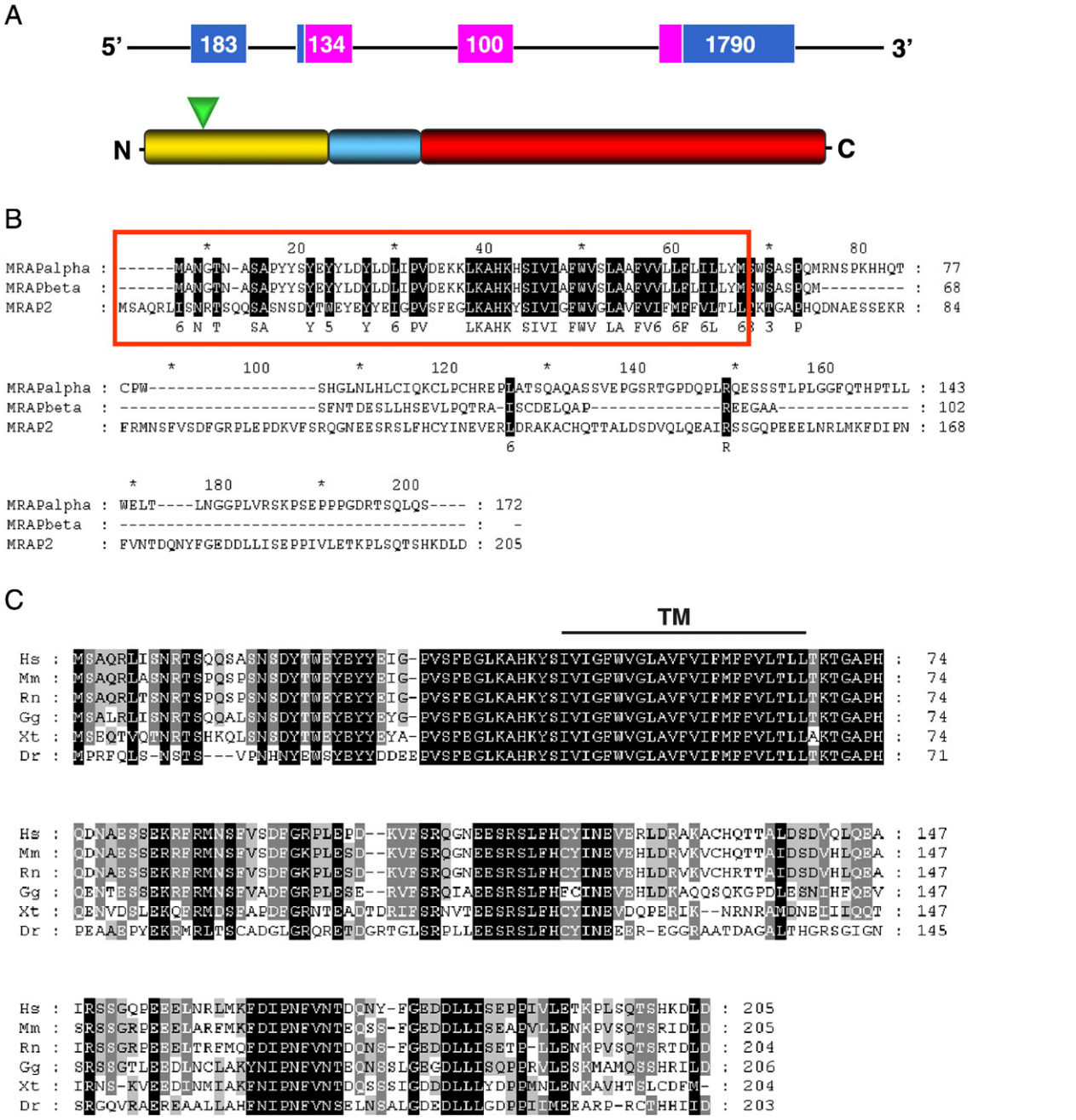

Fig. 3. Diagram of the human MRAP2 gene and protein (A). Length of exons is shown as numbers in boxes. Coding region is in pink, non-coding in blue. N-linked glycosylation site is shown as a green triangle. (B) Protein alignment of human MRAPα, β and human MRAP2. The N-terminal and transmembrane domain shows the highest levels of sequence identity (boxed). (C) MRAP2 is highly conserved as shown by protein alignment of MRAP2 orthologues. Abbreviations: Hs; *Homo sapiens*, Mm; *Mus musculus*, Rn; *Rattus norvegicus*, Gg; *Gallus gallus*, Xt; *Xenopus tropicalis*, and Dr; *Danio rerio*.

concentration of 10^{-6}M ACTH enabled cAMP generation (Chan et al., 2009b) whilst a concentration of 10^{-7}M ACTH was insufficient to generate a signal (Agulleiro et al., 2010; Sebag and Hinkle, 2009b; 2010). However, the failure of MRAP2 to support a functional MC_2 receptor at 10^{-7}M ACTH was reversed with the insertion of MRAP residues 18–21 (LDYI) into MRAP2 which naturally lacked this motif (Sebag and Hinkle, 2009b).

It has recently been shown that MRAP2 can act as an endogenous competitor of MRAP binding to MC_2 receptor, hence reducing the MC_2 receptor potency to ACTH (Sebag and Hinkle, 2010). This dominant negative effect was not reported in other studies, where the presence of MRAP2 either increased MC_2 receptor cAMP generation in response to ACTH (Agulleiro et al., 2010) or had no effect (Chan et al., 2009b).

MRAP is essential for MC_2 receptor mediated steroidogenesis and mutations in MRAP result in familial glucocorticoid deficiency type 2. The physiological role of MRAP2 in the adrenal gland is unclear. Although the protein shares many similarities there is no evidence that MRAP2 mutations cause familial glucocorticoid deficiency, and the presence of MRAP2 does not rescue the phenotype of isolated cortisol deficiency. The possibility of a more complex regulatory MRAP2 role is interesting but requires greater understanding of the temporal and spatial expression pattern of MRAP and MRAP2 which may provide insight into the possible differential or synergistic actions of these MC_2 receptor accessory proteins *in vivo*.

MC_2 receptor is primarily expressed in the adrenal gland, although expression has also been noted in a number of other tissues. MRAP

and MRAP2 are expressed more widely than MC_2 receptor, suggesting that both may have MC_2 receptor-independent function.

5.2.2. MRAP2 regulation of other melanocortin receptors

MRAP2 can interact with all five melanocortin receptors demonstrated by reciprocal co-immunoprecipitation. Unlike MC_2 receptor, the other melanocortin receptors do not require additional factors to traffic to the cell surface. Intriguingly, it appears that MRAP2, as either homodimers or heterodimers with MRAP, act as negative regulators of melanocortin receptor function. MRAP2 is capable of reducing the cell surface expression of MC_4 receptor and MC_5 receptor when co-expressed in CHO cells. MRAP2 did not change the cell surface expression of MC_1 receptor or MC_3 receptor. Reduced signalling, shown by a reduced cAMP response to NDP-MSH stimulation was identified with MC_4, MC_5 receptors. For MC_3 receptor decreased cAMP production was seen when co-expressed with MRAP2 or both MRAPs, and for MC_1 receptor when co-expressed with MRAP and MRAP2 together.

MRAP2 is principally expressed in the brain within the hypothalamus and PVN (Chan et al., 2009b; Lein et al., 2007). MC_4 receptor is highly expressed in the paraventricular hypothalamus and MC_3 receptor in the ventromedial nucleus of the hypothalamus (Balthasar et al., 2005; Chen et al., 2000). The MC_3 and MC_4 receptors are critical to the control of energy homeostasis and appetite regulation (Farooqi, 2008). The pattern of brain expression could imply that MRAP2 may be a potential candidate regulator to other central melanocortin receptors, such as the MC_4 receptor. *MRAP* expression has also been reported in a number of tissues including both brain and hypothalamus (Gardiner et al., 2002; Metherell et al., 2005). MRAP could also have a central role, although there is little suggestion of this in patients with familial glucocorticoid deficiency type 2 (Chung et al., 2010), with the exception of a case report of obesity in a family with a homozygous MRAP mutation (Rumie et al., 2007).

The ability of MRAP to reduce surface expression of MC_5 receptors was also shown by others (Sebag and Hinkle, 2007, 2009a), where the presence of MRAP was thought to disrupt MC_5 receptor dimerisation and hence receptor surface localisation (Sebag and Hinkle, 2009a). Another study demonstrated a reduction of MC_4 receptor at the cell surface in the presence of MRAP and MRAP2, (Sebag and Hinkle, 2010) although in this case little effect on MC_4 receptor signalling was observed. To date, there is little suggestion that MRAPs can affect other GPCRs and in-vitro studies have shown that MRAP2 has little effect on β_2-adrenergic receptor cAMP generation (Chan et al., 2009b; Sebag and Hinkle, 2009a).

Importantly, these studies indicate that MRAP and MRAP2 are able to modulate the melanocortin receptor family members in a bidirectional manner in heterologous cells. The potential central role of MRAP2 on MC_3 or MC_4 receptor mediated energy homeostasis is intriguing. Future studies will ascertain the physiological significance of MRAPs in the regulation of the melanocortin system.

6. Conclusions

Over the last two decades, the understanding of ACTH action on adrenal physiology has depended on key discoveries including the cloning of the MC_2 receptor in 1992 and the identification in 1993 of the first MC_2 receptor mutations causing familial glucocorticoid deficiency. The discovery of MRAP in 2005 as an essential MC_2 receptor accessory protein provides further insight. Research will now focus on discovering novel components of this system with the aim of understanding mechanisms by which ACTH can tightly regulate adrenal steroidogenesis in a dynamic manner, such as the regulation of the critical components of the HPA axis including MC_2 receptor and MRAP. Furthermore, the possibility that MRAP2 is a negative regulator of adrenal steroidogenesis needs exploration.

The melanocortin receptor family has diverse roles in normal physiology and disease processes, with the MC_3 and MC_4 receptors playing a critical function in energy homeostasis. Both MRAP and MRAP2 can interact with the other melanocortin receptors regulating their expression at the cell surface and subsequent signalling. Recent in-vitro data points to a possible central role of MRAP2. Establishing the physiological importance of this highly conserved transmembrane accessory protein is now dependent on future description of MRAP2 transgenic animals and/or patients harbouring MRAP2 mutations. Research in melanocortin receptor biology should therefore take account of these novel accessory proteins to the melanocortin receptor family.

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