Functional analysis of melanocortin 2 receptor variants and dimer formation

A thesis presented for the degree of

Doctor of Philosophy

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Abstract

The adrenocorticotropic hormone (ACTH) receptor, also known as the melanocortin 2 receptor (MC2R) is primarily expressed in the adrenal gland and binds to ACTH to activate a signalling cascade that leads to steroidogenesis. The characterisation of the melanocortin 2 receptor has previously been hindered by the failure of cell surface expression in heterologous cells. Unlike other melanocortin receptors, MC2R surface expression requires an accessory protein called melanocortin 2 receptor accessory protein (MRAP). MRAP has been shown to facilitate trafficking of MC2R from the endoplasmic reticulum to the plasma membrane and it also has a role in MC2R signalling.

Naturally occurring MC2R mutations occur in Familial Glucocorticoid Deficiency (FGD) type 1. The functional defect remains uncertain for the majority, mainly due to the difficulties in characterisation which has been limited in Y6 cells (mouse derived adrenal cells which are free of MCRs) because of poor transfection efficiency. The first two results chapter of this thesis focus on the molecular biology of FGD mutations and the genotype-phenotype relationship of the disease. The functional defects of these mutations have been analysed in transfectable heterologuous cells stably expressing MRAP. The majority of the missense mutations are trafficking-defective, and yet all appear to interact with MRAP, but some mutations are trafficking-competent and fail to signal.

Clinical details including the age of presentation; presenting plasma ACTH and cortisol; height and weight were examined in FGD type 1 and 2 (mutation of MC2R or MRAP respectively) to determine whether there is a genotype-phenotype correlation. Within FGD type 1, there was no clear clinical distinction between the functional defects *in vitro* and *in vivo* data. However, there were phenotypic differences between

FGD type 1 and 2, with FGD 2 appearing to be more severe with a younger age and a lower height SDS at presentation.

The final part of this thesis focuses on MC2R dimerisation and signalling of the receptor. Co-immunoprecipitation studies showed that MC2R homodimerisation is constitutive. This interaction was not enhanced in the presence of MRAP or ACTH. No effect on signalling was observed. When a signalling mutant (R128C) and a ligand binding mutant (D103N) were co-transfected with MRAP and stimulated with ACTH, signalling was not restored, suggesting no cross talk between the two receptors.

In the final discussion the implication of these findings are considered with respect to their relevance to disease and novel therapeutic strategies.

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Grants, prizes, and publications related to this thesis

Grants

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Prizes

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Best poster prize at ESPE, New York 2009 for "Genotype:phenotype relationships in Familial Glucocorticoid Deficiency types 1 and 2"

Publications

Chung TT, Webb TR, Chan LF, Cooray SN, Metherell LA, King PJ, Chapple JP, Clark AJL. The majority of ACTH receptor (MC2R) mutations found in Familial Glucocorticoid Deficiency type 1 lead to defective trafficking of the receptor to the cell surface. *Journal of Clinical Endocrinology and Metabolism* 2008 93: 4948-4954.

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*Chan LF, *Chung TT, Massoud AF, Metherell LA, Clark AJL. Functional consequence of a novel Y129C mutation in a patient with two contradictory MC2R mutations. *Eur J Endocrinol*. 2009 Apr;160(4):705-10. *Equal first author

*Chan LF, *Webb TR, Chung TT, Cooray SN, Guasti L, Chapple JP, Elphick MR, Cheetham ME, Metherell LA, Clark AJL. MRAP and MRAP2 are bidirectional regulators of the Melanocortin receptor family**.** *Proc Natl Acad Sci U S A*. 2009 Apr 14;106(15):6146-51. * Equal first author

Review Articles

Chung TT, Grossman AB, AJL Clark. Adrenal insufficiency in "Williams Textbook of Endocrinology" edited by LJ De Groot (in press).

Clark AJ, Chan LF, Chung TT, Metherell LA. The genetics of Familial glucocorticoid deficiency. *Best Pract Res Clin Endocrinol Metab.* 2009 Apr; 23(2): 159-65.

Abbreviations

AC	Adenylate cyclase
ACTH	Adrenocorticotrophin hormone
ATP	Adenosine triphosphate
BSA	Bovine serum albumine
cAMP	Cyclic adenosine monophosphate
cAMP	Adenosine 3:5-cyclic monophosphate
cDNA	Complementary DNA
CHO	Chinese Hamster Ovart
CLIP	Corticotropin-like intermediate peptide
Co-IP	Co-immunoprecipitation
CREB	cAMP response element
C-terminal	Carboxyl terminal
DAPI	4,6-diamidino-2-phenylindole
dATP	2-deoxyadenosine 5-triphosphate
ddH2O	Distilled water
DM	n-Dodecyl β-D-maltoside
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
dTTP	2-deoxythymidine 5-triphosphate
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FGD	Familial Glucocorticoid Deficiency
FLAG	DYKDDDDK epitope tag
G418	Geneticin
GFP	Green Fluorescent protein
GPCRs	G protein-coupled receptors
HA	YPYDVPDYA epitope tag
HEK 293	Human Embryonic Kidney 293
HPA axis	Hypothalamic-pituitary-adrenal axis
Kb	Kilobase pairs
kDa	Kilodaltons
LB	Luria Bertoni
MC1R	Melanocortin 1 receptor
MC2R	Melanocortin 2 receptor
MC2R	Melanocortin 2 receptors
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MC5R	Melanocortin 5 receptor
MCR	Melanocortin receptor
MRAP	Melancocortin 2 receptors accessory protein

mRNA	Messenger ribonucleic acid
MSH	Melanocyte stimulating hormones
PBS	Phosphate buffered saline
PC	Prohormone convertase
PCR	Polymerase chain reaction
PFA	Formaldehyde
PKA	Protein kinase A
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase-polymerase chain reaction
StAR	Steroidogenic acute regulatory
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Chapter 1

Introduction

1.1 The Adrenal Gland

The adrenal gland is an important endocrine organ which produces hormones including glucocorticoids, mineralocorticoids, sex steroids and catecholamines which are essential to life. The physiological increase in production of glucocorticoid is vital during stress and acute illness which is under the regulation of the hypothalamic-pituitary-adrenal axis (HPA).

1.1.1 The Adrenal morphology

The human adrenal cortex consists of three concentric zones which are morphologically and functionally distinct. The outermost is the zona glomerulosa which produces aldosterone, followed by the zona fasciculata which produces glucocorticoid (cortisol in humans and primates and corticosterone in rodents) and then the zona reticularis which produces adrenal androgens. ACTH is the principal regulator of cortisol production by the zona fasciculata, but it has secondary importance in aldosterone and adrenal androgen production.

1.1.2 The adrenal steroidogenic pathway

The common rate limiting step in steroid biosynthesis is the uptake of cholesterol from cellular stores to the inner membrane of the mitochondrial matrix; this is regulated by StAR (steroidogenic acute regulatory) protein. Once inside the mitochondria, the cholesterol side chain is cleaved to form pregnenolone by cholesterol desmolase (CYP11A1, P450scc). Pregnenolone is the common precursor for all other steroids and is metabolised to mineralcorticoids, glucocorticoids or sex hormones by several other enzymes (see Figure 1.1).

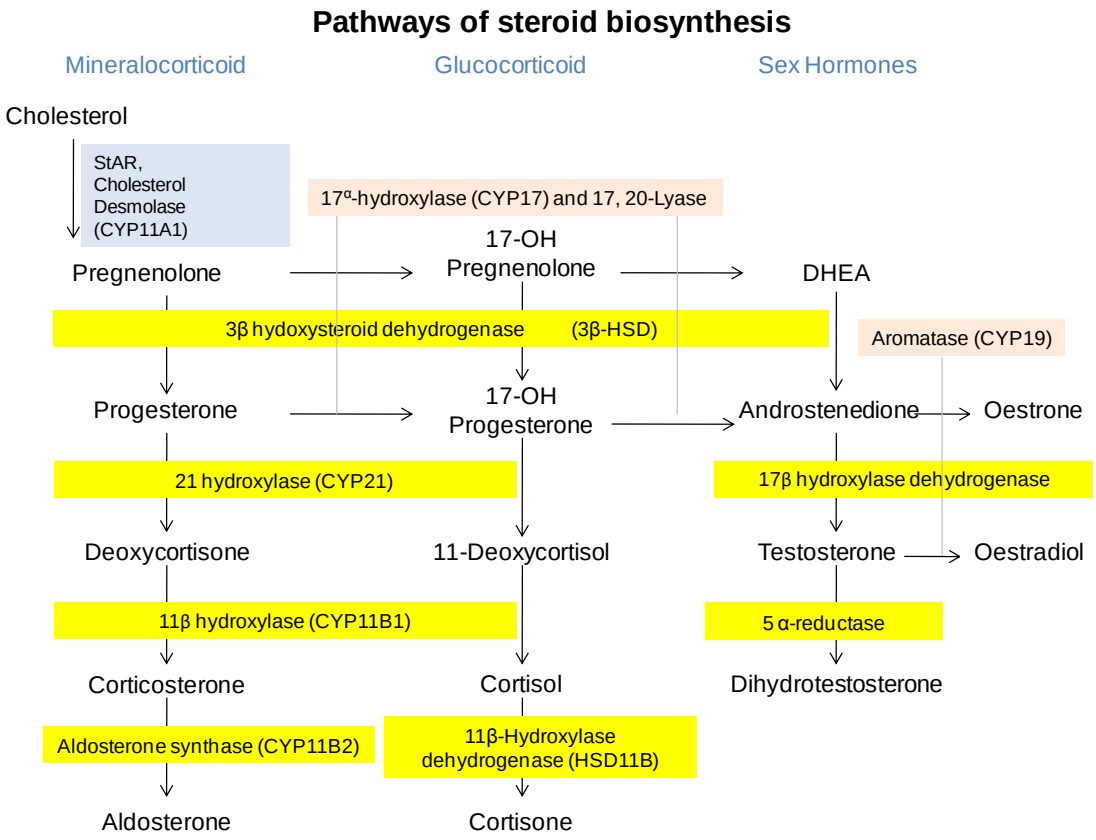

Figure 1.1: The steroidogenic pathway for the synthesis of mineralocorticoids (aldosterone), glucocorticoids (cortisol) and sex hormones (testosterone, dihydrotesterone and oestrogens). The enzymatic activities catalysing each bioconversion are highlighted in colours. Figure adapted from *Endotext* (Nimkarn & New 2008).

1.2 Hypothalmic-Pituitary-Adrenal (HPA) axis

Adrenocorticotrophic hormone (ACTH) is released by corticotroph cells in the anterior pituitary where it is cleaved from the larger peptide pro-opiomelanocortin (POMC). ACTH release is stimulated by corticotrophin-releasing hormone (CRH) secreted by the hypothalamus. CRH acts on specific pituitary receptors and exerts trophic and releasing actions on corticotroph cells. The circadian release of ACTH will stimulate the production of cortisol in the adrenal gland, which is regulated by a negative feedback action at the pituitary. Production of other adrenal cortex hormones (such as androgens) does not exert this regulatory feedback. Figure 1.2 illustrates the regulation of the hypothalamic-pituitary-adrenal axis.

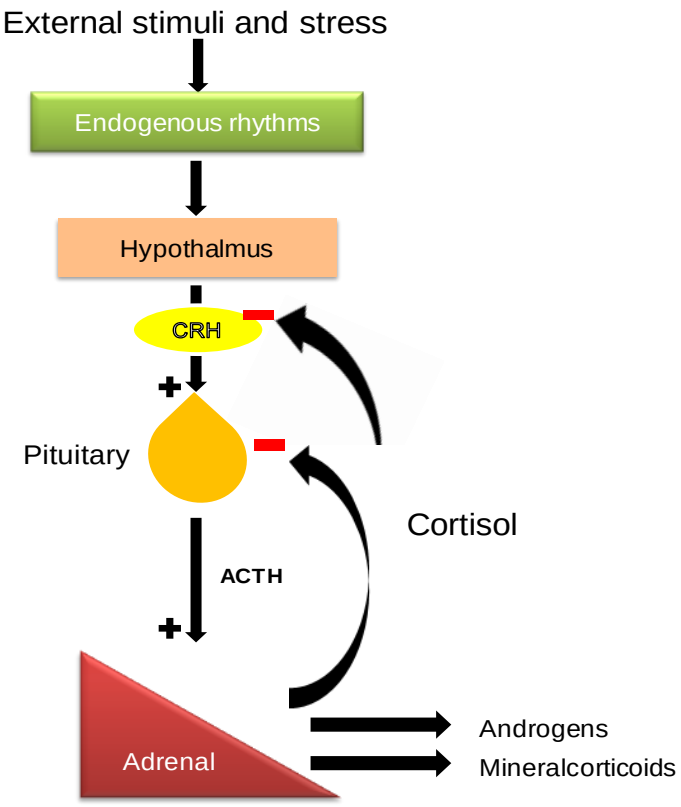

Figure 1.2: General scheme of regulation of the pituitary-adrenal axis. CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotrophic hormone.

1.2.1 Pro-opiomelanocortin

Pro-opiomelanocortin (POMC) is a polypeptide precursor that undergoes extensive, tissue specific post-translation modification to generate a range of smaller, biologically active peptides (Bertagna 1994). These include ACTH and the α-, β-, and γ-melanocyte-stimulating hormone, collectively known as melanocortins. The *POMC* gene is primarily expressed in the pituitary gland, but has also been detected in numerous non-pituitary tissues, including hypothalamus, testes, ovary, placenta, duodenum, liver, kidney, lung, thymus and lymphocytes (Smith & Funder 1988).

Both ACTH and α-melanocyte-stimulating hormone (α-MSH) were first purified and sequenced from the pituitaries of other species. The two peptides shared the first 13 amino acids, but the importance of this was not realised until the isolation of corticotrophin-like intermediate peptide (CLIP) (Scott *et al.* 1974), the peptide that comprises the C-terminal of ACTH. With the identification of the high molecular weight forms of immunoreactive ACTH (Orth *et al.* 1973; Mains & Eipper 1976) it was then realised that ACTH and α-MSH were derived from the same molecule.

The existence of a common precursor to a number of pituitary hormones was uncovered with the discovery of another pituitary peptide, β-endorphin which shares the same sequence as the C-terminal of β-lipotrophin (β-LPH) (Chretien *et al.* 1976; Bradbury *et al.* 1976). This is yet another pituitary hormone released under the same conditions as ACTH. It was later recognised that ACTH and endorphin were derived from the same protein (Eipper & Mains 1978). The true structural relationship between ACTH and endorphin was revealed when the cDNA encoding POMC was subsequently cloned from both bovine intermediate lobe (Nakanishi *et al.* 1979) and from the mouse pituitary cell line AtT-20 (Roberts *et al.* 1979).

1.2.2 Structure of the POMC gene

The structure of the *POMC* gene has been characterised in humans (Takahashi *et al.* 1981; Cochet *et al.* 1982b) and other species and is well conserved. It is located on the short arm of chromosome 2 at 2p23.3 and spans over 8kb. The *POMC* gene consists of three exons separated by two large introns (Takahashi *et al.* 1981; Cochet *et al.* 1982a). As illustrated in Figure 1.3, the first exon contains no coding sequence and the second exon codes for the N-terminal part of the coding sequence for the POMC peptide. The third exon encodes most of the mature protein.

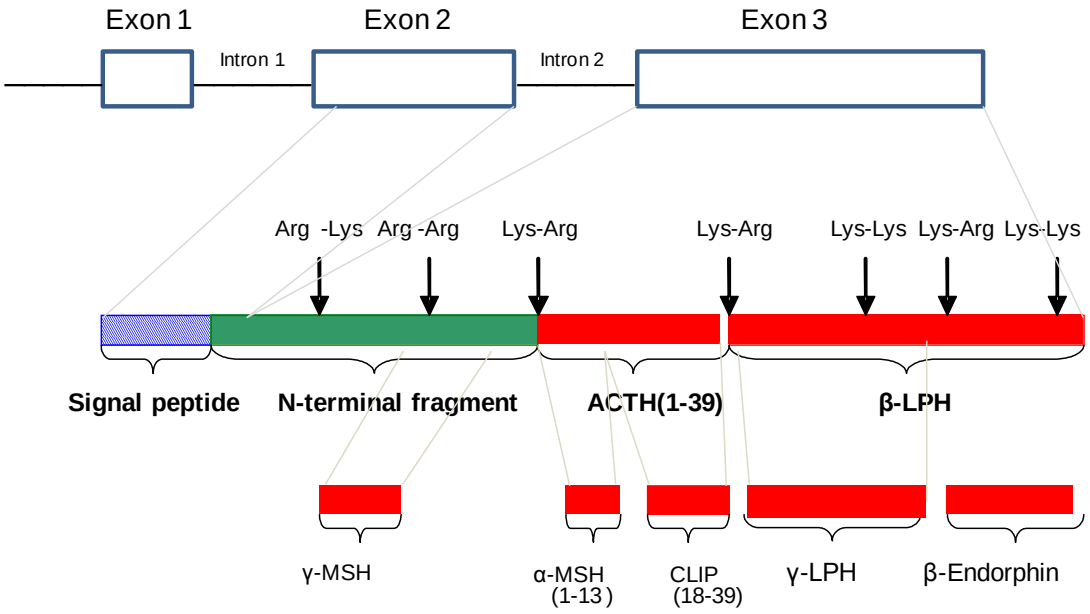

Figure 1.3.: Structure of the POMC gene and protein. The locations of the various peptides together with the dibasic amino acid residue cleavage sites are shown. Adapted from *Journal of Neuroendocrinology* (Bicknell 2008). MSH, melanocyte-stimulating hormone; CLIP, corticotrophin-like intermediate lobe peptide; LPH, lipotropin.

1.2.3 POMC derived peptides

The discovery of the structure and biosynthesis of POMC led to the understanding that a number of bioactive peptides were derived from this large precursor molecule (Eipper & Mains 1980). POMC can be cleaved enzymatically into the following peptides:

1. Adrenocorticotropic hormone (ACTH) and β-lipotrophin (β-LPH) in the anterior pituitary
2. Corticotrophin-like intermediate lobe peptide (CLIP), γ- lipotrophin (γ-LPH),α-melanocortin stimulating hormone (α-MSH) and β –endorphin in the intermediate lobe
3. γ-melanocortin stimulating hormone (γ – MSH)

POMC is expressed in both the anterior and intermediate lobes of the pituitary (the intermediate lobe is absent in the human). In the anterior lobe, POMC is produced in corticotrophs (approximately 1% of the total protein) and in the intermediate lobe, it is produced in melanotrophs. (Bicknell 2008). In rodent pituitaries, the exclusive presence of prohormone convertase, PC2 in the melanotrophs of the intermediate lobe (not present in humans) is responsible for the specific conversion of ACTH to α-MSH and CLIP. This is in contrast with corticotrophs of the anterior lobe, which do not contain PC2, and will therefore only produce ACTH (Vieau *et al.* 1994).

As already described, prohormone convertase enzymes PC1 and PC2 process POMC directly at sites of dibasic residues (Lys-Lys or Lys-Arg) (Benjannet *et al.* 1991). This process generates ACTH, the N-pro-opiomelanocortin, joining peptide, and beta-lipotropin. ACTH can be further processed to generate α-MSH and CLIP,

whereas β-LPH can be processed to generate γ-LPH and β-endorphin (Benjannet *et al.* 1991). Figure 1.4 illustrates the cleavage of POMC related peptides.

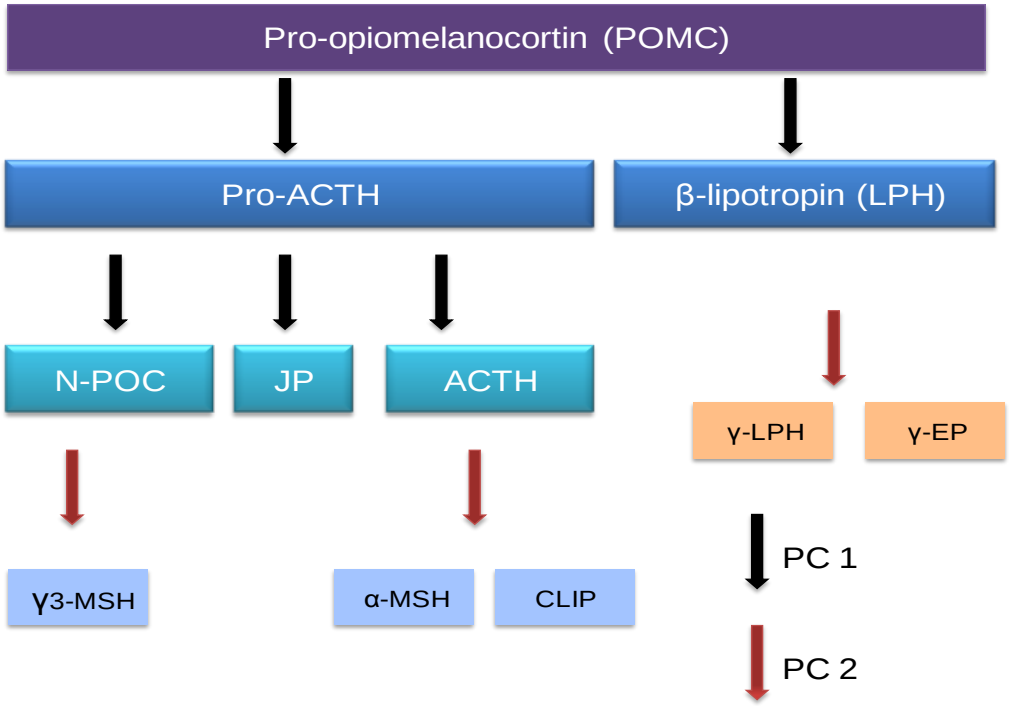

Figure 1.4: Processing of pro-opiomelanocortin and its derivatives. The cleavage of POMC into smaller fragments by prohormone convertases PC 1 and 2. N-POC, N-proopiomelanocortin; JP, joining peptide; EP, endorphin; CLIP, corticotrophin-like intermediate peptide. Adapted from *JBC* (Zhou *et al.* 1993).

1.2.3.1 Adrenocorticotropic hormone

The adrenocorticotropic hormone, ACTH, is released from corticotrophs in the anterior pituitary. In the human pituitary POMC is enzymatically processed to pro-ACTH, which is then cleaved to give ACTH. This process is accomplished by PC1 located within the mature dense core granules (Benjannet *et al.* 1991).

ACTH is a 39 amino acid (aa) peptide, which has a molecular weight of 4.5kDa and is highly specific for the melanocortin 2 receptor (MC2R) in the adrenal gland. The N terminal 12 amino acids are highly conserved between species, thus reflecting

the importance of this region for biological activity. ACTH is part of the melanocortin family of peptides because it contains the melanotropic His-Phe-Arg-Trp structural component at position 6-9.

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe

Figure 1.5: ACTH sequence in three letter code.

ACTH	SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF
α- MSH	SYSMEHFRWGKPV
β-MSH	AEKKDEGPYRMEHFRWGSPPKD
γ-MSH	YVMGHFRWDRFG

Table 1.1: Summary of single letter peptide sequences of ACTH and other MSHs.

The amino acid sequence of ACTH is 39 residues long with a serine at the amino-terminal and phenylalanine at the carboxyl terminal in all mammalian species studied. The sequences of the NH_2–terminal [1-24] and the COOH-terminal [34-39] are identical, whereas some differences are observed between position 25 and 34, suggesting that the first 24 aa contain the biologically important part of the molecule.

This has been confirmed by the observation that the synthetic nonadecapeptide corresponding to the first 19 residues of ACTH exhibited all the biological activity of ACTH (Ramachandran & Choh 1967). Further studies suggest that residues 15-18 containing the positive charge Lys-Lys-Arg-Arg (Figure 1.5) could play an important role in the binding of the hormone to its receptor, whereas the NH_2 – terminal, in particular residue 4-10, forms the “active core” of the molecule for steriodogenesis (Ramachandran 1973).

1.2.3.2 α – Melanocyte stimulating hormone

α –MSH consists of ACTH 1-13 and is derived from ACTH (sequence shown in table 1.1). ACTH is cleaved with PC2 in the melanotroph cells in the intermediate lobe of the pituitary in species such as rat and mouse. The N-terminal serine residue of α-MSH is N-acetylated in many species and undergoes amidation at the C-terminal valine. The adult human pituitary does not have this distinct intermediate lobe and therefore this is not a source of α-MSH in humans. However, α-MSH produced in the skin or hypothalamus may access the circulation, but it is not clear if α-MSH is in the circulation normally (Kortlandt *et al.* 1986; Croughs *et al.* 1991).

1.2.3.3 γ – Melanocyte stimulating hormone

γ-MSH is a proteolytic cleavage product of the N-terminal fragment of POMC, which has a much more limited spectrum of biological activities than does ACTH or α –MSH. It selectively stimulates MC3R, being approx 100X more potent at MC3R than the MC4R.

1.2.3.4 Other derivative peptides

Corticotropin-like Intermediate Lobe Peptide (CLIP) is produced as the residue after the cleavage of α-MSH. CLIP, which is cleaved from ACTH 18-39 not thought to circulate in humans and does not have a bioactive role.

β–lipotropin lies at the C-terminus of POMC and is cleaved into γ-lipotropin and β-endorphin. In the human anterior pituitary, the cleavage is limited. In the circulation there is much more β-lipotropin than β-endorphin, which is present at very low levels (Stewart *et al.* 1994).

β–endorphin is a 31 amino acid peptide containing the sequence for metencephalin as the first 5 amino acids at its N terminus. It has been demonstrated

to play a role in certain behavioural patterns, in obesity, diabetes and psychiatric diseases (Dalayeun *et al.* 1993).

1.2.4 ACTH action

Circulating ACTH binds with high affinity receptors located on the surface of adrenocortical cells. ACTH binds to the melanocortin-2 receptor (MC2R) in the adrenal gland (Catalano *et al.* 1986; Clark 2000). This results in an increase in intracellular cAMP levels (Haynes 1958), which in turn activates the steroidogenic pathway (see section 1.1.2). Activation of the MC2R by ACTH leads to uptake of cholesterol into the cell, activation of the enzymes which mediate conversion of cholesterol esters to free cholesterol and the translocation of cholesterol into mitochondria. Furthermore, ACTH also upregulates the expression of mitochondrial enzymes converting cholesterol to corticosterone (Ney *et al.* 1967; Garren *et al.* 1971; Gill 1972; Mukai *et al.* 1998).

There are several key genes known to be regulated by ACTH stimulation of cAMP in the adrenal (listed in table 1.2). These target genes include steroidogenic acute regulatory (StAR) protein, which is involved in mitochondrial cholesterol import, steroidogenic enzymes involved in conversion of cholesterol into steroid, and the *MC2R* gene itself. The steroidogenic pathway has already been illustrated in Figure 1.1.

Gene name	Alternative or common name
CYP11A1	P450 side chain cleavage enzyme
CYP17	17α- Hydroxylase
CYP21	21 Hydroxylase
CYP11B1	11β-Hydroxylase
CYP11B2	Aldosterone synthase
MC2R	ACTH receptor

StAR
c-FOS
c-JUN
JUN-B
c-MYC

Table1.2: Key genes regulated by ACTH stimulation of cAMP in the adrenal gland. (Clark 2000)

1.3 Primary adrenal insufficiency

It has now been more than 150 years since Thomas Addison first described the clinical features of adrenal failure (Addison T 1868). It is well known that this clinical syndrome could be caused by a diversity of diseases, which have in common the impaired secretion of glucocorticoids, mineralocorticoids and androgens. The principal symptoms are fatigue, reduced appetite, weight loss and many patients experience salt craving and dizziness. The most obvious clinical sign is hyperpigmentation owing to the high level of circulating ACTH.

The presentation of disease varies significantly between individuals. Many patients have symptoms for many years prior to diagnosis and others develop adrenal crisis more acutely. Untreated disease could be fatal, and correctly managed patients have been reported to live near-normal lives (Mason *et al.* 1968). However, a retrospective population-based study performed in Sweden over 14 years, had shown that the relative risk of death is more than 2-fold greater in patients with adrenal insufficiency (Bergthorsdottir *et al.* 2006). The higher rate of death was attributed to cardiovascular, malignant and infectious diseases.

Biochemical features include electrolyte disturbances (hyponatraemia and hyperkalaemia), serum cortisol is typically below the reference range, and ACTH is grossly elevated (Arlt & Allolio 2003). Low serum aldosterone levels and elevated renin activity and low dehydroepiandrosterone sulphate levels usually accompany the diagnosis.

The various aetiologies of primary adrenal insufficiency can be categorised into adrenal dysgenesis/ hypoplasia, adrenal destruction and impaired steroidogenesis (Ten *et al.* 2001). Adrenal dysgenesis due to MC2R mutations is the focus of this thesis.

1.3.1 Familial Glucocorticoid Deficiency

Familial glucocorticoid deficiency (FGD), also known as hereditary unresponsiveness to ACTH was first recognised half a century ago (Shepard *et al.* 1959). Inactivating MC2R mutations in Familial Glucocorticoid Deficiency (FGD) were first described over 15 years ago (Clark *et al.* 1993; Tsigos *et al.* 1993). This is a rare autosomal recessive disease which is characterised by isolated glucocorticoid deficiency and ACTH resistance (OMIM 202200). Inactivating mutations of the G-protein coupled receptor, MC2R are found in 25% of FGD kindred, known as FGD type 1 (Clark *et al.* 1993; Tsigos *et al.* 1993; Weber *et al.* 1995; Clark *et al.* 2005). FGD type 2 is due to mutations of the MRAP gene and accounts for 20% cases and FGD type 3 is due to *STAR* mutation which accounts for 5% (Metherell *et al.* 2009). The cause of the remaining ~50% of cases is yet to be found.

In general, many cases present in the neonatal period, when persistent or severe hypoglycaemia prompts further investigation. If diagnosis is not recognised at an early age, patients usually present over the next few years with failure to thrive, collapse, recurrent infective and hypoglycaemic episodes with skin pigmentation and even coma. Long term neurological sequelae of FGD could vary from learning difficulties to spastic quadriplegia, which are likely to be due to the numerous episodes of severe hypoglycaemic attacks during childhood (Modan-Moses *et al.* 2006). The adrenal pathology of affected individuals reveals the absence of fasciculata or reticularis cells together with disorganisation of glomerulosa cells (Shepard *et al.* 1959; Migeon *et al.* 1968; Kelch *et al.* 1972; Clark *et al.* 2005).

The diagnosis is confirmed with a low plasma cortisol in the presence of elevated ACTH (above 1000pg/ml [normal range <80pg/ml]) (Clark & Weber 1998). One distinguishing feature which separates this disease from other forms of primary

adrenal deficiency, is the preservation of the renin-angiotensin-aldosterone system (RAS), and these patients have normal electrolytes. It has been recognised that minor transient impairment of the RAS is seen in some patients at the time of presentation. This may reflect volume depletion at this time and therefore reassessment of the renin and aldosterone levels after stabilization may be valuable. A recent report suggested that a partial mineralocorticoid deficient state exists in those patients with a more "severe genotype" (Lin *et al.* 2007). The physiological explanation of this observation is unclear. In contrast, subsequent study of FGD patients with homozygous nonsense and frameshift mutations predicting a significantly truncated protein hence severely disrupting MC2R function, did not have mineralocorticoid deficiency (Chan *et al.* 2009b).

1.3.2 Familial Glucocorticoid Deficiency Type 1

Since the cloning of the MC2R in 1992 by Mountjoy, numerous homozygous or compound heterozygous mutations of MC2R have been identified worldwide (Clark *et al.* 2005). Interestingly, the majority of these were missense mutations, and nonsense mutations were usually compounded with a missense mutation on the opposing allele. Homozygous nonsense and frameshift mutations are extremely rare (Chan *et al.* 2009b). The expected outcome would be a significantly truncated protein severely disrupting the function of MC2R (Tsigos *et al.* 1995), which could lead to the possibility of reduced survival in utero, or that it could present as a very different phenotype (Clark *et al.* 2009).

In FGD type 1, mutations of the MC2R are widespread and scattered throughout the coding region of the gene (Figure 1.6).

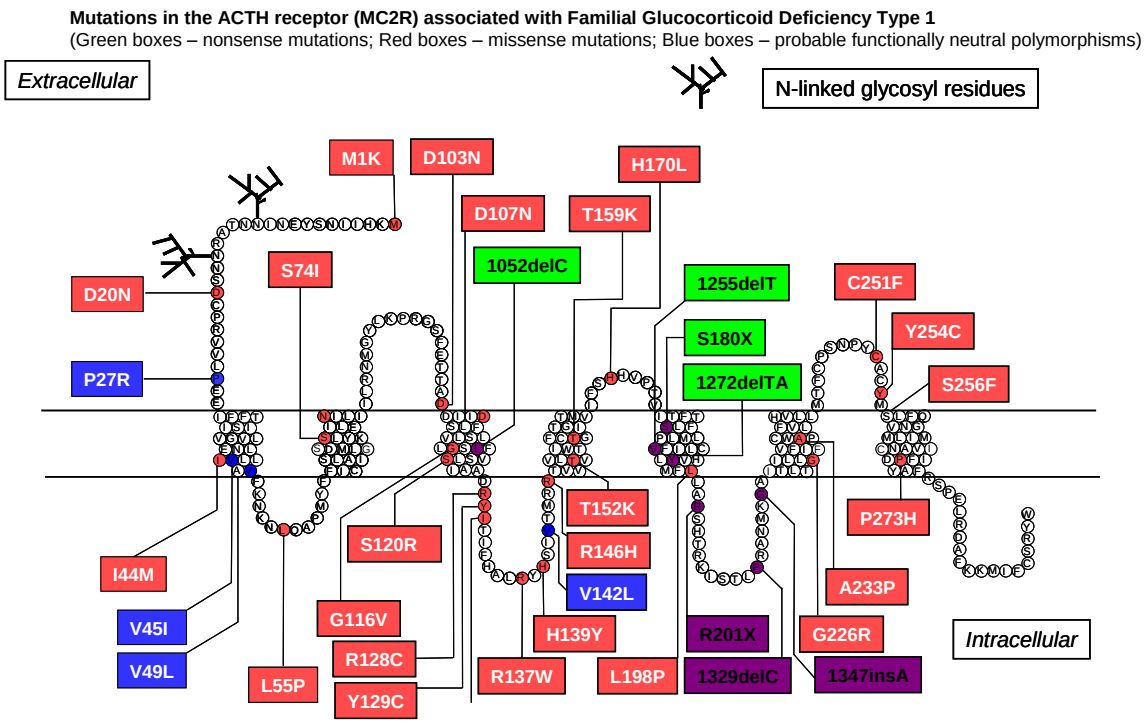

Figure 1.6: Schematic diagram of the MC2R showing the positions of all known mutations found in FGD type 1. Adapted from *Trends in Endocrinology and Metabolism* (Clark *et al.* 2005).

1.3.3 Familial Glucocorticoid deficiency type 2

Originally, the many patients who did not have mutations within the coding exon of the MC2R gene were classified as having FGD type 2. Genetic studies using homozygosity mapping revealed a potential locus for FGD on chromosome 21, and this was subsequently identified as the *MRAP* gene (this is discussed in section 1.7).

Several mutations in *MRAP* have been described in FGD type 2. These mutations appear to cluster around the first coding exon (exon 3), particularly at the splice donor site. The same mutations occur in unrelated families, suggesting that this is a common region for mutation.

The majority of MRAP mutations are homozygous nonsense mutations, which probably result in the complete absence of the truncated protein, in contrast with MC2R mutations. To date, only two full length missense MRAP mutations have been identified, V26A and Y59D. Functional analysis demonstrates impairment of MC2R function with late age of onset of disease (Hughes *et al.* 2009).

1.4 G-protein coupled receptors (GPCRs)

The clinical focus of the thesis is the defective function of MC2R in FGD type1. MC2R belongs to a family of seven transmembrane domains G-protein-coupled receptor and is the smallest member of the melanocortin family subgroup.

1.4.1 General features of GPCRs

GPCRs also known as seven transmembrane domain receptors, are the largest group of integral membrane proteins. These proteins function to act as a link between extracellular signals and intracellular processes. There are over 890 GPCRs in the human genome of which approximately 450 are predicted to be receptors for a vast range of endogenous ligands (Devi 2001; Takeda *et al.* 2002) including biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions and proteases. GPCRs have been named based on their ability to recruit and regulate the activity of intracellular heterotrimeric G proteins.

Important studies over the last decade have led to the resolution of four GPCR structures. The first structure to be crystallized was the rhodopsin receptor in 2000 (Palczewski *et al.* 2000), it was not until eight years later that the model for opsin (the ligand-free form of GPCR rhodopsin) was achieved (Park *et al.* 2008; Scheerer *et al.* 2008). In 2007 the first structure of a ligand-activated GPCR, β_2–adrenergic receptor, was crystallised (Cherezov *et al.* 2007). This was closely followed by the β_1 adrenergic receptor (Rasmussen *et al.* 2007) and the adenosine receptor (Warne *et al.* 2008) the following year.

GPCRs do not share any overall sequence homology (Probst *et al.* 1992; Kolakowski, Jr. 1994). The presence of seven transmembrane-spanning α-helical

segments (Figure 1.7) connected by alternating intracellular and extracellular loops, with the amino terminus located on the extracellular side and the carboxyl terminus on the intracellular side is the only common structural feature in all GPCRs. These helices are thought to be organised sequentially in an anti-clockwise fashion traversing the phospholipid bilayer 7 times.

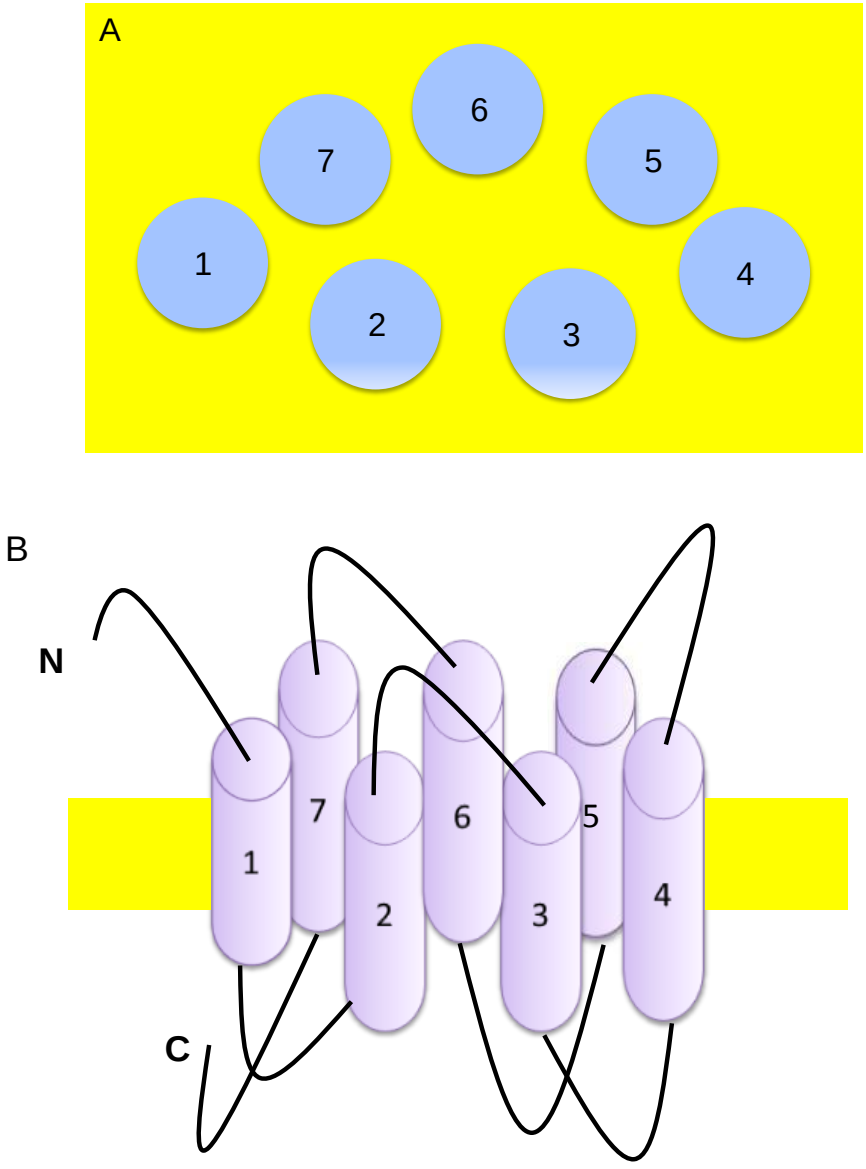

Figure 1.7: Schematic structure of a seven helical GPCR. A: Aerial view of the GPCR with the helices transversing the lipid membrane in an anti-clockwise manner. B: Showing the transmembrane domains connected by intra- and extracellular loops.

1.4.2 GPCR classification

There are three major subfamilies where significant sequence homology is found (Gether 2000). These include the receptor related to the rhodopsin and the β_2 adrenergic receptor (family A), the receptors related to the glucagon receptor (family B), and the receptors related to the metabotropic neurotransmitter receptors (family C). The yeast pheromone receptors make up two minor unrelated subfamilies, family D (STE2 receptors) and family E (STE3 receptors). Table 1.3 summaries the three major subfamilies.

The subfamily of rhodopsin/ β_2 adrenergic receptor-like receptor (family A) is by far the largest and the most studied. Melanocortin receptors and MC2R, which are the main focus of this thesis belong in this group. Family A is phylogenetically subdivided further into six major subgroups. The overall homology among all type A receptors is low, and the only residue that is conserved among all family A receptors is the arginine in the Asp-Arg-Tyr (DRY) motif at the cytoplasmic side of transmembrane domain (TM3) (Bourne *et al.* 1991; Kolakowski, Jr. 1994; Gether 2000).

Family A: Rhodopsin / β2 adrenergic receptor-like
Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)
CCK, endothelin, tachykinin, neuropeptide Y, TRH, neurotensin, bombesin and growth hormone secretagogues receptors
Adenosine, cannabinoid, melanocortin and olfactory receptors
Chemokine, FSH, LH, TSH, opioid, oxytocin, vasopressin, plus others
Melatonin receptors and other non-classified
Invertebrate opsins and bradykinin receptors
Family B: Glucagon, vasoactive intestinal polypeptide (VIP) receptors, calcitonin-like receptors
Calcitonin, CGRP, CRF receptors
PTH and PTHrP receptors

Glucagon, glucagon-like peptide, GiP, GHRH, PACAP, VIP and secretin receptors
Family C: Metabotropic neurotransmitter/ calcium receptors
Metabotropic glutamate receptors
Metabotropic GABA receptors
Calcium receptors
Vomeronasal pheromone receptors
Taste receptors

Table 1.3: Table of the three major subfamilies division in GPCR and some of the major subgroups. Adapted from *Endocrine Reviews* (Gether 2000).

1.4.3 The GPCR signalling pathway

1.4.3.1 Heterotrimeric G proteins

Transmembrane GPCRs activate G-proteins, guanine-nucleotide exchange factors, on the inner surface of the cell membrane and continue the signal transduction initiated by exogenous stimuli (Kolakowski, Jr. 1994; Hoon *et al.* 1999). The G-proteins are heterotrimeric and are composed of three subunits, namely the α-, β and γ subunits. The activated receptor induces a conformational change in the associated G protein α-subunit leading to the release of GDP (guanosine diphosphate) followed by binding of GTP (guanosine triphosphate) (Bourne *et al.* 1991). In the inactive state, the α-subunit of the G-protein is bound to GDP. The complex formation between the active receptor state and the G protein is followed by the release of GDP from α-subunit of the G protein, subsequently, allowing for a GTP molecule to bind. This allows the α- and βγ-subunits to dissociate which then results in the activated form of G protein (Figure 1.8). The active GTP-bound α-subunit is sometimes referred to as $G\alpha_{(GTP)}$ and the βγ-subunit as Gβγ. These mobile subunits

influence cell functions and the consequent production of second messangers (eg. cAMP or inositol-1,4,5-triphospate [IP_3] or to alter ion channel functions) (Hamm 1998; Wieland & Chen 1999). Within moments, the GTP on $G\alpha_{(GTP)}$ is hydrolysed to GDP by GTPase on the Gα and this allows for the α- and βγ-subunits to re-unite to form inactive G-protein complexes again.

There are a number of different Gα genes, the four main subfamilies of Gα-protein, shown in Table 1.4 (Sierra *et al.* 2000), GPCRs appear to show selectivity for coupling to these respective G-protein families.

Subclass	Functions
Gs	activate adenylyl cyclase, regulate Ca 2+ channels
Gi	inhibit adenylyl cyclase, regulate K+ and Ca 2+ channels,
	activate cGMP phosphodiesterase
Gq	activate phopholipase C
G12	regulate small GTP binding proteins

Table 1.4: Four main subclasses of α-subunits.

Numerous GPCRs signal via activation of α-subunit (G_s) which activates the effector molecule adenylyl cylase and subsequent signalling processes as seen in figure 1.8. Melanocortin 2 receptor is one of these GPCRs which signals via adenylyl cyclase.

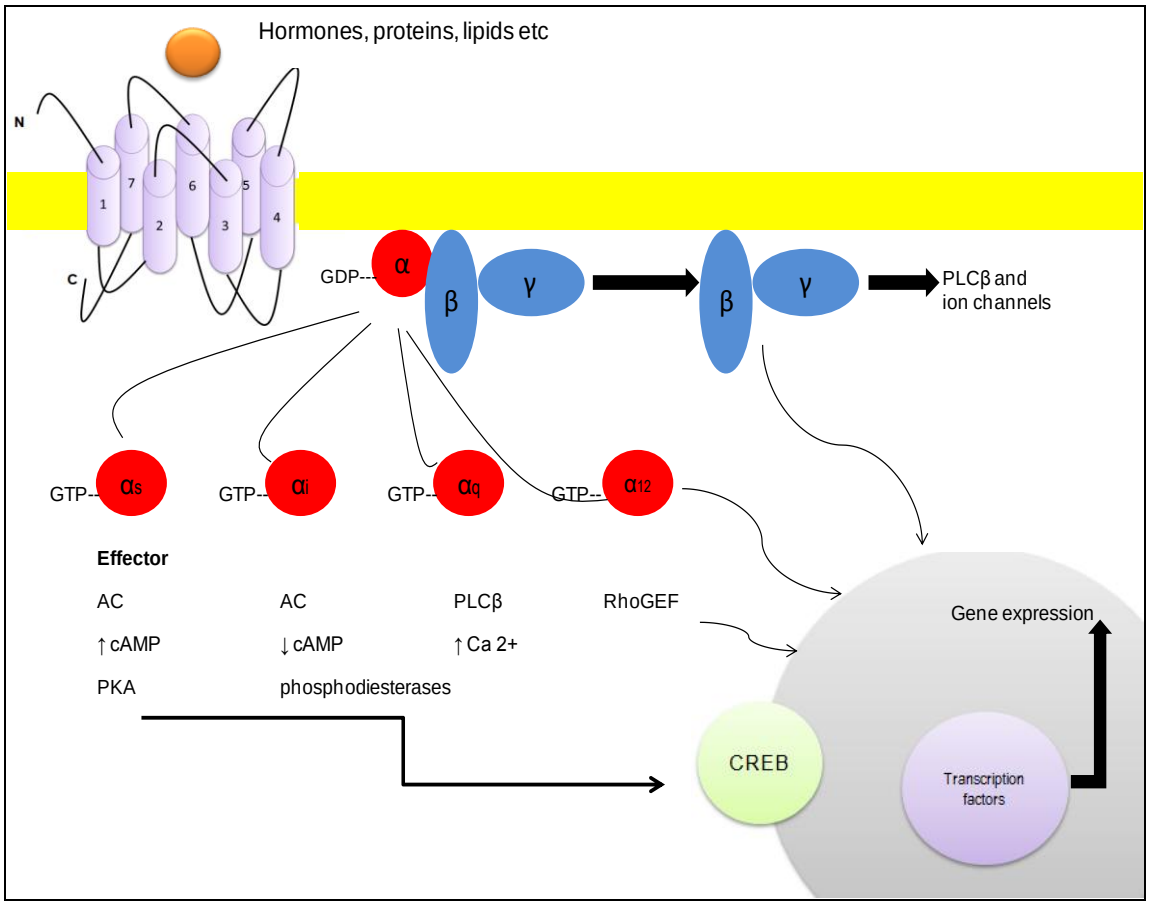

Figure 1.8: Diagram of GPCRs interaction with heterotrimeric G proteins. The activation of the receptor triggers a conformational change in the receptor, which then catalyses the dissociation of the G proteins and activates several downstream effectors. Adapted from *Nature Reviews* (Dorsam & Gutkind 2007).

1.4.3.2 Adenylyl Cyclases

Adenylyl cyclase (AC) is the effector enzyme responsible for converting adenosine triphosphate (ATP) to adenosine 3, 5-monophosphate (cAMP). It is a ubiquitous second messenger that mediates various cellular responses by activation of cAMP-dependent protein kinase (Tesmer *et al.* 2002). There are nine isoforms of adenylyl cylcase which have been identified (ACI to ACIX). They all share several

features including activation by the α-subunit of the heterotrimeric G protein G_s, activation by the diterpene forskolin and inhibition by a class of adenosine analogues known as P-site inhibitors (Tesmer *et al.* 2002). This is process is illustrated in Figure 1.9.

1.4.3.3 Adenosine 3, 5-monophosphate (cAMP)

cAMP is recognised as a versatile regulatory agent that acts to control the rate of a number cellular processes (Robison & Sutherland 1970). It is used as a second messenger widely throughout the animal kingdom, and has a principal role in mediating the effects of a great variety of hormones (Robison *et al.* 1968; Butcher *et al.* 1968).

As mentioned before, the formation of cAMP from ATP is catalyzed by adenylyl cyclase. cAMP is one of the most important second messengers involved as a modulator of physiological processes (Rall & Sutherland 1958; Sutherland & Robison 1966), and is involved in regulating the neuronal, endocrine, cardiovascular systems along with other functions and actions.

The level of cAMP in cells is influenced by the relative specific phosphodiesterase, which catalyzes the hydrolysis of cAMP to 5'-AMP (Butcher & Sutherland 1962). Cyclic nucleotide phosphodiesterases (PDEs) function in conjunction with adenylyl cyclases to regulate the amplitude and duration of cell signalling mediated via cAMP. Therefore, PDEs serve to regulate a range of biological responses to the first messengers such as light, hormones or neurotransmitters, making this step a potential therapeutic target.

1.4.3.4 cAMP-dependent protein kinase

cAMP-dependent protein kinase (PKA), a serine/threonine kinase, is the mediator of cAMP signalling in mammals (Scott 1991). Activation of a GPCR and subsequently an adenylate cyclase as described in section 1.4.3.2, leads to the production of cAMP. The cAMP-dependent kinase is activated by binding of cAMP. The PKA holoenzyme is composed of the genetically distinct catalytic (C) and the regulatory (R) subunits, which bind cAMP (Figure 1.9). These form the tetrameric holoenzyme R2C2 that dissociates in the presence of cAMP into R2(cAMP)4 dimer and two free catalytically active C subunits (McKnight *et al.* 1988). These catalytic subunits, released after conformational changes in the regulatory subunit following cAMP binding, will then phosphorylate cytoplasmic and nuclear target proteins. Cross-talk with other intracellular signalling pathways such as the MAPK (mitogen-activated protein kinase) pathway has been shown (Naor *et al.* 2000). In the nucleus, PKA catalytic subunits phosphorylate the cAMP-responsive element-binding protein (CREB), resulting in activation of transcription of cAMP-responsive element (CRE)-containing genes (Schenk & Snaar-Jagalska 1999).

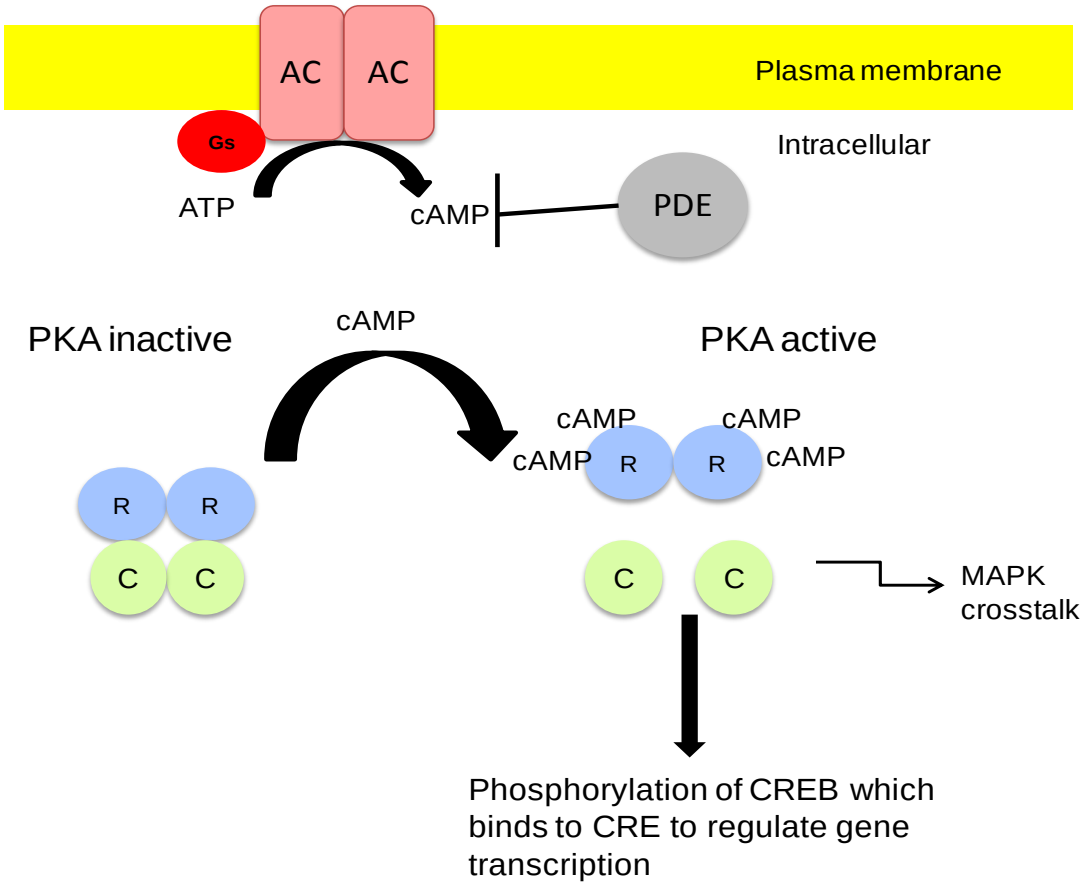

Figure 1.9: Cartoon summarising the activation of cAMP and protein kinases. cAMP activates protein kinase A (PKA). The catalytic subunit of PKA enters the nucleus and phosphorylates CREB which then binds to CRE containing genes and transcription. PDE, phosphodiesterases; AC, adenylyl cyclase; MAPK, mitogen activated protein kinase.

1.4.4 GPCR dimerisation

Many types of GPCRs are known to exist predominately as both homodimers and heterodimers, rather than as monomers (Bouvier 2001; George *et al.* 2002). Recent studies also suggest that the existence of this oligomeric assembly could have functional roles for receptor pharmacology, signalling and regulation (Terrillon & Bouvier 2004; Milligan 2004; Prinster *et al.* 2005). Even though their existence is widely accepted, the functional importance had been met with some scepticism.

It has been shown that the essential step for the biosynthesis and expression of proteins along the secretory pathway is the ability to exit from the ER (Petaja-Repo *et al.* 2000). Only correctly folded receptors can escape the ER quality control system, whereas the incorrectly folded proteins are retained and degraded (Petaja-Repo *et al.* 2001). Previous studies have shown that GPCRs like the metabotropic γ-aminobutyric acid b receptor ($GABA_B$) requires heterodimerisation of $GABA_{B1}$ and $GABA_{B2}$ subunits in order for correct transport to the plasma membrane, as, when expressed alone, $GABA_{B1}$ is retained intracellularly (Marshall *et al.* 1999; Margeta-Mitrovic *et al.* 2000).

Using newer methods such as cellular fractionation and fluorescence or bioluminescence resonance energy transfer studies (FRET and BRET respectively) there is increasing evidence that GPCR dimerisation occurs in the ER (Issafras *et al.* 2002; Overton & Blumer 2002; Terrillon *et al.* 2003). Other supporting evidence for GPCR dimerisation occurring early in the secretory pathway is seen with mutant receptors behaving as dominant-negatives in the presence of the wild type receptors and preventing any cell surface expression. These observations were made with the truncated mutations of the vasopressin receptor V2R (Zhu & Wess 1998), rhodopsin mutants (Colley *et al.* 1995) and GnRH receptors (Grosse *et al.* 1997) among others.

There is conflicting data as to whether receptor activation can promote or inhibit dimerisation. Some studies suggest that ligand binding is able to promote dimer formation (Rodriguez-Frade *et al.* 1999; Angers *et al.* 2000; Horvat *et al.* 2001) or inhibition of its formation (Gines *et al.* 2000; Cheng & Miller 2001), whereas many others believe that homodimerisation and heterodimerisation are constutitive processes which are not affected by ligand binding (Overton & Blumer 2000; Issafras *et al.* 2002; Ayoub *et al.* 2002; Terrillon *et al.* 2003; Guo *et al.* 2003; Stanasila *et al.* 2003). It is possible that different receptors behave differently or it may be that the

differences in findings could be due to the interpretation difficulties linked to the techniques used, such as FRET and BRET (Terrillon & Bouvier 2004). These techniques depend on the relative distance and orientation between the donor and acceptor fluorophores, and, if there is a change in energy transfer after ligand stimulation, this can suggest either that there is change in the dimerisation process or, alternatively, a change in the conformation of the existing dimers.

The studies on $GABA_B$ imply the first evidence that GPCR dimerisation may have a crucial role in signal transduction. $GABA_{B1}$ binds γ-aminobutyric acid (GABA) at the cell surface, but it is only functional when co-expressed with $GABA_{B2}$ (Margeta-Mitrovic *et al.* 2000; Galvez *et al.* 2001). This was explained by a transactivation model in which $GABA_{B1}$ binds to GABA while $GABA_{B2}$ activates a G protein for signalling (Galvez *et al.* 2001). Signalling as a result of heterodimerisation has also been suggested in other receptors, for example the chemokine CCR5/CCR2 (Mellado *et al.* 2001), somatostatin receptors SST2a/SST3 (Pfeiffer *et al.* 2001) and taste receptors (Nelson *et al.* 2001).

Indeed, the artificial over-expression system in heterologus cells would not be of any biological relevance unless these receptors are expressed in the same cells *in vivo. In vivo* studies with mouse cardiomyocytes, shows that blockade of either AT_1 receptor or the β_2-adrenergic receptor with selective antagonists inhibits the signalling of both receptors simultaneously (Barki-Harrington *et al.* 2003). Suggesting in a physiological model that two GPCRs could heterodimerise.

1.4.5 Ligand-Binding domains of Class A GPCRs

Class A GPCR binding sites to endogenous “small molecule” ligands are well characterised for the retinal photochromophore in rhodopsin and for catecholamines in the adrenergic receptors and others (Savarese & Fraser 1992; Kobilka 1992; Ji *et al.* 1998). The binding sites for these classical neurotransmitters such as epinephrine, dopaimine, serotonin etc are thought to be contained in a binding crevice which is formed by the transmembrane helices (Gether 2000). For example, the binding site for β_2 adrenergic receptor agonists and antagonists is found in TM 3, 5 and 6. By spectroscopic analysis of the fluorescent antagonist carazolol to β_2 adrenergic receptor, there was evidence that the binding crevice is deeply buried in the receptor molecule (Tota & Strader 1990). Interestingly, the most important interaction is thought to be a salt bridge between the charged amine of adrenergic ligands and the carboxylated side chain of Asp113 located at TM3 (Strader *et al.* 1991).

For the majority of Class A receptors, such as angiotensin (AT_1 and AT_2) (Hjorth *et al.* 1994; Heerding *et al.* 1997), chemokines (Leong *et al.* 1994), vasopressin (Cotte *et al.* 1998), gonadotropin-releasing hormone receptors (GnRH) (Davidson *et al.* 1997) and others, there is evidence that the major interactions between ligand and receptor are in the amino terminus and predicted extracellular loop regions. It has also been reported that in the case of high affinity binding of glycoprotein hormones, for example luteinizing hormone (LH) and follicle stimulating hormone (FSH), to their receptors, binding initially occurs at the extracellular domain, and is followed by a conformational change at the amino-terminal part of the hormone which will lead to secondary contacts with the extracellular loop regions of the membrane-associated part of the receptor for activation (Ji *et al.* 1998).

1.5 The Melanocortin receptor family

The natural melanocortins α-, β-, and γ-MSH and ACTH bind to melanocortin receptors (MCR), which are expressed on the cell surface and belong to the superfamily of GPCRs (Mountjoy *et al.* 1992; Cone *et al.* 1996). Five melanocortin receptors have been cloned (Mountjoy *et al.* 1992; Chhajlani *et al.* 1993; Gantz *et al.* 1993a). They have a sequence homology of 39 to 61% to one another at the amino acid level and bind to the natural melanocortin peptides with differential affinity (Table 1.5). MC2R is selective for ACTH, α-MSH is the preferred, though not exclusive agonist for MC5R whereas MC3R is the least selective receptor of the family (Brzoska *et al.* 2008).

Receptor	Ligand specificity	Site of expression	Action
MC1R	α-MSH > ACTH > γ-MSH	Skin, melanocyes, macrophage	Skin and hair pigmentation
MC2R	ACTH	Adrenal cortex, adipose tissue	Steroidogensis
MC3R	γ-MSH > ACTH > α-MSH	Brain, placenta, pancreas, stomach	Energy homeostasis
MC4R	α-MSH > ACTH > γ-MSH	Brain, spinal cord	Control of appetite, energy homostasis, erectile function
MC5R	α-MSH > ACTH > γ-MSH	Exocrine glands, lymphocytes	Regulation of excorine function, immunoregulatory function

Table 1.5: Melanocortin receptors, ligand specificity, site of expression and action.

1.5.1 Cloning of the Melanocortin receptors

MC1R was the first melanocortin receptor to be identified by PCR using primers designed to the highly conserved TM3 and TM6 domains of GPCRs with cDNA from a human melanoma specimen (Mountjoy *et al.* 1992). The sequence of this PCR product indicated a novel 372 nucleotide product with GPCR characteristics

(Mountjoy *et al.* 1992; Chhajlani *et al.* 1993). This novel product was then used as a probe to perform Northern hybridization to screen for its expression in several tissues such as brain, adrenal gland, testis, WM 266-4 melanoma cells and others. A specific RNA band was exclusively seen in the melanoma cell line sample. A melanoma cell cDNA library was then screened using this novel DNA fragment that resulted in the isolation of a cDNA clone, which encodes a 317 amino acid protein which was subsequently found to bind MSH peptide, now known as MC1R.

The human MC2R gene was cloned in parallel with the human MC1R by Mountjoy in 1992. Its identity was based on homology with the MC1R and its specific site of expression in the adrenal cortex. Soon after, the other three members of the melanocortin family (MC3, 4 and 5R) were cloned using degenerate oligonucleotides based on the conserved amino acid sequences of the MC1R and MC2R (Gantz *et al.* 1993a; Gantz *et al.* 1993b; Gantz *et al.* 1994).

1.5.2 Melanocortin 1 receptor

The MC1R is the principal melanocortin receptor in the skin where it regulates pigmentation (Haskell-Luevano *et al.* 1994) with high affinity to α-MSH. It is also present in the mucosal cells of the human gastrointestinal tract (Colombo *et al.* 2002), and various cell types of the immune system including human monocytes (Bhardwaj *et al.* 1997; Cooper *et al.* 2005), lymphocytes, and neutrophils(Catania *et al.* 1996). Due to the wide distribution of the receptor, it has been shown to be involved in a number of biological functions including pigmentation, anti-pyretic and anti-inflammatory actions (Catania *et al.* 1996; Bhardwaj *et al.* 1997).

Mutations of MC1R have profound effects on pigmentation. MC1R is highly polymorhphic in humans (Schaffer & Bolognia 2001). Certain allelic variants of the gene in humans are associated with red hair and pale skin (Valverde *et al.* 1995).

1.5.3 Melanocortin 2 receptor

MC2R is the primary ACTH receptor which is responsible for steriodogensis in the adrenal cortex. Mutation in the MC2R may result in familial glucocorticoid deficiency characterised by the resistance to ACTH. MC2R is also expressed in adipose tissue in mice and minimal in humans (Wikberg 1999). Although ACTH is lipolytic in mice, it is not so in humans, and the function of MC2R in human adipose tissue is presently unclear (Gantz & Fong 2003). See section 1.6 for MC2R for more detailed discussion of MC2R function.

1.5.4 Melanocortin 3 receptor

MC3R is expressed in many areas of the CNS and in several peripheral tissues including the gastrointestinal tract and placenta (Chhajlani 1996). MC3R is expressed predominately in the hypothalamic nuclei (Gantz *et al.* 1993a) and is known to regulate food intake and energy homeostasis. MC3R-null mice have a loss of lean body mass and an increase in subcutaneous fat while maintaining a relatively normal body weight (Chen *et al.* 2000). Among the MCR subtypes, γ-MSH has its greatest affinity at MC3R, an observation which is assumed to be physiological importance (Gantz & Fong 2003).

1.5.5 Melanocortin 4 receptor

MC4R is expressed in the CNS, mainly in the hypothalamus (Gantz *et al.* 1993a). Its principal role is in food intake regulation (Butler *et al.* 2001). Inactivating mutations of MC4 cause obesity both in mice and humans (Farooqi *et al.* 2001; Mergen *et al.* 2001). MC4R negatively regulates appetite and food intake in response to agonist thought to be α-MSH, and conversely its inverse agonist/ antagonist AgRP provides an orexigenic signal (Huszar *et al.* 1997; Breit *et al.* 2006).

1.5.6 Melanocortin 5 receptor

MC5R is expressed in numerous peripheral tissues including the adrenals, adipocytes, skin and many others (Chhajlani 1996). It is thought to have a role in exocrine function especially sebaceous gland secretion, as evidenced by MC5R knockout mice having decreased sebaceous lipid production (Chen *et al.* 1997). It may also have a role in tear secretion by the lacrimal gland (Entwistle *et al.* 1990) and in immune regulation (Taylor & Namba 2001).

1.6 Melanocortin 2 receptor

This is the smallest of all G protein coupled receptors (GPCR) consisting of 297aa and is primarily expressed in the adrenal cortex (Mountjoy *et al.* 1992; Naville *et al.* 1994; Cammas *et al.* 1997). It is highly specific for ACTH and unlike other melanocortin receptors, it does not have affinity for other melanocortins (α, β and γ-MSH).

1.6.1 Structure of MC2R gene

The human MC2R gene is located in chromosome 18p11.2- and consists of two exons; 49 bp of the 5' untranslated region of the MC2R is located in exon 1 and is 18 kb upstream of exon 2 (Naville *et al.* 1994). Exon 2 contains 128 bp of 5' untranslated sequence and the full length coding sequence. The short first exon lies downstream of a promoter which includes an initiator element and several steroidogenic factor-1 (SF-1) sites (Naville *et al.* 1994).

1.6.2 Expression of the MC2R

Using *in situ* hybridization, the MC2R mRNA has been shown to be expressed in all three zones of the human adrenal. The highest expression level was found in the zona glomerulosa and fasciculata (Figure 1.10) (Mountjoy *et al.* 1992; Xia & Wikberg 1996; Thomas *et al.* 2003). Other extra-adrenal tissue expression in humans has been shown in the ovary (Chhajlani *et al.* 1993), skin (Slominski *et al.* 1996) and vascular endothelial cells (Hatakeyama *et al.* 2000). It has also been found to be present in adipose tissue (Boston & Cone 1996), pituitary (Morris *et al.* 2003), sympathetic ganglia (Nankova *et al.* 2003) and skin (Slominski *et al.* 1996).

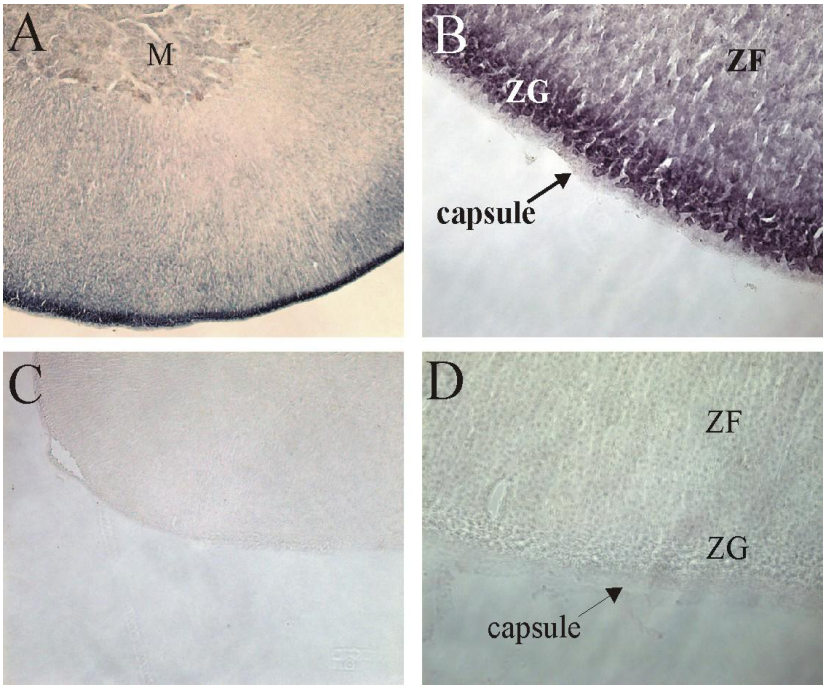

Figure 1.10: MC2R expression in adrenal cortex. In situ localisation of MC2R in rat adrenal sections in zona glomerulosa (ZG) and fasciculata (ZF). A and B: MC2R localised with anti-MC2R antibody (H70, Santa Cruz). C and D: controls, no antibody used. M=medulla. Courtesy of Leonardo Guasti.

1.6.3 Melanocortin 2 receptor signalling pathway

The mechanism of GPCR signalling has already been discussed in section 1.4.3. Briefly, the MC2R is known to signal via the adenylyl cyclase – cAMP signalling cascade. The first step is for Gsα dissociation from the receptor-ligand complex, which leads to adenylyl cyclase activation. This catalyses the production of cAMP which then in turn leads to cAMP dependent protein kinase A (PKA) activation and hence cAMP response element binding protein (CREB) phosphorylation. The cascade will eventually leads to the induction of the enzymes for steroidogenesis, and ultimately cortisol release. The schematic illustration in Figure 1.11, outlines the cAMP signalling cascade and the essential features of the mechanism of action of ACTH.

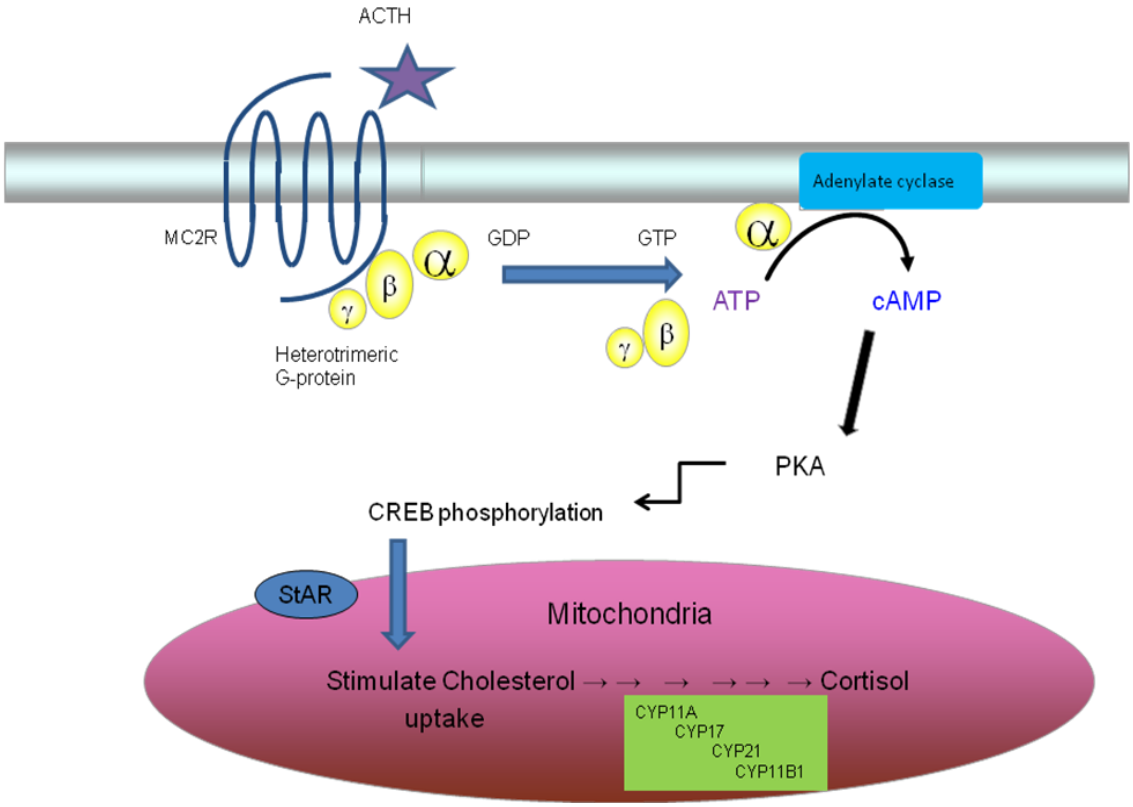

Figure 1.11: The essential features of ACTH action in stimulating steroidogenesis in adrenal cells. ACTH binds and activates the MC2R at the cell surface, resulting in the activation of the heterotrimeric G protein by exchange of GDP for GTP and dissociation of the α-subunit from the βγ-subunits. Gsα then stimulates membrane-associated adenylate cyclase to produce cAMP, which in turn activates PKA in the cytosol. The catalytic subunits of PKA dissociate and phosphorylate target factors, which include the cAMP response element binding protein (CREB). CREB act as a transcription factor that increases the expression of steroidogenic acute regulatory protein (StAR) which stimulates cholesterol uptake into the mitochondria. The transcription, translation and activation of StAR are regulated by PKA in response to trophic hormone. The cholesterol uptake will induce the transcription of the steroidogenic enzymes CYP11A (P450 scc), CYP17 (17α-hydroxylase), CYP21 (21-hydroxylase), and CYP11B1 (11β-hydroxylase), which ultimately results in an increased rate of cortisol synthesis.

1.6.4 MC2R knockout

The specific role of MC2R in adrenal gland development, steroidogenesis and carbohydrate metabolism was tested with an inactivation mutation of the MC2R gene by Chida (2007). They found that MC2R knockout (MC2R-KO) in mice led to three-quarter neonatal lethality, possibly from the result of hypoglycaemia. MC2R-KO mice developed hypoglycaemia during fasting which suggests that they have defective mechanisms in maintaining gluconeogenesis. Neonatal hypoglycaemia in MC2R-KO mice might be secondary to the low level of circulating corticosterone (the main form of glucocorticoid in mice and other species) and epinephrine.

Adult MC2R-KO mice had significant adrenal hypoplasia when compared to the wild type siblings (Chida *et al.* 2007). The surviving adult MC2R-KO mice displayed marked atrophy of the adrenal glands with markedly atrophied zona fasciculata. The zona glomerulosa and the adrenal medulla remained fairly intact. The authors suggest that MC2R is not required for proper development of zona glomerulosa but is required for the zona fasciculata. There were also deficiencies in hormone production, in that not only was the corticosterone level undetectable, but aldosterone and catecholamine levels were also significantly reduced.

The model of MC2R-KO mice is different from the human phenotype of famililal glucocorticoid deficiency type 1 (FGD). In contrast to the human, these mice have low aldosterone levels and unaltered body length. This disparity was explained by the fact that it is very rare for FGD patients to have a homozygous nonsense mutation, as most of them have one or two missense alleles instead.

1.6.5 Expression of MC2R in heterologous cells

There have been significant difficulties in the functional expression of MC2R in a system that is free of any endogenous MCR expression (Naville *et al.* 1996b; Noon *et al.* 2002; Rached *et al.* 2005). Transfected plasmids encoding the MC2R can be transcribed and translated, however, the receptor, fails to exit the endoplasmic reticulum. Several groups have been unsuccessful in the functional expression of MC2R in HEK 293 or CHO cells. There is convincing evidence of functional expression of MC2R in the Cloudman M3 melanoma cell line (Naville *et al.* 1996b). There is also evidence of functional expression seen with African green monkey kidney cell line, Cos-7 cells (Weber *et al.* 1993), but these results were confounded by the presence of other endogenous melanocortin receptors.

Two cell lines which can provide reasonable expression of the MC2R with no other interfering melanocortin receptors are the Y6 and OS3 cell lines. These two mutant cell lines were derived from the ACTH-responsive Y1 mouse adrenocortical cell line (Schimmer *et al.* 1995). Y1 cells endogenously respond to ACTH with an increased adenylyl cyclase activity. Y6 and OS3 mutants fail to express the gene encoding MC2R (Schimmer *et al.* 1995). Studies using these cell lines enabled the functional characterisation of the MC2R using radioligand binding assays as well as cAMP assays (Elias *et al.* 1999). Therefore, this observation that human MC2R could be expressed in cells with endogenous melanocortin receptors led to the speculation that a cofactor for expression is needed which was present in these cell lines.

1.6.6 MC2R desensitisation and internalisation

Desensitisation of the MC2R in the Y1 adrenocortical cell line has been previously investigated (Baig *et al.* 2001). It is well established that the MC2R is

coupled to a Gαs subunit and that activation of adenylate cyclase is a major primary signalling event (Clark *et al.* 2003). Like other GPCRs, after the initial ligand receptor interaction and signalling, the endogenously expressed MC2R in Y1 cells is desensitised. Baig has shown that approximately 60% of MC2R signalling is desensitised within 30 min of ACTH stimulation. The desensitisation process persists for several hours. Interestingly, other factors such as forskolin do not desensitise MC2R.

cAMP-dependent protein kinase (PKA) is activated after ACTH stimulation via binding of cAMP to the PKA regulatory subunits (as described in section 1.4.3.4). When PKA is blocked, using the PKA inhibitor H89 there is a complete loss of desensitisation. Further supportive evidence indicating that PKA plays an important role in desensitisation is seen with site-directed mutagenesis of the single consensus PKA phosphorylation site in the third cytoplasmic loop at serine 208. This mutant receptor has the same cell surface expression as the wild-type MC2R, however, it displayed almost complete loss of desensitisation for the entire 120 min study period (Baig *et al.* 2001).

1.7 Melanocortin 2 receptor accessory protein (MRAP)

The observation that human MC2R could be expressed only in cells with endogenous melanocortin receptors led to speculation that a cofactor for expression was required. This hypothesis was proven correct when MRAP (melanocortin 2 receptor accessory protein) was identified in 2005 (Metherell *et al.* 2005) in an effort to find another causative gene for FGD (Familial glucocorticoid deficiency). Genetic studies using homozygosity mapping revealed a potential locus for disease on chromosome 21q22.1. Sequence analysis of this gene in the index family showed the presence of a splice site mutation that was highly likely to lead to a defective protein. Similar defective MRAP mutations were shown in other cases (Metherell *et al.* 2005).

MRAP was originally identified as FALP (fat tissue specific low molecular weight protein) a small protein of unknown function containing a single hydrophobic transmembrane domain protein which is upregulated during differentiation of the mouse pre-adipocyte cell line 3T3-L1 into adipocytes (Xu *et al.* 2002). Interestingly, MC2R is also upregulated in this cell line during differentiation (Noon *et al.* 2002). MRAP mRNA is found highly expressed in the adrenal, as well as lymph nodes, brain, testis, breast and adipose tissue (Xu *et al.* 2002; Metherell *et al.* 2005) as seen in Figure 1.12.

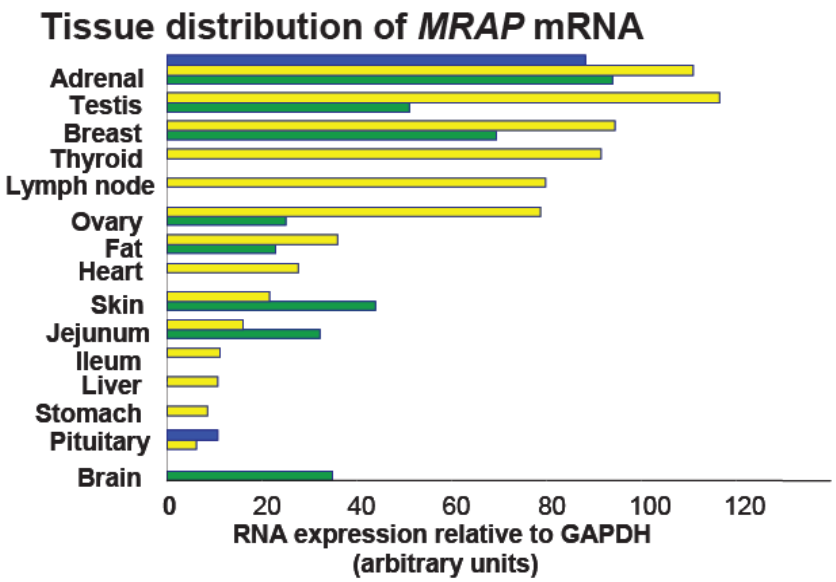

Figure 1.12: Tissue distribution studied by Metherell et al. demonstrates that MRAP expression extends beyond the adrenal gland (Metherell *et al.* 2005). MC2R expression is shown in blue, MRAPα in yellow and MRAPβ in green.

The human MRAP gene consists of 6 exons. Exons 5 and 6 are alternatively spliced to encode MRAPα or MRAPβ. MRAPα is 172 amino acids in length, and predicted to have a molecular mass of 19kDa. MRAPβ is 102 residues and is approximately 14kDa. MRAPα is encoded by exons 1-5 and MRAPβ by exons 1-4 and 6. The MRAP gene structure and its alternative splicing have been illustrated in Figure 1.13.

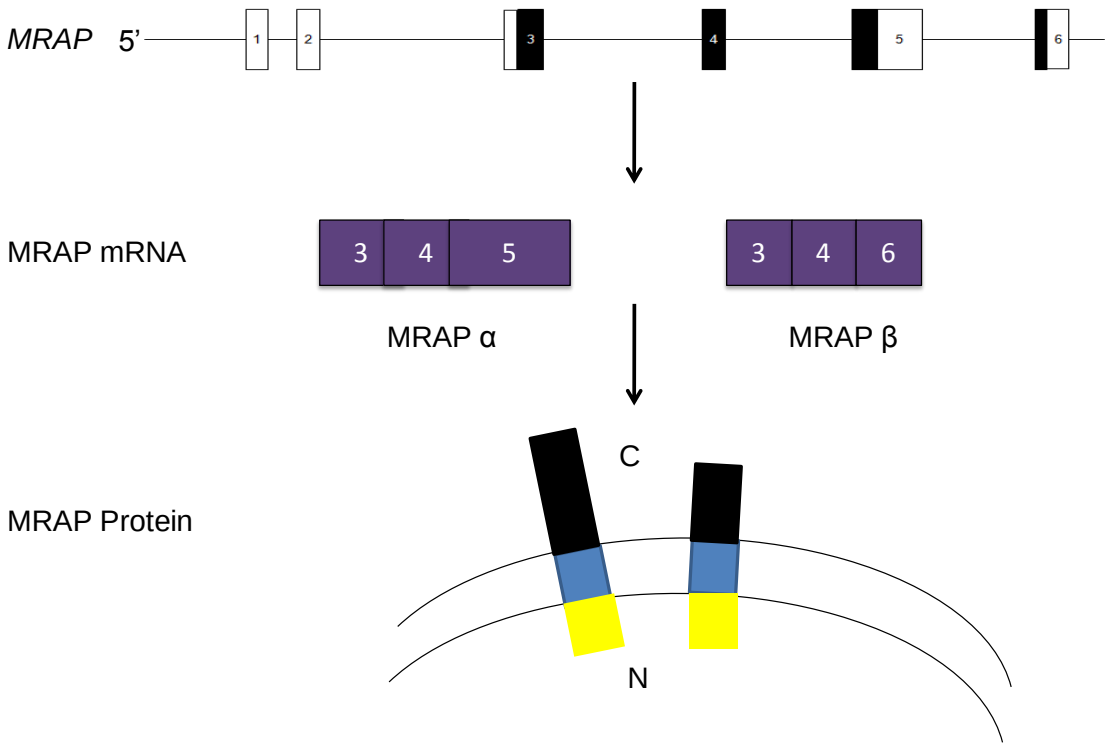

Figure 1.13: Diagram of MRAP gene, transcription and translation. Alternative splicing leads to the inclusion of exon 5 or 6 to generate the MRAPα and MRAPβ isoforms respectively. The N-terminus (yellow), transmembrane domain (blue) and C-terminus (black) of the MRAP protein is shown.

MRAP joins a group of single transmembrane domain proteins that supports expression of GPCRs. These include the small single transmembrane domain receptor activity modifying proteins (RAMPs), which support the expression of the calcitonin and calcitonin-like receptor (McLatchie *et al.* 1998; Morfis *et al.* 2003); the dopamine receptor interacting protein (DRiP78) which is required for the trafficking of the D1 dopamine receptor (Bermak *et al.* 2001); and the RTP proteins, which traffic olfactory receptors (Saito *et al.* 2004). Despite similar protein structures and functional parallels, there is no sequence conservation and no indication that they function by a common mechanism.

The identification of MRAP provided a molecular explanation for the previous difficulties in expression of MC2R. MRAP interacts with MC2R and facilitates cell surface expression and produces a functional ACTH responsive receptor when transfected in non-adrenal cell lines. On confocal imaging MRAP co-localises with MC2R at both the endoplasmic reticulum and at the plasma membrane (Metherell *et al.* 2005).

The mouse adrenocortical cell line, Y1 endogenously expresses both MC2R and MRAP, and is responsive to ACTH. When MRAP is knocked down in Y1 using RNA interference, the response to ACTH was significantly reduced (Cooray *et al.* 2008). When human MRAP was re-transfected into the knockdown Y1 cells, the MC2R function was restored.

MRAP has been shown to exist as an antiparallel homodimer where one copy of MRAP has a "C-terminus out" and the other has "N-terminus out" orientation (Sebag & Hinkle 2007). The MRAP dimer structure was also suggested by Cooray (2008) when a significantly higher molecular weight band was seen on gel electrophoresis using Y1 adrenocortical cells after transfection of hMRAP. This was confirmed with mass spectroscopic analysis.

The interaction site between MRAP and MC2R has recently been identified (Webb *et al.* 2009). The tyrosine rich N-terminus of MRAP is required for trafficking MC2R to the cell surface and the MRAP transmembrane domain is sufficient for the MRAP/MC2R interaction. Whilst the C-terminus of MRAP is not required for MC2R interaction or trafficking of the receptor, it may be important in the regulatory role of cell surface expression (Roy *et al.* 2007; Webb *et al.* 2009).

Previous studies have shown that MRAP interacts with MC2R and has a role in the trafficking of the receptor which facilitates cell surface expression (Metherell *et*

al. 2005; Roy *et al.* 2007; Cooray *et al.* 2008). MRAP allows the MC2R to be glycosylated and localise to the plasma membrane; in the absence of MRAP, MC2R is nonglycosylated and is degraded in the ER (Sebag & Hinkle 2007). However, contrary findings of Roy et al. (2007), showed that N-terminus myc tagged MC2R localised on the cell surface in the absence of MRAP in HEK293-FRT cells, whilst MRAP is required for the GFP-MC2R cell surface expression. They suggest that MRAP enhanced MC2R expression at the cell surface but MRAP is not necessary for MC2R cell surface localisation. It is possible that the observed discrepancy in result may be due to the differences in MC2R constructs and it is possible that the cell line used may express endogenous MRAP.

There is evidence that MRAP is required for ACTH binding and MC2R signalling. Following knockdown of MRAP mRNA with siRNA, there was a marked reduction of signalling in response to ACTH in Y1 cells (Cooray *et al.* 2008). There is published data suggesting the MRAP amino acids 18-21 (LDYI) are essential for MC2R cAMP signalling and binding to ACTH (Hinkle & Sebag 2009; Sebag & Hinkle 2009b). This finding was revealed in the characterisation of MRAP2, an MRAP homologue which lacks the critical LDYI domain. In their hands, MC2R had a marked reduction in ACTH binding and in cAMP production when co-expressed with MRAP2 and stimulated with ACTH compared with MRAP. They therefore concluded that the LDYI region in MRAP is required for ACTH binding to MC2R. This is somewhat conflicting to the finding from Chan *et al* (Chan *et al.* 2009c) who showed that MRAP and MRAP2 independently assist MC2R signalling. There was also the interesting observation that the disruption of the single MRAP2 glycosylation site leads to failure of cAMP signalling in MC2R after ACTH stimulation. The exact functional mechanism of MRAP in MC2R signalling therefore remains unclear.

1.8 Hypothesis and Aims:

The overall aim of this thesis is to study the molecular mechanisms responsible for ACTH insensitivity in FGD and to investigate MC2R dimerisation in the presence of MRAP. This thesis exploits the availability of MRAP, using it as a tool for the detailed investigation of MC2R for the first time.

The first aim of this thesis was to identify the molecular defects in all naturally occurring mutations found in FGD and analyse the genotype-phenotype relationship. In other melanocortin receptors such as MC4R, some of the defective mutations are known to have defective trafficking and are therefore non-responsive to agonist (Ho & MacKenzie 1999). The hypothesis is that mutations in the MC2R cause loss of function in many cases because of interference with trafficking and folding of the receptor rather than ligand binding in itself. To test this hypothesis, the functional consequences of the missense mutations in the trafficking of the receptors or signalling were tested in stably expressing MRAP cell lines and potential loss of interaction with MRAP was tested by co-immunoprecipitation.

Secondly, the functional data of the different MC2R genotypes were compared with the phenotype data and judged for their severity. Correlations between FGD type 1 and 2 were also made to compare any phenotypic differences.

The final aim of this thesis was to investigate the homodimerisation and heterodimerisation of the MC2R using co-immunoprecipitation. The functional consequences of dimerisation were also assessed *in vitro.*

Chapter 2

Materials and Methods

2.1 Equipment, reagents and plasticware

The details of laboratory equipment are listed in the Appendix 8.1. General laboratory chemicals, tissue culture reagents and antibodies stated (listed in Appendix 8.2) were purchased from Sigma-Aldrich (Poole, Dorset, UK) and general plasticware from VWR, UK unless otherwise stated. General solutions and buffers were prepared in deionised water and autoclaved or filtered where necessary.

2.2 Cell culture protocols and stable cell lines

All cell culture procedures were performed under sterile conditions in a laminar airflow hood. Cells were incubated in a humidified atmosphere of 5% CO_2, 95% air (LEEC Ltd. Research Incubator, Nottingham, UK). The cell lines used in this study were HEK 293T (human embryonic kidney cells) and CHO (Chinese hamster ovary cells). The medium was changed every 2-3 days. When cells became 90-100% confluent they were passaged and placed in new tissue culture flasks or plated out for experiments.

2.2.1 Cell culture maintenance

CHO cells were grown in 50% Dulbecco's Modified Eagles Medium with L-glutamate (DMEM) (GIBCO,UK) and 50% Ham's F12 (GIBCO, UK) media supplemented with 10% foetal bovine serum (Sigma-Aldrich, UK), 1% penicillin /streptomycin (Invitrogen Ltd, UK).

HEK 293T cells were grown in the presence of DMEM media with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin.

The media was pre-warmed to 37ºC before use. Passage numbers were recorded on each flask.

2.2.2 Passaging cells

Materials

Phosphate buffer solution (PBS) - 7.5mM Na_2HPO_4, 2.5mM NaH_2PO_4 and 150 mM NaCl (Sigma-Aldrich, UK)

Trypsin (2.5g porcine trypsin/EDTA)

DMEM/ Ham's F12 (1:1) or DMEM medium as stated above

Method

Cells were serially passaged in 75cm^2 tissue culture dishes or plates (Greiner, Gloucestershire, UK) in a 37^{o}C, 5% CO_2 incubator. When cells reached 90% confluence, they were split in a 1:5 to 1:10 ratio. Cell passage numbers were noted at all times. The medium was first aspirated and cells washed in PBS before incubation with 3mls of trypsin/EDTA, to destroy adherence. Fresh medium was then added to the cell suspensions and these were transferred to a new tissue culture dish.

2.2.3 Cell storage

Cells were grown to confluence to be stored in liquid nitrogen. After cells were trypsinised, equal volumes of fresh medium were then added and centrifuged at 1000Xg for 5 min. The pellets were then resuspended in FBS containing 10% (v/v) dimethyl sulphoxide (DMSO) [Sigma-Aldrich, UK]. 700µl was transferred to cryotubes. The cryotubes were wrapped in multiple layers of paper and bubble wrap to allow slow cooling in a -70^{o}C freezer overnight. Within 48-72hours, the cryotubes were transferred to liquid nitrogen for long term storage.

2.2.4 Cell counting

Materials

Neuberg Haemocytometer

Method

Cells were counted in a Neuberg Haemocytomer to determine cell concentration. 25µl of cell suspension (after trypsinisation) was added to 25µl trypan blue and 150µl medium. 50µl of this mixture was then applied to the cytometer by pipetting into the counting area. A 10X lens was focused on the grid lines of the 1mm^2 area and clear cells visible in the region (dead cells take up the trypan blue and are hence blue coloured) were counted.

Cell concentration was determined as the average of 4 corners counted (n).

Cell concentration (cells/ml) = n X dilutional factor (200/25) X 10 000

2.2.5 Transfection into mammalian cell lines

Lipid based transfection of mammalian cells was performed using Lipofectamine™ 2000 (Invitrogen Ltd, UK) according to the manufacturer's instructions.

Materials
Lipofectamine™ 2000 (Invitrogen Ltd,UK)
Cell culture medium
Opti-MEM® I reduced serum medium (Invitrogen Ltd, UK)

Methods

Cells were grown in tissue culture plates and were at 60-80% confluence before transfection. The amount of DNA and lipofectamine used was as suggested by the manufacturer (Invitrogen).

Culture vessel	DNA transfection per well (ng)	Lipofectamine (µl/well)
24 wells	200	1
12 wells	800	2
6 wells	2000	5
10 cm^2 plate	4000	20

Table 2.1: Table of transfection regent and DNA used for specific tissue culture vessel.

In two separate tubes DNA was added to a suitable volume of Opti-MEM® I reduced serum medium, gently mixed and incubated at room temperature for 5 min. In another centrifuge tube the appropriate amount of lipofectamine was added to Opti-MEM and left to stand for 5 min. Thereafter, the two mixtures were combined and left for 25min at room temperature for complex formation. The mixture was then gently pipetted onto the cell culture flask and incubated at 37°C in a CO_2 incubator for 24 to 48 hrs.

The transfection efficiency was assessed by transfection of cells under the same condition with a pEGFP-N1 (Clontech) empty vector. The percentage of cells expressing GFP was determined by epi-fluorescence using a Nikon Eclipse TS100 inverted microscope, equipped with a Nikon C-SHG and T1-FM epi-fluorescence module through a FITC filter (Ex 465-495, Dm 505, Bar 155/555) [Nikon Instruments Europe B.V., Netherlands). The transfection efficiency achieved in HEK 293T cells was 90% and 60% for CHO cells.

2.2.5 Stable cell line generation

The first step for the generation of a G418 resistant stable cell line was to determine the concentration G418 leading to cell death of the non-transfected cells, over 10 day period. This was determined using an antibiotic titration curve.

2.2.5.1 Antibiotic Titration Curves

Materials
Geneticin (G418) (Sigma-Aldrich, UK)
CHO Cell medium or HEK 293 cell medium

Methods

Cells were plated in their standard medium to achieve 25% confluence in 24 well plates. Wells were treated in triplicate with varying amounts of G418 antibiotic: 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 µg/ml in medium. Cells were then incubated for 10-14 days with the medium and antibiotic refreshed every 3 days. The plates were inspected daily for signs of toxicity and the presence of viable cells. The lowest concentration of G418 that began to give notable cell death at approximately 7-10 days and killed all cells within 2 weeks was identified and this concentration was then used to select cells containing MRAP α or β pcDNA3.1 plasmid after transfection.

2.2.5.2 Creating stable cell lines expressing MRAPα and MRAPβ

Cells were grown in 100cm^2 tissue culture plates until 60% confluence was achieved. Constructs made from pcDNA3.1(+) vectors containing either MRAP α, MRAP β or empty vector were transfected individually into both CHO and HEK293 cells. 12 µg DNA was transfected in each plate of cells. Cells were transfected for 24 hours before they were split into a 1:5 and 1:10 ratio. New medium with an appropriate dose of fresh G418 was added the following day. Untransfected cells were treated with G418 and used as a negative control. Once the cells in the negative control were all killed off, the selection was considered to be complete and the cells were grown in media with G418 until distinct colonies were visible (14-21 days). The selection medium was changed every 3 days during this process. Individual colonies were isolated by the dilutional method (see section 2.2.5.3). All subsequent tissue culture was performed in medium containing antibiotic at the same concentration as in the selection process.

2.2.5.3 Isolation of colonies after antibiotic selection

Monoclonal stable cells were selected by the dilutional method. Cells were diluted to 4 cells/ 1000µl and 200 µl of diluted cells with antibiotic medium were plated into 96 well plates. Cells were incubated at 37°C and visually inspected daily. Wells with more than one colony were discarded. Wells with one colony only were selected and grown into a bigger population, as potential stable cell lines.

2.3 Nucleic acid extraction, purification, amplification and modification

2.3.1 Extraction of RNA from cells

This extraction technique is based on selective binding of RNA to a silica gel based membrane, hence enabling wash steps to remove contaminants such as DNA and protein.

Materials

RNaseZAP®

QIAGEN RNeasy® mini kit

RNAse free water

Phosphate buffered solution (PBS)

Method

Equipment and work surfaces were treated with RNaseZAP® to minimise the degradation of extracted RNA by RNases. Cell were grown to 80% confluence in 6 well plates, washed twice in PBS and then harvested by cell scraping in 1.5ml ice cold PBS. Cells were then pelleted by a 2minute centrifugation at 300xg at 4^{o}C and the supernatant discarded. The rest of the protocol is as per manufacturer's instruction for QIAGEN RNeasy® Mini Kit. Briefly, the cell pellet was then resuspended and homogenised in 350µl buffer RLT lysis buffer for lysing pelleted cells. One volume of 70% ethanol was added to the homogenized lysate, and mixed well by pipetting. Up to 700µl of the sample was transferred to an RNeasy spin column which was placed over a 2ml collection tube and spun for 15sec at 13 000 rpm. The flow through was discarded, 700µl buffer RW1 (containing a guanidine salt, for washing the membrane bound RNA) was added to RNeasy spin column it was then spun at 13 000 rpm for

another 15secs. Flow through was discarded, 500µl buffer RPE (wash buffer) was added to RNeasy spin column. This was spun and flow-through discarded. Another 500µl buffer RPE was added to RNeasy spin column, then spun and flow-through was discarded. Finally RNeasy spin column was placed in a new 1.5ml collection tube, 30-50µl RNase-free water was added directly to the spin column membrane. This was then spun at 13 000 rpm for another one minute in order to elute RNA.

The RNA collected was quantified using the Nanodrop ND 1000 spectrophotometer.

2.3.2 DNAse treatment of RNA

Materials

DNase I - 6.4 µg/ml , 18.6 unit/ µg (Promega)

RNase inhibitor - 40units/µl (Promega)

M-MLV RT 5X buffer (Promega)

70% ethanol

RNase free water

Method

To ensure that the RNA was not contaminated with genomic DNA, all RNA extracted underwent DNase treatment. 2 µg RNA was treated with 1µl DNase, 1 µl RNase inhibitor and 4 µl buffer (5X) M-MLV RT was reconstituted in total volume of 20 µl with water. The mixture was left at 37°C for 15 min.

2.3.3 Phenol extraction of nucleic acids

To remove proteins from nucleic acid, for example following enzymatic treatments, an equal volume of phenol equilibrated to pH 8.0 was added to the solution which was then vortexed well and centrifuged at 13 000 rpm for 2min. The contaminating protein, lipids and carbohydrates are preferentially soluable in the non-aqueous phenol whilst the nucleic acid remains in the aqueous upper phase. The separated upper aqueous phase was removed by pipetting to a clean tube.

2.3.4 Precipitation of nucleic acids

The precipitation of nucleic acid (50µl) was performed by adding 1/10 volume (5µl) of 3M Na acetate, 2.5 volume (125µl) absolute ethanol and 2µl glycogen. The mixture was mixed and incubated at -70ºC for a minimum of 15 min and a maximum of overnight to precipitate nucleic acids. Precipitated products were centrifuged at 13 000 rpm for 10 min. The RNA pellet can then be visualised at the bottom of the microcentrifuge tube. The supernatant was carefully removed by pipetting. The pellet was washed with 70% ethanol and centrifuged again for further 10 min at 13 000 rpm. After removal of all ethanol, the pellet was air-dried and resuspended in 10 µl water.

2.3.5 Determination of Nucleic acid concentration and purity

The concentration and purity of DNA or RNA was determined using the NanoDrop (ND-100) spectrophotometer (Labtech International, UK) in accordance with the manufacturer's instructions.

The optical density (OD) absorbance reading at 260nm was used to determine the concentration of the DNA or RNA, OD 260/ OD 280 ratios were used to determine the purity of the sample. Pure DNA and RNA have ratios of 1.8 and 2.0 respectively. Ratios less than 1.8 indicate that the sample may be contaminated with protein whereas ratios greater than 2.0 may mean that the sample is contaminated with chemicals.

2.3.6 cDNA synthesis from mRNA using reverse transcriptase

Materials

RNA

Random primer (0.5μg/μl) , Promega

M-MLV RT buffer (5X), Promega

Dithiothreitol (DTT)

dNTP 10mM (equal volume of dATP, dCTP, dGTP, dTTP)

Reverse transcriptase (Promega)

RNAse® ribonuclease inhibitor (Promega)

Method

2μg RNA was added with 1μl random primer master mix to make the final volume to 12μl. The mixture was heated to 70°C for 10 min to break any secondary structure of RNA. This was immediately placed on ice for a few minutes to anneal the random primers. The reverse transcriptase reaction was typically made up to a total volume of 20μl as follows:

1 μl into freezer was removed and stored at -20ºC (a negative RT sample).

To the remaining 11 µl was added: RT buffer (5X) : 4 µl
0.1M DTT : 2µl (dithiothreitol)
10mM dNTP: 1µl
Reverse Transcriptase: 1 µl (MMLV- RT)
RNAse inhibitor: 1 µl

The reaction was mixed gently, spun briefly and incubated at 37ºC for 1 hour. Samples were stored at -20ºC.

2.3.7 Designing oligonucleotides

The general principles for designing oligos are that:

1. The optimal primer length is 18-25 base pairs (bp).

2. The G/C content should be designed to be 40-60%

3. Primers used in RT-PCR should be intron-spanning to ensure the PCR reaction amplifies only cDNA and do not have contaminated genomic DNA.

   Forward and reverse primers should anneal at approximately the same temperature. 52-58 ºC generally produces the best results- a rough calculation for the melting temperature, T_m of oligonucleotides can be made using is the following formula:

   T_m (ºC) = (number of C/ G bases) X 4 + (number of A /T bases) X2

   In practice however most primer sequences were designed using Primer 3 software with DNA sequences of interest from NCBI or ENSEMBL databases.

For site directed mutagenesis (SDM) two complementary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence were designed and synthesised. 12-17 bases are incorporated on either side of the

mutated nucleotide, giving a primer length of 25-35 bp (Figure 2.1). The primers used for SDM are shown in Appendix 8.3.

Normal sequence: ....TCTCCCTGCTTG*G*CTCCATCTTCAG.....

G116V ....TCTCCCTGCTTG*T*CTCCATCTTCAG....

Figure 2.1: Example of oligonucleotide design for site directed mutagenesis. Normal *MC2R* sequence at the region of interest is shown. To perform SDM, the glycine is exchanged for valine by the exchange of GGC for GTC.

2.3.8 Polymerase Chain Reaction (PCR)

PCR is a technique used to amplify the gene of interest. PCR consists of a series of 20-40 cycles of repeated primer extensions using a thermostable DNA polymerase. The basic principle involves an initiation step when DNA is denatured at 95ºC. This is followed by annealing of sequence-specific oligonucleotide primers at reaction temperature is usually about 3-5 ºC below the T_m of the primers used. This temperature is usually in the ranges of 50-65 ºC for 20-40 seconds. Following annealing the extention reaction takes place at 72ºC with synthesis of DNA occurring at a rate of approximately 1-2kb/min.

PCR reaction is typically carried out in a total volume of 25µl as follows:

Materials

Template	0.5µl
Sense primer (10 µM)	0.5µl
Anti Sense primer (10 µM)	0.5µl
10X buffer	2.5µl
Taq DNA polymerase (5 U/µl)	0.25µl
dNTPs (10mM)	0.5µl
ddH2O	20.25µl

Total volume 25µl

Method

PCR automated cycling was typically carried out in the following condition shown below.

1. initial template denaturation 94°C for 5 min ------1 cycle
2. Subsequent denaturation 94°C for 30 secs
   primer annealing 55°C for 30 secs
   primer extension 72°C for 60 secs-----25 cycles
3. extension of incomplete products 72°C for 7 min ------1 cycle

2.3.9 Agarose gel electrophoresis

Materials:

Agarose (electrophoresis grade)

Ethidium Bromide (10mg/ml)

Loading dye solution (40% w/v surose, 0.25% w/v bromophenol blue or Orange G, DEPC water)

10X TAE (40mM Tris-acetate, 2 mM disodium ethylenediaminetetraactete (Na_2EDTA), pH 8.3) National Diagnostics

DNA markers (GeneRuler ^{TM} DNA ladder Mix, 0.5mg DNA/ml, Fermentas)

Method:

All PCR products were run on 1-2% agarose gels in 1X TAE buffer and visualised alongside DNA markers with ethidium bromide staining. The PCR reaction was mixed with loading dye at a 1:5 ratio prior to loading into the wells of the agarose gel. In the laboratory gel tank, the electrophoresis was carried out at 120 V for 30 min or until clear separation of bands was achieved. Ethidium bromide

intercalated into DNA will fluoresce under UV light at 340 nm, allowing visualisation of the DNA. An Uvitec transluminator was used to visualise the bands, and capture a digital image of the gel.

2.3.10 Extraction of DNA from agarose gels

Materials:

The QIAquick Gel extraction kit, QIAGEN

The exact composition of the buffers used is unavailable but the principle of QIAquick is based on binding properties of DNA to a silica membrane in the presence of high salt buffer. This allows washes with high salt buffers to remove impurities and contaminants, and elution of DNA from the membrane is then accomplished under low salt condition.

Method:

The DNA fragment of interest was visualised under UV light, and excised with a sharp clean blade. The gel slice was weighed in a microcentrifuge tube and the volume was estimated, assuming 100mg = 100 µl. 3 x volume of buffer QG (binding and solubilisation buffer) was added to 1 volume of gel. The samples were incubated at 50ºC, vortexing intermittently until complete gel solubilisation was achieved. The sample was applied to the QIAquick column and centrifuged for 1 min at 13 000 rpm. The flow-through was collected and discarded. The column was washed with 750 µl of buffer PE and centrifuged at 13 000 rpm for a further minute. The column was placed in a clean microcentrifuge tube and allowed to dry at room temperature for 1 min. 30 – 50 µl water was added to the membrane to elute DNA sample by centrifugation at 13 000 rpm for 1 min. The DNA was stored

at -20 ºC.

2.3.11 DNA sequencing

Sequencing of PCR products or clones was performed to ensure that they were specific. Sequencing was performed by the Genome Centre (Bart's and the London School of Medicine and Dentistry, Queen Mary University of London) using BigDye 3.1 chemistry (Applied Biosystems, UK), which is based on the Sanger dideoy-mediated chain termination method. Analysis of sequence chromatograms was carried out using BioEdit (Hall, 1999).

2.4 Cloning and plasmid preparation

2.4.1 Basic steps of subcloning

Subcloning is a basic molecular biology procedure which involves the introduction of DNA sequences typically into bacterial plasmid vectors. Inserts of interest could be cut out from parental vectors or could be generated by PCR, from the relevant tissue cDNA. Figure 2.2 outlines the principal steps involved in subcloning.

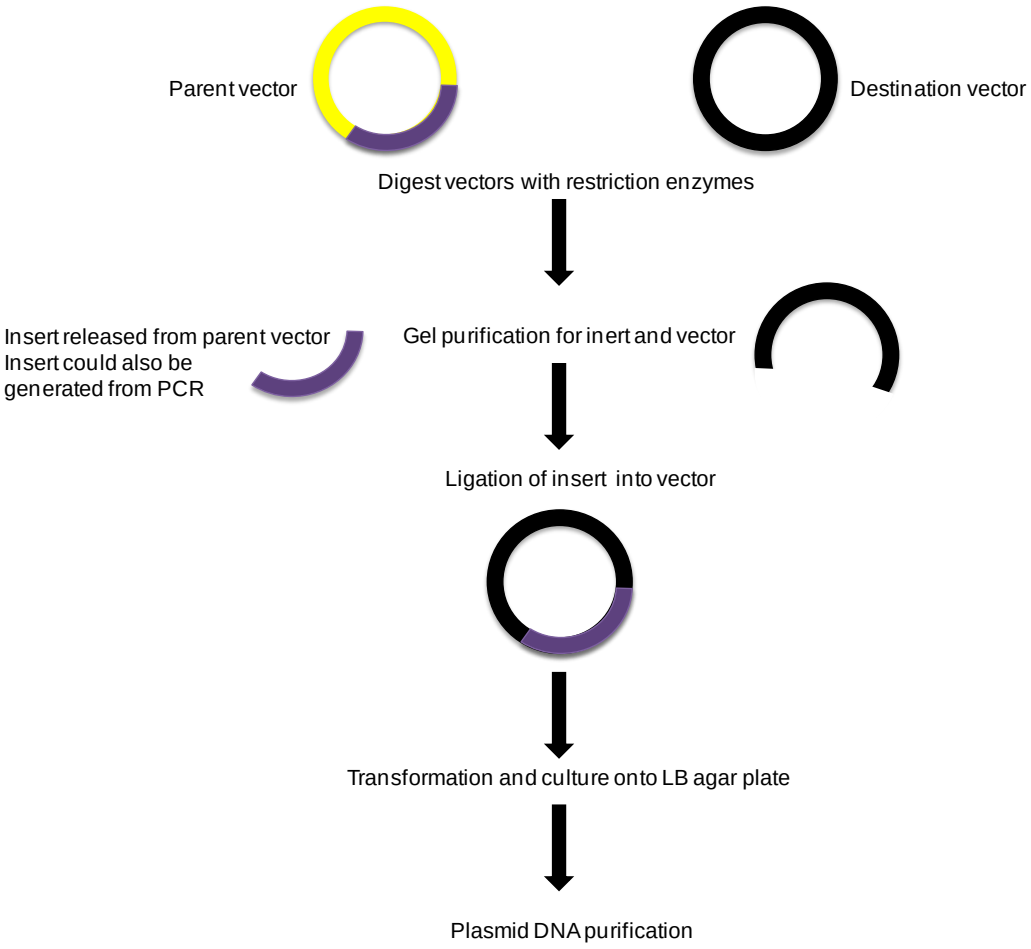

Figure 2.2: Cartoon diagram illustrating the basic steps of subcloning.

2.4.2 MRAP α and β plasmid constructs

Two sets of primers to human MRAP α and β were designed with *EcoR-I* and *Hind-III* restriction sites in place. These restriction sites were incorporated into the primer sequences to allow direct cloning into the mammalian vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) in the correct orientation. MRAP α and β share the same sequence for the first 200 bp, and therefore the same forward primer was used.

The forward primer was designed with a *Hind-III* (underlined) restriction site at the 5' end:

MRAP αβ forward primer:

5' GCA AAG CTT ATG GCC AAC GGG ACC AAC 3'

The reverse primers were designed with a *EcoR-I* (underlined) restriction site at the 5' end:

MRAP α reverse primer:

5' TAC GAA TTC TCA GCT CTG CAA TTG AGA 3'

MRAP β reverse primer:

5' TAC GAA TTC TCA GGC CGC CCC TTC CTC 3'

RT-PCR was performed as previously described (section 2.3.8) using human adrenal cDNA as a template (made from adrenal cortex total RNA, bought from Ambion). The PCR product was cloned into the mammalian expression vector (pcDNA3.1).

Previously cloned by another investigator in the lab, human MRAP α in pGEM®T-

easy (Promega, UK) was digested with *EcoR-I* and *Not-I*, which are common restriction sites to both pGEM T-easy and pcDNA3.1(+) vectors. This can be seen in the vector maps (Appendix 8.4).

2.4.3 Restriction digest

Restriction endonuclease digestion is part of the cloning strategy, to linearise vectors and to verify the presence and orientation of a given insert in a particular vector. Enzymes were obtained from New England Biolabs (NEB) or Promega, UK. A typical reaction is shown below.

Materials

DNA	1µg
10X buffer	5µl
Restriction enzyme	1µl (2-10 units)
ddH2O	x µl
total volume reaction	50µl

Typically the digest was performed at 37 ºC for 2 hours. The program NEB CUTTER V2.0 was used to select the most suitable restriction sites in the desired vector. Double digests were performed in a similar manner in a two step procedure; the reaction buffers were selected using information available from manufacturers.

2.4.4 Ligations

The inserts were incorporated into the plasmid vectors by ligation using T4 DNA

ligase and reactions were set up as following:

T4 DNA Ligase (1-3U/µl; Promega)	1µl
T4 DNA ligase buffer 10X	1µl
(300mM Tris buffer [pH 7.8 at 25ºC], 100mM $MgCl_2$, 100mM DTT and 10mM ATP)	
Vector	1µl
Insert	xµl
ddH2O to a final volume of 12µl	yµl

Method

The digested vector was gel cleaned as described in section 2.3.10, prior to ligation. Ligation reactions were left overnight at 4ºC. The ratio of insert/vector DNA was calculated according to following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Optimal molar ratios of insert/vector were 3: to 5:1

2.4.5 Preparation of ampicillin antibiotic plates

Materials

Luria Bertani (LB) Agar	9g LB, 8g Agar in 400ml ddH_2O
Ampicillin	final concentration 100µg/ml
Petri dishes	

Method

400ml LB agar was freshly prepared and autoclaved. The broth was left to stand at room temperature and was allowed to cool to approximately 37^{o}C.

100µg/ml ampicillin was then added and the broth mixed. Approximately 15-20ml broth was then poured into 10cm Petri dishes under a Bunsen burner flame. The plates were then to dried in a 37ºC cabinet and stored at 4^{o}C in a sealed bag for up to 1 month.

2.4.6 Transformation of competent bacteria

Materials

LB agar plates with 100µg/ml ampicillin

S.O.C medium (Invitrogen Ltd, UK) or LB broth

JM 109 *E-coli* competent cells (Promega, UK)

DNA sample

Method

A 15ml round bottomed polypropylene culture tube was chilled on ice. 100µl of JM 109 competent cells, thawed on ice, were pipetted into the 15ml tube. 50ng of DNA was added to JM 109 cells and placed on ice for 10 minutes. The cells were heat shocked at 42°C for 45-50 seconds and returned to the ice for another 2min before 900 µl SOC medium was added. The tube was incubated at 37°C for 60min with shaking at 225 rpm.

The cells were then centrifuged for 5 min at 2000 rpm and the supernatant discarded. The cells were then plated onto an LB ampicillin agar plate and incubated overnight at 37°C. The vector pGEM®-3Z (Promega, UK) was transformed as a control plasmid to assess the transformation efficiency of the cells.

2.4.7 Bacterial culture

Materials

LB broth (9g per 400ml ddH2O)

Ampicillin (final concentration 100 µg/ml)

Method

Each bacterial colony was selected from the LB agar plate with a sterile pipette tip and transferred to 5ml of sterile LB broth containing ampicillin. The transfer of the colony to culture has to be sterile and performed under a burning flame. The cultures were incubated overnight at 37ºC in a shaking incubator at 225rpm.

2.4.8 Glycerol stock

500 µl of cultured transformed bacterial cells were pipetted into a sterile centrifuge tube containing 500µl sterile glycerol (Sigma-Aldrich, UK). The mixture was vortexed well and stored at -70°C.

2.4.9 Plasmid DNA purification

The principle of the QIAprep procedure is based on the alkaline lysis method of isolation of low molecular weight (plasmid) DNA from bacterial cells. QIAprep columns use a silica membrane for selective adsorption of DNA in the presence of high salt buffer. The DNA is eluted in low-salt buffer or water. The exact composition of the reagent is unavailable.

Materials

QIAprep miniprep kit, QIAGEN

Method

Plasmid DNA was purified according to manufacturer's instruction. The 5ml bacterial culture was centrifuged in a 15ml Falcon tube for 15min at 3000 rpm, at 4ºC. After removing the media, the pellet was suspended in 250µl of ice cold Buffer P1 (cell suspension solution) and transferred to a 1.5 ml microcentrifuge tube. Then 250µl P2 (cell lysis solution) was added and mixed gently. 350µl N3 (neutralization solution) was added and mixed gently.

The microcentrifuge tube was spun at 13000 rpm for 10minutes. The supernatant was applied to a QIAprep spin column and centrifuged at a maximum speed for 30-60seconds. The flow through was discarded and the column washed with 500µl PB solution and then 750µl buffer PE. The DNA was then eluted in 30-50 µl ddH2O and collected in a clean microcentrifuge tube, which was centrifuged for 2 min at 13 000 rpm.

2.4.10 Site directed mutagenesis

DNA mutations, for example to change amino acids in a protein, were created by deleting or inserting single or multiple nucleotides.

Materials

Clontech Advantage® HD Polymerase Mix (2.5 unit/µl)

Advantage® 5X reaction Buffer (containing 5mM MgCl2)

Dpn-I restriction enzyme 20, 000 units/ml

dNTP mix 10mM

Competent JM109 *E.coli* cells (Promega, UK)

Primer design

Two complementary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence were synthesised (see Appendix 8.3 for primers sequences). Primers were between 25-35 bases in length. The desired mutation should be in the middle of the primer with ~12-17 bases of correct sequence on both sides.

The sample reaction was prepared as follows:

DNA template	50ng
Reaction Buffer 5X	10µl
Oligonucleotide primer 1	125ng
Oligonucleotide primer 2	125ng
dNTPs	1µl
Advantage® HD Polymerase Mix	0.4µl
ddH2O	xµl
Total volume	50µl

Methods

The reaction mixture was subjected to thermal cycling based on the following cycling parameters. Segment 1 was 1 cycle at 95ºC for 30 seconds, followed by segment 2 which had 12 cycles of 95ºC for 30 seconds, 55ºC for 1 minute, 68ºC for 6.5 minutes (1 min/kb of plasmid). Finally, segment 3 was 1 cycle of at 68°C for 7 minutes. This reaction mixture then contained new mutated plasmid containing staggered nicks, as well as the original native plasmid. The mixture was transferred to ice for 2 minutes, to ensure that it was below 37ºC, and then 1µl *Dpn-I* restriction endonuclease was added, and the mixture mixed by pipetting, and centrifuged 15 secs, at 6000X g. This was then incubated at 37ºC for 60 minutes to digest the

parental (nonmutated) DNA leaving only the mutated plasmid product. 5µl of the treated DNA was then used for standard bacterial transformation (described in section 2.4.6). The transformed plasmid was then purified (see section 2.4.9) and the presence of the desired mutation confirmed by direct sequencing (see section 2.3.11).

2.5 Protein analysis and protein-protein interaction

2.5.1 Protein extraction

To detect mutant MC2R protein in transfected cells and for co-immunoprecipitation assays, total protein was extracted from confluent cells, grown in 75 cm^2 plates.

Materials

n-Dodecyl-β-D-maltoside - Sigma-Aldrich, UK

Protease inhibitor cocktail (Roche, cOmplete) [chymotrypsin 1.5μg/ml, thermolysin 0.8μg/ml, papain 1mg/ml, pronase 1.5Mg/ml, pancreatic extract 15μg/ml, trypsin 0.2μg/ml, one table dissolved in 50 ml PBS]

PBS pH 7.4

Sample buffer (see section 2.5.2)

Method

Cell lysis buffer was prepared from chilled PBS + 0.1% n-Dodecyl-β-D-maltoside + protease inhibitor cocktail, a fresh buffer for each cell lysate preparation. Cells in 75cm^2 tissue culture dish were washed in cold PBS three times and aspirated. Cells were then frozen at -70ºC for at least 15 min to assist with cell lysis. 500-800μl ice-cold cell lysis buffer was then added to the dish. Cells were scraped and collected in a 1.5ml microcentrifuge tube, followed by dispersion with a syringe and G23 needle. Cells were incubated on ice for 30min. The lysed cells were then centrifuged at 13 000 rpm at 4^0C for 30minutes. Supernatant was collected and stored at -70°C until required.

2.5.2 Preparation of buffers

2X Sample buffers (SDS reducing buffer)

Distilled water	2.8ml
0.5M Tris-HCl, ph 6.8	1ml
Glycerol	2ml
10% (w/v) SDS	1.6ml
2-β-mercaptoethanol	0.4ml
0.05% Bromophenol blue	2µl

Alloquot into 1.5 ml centrifuge tubes and freeze -20°C.

Washing buffer

PBS tablet	5 tablets + 1L water
Tween-20 (Sigma-Aldrich)	0.5ml

Mixed together and stored at room temperature

5X Transfer buffer

For 400ml mixed in ddH_2O

Tris base	6g
Glycine	28.8g

Dissolved the mixture and stored at room temperature
Add 20% methanol and dilute in water immediately prior to use.

Blocking buffer

5% (w/v) reduced fat milk powder (Marvel)
Washing Buffer

2.5.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The specific composition of the Invitrogen running buffers or Bis- Tri Gels were not available.

Materials

Protein ladders – Prestained protein marker, broad range (6-175 kDa) – New England Biolabs, UK or rainbow molecular weight markers (10-250 kDa) – Amersham, UK

MES (2-[N-Morpholino]ethanesulfonic acid) or
MOPS (3-[N-morpholino]propanesulfonic acid) SDS Running buffer (20X)- Invitrogen NuPAGE®
MOPS/SDS is for proteins greater than 200 kDa to 14 kDa or MES/SDS for proteins between 25 to 60 kDa.
10-12% Bis-Tri Gel – Invitrogen NuPAGE® 1.00mm X 10-12 wells

XCell SureLock® Mini-Cell – Invitrogen, UK

Method

The protein samples were mixed with an equal amount of 2X loading buffer in a 1.5ml centrifuge tube. SDS is an ionic detergent which coats the protein proportional to their molecular weight and the resultant negative charge allows the proteins to separate on a gel based on their size. The samples were heated at 95ºC for 5min, to denature the protein complexes, centrifuged briefly and loaded into pre-cast 12% gels (when blotting for MRAP-Flag). In some cases denaturing was performed at room temperature for 30 min, specifically to improve resolution of GPCRs on gels. Samples were run alongside protein markers to enable an approximation of the molecular weight of identified proteins. Using a XCell SureLock® Mini-Cell, proteins were separated by SDS-PAGe at 120 V, until the dye front reached the bottom of the gel. MES/SDS buffer was used for MRAP blotting and MOPS/SDS buffer was used for GPCR blotting.

2.5.4 Protein transfer

Materials

Washing buffer (see section 2.5.2)

Hybond-P TM hydrophobic polyvinylidene Difluoride (PVDF) membrane – Amersham, UK

Extra thick blotting paper- Bio-Rad Laboratories, CA, USA

Transfer buffer (see section 2.5.2)

Trans-Blot SD Semi Dry Transfer Cell – Bio-Rad Laboratories Ltd, UK

Ponceau S stain (0.2% (w/v) Ponceau S + 1% Acetic acid)

Method

After running the gel, protein transfer was effectively achieved using a semi dry-transfer technique. Two sheets of Bio-Rad thick blotting paper were soaked in transfer buffer. A piece of PVDF membrane, cut to size was soaked in water. A Trans-Blot SD Semi Dry Transfer Cell was used for protein transfer according to the manufacturer's instructions. The semi-dry blotter was run for approximately 30 min at 15V with a current of 0.4 A per gel. After transfer the membrane was removed and the transfer of proteins was determined using Ponceau S stain.

2.5.5 Immunoblotting

Materials

Blocking buffer (see section 2.5.2)

Monoclonal anti-Flag M2 antibody produced in mouse (Sigma-Aldrich, UK)

Monocloncal anti-HA antibody produced in mouse (Sigma-Aldrich, UK)

Rabbit Anti-GAPDH polyclonal antibody (Santa Cruz Biotechnology Inc, Germany)

IRDye 800CW goat anti-mouse antibody, 800CW goat anti-rabbit or 680CW goat anti-mouse (LICOR, bioscience)

LICOR Odyssey reader -a fluorescence-based detection method for western blotting

Method

The transferred membrane was incubated with blocking buffer over night at 4ºC or at least 2hrs at room temperature, with gentle agitation. The membrane was incubated with primary antibodies for 1.5 – 24 hours (mouse anti-Flag mouse or mouse anti-HA or rabbit anti-GAPDH antibody) at 1:1000. The membrane was washed thoroughly with washing buffer and then incubated with the appropriate secondary antibodies for 1 hour (for example, 800Hz mouse 1:10000), in the dark in a blacked out box. The membrane was then washed thoroughly with washing buffer and was ready for scanning with the LICOR Odyssey reader.

2.5.6 Re-probing Western blots

To determine the total protein loaded in each lane, blots required further probing with rabbit polyclonal GAPDH antibody. The probed membrane was washed in washing buffer for 30 min with gentle agitation. This was follow by immunoblotting with GAPDH antibody as described in section 2.5.5.

2.5.7 Co-immunoprecipitation

Materials

Cell lysis buffer (see section 2.5.1)
Monoclonal anti-HA agarose conjugate, Clone HA-7 (Sigma-Aldrich, UK)
Anti-Flag M2 Affinity gel (Sigma-Aldrich, UK)
Monoclonal anti-Flag M2 antibody produced in mouse (Sigma-Aldrich, UK)
Monocloncal anti-HA antibody produced in mouse (Sigma-Aldrich, UK)
Sample buffer (see section 2.5.2)
Washing buffer (see section 2.5.2)

Method

The agarose was washed 3 X in 1ml PBS and after each wash the agarose was centrifuged at 4 000 rpm for 1 min. The PBS was discarded carefully leaving the agarose intact. 500µl of cell lysate previously prepared (as described in 2.5.1) was added to 30µl agarose beads in a microcentrifuge tube and placed on a rolling rotor overnight at 4ºC. The following day, the agarose was washed 5 X in 1ml PBS. With the last two washes lasting for 20 – 30 min. On the last wash approximately 10µl supernatant was left after removing the PBS from the agarose. 40µl sample buffer was added and to the sample and boiled for 3 min. The samples were then centrifuged at 13 000 rpm for 1 min. The supernatant was then ready for immunoblotting as described in section 2.5.3.

2.6 Functional studies

2.6.1 Cell surface assay

Materials

For transfection (refer to section 2.2.5)

200ng plasmid

2µl lipofectamine per well (in 24 well plate)

For cell surface assay

Triton 0.025% (Sigma-Aldrich, UK, Triton X-100) diluted in PBS

Monoclonal Anti-HA (mouse IgG1 isotype) (Sigma-Aldrich, UK)

LI-COR® Bioscience Blocking Buffer

LI-COR® Bioscience IRDye®800CW Conjugated Goat Anti-Mouse IgG

PBS (PBS tablets, Sigma-Aldrich, UK)

3.7% paraformaldehyde (PFA) (BDH,UK)

Method

After cells were transfected for 24-48hrs in 24 well plates, all medium was discarded, and the cells were washed with PBS for 5 min twice. The cells were fixed with 200 µl 3.7% PFA for 10min and then washed for 5 min twice with PBS. To permeabilise cells, 200 µl 0.025% triton was added for 10 minutes. Again cells were washed twice in PBS. The plates were then blocked for 1 hour with LI-COR® blocking buffer at room temperature, followed by incubation with 200µl mouse anti-HA (1:1000) antibody diluted in blocking buffer for one hour. After two further washes with PBS, cells were then incubated with 200µl of the secondary antibody IRDye®800CW (1:1000) diluted in blocking buffer. This step was performed at room

temperature but in darkness (plates were foiled). Again they were washed with PBS (2X) and then the plates were scanned using LI-COR® Odyssey plate reader.

The quantification of cell surface expression of the receptors was calculated by the ratio of the signal from unpermeabilised and permeabilised cells. The theory is outlined in figure 2.3.

$$\text{Cell expression ratio} = \frac{\text{Unpermeabilised reading – background reading}}{\text{Permeabilised reading – background reading}}$$

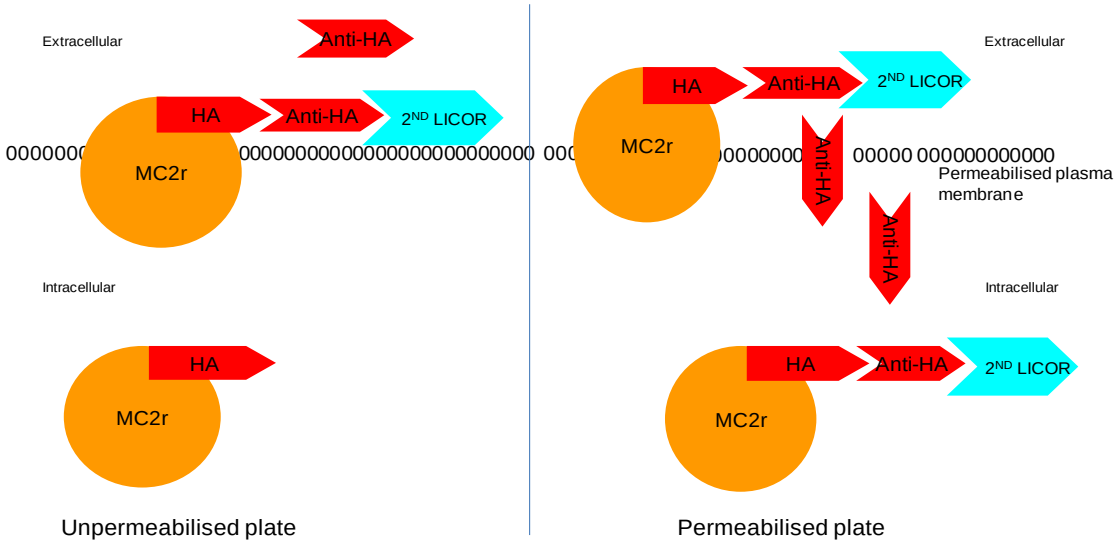

Figure 2.3: Cartoon illustration of cell surface assay. Cell surface expression was quantified by cell surface assay and assyed using a LICOR Odyssey plate reader. Cell surface expression was normalised for transfection efficiency by determining the ratio of the unpermeabilised to the permeabilised signal.

2.6.2 Luciferase Assay

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Applications include the study of receptor activity,

transcription factors, intracellular signalling, mRNA processing and protein folding. Dual reporters are commonly used to improve experimental accuracy. Promega's Dual-Luciferase Reporter Assay System assays the activities of firefly (*Photinus pyralis*) and Renilla (*Renilla reiformis*, also known as sea pansy) luciferases which are measured sequentially from a single sample.

After stimulation of a Gs linked 7 transmembrane receptor, intracellular signalling leads to cAMP production and the subsequent phosphorylation of the transcription factor CREB (cAMP response binding protein). Phosphorylated CREB in turn upregulates the transcriptional activity gene promoters containing cAMP response elements (CRE). By using a construct containing a luciferase gene under the control of a promoter containing a CRE, the subsequent luciferase activity produced gives a direct indication of the amount of cAMP produced by the stimulus. The transfected renilla plasmid is constitutively active and the renilla activity serves as a control for transfection efficiency for each sample.

Cell extractions were carried out with 150µl Passive lysis buffer. The luciferase assay was performed by adding 80µl of the dissolved luciferase substrate to the cell lysate. Luciferase in cell lysate catalyzes the chemiluminescent reaction, which emits light (560nm) constantly for several minute, the emitted light is measured with a luminometer, Lumistar Omega (BMG Labtech, Germany). After recording the luciferase activity, 80µl of the Stop and Glo reagent was added, which simultaneously quenched the luminescence from the firefly luciferase and activated the luminescence from the renilla luciferase, which was then quantified. Because of the different substrate requirements of the two types of luciferase, it was possible to distinguish between the different bioluminescent reactions.

Materials

For transfection in 6 well plates

pRL-CMV Renilla luciferase plasmid (Promega, UK) - 100ng/well

α GSU-846 luciferase plasmid – 1000ng/well

DNA plasmid of interest – 1000ng/well

For luciferase assay

Luciferase assay buffer

Luciferase assay substrate

Stop & Glo® buffer (Promega)

Stop & Glo® substrate (Promega)

Passive Lysis Buffer (5X) (Promega)

Luciferase assay reagent was prepared by resuspending the lyophilized Luciferase assay substrate in 10ml Luciferase assay buffer. This was stored at -70ºC. Stop & Glo substract was added to Stop & Glo buffer and stored -70ºC.

Method

Cells were co-transfected with αGSU-846 luciferase and pRL-CMV Renilla luciferase plasmid along with MC2R or mutant MC2Rs. After 24-48hours of transient transfection, cells were washed with PBS. Stimulations were carried out using ACTH (10^{-7}M) for 7 hours. The medium was removed and the cells were washed in PBS before harvesting with 150µl/well lysis buffer. The samples were collected in a centrifuge tube and centrifuged at 13 000 rpm for 10 min. 25 µl samples were plated in opaque black 96 well plates. Using the Lumistar Omega with the appropriate setting, 80µl luciferase and 80µl Stop and Glow reagent were used for each sample.

2.7 Confocal microscopy

The key feature of confocal microscopy is its ability to produce in-focus images of thick specimens, a process known as optical sectioning. It produces images of the cells in a single plane.

Materials

3.7% formaldehyde
0.25% Triton-X-100 (Sigma-Aldrich, UK)
PBS
Blocking buffer - 3% BSA, 10% donkey serum in PBS
Monoclonal mouse Anti-HA antibodies (Sigma-Aldrich, UK)
Cy ™ 3-conjugated AffiniPure Donkey anti-mouse antibody (1:100) – Jackson ImmunoResearch
4', 6-diamidino-2-phenylindole (DAPI)
Fluorescent mounting media

Method

CHO α1 cells were seeded into 12 well plates with a sterile cover slip previously placed in each well bottom. Transfection was performed for 24 hours with wt and mutant MC2R constructs N terminally -3XHA tagged and empty vector. The media was removed and the cells were washed 3X with PBS. Cells were then fixed using 3.7% formaldehyde in PBS for 10 min. The cells were permeabilised after incubation with 0.25% Triton for 10 min. After three more washes with PBS (10 min per wash), the cells were incubated with blocking buffer (3% (w/v) BSA, 10% (v/v) donkey serum in PBS) for 1 hour. Incubation with appropriate primary antibody (for example, mouse anti-HA, polyclonal rabbit anti-Calnexin antibody) was performed at a dilution of 1:200 at room temperature for 90 min. The cells were rinsed for 3X 10 min washes in PBS, followed by incubation with appropriate secondary antibody (for example, Cy2/Cy3-conjugated donkey secondary antibody) at a dilution of 1:100 for 1 hour at room temperature. Thorough washes with PBS were critical between each

step. The nuclei were stained for 1 min with PBS containing 2 µg/ml DAPI (1 in 5000). The cover slip was then removed carefully onto a clean slide and mounted it with drops of fluorescent mounting media, DakoCytomation Fluorescent Mounting Medium (DAKO). Fluorescence images were taken using a Zeiss LSM 510 confocal microscope. The slides were stored at 4ºC in the dark.

Chapter 3

Naturally occurring MC2R mutations in Familial Glucocorticoid Deficiency type 1

3.1 Introduction

Familial Glucocorticoid Deficiency (FGD) is a rare autosomal recessive disease which is characterized by isolated glucocorticoid deficiency and ACTH resistance; presenting features include severe neonatal hypoglycaemia, frequent childhood infection and excessive skin pigmentation (Shepard *et al.* 1959; Franks & Nance 1970; Clark & Weber 1998). This is a genetically heterogeneous disorder with mutations in the ACTH receptor (Melanocortin 2 receptor, MC2R) accounting for approximately 25% of cases of FGD (Clark *et al.* 1993; Tsigos *et al.* 1993; Weber *et al.* 1995; Clark *et al.* 2005) and this is referred to as FGD type 1 (OMIM 202200).

MC2R is one of five melanocortin receptors (Mountjoy *et al.* 1992). It is a 297 amino acid seven transmembrane receptor, the smallest of all G protein-coupled receptors (GPCRs); it is expressed predominantly in adrenocortical cells and is highly specific for ACTH, which stimulates the production of glucocorticoids. Earlier studies on MC2R were hampered by significant difficulties in expressing this GPCR in heterologous cells. The MC2R cDNA is transcribed and translated from transfected expression plasmids, but the protein product fails to traffic from the endoplasmic reticulum (ER) (Noon *et al.* 2002). The observation that human MC2R could be expressed in cells of adrenal origin but which lack endogenous melanocortin receptors (the mouse Y6 and OS3 cell lines) (Yang *et al.* 1997; Elias *et al.* 1999; Swords *et al.* 2002), has led to the hypothesis that a cofactor for expression is required. Identification of the genetic cause of a second form of FGD (FGD type 2) led to the identification of a novel gene encoding a single transmembrane domain protein, named Melanocortin 2 receptor accessory protein (MRAP) which appears to fulfil this role. MRAP interacts with MC2R and facilitates the trafficking of the receptor from ER to the cell surface (Metherell *et al.* 2005; Cooray *et al.* 2008).

The discovery of MRAP now provides the opportunity to characterise fully the MC2R in readily transfected and maintained cell lines, such as Chinese hamster ovary (CHO) and human embryonic kidney 293 (HEK293) cells, both of which are known not to endogenously express melanocortin receptor (MCR) and to be unresponsive to ACTH and NDP-MSH (Blondet *et al.* 2004; Rached *et al.* 2005). This MCR-free system allows the complete characterization of naturally occurring mutations enabling the analysis of cell surface expression and signal transduction.

Previous studies have shown that some of these mutations have impaired ligand binding and cAMP signalling, but the mechanisms for this defect were not investigated. This comprehensive analysis of MC2R mutations is the first study to examine the effect of the clinically identified mutations on both receptor trafficking and signal transduction.

3.2 Aims

Naturally occurring MC2R mutations found in FGD type 1 have been known for some time, however the functional consequences of these mutations remains poorly understood. The main aim of this chapter was to establish the molecular basis in the impairment of function of MC2R mutations found in FGD. In order to achieve this goal the following steps have been taken.

1. The first objective was to establish a stable cell line expressing MRAP in non-adrenal cells (CHO and HEK293), which would gives a readily transfectable system that is free of other MCRs. This allows complete characterisation of MC2R mutations to begin.

2. Assessment of cell surface expression of the epitope-tagged MC2R missense mutations was to be determined by using cell surface assay and observing the localisation of these receptors using confocal microscopy. This will determine the group of mutations which are either trafficking-defective or trafficking-competent.

3. For those receptors that reach the cell surface efficiently, receptor function will be characterised by ACTH binding and cAMP generation in response to ACTH.

4. For the mutations which are trafficking-defective, it is possible that there could be interference with MRAP interaction, which could be investigated by co-immunoprecipitation of MC2R with MRAP.

3.3 Materials and Methods

All methods below have been outlined in detail in Chapter 2. The techniques used and the specific antibodies for this study are briefly mentioned below.

3.3.1 CHO and HEK293 stable cell lines expressing human MRAPα

Human MRAPα amplified from human adrenal cDNA (Ambion) was subcloned into pcDNA3.1(+) vector (Invitrogen). CHO cells were grown in DMEM/Ham's F12 (1:1); HEK293 grown in DMEM, both with 10% foetal bovine serum and penicillin/streptomycin. Expression of MRAPα mRNA in the selected stable cell lines was confirmed using RT-PCR. In addition, the cell surface assay with wild-type hemagglutinin (HA) epitope tagged at the N-terminus MC2R (Wt-HA-MC2R) was performed to ensure cell surface expression of MC2R.

3.3.2 Site directed mutagenesis

Mutant MC2R expression constructs were generated by site-directed mutagenesis (QuickChange®II XL) using human Wt-HA-MC2R (Missouri S&T cDNA Resource Center) as template. Primer sequences are specified in the appendix 8.3. The entire mutant MC2R constructs were then sequenced to confirm the presence of specific mutations and ensure the absence of unwanted errors.

3.3.3 Cell surface assay

Briefly, CHO cells stably expressing MRAPα (CHOα1) were seeded in 24 well plates and transfected with Wt-HA-MC2R or mutated HA-MC2R. Both permeabilised and unpermeabilsed plates were blocked with Odyssey® Blocking buffer (LI-COR) for 1 hour, followed by incubation with anti-HA antibody (1:1000) for 1 hour. Infrared-labelled secondary goat anti-mouse antibody was then added at 1:1000 (Odyssey® Goat anti-mouse IR Dye @800CW) for one hour. The plates were then analysed with

the LI-COR Odyssey® plate reader (800nm) after thorough washes. The relative values of cell surface expression of the receptors were calculated by the ratio of unpermeabilised and permeabilised cells which was then normalised to wild type expression in each experiment.

3.3.4 Immunoblotting and co-immunoprecipitation

Interaction of mutated MC2R with MRAPα was assessed by co-immunoprecipitation (Co-IP) and immunoblotting as previously described (Metherell *et al.* 2005; Cooray *et al.* 2008) CHO cells were transiently transfected with MRAPα-Flag, and mutant HA-MC2R constructs and twenty four hours after transfection, cells were lysed with 0.1% D-dodecyl-β-maltoside in PBS in the presence of protease inhibitors. Anti-HA agarose conjugate beads (Sigma) were added to the cell lysate and incubated overnight, at 4°C. The beads were collected, washed thoroughly and resuspended in SDS sample buffer before heating at 95ºC for 10 minutes. The samples were then subjected to immunoblotting using anti-Flag (Sigma) antibodies at a dilution of 1:2000, followed by secondary antibody incubation (IRDye 800CW Goat Anti-Mouse IgG, LI-COR) at 1:10000 for 1 hour and imaged with LI-COR's Odyssey Infrared Image System.

3.3.5 Confocal microscopy

CHOα1 cells were seeded into 12 well plates containing sterile cover slips and transfected with either Wt-HA-MC2R, empty vector or mutated HA epitope-tagged MC2Rs for 24 hours. After non-specific blocking, the cells were then incubated with mouse anti-HA antibody (1:200 dilution in buffer A) for 90 minutes. This was followed by incubation with Cy™3-conjugated Donkey anti-mouse antibody 1:100 (Jackson ImmunoResearch, PA, US) for 1 hour. For co-localization with the ER, fixed and permeabilised cells were incubated with rabbit anti-calnexin antibodies 1:300 (Sigma)

and anti-HA antibody 1:200 in blocking buffer for 90 minutes. Secondary antibody incubation was performed with Cy^{TM}3 (mouse) at 1:100 and Cy^{TM}2 (rabbit) at 1:50 (Jackson ImmunoResearch, PA, US) for 1 hour. Nuclei were stained with PBS containing 2µg/ml DAPI, 4',6-diamidino-2-phenylindole. The cover slips were then carefully placed onto a clean slide and mounted with drops of fluorescent mounting media. Fluorescent images were taken using a Zeiss LSM 510 confocal microscope.

3.3.6 cAMP reporter assay

Stable HEK293 MRAPα (HEKα7) cells grown to 70-80% confluence in 6 well plates were transfected with 1000ng of receptor expression plasmids along with 900ng αGSU-846 luciferase and 100ng pRL-CMV *Renilla* luciferase plasmid constructs (Cooray *et al.* 2008). 24-48 hours after transfection, cells were stimulated with ACTH (10^{-7}M) for 6 hours. Cell lysates were collected and assayed using the Dual luciferase reporter assay system (Promega). Luciferase activity was measured using a multiplate reader (Lumistar Omega BMG Labtech), and values were normalised to the *Renilla* luciferase activity.

3.3.7 Statistical analysis

The data reported are the mean ± SEM of at least three independent experiments, performed in duplicate. Statistical comparison was performed using unpaired two-tailed Student's t-test, and *P* values are indicated as *, $P<0.05$; **, $P<0.01$; ***, $P<0.0001$.

3.4 Results

3.4.1 Establishing stably expressing MRAP cell lines

The rationale for creating stable hMRAP cell lines is to establish a system free of other melanocortin receptors which has consistent hMRAP expression and have good transfection efficiency. As discussed in Chapter 1, Y6 and OS3 cell lines, derived from the mouse Y1 adrenal cell line (Schimmer *et al.* 1995; Rainey *et al.* 2004) are the only cell lines which provide reasonable expression of the MC2R with no other interfering melanocortin receptors. The disadvantage is that these cells transfect poorly. Both CHO and HEK293 cells have been readily used in the studies of other GPCRs, and they have no endogenous MCRs or hMRAP (PCR shown in figure 3.3) (Noon *et al.* 2002; Rached *et al.* 2005). In order to use CHO and HEK293 cells to study MC2R mutations, it was first necessary to introduce MRAP into them to allow cell surface expression and activity of the introduced receptor. Transient transfection of hMRAP into these cells is possible, but will introduce transfection variability between experiments and is also time consuming. Stable cell line is therefore thought to be a better and more efficient alternative.

The hMRAP expression vector contained a neomycin resistant gene and cells containing this plamid were selected for their resistance to G418. As a first step, a cytotoxicity titration curve was required to establish the minimal concentration leading to cell death at approximately 7-10 days.

3.4.1.1 Antibiotic titration curve for CHO cells

G418 dose (µg/ml)	Day on which no viable cells seen
0	No cell death
600	14

800	9
1000	7

Table 3.1: G418 cytotoxicity titration curve for CHO cells. Cytotoxicity was noted daily for each antibiotic dose.

Cells were plated out into 6 well plates and were treated with various doses of G418. Cytotoxicity was judged by daily microscopic inspection. The optimal cytotoxic dose of G418 is when complete cell death is evident between days 7 to 10. In CHO cells, 700 µg/ml G418 dose was chosen for stable cell selection.

3.4.1.2 Antibiotic titration curve for HEK 293 cells

G418 dose (µg/ml)	day on which no viable cells seen
1000	No cell death
1200	No cell death
1400	14
1600	12

Table 3.2: G418 cytotoxicity titration curve for HEK 293

Cytotoxicity was more difficult to achieve in HEK 293 cells compared to CHO cells. A higher dose of antibiotic was required to attain complete cell death. A concentration of 1500 µg/ml G418 was chosen for stable cell selection in HEK 293 cells.

The cloning of the constructs for hMRAP α cDNA in pcDNA3.1(+) have been described in Materials and Methods section (Chapter 2). These constructs were confirmed on sequencing.

CHO and HEK 293 were transfected with hMRAP α, as well as empty pcDNA3.1(+). Antibiotic selection with G418 700 µg/ml for CHO transfected cells and for HEK 293 1500 µg/ml were used and colonies were visible after 21 days of antibiotic treatment.

Clones of CHO hMRAP α were selected with the dilutional method (as described in Chapter 2.2.5.3). After the colonies were selected, they were tested based on:

1. To confirm the function of the stable cell line, cell surface assay was used. The function was tested using cell surface expression of wild type 3XHA-MC2R (N terminal). A functional colony should express MC2R at the cell surface adequately. This cell surface expression should be comparable to wild type cell lines (CHO or HEK293) transiently co-transfected with MRAP α and HA-MC2R.

2. The mRNA expression of these cells was confirmed with RT-PCR for hMRAP.

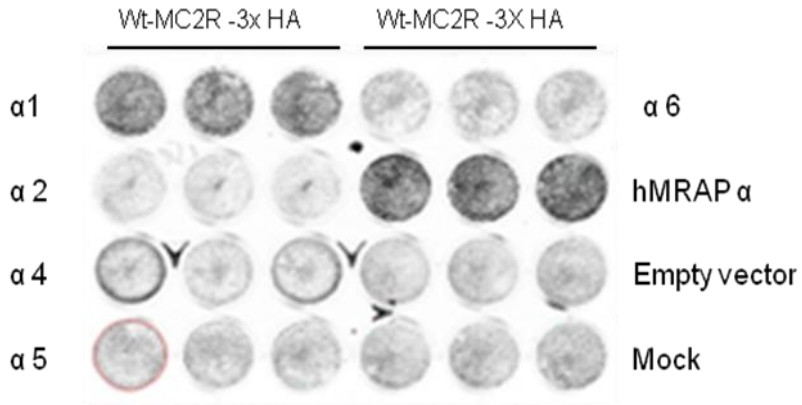

Figure 3.1: Selection of stable MRAPα CHO cells. Wt-HA-MC2R transiently transfected (in triplicate) into various potential stable cell lines (α1, 2, 4, 5 and 6). Transient transfection of Wt-HA-MC2R in wild type CHOs cotransfected with hMRAPα acts as the

positive control and empty vector as negative control. Additional negative controls with mock transfection in wild type CHOs were also used. As this experiment was a screening technique to select the best stable cell line it was only performed once.

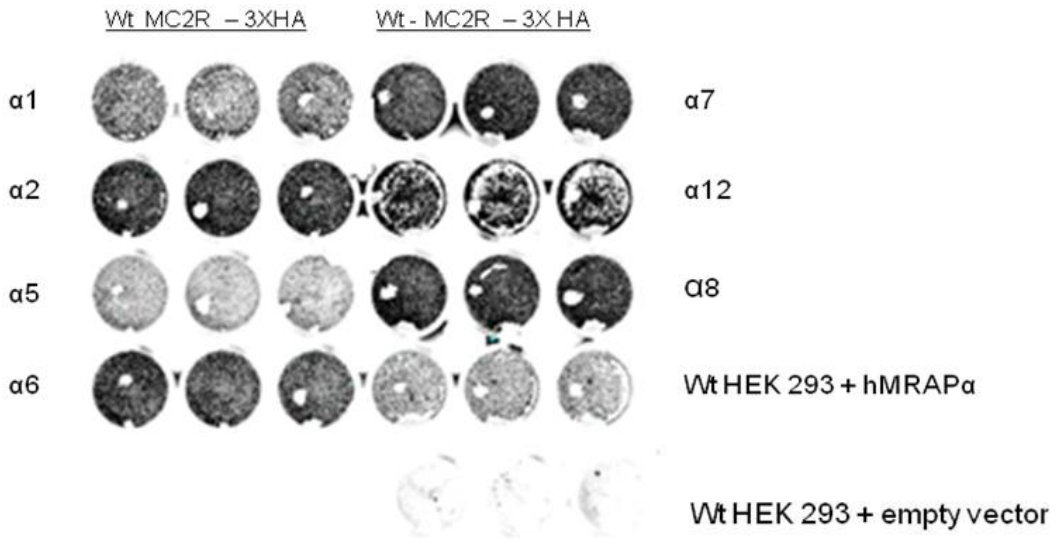

Figure 3.2: Selection of stable MRAPα HEK 293 cells. Wt-HA-MC2R transiently transfected (in triplets) into various potential stable cell lines (α1, 2, 5, 6, 7, 8 and 12). Transient transfection of Wt-HA-MC2R in wild type wild type HEK 293 and cotransfected with hMRAPα act as the positive control and empty vector as negative control.

The cell lines which demonstrated the best functionality when compared with transiently transfected MRAP α were selected and used for the rest of the experiments (Figure 3.1 and 3.2).

3.4.1.3 RT-PCR confirmation of MRAP expression

In the absence of an antibody to detect human MRAP, the expression of MRAP in these cell lines was confirmed by RT-PCR.

cDNA was made from each of the selected stable cell lines and PCR was performed using MRAP specific primers.

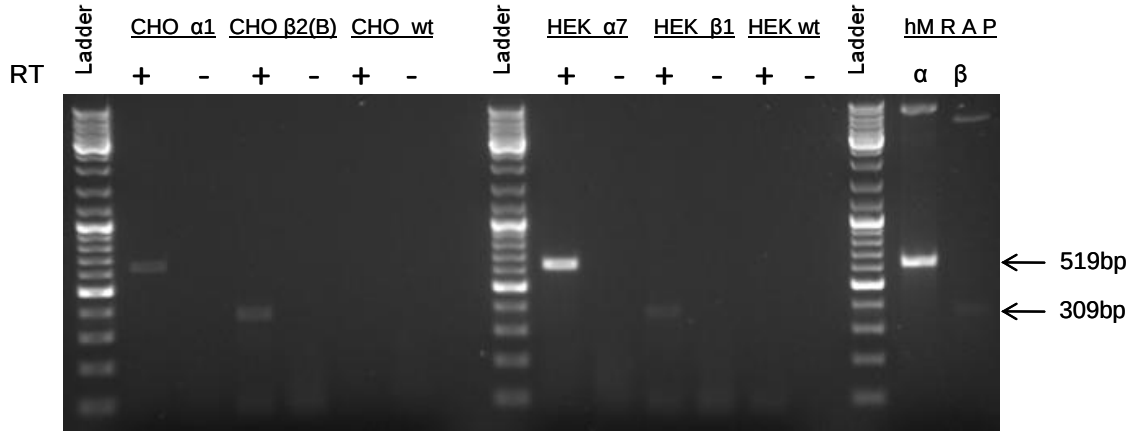

Figure 3.3: RT-PCR showing MRAP expression. 1% agarose gel showing RT-PCR using primers specific for hMRAPα and hMRAPβ. Lanes are labelled with various stable cell lines, CHOα1, CHOβ2(B), HEKα7 and HEKβ1. The corresponding RT +/- (reverse transcriptase), RT- is to demonstrate the lack of genomic DNA in each sample. Positive controls are MRAPα and β vectors and negative control are wild type CHO and HEK 293.

All the selected cell lines demonstrated mRNA expression for the relevant MRAP. The wild type CHO and HEK confirmed to have no MRAP expression. The presence of human MRAP α is indicated by gel electrophoresis band at 519 bp and MRAP β at 309 bp. Only MRAP α cell lines were used, as MRAP α is thought to have the similar action to MRAP β.

The selected stable cell lines, CHO α1 and HEK α7 were confirmed to have both mRNA expression and the best functionality of hMRAP α that is comparable with transiently transfected MRAP.

3.4.2 Site directed mutagenesis of missense mutations

Having established a heterologus MCR free system in which to study MC2R receptor activity it was now necessary to generate expression clones for the different MC2R mutants to be tested. Wild type HA tagged MC2R was used for site directed mutagenesis for all naturally occurring missense mutations, except for M1K (as this is a non translatable protein). A schematic diagram demonstrating the position in the receptor of all 24 missense mutations used in this study has previously been shown in Figure 1.6. The list of the mutations is shown in Table 3.3. The introduction of each mutation was confirmed by sequencing.

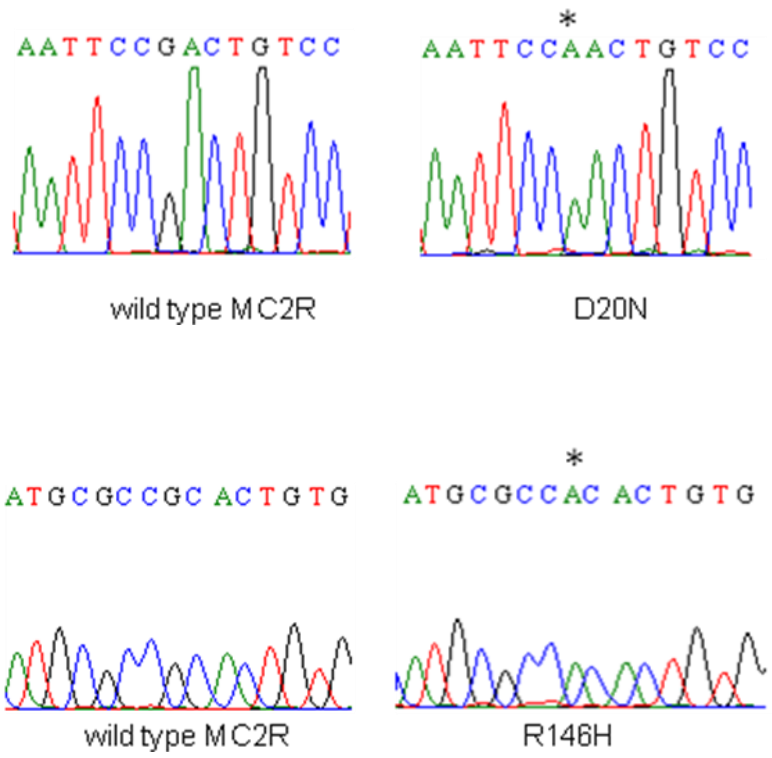

Figure 3.4: Sequencing for D20N and R146H mutations. Illustration of sequencing results for D20N and R146H which verifies the presence of introduced mutations. The other 22 mutations were also confirmed by sequencing (sequencing data not shown).

D20N	R128C	H170L
I44M	Y129C	L198P
L55P	I130N	G226R
S74I	R137W	A233P
D103N	H139Y	C251F
D107N	R146H	Y254C
G116V	T152K	S256F
S120R	T159K	P273H

Table 3.3: List of all missense mutations in MC2R studied.

3.4.3 Cell surface expression of MC2R mutations

CHOα1, described in section 3.4.1, was chosen for its adherence properties and good transfection efficiency compared to HEKα7 (which also have good transfection efficiency, but poor adherence). Adherence of cells is important, as this assay requires multiple washes.

CHOα1 was used as a host for transient transfection of either N-terminal tagged HA Wt-MC2R or each of all 24 HA-MC2R missense mutations. A cell surface assay was used to monitor cell surface expression of epitope-tagged MC2Rs to the plasma membrane by detection of fluorescent signals as described in chapter 2. Assays were performed in duplicate and in at least three independent experiments. The transfection efficiency was corrected to overall expression of receptors in permeabilised cells. Each experiment was performed with wild type MC2R and expressed as a relative ratio to wild type, this is to correct for the variability of fluorescent readings between experiments.

An occasional problem with this assay was the variability of the result between assays, for example for wild type MC2R, the variable readings of the unpermeabilised

/ permeabilised ratio (range 0.34 – 0.76; n=5) were noted. Visual inspection of the emitted fluorescence from the plate, indicated that cell surface expression was definitely present in the wild type and was more prominent when compared with mutants. The relative ratio of expression were normalised to wild type in order to compare between experiments. The pitfalls of the assay were poor cell adherence after multiple washes, inadequate washes giving a high background reading and variable transfection efficiencies between experiments. Therefore gentle but thorough washes were essential to perfect this technique.

The data presented in Figure 3.5 shows that the majority of the mutations failed to reach the cell surface. Eighteen out of 24 MC2R mutations demonstrated varying degrees of intracellular retention when compared with wild type. These are subsequently referred to as the trafficking-defective mutations. S74I and T152K showed some degree of intracellular retention which did not reach statistical significance. The trafficking defective mutations appear to be located throughout the receptor but those located towards the C-terminus appear to be more affected. Furthermore, with the exception of R128C, majority of the trafficking-competent mutations are located in close proximity to or within the extracellular loops.

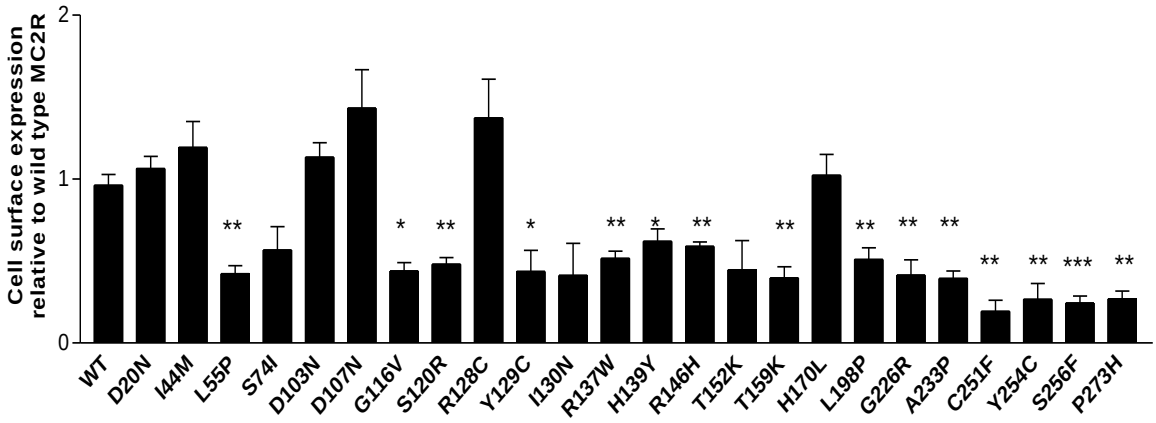

Figure 3.5: Results of cell surface assay for all 24 missense mutations. The cell surface assay was used to quantify cell surface expression comparing Wt-HA-MC2R with all 24 MC2R missense mutations. The quantified relative values of cell surface expression are shown (n=3). *,*P*<0.05; **, *P*<0.01; *, *P*<0.0001, relative to Wt-HA-MC2R. The majority of MC2R mutations had significant reductions in cell surface expression. There is only partial cell surface expression in S74I, I130N and T152K, but there is no significance difference when compared with wild type.**

To independently verify the results of the cell surface assay, confocal analysis of CHOα1 cells expressing HA-MC2R mutants was performed (Figure 3.6). Wild type MC2R displayed efficient cell surface expression in permeabilised cells. Similarly D103N and R128C showed substantial plasma membrane expression, as did the other mutants which reached the cell surface, (D20N, I44M, D107N and H170L-data shown in appendix 8.5A and 8.5B). The trafficking-defective mutants such as R146H, T159K and Y254C (Figure 3.6 D, E and F) exhibited marked intracellular retention consistent with the results from the cell surface assay. These mutants demonstrate ER retention, as exemplified by T159K which co-localises with the ER marker calnexin (Figure 3.6 J, K and L). The other trafficking-defective mutants displayed similar findings (data not shown). The majority of these mutations lie within the

transmembrane domain (10/18) and second intracellular loop (5/18) (Figure 3.7 A). The S74I, I130N (Figure 3.7 G and H) and T152K demonstrated partial trafficking impairment, which did not reach a statistically significant difference on cell surface assay.

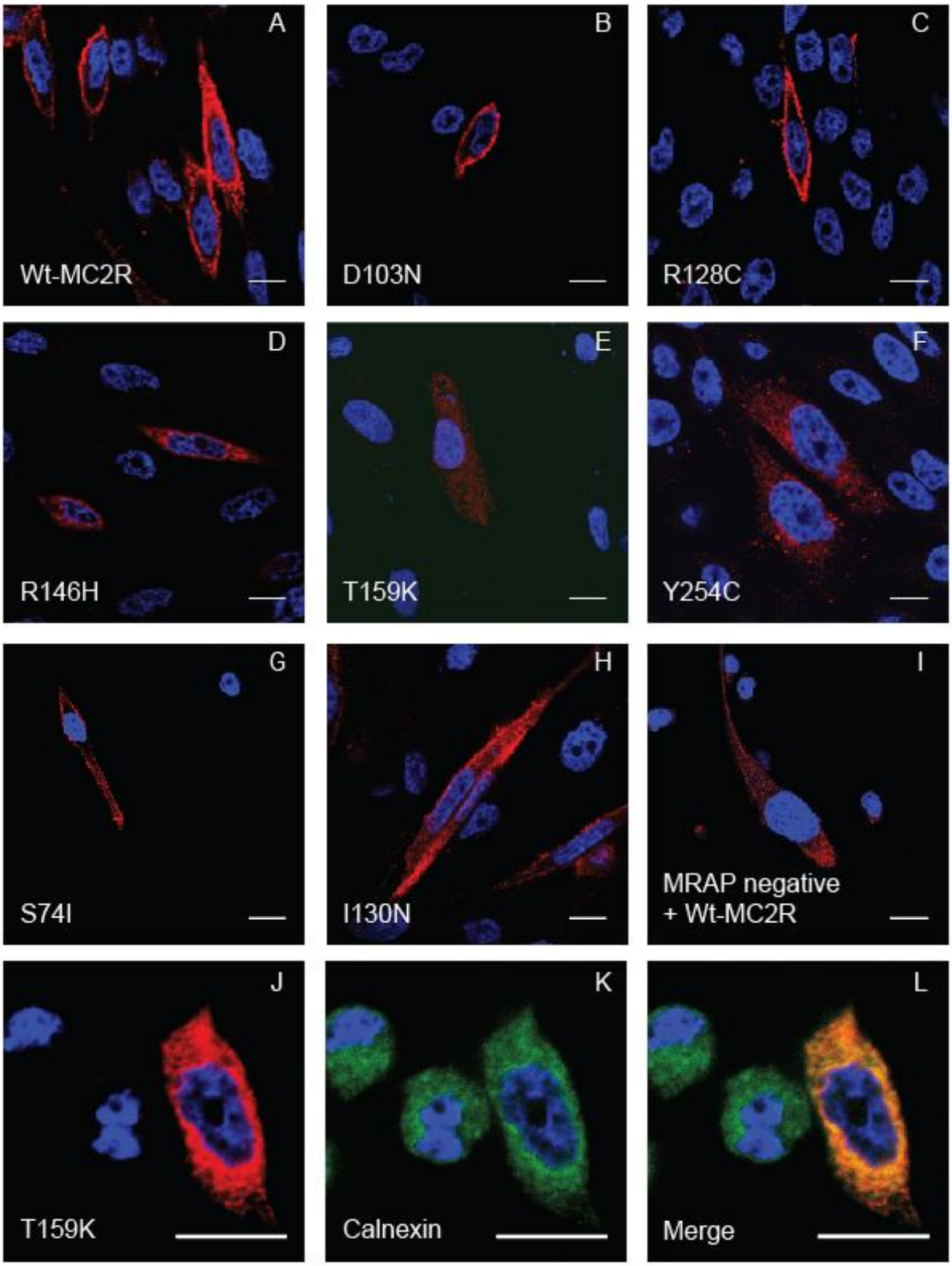

Figure 3.6: Confocal analysis of MC2R mutants in permeabilised stable CHOα1. CHOα1 cells were transiently transfected with A: Wt-HA-MC2R (positive control); B: D103N; C: R128C; D: R146H; E: T159K and F: Y254C; G: S74I and H: I130N. I: Wt-HA-MC2R in

MRAP negative cells was used as the negative control. J, K and L: T159K mutation (red) co-localises with ER marker, calnexin (green). Cells were incubated with anti-HA antibody (red) and DNA was stained with DAPI, 4',6-diamidino-2-phenylindole (blue). Representative images are shown. The scale bar represents 10μm.

The confocal analysis of MC2R mutants correlated with the results from cell surface assay. In the confocal study, all trafficking-competent mutations showed convincing evidence of receptor expression at cell surface, such as D103N and R128C. All the trafficking-defective mutations identified in Figure 3.6 failed to display convincing receptor plasma membrane localisation eg: I 130N, R146H, T159K and Y254C. Furthermore, the trafficking defective mutations eg T159K colocalised with calnexin, which suggests that they are localised within the ER. The confocal images of the other mutations can be found in the appendix (8.5A and B).

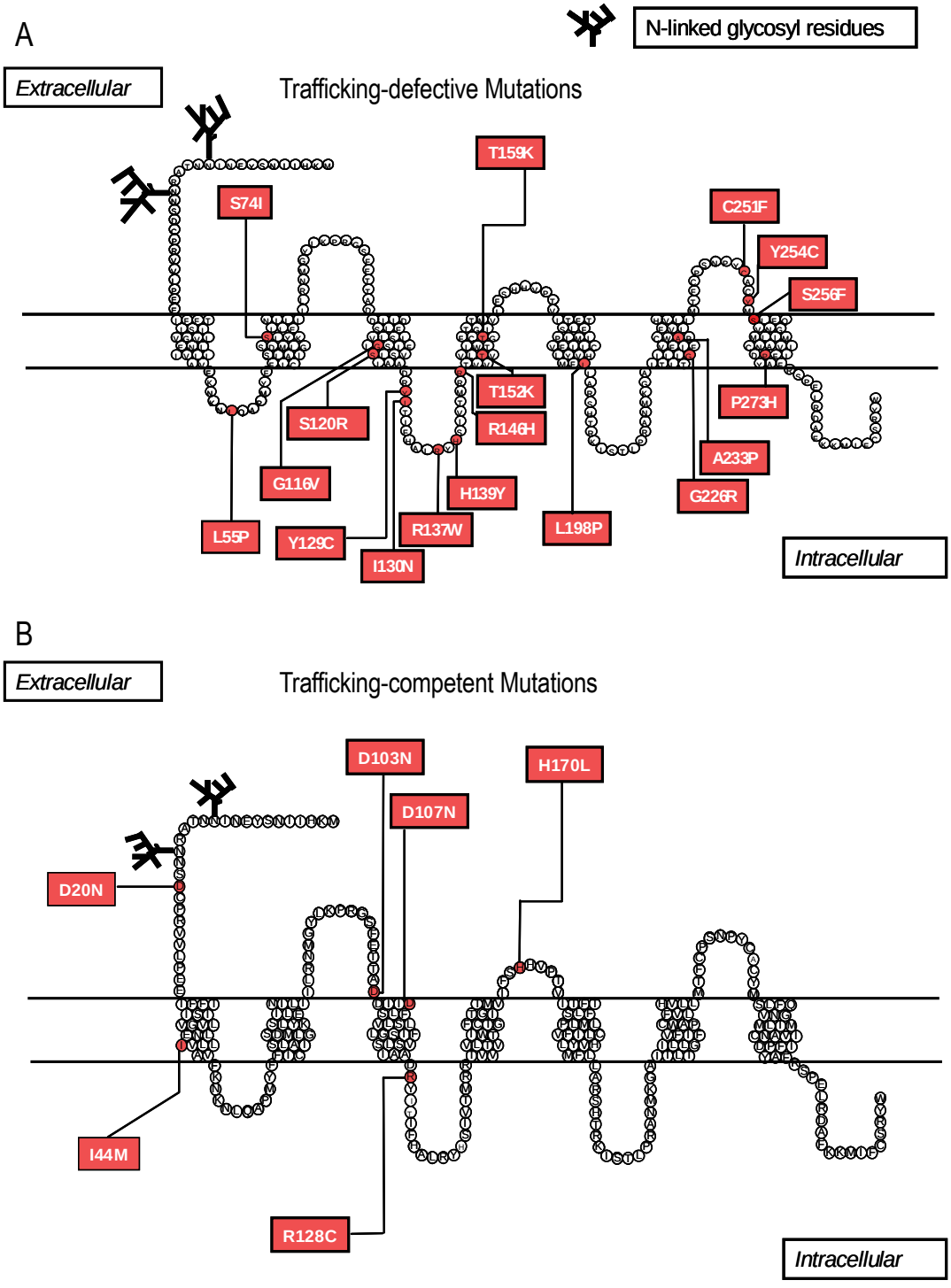

Figure 3.7: Pseudostructural plot of the MC2R mapping the locations of the 18 trafficking-defective mutations in A and the 6 trafficking-competent mutations in B.

The positions of the trafficking-defective and trafficking-competent mutants were mapped onto the MC2R. Of the six mutants with intact trafficking function, three are located in the extracellular domain of MC2R (Figure 3.7 B). Interestingly, one of the trafficking-competent mutation located within the 2nd intracellular loop of the MC2R is the R128 substitution (R128C), which lies within the highly conserved DRY (Asparagine-Arginine-Tyrosine) motif, which is required for G protein coupling and activation (Zhu *et al.* 1994; Jones *et al.* 1995).

3.4.4 Signalling function of the trafficking-competent mutants

To determine whether there is a loss of MC2R signalling in the mutants expressed at the cell surface, HEKα7 cells were transfected with either Wt-HA-MC2R, untagged Wt-MC2R or mutant HA-MC2R and stimulated with ACTH 10^{-7} M. Cellular cAMP accumulation was analysed to assess receptor activity. The stable HEK cells were chosen in place of the CHOα1 because of their high levels of transfection efficiency, protein expression and good cAMP responsiveness, but less adherence. Interestingly, results showed that the untagged Wt-MC2R generated 40% more cAMP than Wt-HA-MC2R. Furthermore, both D20N and I44M demonstrated similar function when compared with Wt-HA-MC2R (Figure 3.8). However, all other mutants (D103N, D107N, R128C and H170L) had significant impairment of cAMP generation, as seen by the decreased luciferase activity.

Previous signalling studies on mutations such as R146H and T159K showed a lack of response to ACTH (Elias *et al.* 1999). Data in Figure 3.5 indicate that these mutants fail to reach the plasma membrane and are located in the ER. For this reason these mutants were not included in this study.

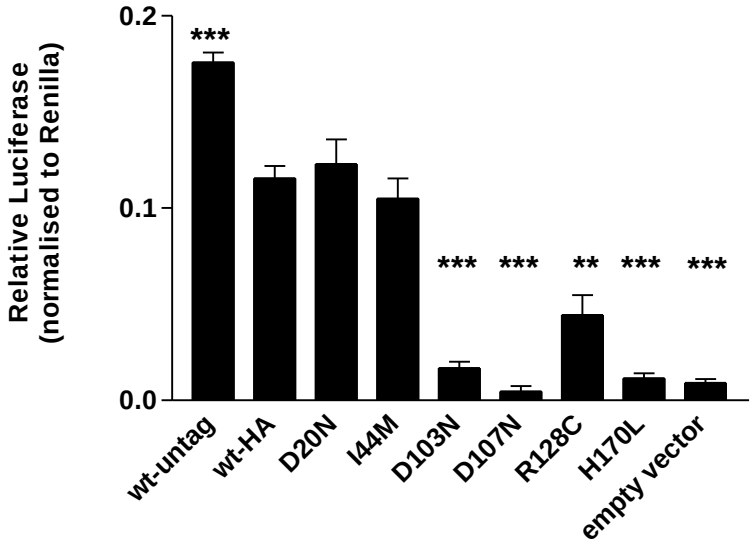

Figure 3.8: Results of luciferase assay for all trafficking-competent mutants. The luciferase assay was employed to assess the effect of MC2R mutations on cell signalling in response to ACTH (10^{-7}M) in the HEKα7 clonal cells. All trafficking-competent mutants were transiently transfected. **,*P*<0.01; *,*P*<0.0001 (compared with Wt-HA-MC2R). Despite good cell surface expression, 4/6 mutants did not respond to stimulation by ACTH after 6 hours treatment.**

3.4.5 Binding studies

Attempts were made to investigate the binding of ACTH to the trafficking-competent but signalling-defective mutants. It was important to dissect if the lack of response to ACTH was due to an ACTH binding defect or whether it was a downstream signalling problem. The major obstacle in this study was the lack of cell adhesion after multiple washes despite modification of the cell culture plates with collagen treatment. Furthermore, the failed experiments had proven to be costly, with the now limited production of [^{125} I] iodo-tyrosyl ^{23} (mono-iodinated ACTH at tyrosine 23). This particular iodination was vital as iodination at tyrosine 23 will not cause

hindrance to ACTH binding to its receptor (Schioth *et al.* 1996). The usual production by Amersham had been discontinued at the time of this study and therefore the iodinated ACTH had to be custom made to order. After six attempts, the experiments were abandoned due to the high cost of the labelled production.

Previous work by Elias (1999) had shown that D103N has impaired ACTH binding and that R128C is most likely involved in failure to signal. These results appear to be consistent with this study.

3.4.6 Co-immunoprecipitation with MRAP

Considering the importance of MRAPα in cell surface expression and receptor function, we investigated whether any mutation in MC2R leads to failure of interaction with MRAPα. Wild type CHO cells were transiently transfected with MRAPα-Flag and either Wt-HA-MC2R or mutant HA-MC2R. Immunoprecipitation of MC2R followed by immunoblotting with anti-Flag antibody provides a qualitative indication of the interaction of Wt-MC2R and MRAPα (Figure 3.9 a). MRAP-Flag has a predicated molecular weight of 24kDa and the major isoform of MRAPα is seen as a define band at approximately at 23kDa. Figure 3.9b demonstrates interaction with MRAPα for L55P, I130N, R137W and G226R. Interestingly, all of the MC2R mutants examined also interacted with MRAP, this is shown in Figure 3.10.

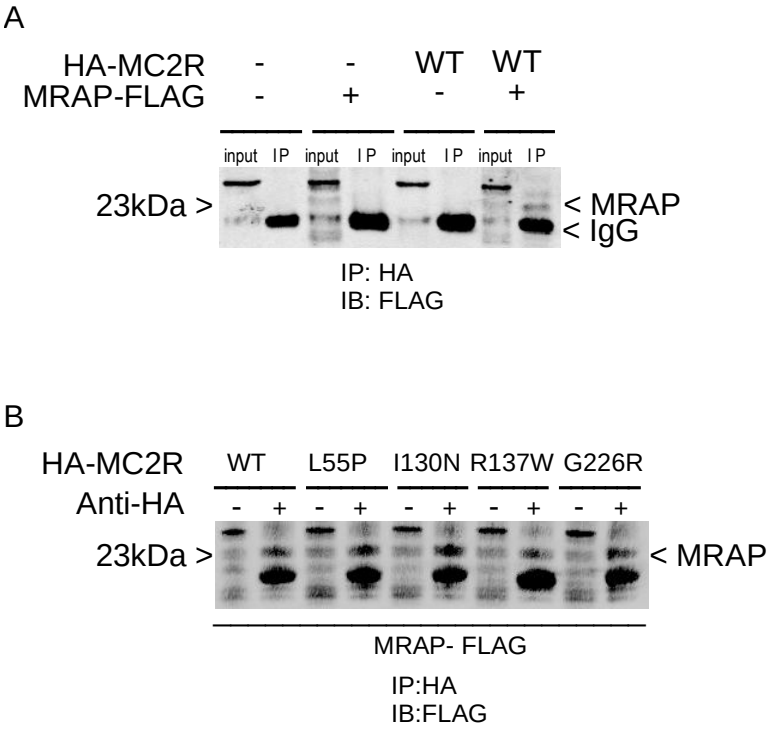

Figure 3.9: Co-immunoprecipitation studies of MC2R mutations with MRAPα. (A): CHO cells transiently transfected with empty vector, MRAPα-Flag, Wt-HA-MC2R or MRAPα-Flag + Wt-HA-MC2R were immunoblotted with anti-Flag antibody. Cell lysates were immunoprecipitated with anti-HA and washed prior to SDS-PAGE and immunoblotting with anti-Flag antibody. The major isoforms of MRAPα were identified as shown by the arrow heads at 23kDa. IP = immunoprecipitation. IgG represents light chain. (B): CHO cells were transfected with Wt-MC2R, L55P, I130N, R137W and G226R mutants and MRAPα-Flag. Cell lysates were immunoprecipitated with anti-HA and washed prior to immunoblotting.

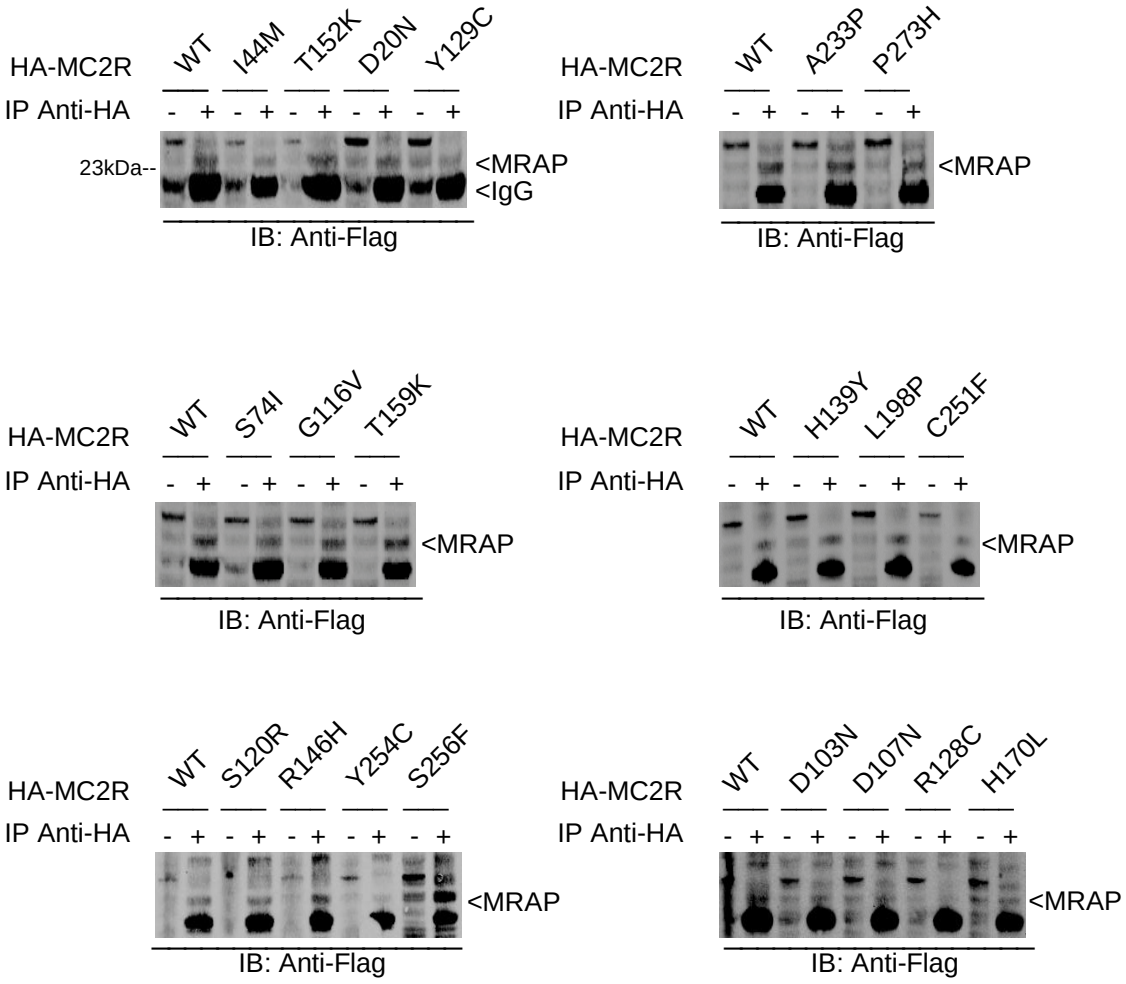

Figure 3.10: Interaction between of the other missense mutations with MRAPα. HA-MC2R mutations and MRAP-Flag co-transfected into wild type CHO cells overnight. Proteins were immunoprecipitated using the anti-HA conjugated beads, and immunoblotted with anti-Flag antibody. The arrowheads depict the MRAP protein (23kDa) and the light chain (IgG at 19kDa).

3.5 Discussion

In this study, a systematic approach was taken to characterise the functional defect of twenty four naturally occurring missense mutations of the MC2R found in FGD type 1. It transpires that the majority of the mutations traffic poorly to the plasma membrane despite direct interaction with MRAP and that most of those that do traffic efficiently fail to signal after stimulation with ACTH. This study provides a novel insight into the molecular mechanisms accounting for the MC2R functional defects, and confirms the problem of MC2R trafficking from the ER as that most likely to cause FGD type 1.

Four out of six trafficking-competent mutations, D103N, D107N, R128C and H170L, failed to signal despite receptor cell surface expression. Previous studies found that both D103N and D107N fail to bind ACTH (Naville *et al.* 1996b; Elias *et al.* 1999).

The only receptor mutation which traffics successfully to the cell surface and is located intracellular region of the receptor is R128C. This is an interesting mutation as it lies in the Asp-Arg-Tyr (DRY) region in the second intracellular loop, which is the most conserved region of all GPCRs (Oliveira *et al.* 1994). Indeed, the arginine residue in this motif is the only amino acid conserved among all subclass 1 receptors (Probst *et al.* 1992). Substitution of the arginine residue within the highly conserved DRY motif will abolish or drastically reduce G protein coupling (Zhu *et al.* 1994; Jones *et al.* 1995) and is proposed to represent the primary trigger for the release of GDP from the receptor-G protein complex (Acharya & Karnik 1996). Interestingly, substitution of the next amino acid in the DRY motif - the Y129C mutant caused significant intracellular retention. Tyrosine is the least conserved of the triad sequence, and in other GPCRs this tyrosine mutation often only marginally affects receptor function, if at all (Rovati *et al.* 2007).

Both D20N and I44M were found to have efficient cell surface expression and cell signalling. I44M was described in a Finnish girl by Weber *et al* (Weber *et al.* 1995), as a compound heterozygous mutation in combination with L192fs. This frame shift results in a nonsense sequence of 54 residues followed by a premature stop codon. It is not clear how the I44M mutation alters receptor function, if at all, as this isoleucine in the first transmembrane domain is not a conserved residue and is substituted by the relatively hydrophobic methionine in the bovine ACTH receptor. No novel splice site was created by either mutation as predicted by analysis using http://www.fruitfly.org/seq_tools/splice.html**.** A further possibility is that both D20N and I44M are in linkage disequilibrium with a more functionally significant mutation elsewhere in the gene and outside the coding region such as the previously reported -2 substitution in the MC2R promoter initiator element (Slawik *et al.* 2004; Tsiotra *et al.* 2006). This is a relatively common polymorphism which is found in 6.5% of a healthy population (Tsiotra *et al.* 2006) and normal subjects homozygous for the rarer C allele displayed higher ACTH/cortisol ratios in response to CRH testing (Slawik *et al.* 2004). This variant has been proposed as a cause of FGD when combined as a compound heterozygote with a frameshift mutation on the other allele (Tsiotra *et al.* 2006).

The majority of the mutant receptors trafficked inefficiently to the plasma membrane. Notably, the most severely affected mutations are located towards the C-terminus of the receptor. Previous functional studies performed for a variety of mutations such as G116V (Collares *et al.* 2008), R137W (Fluck *et al.* 2002), R146H (Weber *et al.* 1995; Elias *et al.* 1999), T159K (Elias *et al.* 1999), C251F (Naville *et al.* 1996b) and Y254C (Fluck *et al.* 2002) all found that there was impaired receptor signalling when stimulated with ACTH and low affinity for ACTH binding. It is now apparent that this was because of impaired cell surface expression of the receptors. We investigated the hypothesis that mutations that affect trafficking do so by interfering with the

interaction between MRAP and MC2R, as the latter plays an important role in facilitating trafficking of the receptor to the cell surface. No mutation was found to block this interaction using co-immunoprecipitation, implying that this may not be the mechanism underlying trafficking failure. However, in a system with over-expression of MC2R and MRAP, it is possible this technique would not be sensitive enough to detect any differences in MRAP interaction at all.

This finding has also been shown for other melanocortin receptors. Similar studies have been performed in MC1R mutants (Sanchez-Laorden *et al.* 2009) with phenotypes which result in poor photoprotective phenomelanins and increased risk of skin cancer. They concluded the naturally occurring mutations in MC1R such as R151C, R160W and D294H demonstrated loss of function and altered cell surface expression. In parallel with mutants in the equivalent position found in MC2R, R151C and R160W were retained within the ER and the proximal Golgi respectively, whilst good cell surface expression was seen in D294H. Similar results are also seen in monogenic obesity. About 130 naturally occurring MC4R gene mutations have been identified, in patients with this condition and intracellular retention of mutated MC4Rs is a common obesity associated defect (Ho & MacKenzie 1999).

Several inherited diseases are now found to result from GPCR trafficking defects. These include, rhodpsin mutations in retinitis pigmentosa (Mendes *et al.* 2005), vasopressin 2 receptor mutations causing nephrogenic diabetes insipudus (Bernier *et al.* 2004) and GnRHR mutations causing hypogonadotrophic hypogonadism (Janovick *et al.* 2002). The strict quality control mechanisms within cells ensures that improperly folded proteins are targeted for degradation via the proteosome or other pathways (Sitia & Braakman 2003). At least two types of quality control systems have been described. A general system involving ER-resident chaperones tests for broad conformational features such as correct disulfide bonding (Ellgaard & Helenius 2003;

Anelli & Sitia 2008). A more specific quality control system, is thought to be able to distinguish the specific motifs interacting with proteins that assist correct folding or escort the correctly folded proteins through the ER and the Golgi (Duvernay *et al.* 2005; Michelsen *et al.* 2005). Only a limited number of these motifs have been identified. The trafficking defective mutants identified in this study which were localised in the ER are likely to be under the scrutiny of the general system where the folding or conformational structure is incorrect.

It has been suggested that some low molecular weight compounds have been shown to inhibit aggregation and/or enable mutant proteins to escape the quality control system and, theoretically, this will result in the "rescue" of their function. These small molecules, named chemical chaperones, are thought to non-selectively stabilise mutant proteins and facilitate their folding (Welch & Brown 1996). Receptor ligands or enzyme inhibitors, which selectively recognise the mutant proteins and rescue conformational mutants, are referred to as pharmacological chaperones, and these present promising therapeutic avenues (Conn *et al.* 2002; Cohen & Kelly 2003). In principle this approach could also be applied for rescuing MC2R mutations with trafficking defects, but this therapy is not likely to be practical, as there already exists a simple and effective treatment in the form of replacement with hydrocortisone.

Chapter 4

Variation of phenotype and genotype in FGD 1 and 2

4.1 Introduction

In FGD type 1, multiple mutations have been identified throughout the MC2R receptor, the majority of which are homozygous or compound heterozygous missense mutations. FGD resulting from MC2R mutations accounts for ~25% of all FGD and is now known as FGD type 1 (OMIM#202200). FGD type 2 (OMIM*609196) describes a group of patients with normal MC2R but with mutations in MRAP which is required for MC2R trafficking and function (Metherell *et al.* 2005) and this accounts for ~20% of FGD and is now known as FGD type 2 (Clark *et al.* 2005).

There are at least twenty-five missense mutations of MC2R identified in FGD type 1. *In vitro* functional studies in Chapter 3 have shown that the majority of MC2R mutants have impaired trafficking from the endoplasmic reticulum (ER) to the cell surface resulting in reduced receptor expression and ACTH signalling. Consequently there is often some protein with residual function. There are also mutations which traffic effectively but fail to signal. It would therefore be of interest to compare the *in vitro* findings with the clinical data of the patients.

Nine mutations of MRAP in FGD type 2 have been reported to date, all of which are splice site or nonsense mutations and are predicted to produce proteins lacking the transmembrane domain essential for interaction with MC2R (Metherell *et al.* 2005; Modan-Moses *et al.* 2006; Rumie *et al.* 2007). MRAP is expressed more widely than MC2R and there is evidence that it may interact with other melanocortin receptors (Chan *et al.* 2009a). For this reason, the phenotype of FGD type 2 may differ from that of FGD type 1, perhaps reflecting a broader spectrum of activity than that of the MC2R. Of particular relevance is the report that a patient with FGD type 2 was significantly obese from childhood, which prompted the speculation that MRAP

may have a role in the activity of the appetite regulator MC4R in the hypothalamus (Rumie *et al.* 2007)

4.2 Aims

The aim of this study was to define the relationship between genotype and phenotype in patients with FGD, by analysing auxological, biochemical and genetic data from all known patients with FGD type 1. Moreover, this project also aimed to compare the phenotypic differences between FGD type 1, caused by MC2R mutations and FGD type 2, caused by MRAP mutations.

4.3 Patients and Methods

4.3.1 Patients

164 DNA samples collected between 1993 to 2008 from patients with a clinical diagnosis of FGD characterised by elevated plasma ACTH, and low or undetectable cortisol in the absence of overt mineralocorticoid deficiency were available for genetic screening. Other causes of adrenal insufficiency such as autoimmune adrenalitis, adrenoleukodystrophy or secondary adrenal failure and others were excluded. From this cohort, 26 patients with FGD type 1 and 19 with FGD type 2 were identified among these. All clinical details and information given by the referring physician were included in the analysis. All mutation analyses of *MC2R* and *MRAP* have previously been performed by others in the lab.

In addition, a PubMed search was performed for all genetically identified FGD type 1 and 2 cases reported until September 2009 and this resulted in a further 19 patients being included in this study. This included seventeen patients with FGD 1 and two patients with FGD 2.

4.3.2 Statistical evaluation

Data were tested for normality on SPSS software Version 16. For normally distributed data, the two groups were compared using independent t-test. Values are stated as the mean ± SD unless otherwise stated. Significance was defined as $P < 0.05$.

4.4 Results

In the analysis, forty-three FGD type 1 patients with eighteen different homozygous and compound heterozygous MC2R mutations and twenty-one FGD type 2 patients with 9 different mutations within MRAP were included (Table 4.1 and 4.2). The majority of the MC2R mutations have been previously reported (Weber *et al.* 1995; Naville *et al.* 1996b; Wu *et al.* 1998; Slavotinek *et al.* 1998; Elias *et al.* 1999; Elias *et al.* 2000; Ishii *et al.* 2000; Fluck *et al.* 2002; Penhoat *et al.* 2002; Collares *et al.* 2008; Artigas *et al.* 2008; Mazur *et al.* 2008; Chan *et al.* 2009a; Chan *et al.* 2009c), and of these the S74I mutation was present in 20 patients. All nine mutations have previously been reported for FGD type 2 (Metherell *et al.* 2005; Modan-Moses *et al.* 2006; Chan *et al.* 2006; Rumie *et al.* 2007).

Auxological data included in the comparisons were the age of presentation, height (in standard deviation score, SDS) of subjects at presentation, biochemical parameters including ACTH and cortisol levels and response to short synacthen testing (where available). Table 4.1 summaries all published and unpublished MC2R and Table 4.2 MRAP mutations and the clinical data.

The phenotypic presentation of FGD type 1 has been summarised in Table 4.1. There appears to be no pattern of clinical correlation with respect to the genotyping. Even within the same mutation such as homozygous S74I, there is a diverse range of age at presentation (Table 4.1).

	Mutations	Age at diagnosis	Basal ACTH (ng/L) [NR: 10-80]	Basal Cortisol (nmol/L) [NR >200]	SST [Norm > 580]	Height (SDS)	Weight (SDS)	Source
I44M	comp hete; I44M and L192fs	1yrs	>1500	undect	NS	4.6	NS	(Weber *et al.* 1995)
S74I	homozygote	6yrs	2700	undect	NS	NS	NS	(Wu *et al.* 1998)
	homozygote	7yrs	648	190	550	1	1.5	(Elias *et al.* 1999)
	homozygote	3yrs	1850	90	NS	0.9	2	(Elias *et al.* 1999)
	homozygote	3.5yrs	NS	undect	NS	2	2	(Elias *et al.* 1999)
	homozygote	4.6yrs	NS	133	NS	2	NS	(Elias *et al.* 1999)
	homozygote	1.33yrs	NS	undect	12	NS	NS	(Elias *et al.* 1999)
	homozygote	3 mths	high	49	194	NS	NS	(Elias *et al.* 1999)
	homozygote	6.5yrs	>1000	30	<20	2.9	NS	(Elias *et al.* 1999)
	homozygote	3 mths	NS	502	461	0.9	NS	(Elias *et al.* 1999)
	homozygote	2yrs	2690	449	613	1	NS	Clark Lab
	homozygote	16yrs	NS	undect	8.9	-1	NS	Clark Lab
	homozygote	14 weeks	2080	undect	<30	0.9	3.1	Clark Lab
	homozygote	18mths	4050	163	F	2	2	(Thistlethwaite *et al.* 1975)
	homozygote	4.6yrs	6888	132	F	2	2	(Thistlethwaite *et al.* 1975)
	homozygote	neonatal	546	106	149	NS	NS	Clark Lab
	homozygote	6.5yrs,	324	undect	F	0.2	7.6	Clark Lab
	homozygote	9mths	900	NS	NS	0.3	NS	Clark Lab
	homozygote	5.66yrs	high	NS	NS	2	2	Clark Lab
	homozygote	10yrs	NS	NS	NS	NS	NS	Clark Lab
	comp hete; S74I and I118fs	3yrs	4310	<30	<30	4	2	(Elias *et al.* 1999)
D103N	homozygote	1.5yrs	277	207	NS	NS	NS	(Penhoat *et al.* 2002)
	homozygote	2mths	1800	<30	F	1.3	1.3	(Kim *et al.* 2009)
	comp hete; T159K and D103N	2 yrs;	4320	undect	29	2	2	(Elias *et al.* 1999; Elias *et al.* 2000)
D107N	homozygote	4 hours old	2520	undect	F	NS	NS	(Naville *et al.* 1996b)
G116V	homozygote	4yrs	940	20	F	2	NS	(Collares *et al.* 2008)

S120R	premature stop R201stop and S120R	1yrs	426	undect	NS	NS	NS	(Tsiotra *et al.* 2006)
A126S	comp hete; G217fs and A126S	2yrs	1047	<27	30.35	3.5	NS	(Artigas *et al.* 2008)
R128C	comp hete; S74I and R128C	8.5yrs	820n	170-190	300/452	4.5	4.5	(Weber *et al.* 1995)
Y129C	comp hete; Y129C and F278C	8mth	>1250	low	low	-1.5	-1.5	(Chan *et al.* 2009a)
R137W	comp hete; D103N and R137W	3mths	396	70	65	0.9	0.9	(Ishii *et al.* 2000)
	homozygote	2.25yrs	1134	<27	F	2	3.7	(Fluck *et al.* 2002)
R146H	homozygote	4.7yrs	790	37	50	4	NS	(Weber *et al.* 1995)
	homozygote	2.25yrs	3240	undect	F	1.2	NS	(Weber *et al.* 1995)
	homozygote	5.75yrs	1409	undect	F	2	2	(Slavotinek *et al.* 1998)
T152K	homozygote	4yrs	>1250	undect	F	2.4	2.4	Clark Lab
T159K	homozygote	8 days;	1000	undect	83	2.2	NS	(Elias *et al.* 1999; Elias *et al.* 2000)
	homozygote	3mths	5160	undect	F	-1.5	NS	(Elias *et al.* 1999; Elias *et al.* 2000)
H170L	comp hete; S74I and H170L	neonate	extremely high	extremely low	NS	NS	NS	Clark Lab
A233P	homozygote	10 day old	4652	<30	NS	NS	NS	(Penhoat *et al.* 2002)
C251F	comp hete; C251F and G217fs	2yrs	250	undect	<28	NS	NS	(Naville *et al.* 1996a)
Y254C	comp hete; Y254C and S74I	1.58yrs	1485	37 & 55	NS	2	1.5	(Fluck *et al.* 2002)
P273H	comp hete; S74I and P273H	12 mth	545	undect	NS	NS	NS	(Wu *et al.* 1998)

Table 4.1: Summary table of all published and unpublished mutations of MC2R. The clinical data including age of onset; cortisol and ACTH at presentation; cortisol response to synacthen testing (SST); weight and height at presentation. NS = not specified; undect = undetectable level; F= failed.

Mutations	Patient	Age at diagnosis (yrs)	Basal ACTH (ng/L) [NR:10-80]	Basal Cortisol (nmol/L) [NR > 200]	SST [Norm >580]	Height (SSD)	Weight (SDS)	BMI	Source
IVS3ds+1delG	1	0.25	108.5	<50	F	1.8	-1.5	12.5	(Metherell *et al.* 2005)
	2	0.25	increased	low	NS	NS	NS	NS	(Metherell *et al.* 2005)
IVS3ds+3insT	3	neonate	>1500	47	NS	1.24	0.9	14.2	(Metherell *et al.* 2005)
	4	NS	NS	NS	NS	NS	NS	NS	(Metherell *et al.* 2005)
	5	birth	1400	undetect	F	-1.3	0.7	18.5	(Metherell *et al.* 2005)
	6	birth	3705	31	NS	-2.3	-0.3	19.7	(Metherell *et al.* 2005)
	7	birth	1703	24	NS	NS	NS	NS	(Metherell *et al.* 2005)
IVS3ds+1G→C	8	neonate	increased	<50	NS	NS	NS	NS	(Metherell *et al.* 2005)
	9	neonate	697	<50	F	-0.5	1	24.7	(Metherell *et al.* 2005)
M1I	10	1.16	4500	200	F	0.9	3.7	36.9	(Metherell *et al.* 2005)
	11	neonate	>1500	<50	UD	0.1	2.6	30.7	(Metherell *et al.* 2005)
	12	NS	273	276	NS	0.3	0.6	16.2	(Metherell *et al.* 2005)
	13	NS	248	469	NS	0.4	0.2	17.8	(Metherell *et al.* 2005)
	14	1.08	NS	NS	F	-1.6	-0.9	23.4	(Metherell *et al.* 2005)
	15	0.11	288	<50	NS	-0.1	NS	NS	(Metherell *et al.* 2005)
compound het M1I, ds+1G→ A	16	neonate	extremely elevated	<50	NS	NS	NS	NS	(Metherell *et al.* 2005)
compound het M1I, IVSds+3 insT	17	1.08	1250	undetect	F	1.4	2.2	19.7	(Metherell *et al.* 2005)
Y11X	18	0.125	1200	33	NS	-1.6	-2.1	15.8	Clark Lab
	19	birth	3275	NS	NS	0.9	0.6	16.5	Clark Lab
V44X	20	0.125	>730	<50	F	1.2	2.5	28	(Rumie *et al.* 2007)
Del 17-23 stop codon L31X	21	1.58	1250	<50	NS	NS	NS	NS	(Modan-Moses *et al.* 2006)

Table 4.2: Clinical summary table of all published and unpublished MRAP mutations. F=failed; NS = not specified.

4.4.1 Age at presentation

FGD type 1 presents with a variable age of onset, mean (± SD) 3.11 ± 3.40 yrs (range 0-16yrs), whilst FGD type 2 presents at an earlier age with mean onset at 0.31 ± 0.51 yrs (range 0-1.6yrs), $p < 0.01$ (Figure 4.1A). Interestingly, the most common MC2R mutation, homozygous for S74I (n=20), appears to display a wide spectrum in age of presentation, with the mean age of onset 3.82 ± 3.81yrs (range 0-16) which does not differ from that of other patients with FGD type 1 (Figure 4.1A).

4.4.2 Height and weight at presentation

The height of patients with FGD type 1 has previously been noted to be unusually tall (Clark & Weber 1998; Elias *et al.* 2000; Imamine *et al.* 2005). In FGD type 1 height SDS at presentation was + 1.76 ± 1.52 (mean ± SD) and in FGD type 2 height SDS at presentation was + 0.12 ± 1.35, $p < 0.001$ (Figure 4.1B). Not all weight measurements were available for FGD 1. Often weight was only available in SDS rather than in metric units. Where data was present, the mean weight SDS for FGD type 1 (n=19) at presentation was + 1.7 ± 1.46 and in FGD type 2 (n=14) the mean weight SDS at presentation was + 0.718 ± 1.62, (NS). (Figure 4.1C). There is no concordance between height variations in relation to the age of presentation in FGD 1, the relationship did not reach statistical significance (Figure 4.1D).

4.4.3 ACTH / cortisol at presentation

The basal cortisol at presentation is shown in Figure 4.1E, with FGD type 1 mean cortisol 78.9 ± 115.9 nmol/L and FGD type 2 mean cortisol 79.0 ± 106.5 nmol/L, (NS). Where tested, the majority of the FGD type 1 and 2 patients failed short synacthen testing. Similarly, the mean plasma ACTH (± SD) at presentation for FGD type 1 was 1920 ± 1685.78 ng/L and 1454 ± 1195.07 ng/L for FGD type 2, (NS) (Figure 4.1F).

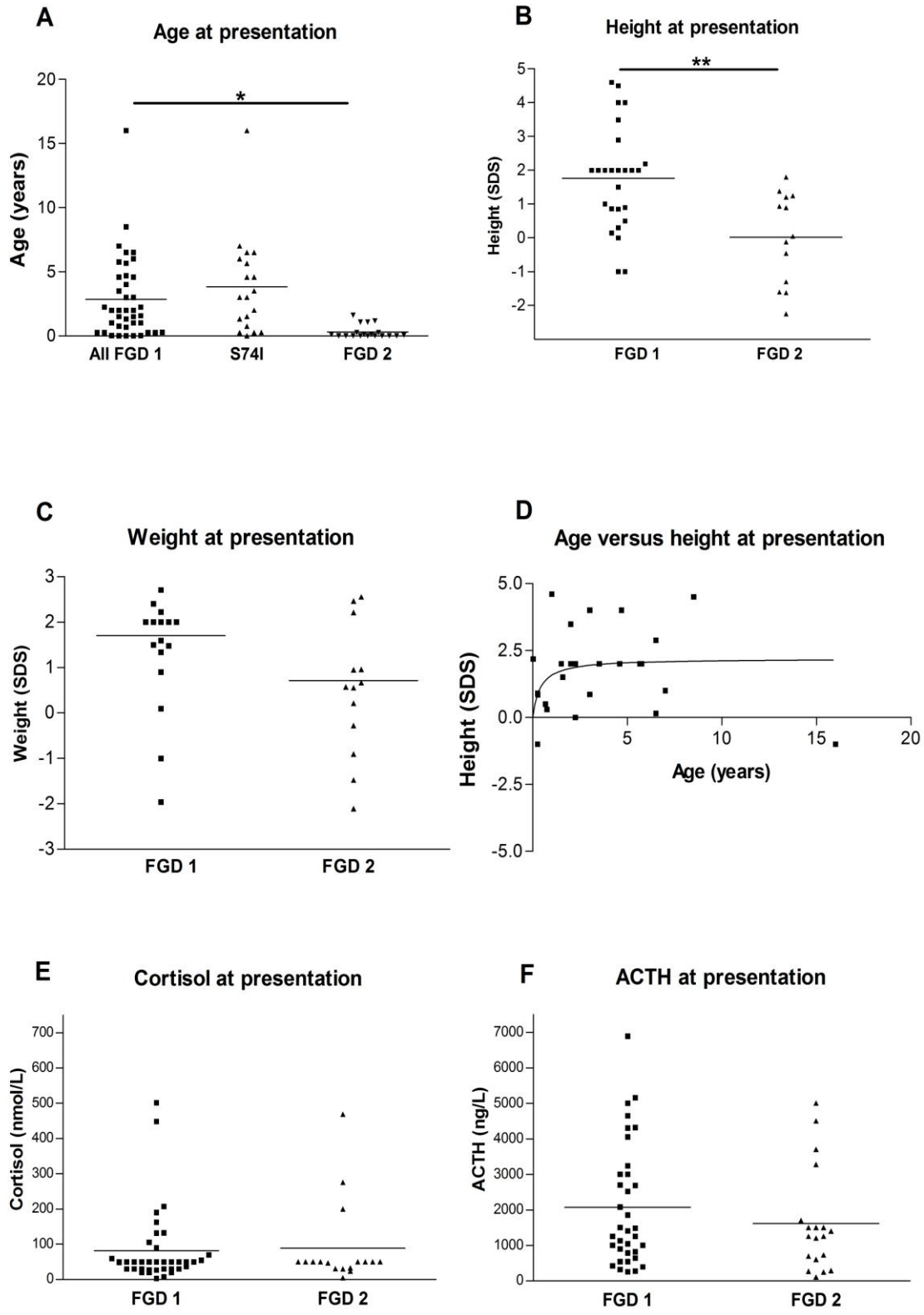
A
Age at presentation
Age (years)
All FGD 1
S74I
FGD 2
B
Height at presentation
Height (SDS)
FGD 1
FGD 2
C
Weight at presentation
Weight (SDS)
FGD 1
FGD 2
D
Age versus height at presentation
Height (SDS)
Age (years)
E
Cortisol at presentation
Cortisol (nmol/L)
FGD 1
FGD 2
F
ACTH at presentation
ACTH (ng/L)
FGD 1
FGD 2

Figure 4.1: Plotted graphs of all auxological and biochemical data for FGD patients at presentation. (A) Age (years) of all patients with FGD type 1, those patients with the S74I mutation, and FGD type 2 are shown. The horizontal line represents the mean. (B) Height (in SDS) of all patients with FGD type 1 and FGD type 2 are shown. The horizontal line represents the mean. ** = p< 0.01; *** = p<0.001. Weight of FGD patients at presentation is seen in C. The weight (in SDS) of all patients with FGD type 1 and 2 are shown. The horizontal line presents the mean. There was no statistical significance detected. (D): Relationship between height SDS at diagnosis and age of presentation in FGD type 1 patients. Plasma ACTH and cortisol at presentation. (E) cortisol (nmol/L) and (F) ACTH (ng/L) and for all patients with FGD type 1 and 2 are shown. The horizontal line represents the mean. No statistical significance was detected in the two groups.

4.5 Discussion

The diagnosis of FGD is based on clinical findings and patients usually present with hypoglycaemia, seizure, jaundice, hyper-pigmentation, failure to thrive and frequent or severe infections. The biochemical findings are a markedly elevated plasma ACTH in the presence of low cortisol but with a preserved mineralocorticoid production and are characteristic of ACTH insensitivity. There are three genetically identified causes in FGD, mutations in the *MC2R, MRAP* and *STAR* known as FGD 1, 2 and 3 respectively. This is the first phenotype-genotype comparison between FGD types 1 and 2.

4.5.1 Age at presentation

The auxological data in FGD type 1 demonstrated a wide variation in disease presentation associated with different mutations for MC2R. Even for the same mutation, such as S74I, which is the most common FGD mutation, the age of presentation ranged from neonate to 16 years old (Table 4.1). This variability was also observed for basal cortisol at presentation (from undetectable cortisol to 502nmol/L), responsiveness to short synacthen testing and height (SDS) at presentation. The *in vitro* data from Chapter 3 (Figure 3.5) suggested that mutations near the C terminus appeared to have a greater effect on trafficking, however this was inconsistent with the clinical data. For example, T159K is functionally less defective than C251F in the cell surface assay (Figure 3.5), nevertheless the age of presentation appear to contradict the *in vitro* data. Therefore, no correlation between genotype and phenotype features in FGD type 1 was found. The results in Chapter 3 has shown in *in vitro* studies that missense mutations in the MC2R have varying degrees of impaired trafficking from the ER to the cell surface resulting in

reduced receptor expression (20-100% when compared with wild-type) and ACTH signalling. Consequently there is often some protein with residual function. Contrasting this to an animal model in the MC2R knockout (KO) mice, approximately three-quarters of MC2R KO pups died within the first 48 hour after birth (Chida *et al.* 2007). Only five out of 70 MC2R KO survived until 12 weeks, this demonstrates that the phenotypic presentation of MC2R KO mice do not represent human equivalent of FGD 1 missense mutations. The homozygous nonsense and frameshift MC2R mutations in FGD may represent a more appropriate comparsion with the MC2R KO model, although such patients are extremely rare. All six children previously reported (Chan *et al.* 2009b) presented during neonatal period or in infancy with severe illness. Some of these patients had some degree of disturbance of the renin-angiotensin-aldosterone axis, but no patient was frankly mineralocorticoid deficient to require fludrocortisone replacement.

FGD 2 had a much earlier age of presentation when compared with FGD 1 and therefore a more severe phenotype. This is in agreement with the view that MC2R mutations have residual protein function and hence later age of presentation.

4.5.2 Weight of FGD patients

There was no statistical difference between weight SDS in FGD 1 and 2 at presentation. If there is a difference in weight then perhaps a better comparison should be body mass index, since FGD 1 patients are comparatively taller. However, due to lack of clinical data including the missing paired weight and height this calculation was not possible. In the plotted data for weight (Figure 4.1C), it could be appreciated in FGD 2 that majority of the weight SDS at presentation were below 1 SDS. FGD 2 patients appear to

show a more severe phenotype with earlier age of presentation and perhaps the more reduced body weight could be the result of failure to thrive in a more severe phenotype.

In vitro study has shown that MRAP and MRAP2 can interact with MC4R (Chan *et al.* 2009c), and this interaction reduced the cell surface expression of MC4R and also caused reduction in signalling. MC4R negatively regulates appetite and food intake in response to agonist, such that there is an increase in energy expenditure and a decreased food intake (Yeo *et al.* 1998; Farooqi *et al.* 2003). MC4R knockout mice displayed hyperphagia and increased adiposity (Huszar *et al.* 1997). Theoretically, interaction of MC4R with MRAP in the hypothalamus would impair the ability of MC4R to regulate appetite and hence it is possible that the loss of function mutations in MRAP seen in FGD type 2, would relieve this negative regulation of MC4R activity resulting in the leaner FGD 2 phenotype.

Rumie *et al* (2007) reported that a patient with an MRAP mutation was markedly obese, and hypothesised that MRAP may play a positive role in MC4R function, given its expression in the brain. The data presented here show that MRAP patients are more commonly underweight and data from Chan *et al* (2009) show that MRAP has a negative effect on MC4R function, indication that the obesity seen in this patient is unlikely to result from the MRAP mutation. These observations assume that MRAP is present in the hypothalamus, but its expression is yet to be determined.

4.5.3 Height of FGD patients

When the clinical features of FGD types 1 and 2 were compared, striking distinctions were discovered in the age of presentation and height, but not in other aspects

of endocrinology or body weight. Due to the incompleteness of the data, such as ethnicity SDS and mid parental height could not be considered. The correlation between the estimated severity of the receptor defect *in vitro* and the degree of clinical severity remains poor. Plotting height versus age was not suggestive of a relationship between length of exposure to high ACTH / low glucocorticoid and tall stature (Figure 4.1D). These data were transformed in various ways including log transformation for age in years and months or charting data divided into quartile age groups (not shown). However, there was no statistically significant relationship between age of presentation and height in FGD type 1.

Unusually tall stature has been described in many FGD type 1 cases in the literature (Shepard *et al.* 1959; Migeon *et al.* 1968; Thistlethwaite *et al.* 1975; Soltesz *et al.* 1985), the cause of which is not known. *In vitro*, ACTH increases the development of a chondrogenic phenotype with an increase in proliferation and differentiation of chondrocyte precursors (Evans *et al.* 2004). Consequently it is a reasonable hypothesis that ACTH at high concentrations could activate melanocortin receptors expressed in bone and the cartilaginous growth plate and stimulate growth (Yeh *et al.* 2006). Alternatively, glucocorticoid inhibits the synthesis of IGF binding protein 5 (IGFBP-5) in the osteoblast (Gabbitas *et al.* 1996). Bone growth is stimulated by IGFBP-5, and thus conceivably cortisol deficiency results in a lack of negative inhibition and the consequent growth spurt seen in FGD type 1. No abnormality in the GH-IGF-I axis has been reported in FGD patients to date. It is interesting to note that MC2R KO mice do not exhibit any significant difference in body length when compared with wild-type (Chida *et al.* 2007).

Clinical observations suggest that replacement of glucocorticoid normalises the advanced growth rate in FGD (Elias *et al.* 2000; Imamine *et al.* 2005). This can be seen when the growth rate is documented in growth charts (Figure 4.2). Two patients presented

with heights at the 97th centile at the time of diagnosis, and the growth rate declined after replacement with hydrocortisone. In the reported cases, despite adequate hydrocortisone replacement ACTH often remains elevated both in FGD 1 and 2, for reason which remain unclear (Elias *et al.* 2000). It is conceivable that the treating clinicians may attempt to normalise the ACTH. Therefore, it is also very difficult to exclude the possibility that overtreatment with glucocorticoids may be responsible for this deceleration in growth. The discordance of the plasma ACTH and bone growth suggest exposure to cortisol could play a role in this. However, if height is partly ACTH driven then the question remains as to why this phenomenon is not observed in FGD type 2. A probable explanation may be that FGD 2 patients present at and are treated at an earlier age.

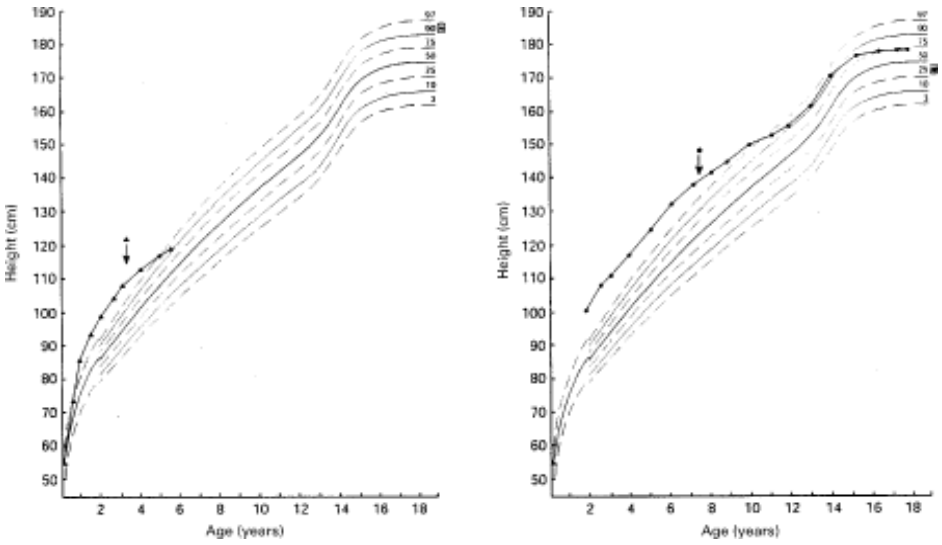

Figure 4.2: Growth chart of two patients with FGD type 1. Both patients were diagnosed with S74I mutations. The vertical arrow indicates the commencement of hydrocortisone treatment (12mg/m^2 and 15mg/m^2). The growth rate slows down after treatment. Adapted from *Clinical Endocrinology* (Elias *et al.* 2000).

4.5.4 Limitation

A limitation of this study is that the available clinical data is far from complete. The majority of the patients were only referred to us for genotyping by the treating clinicians, both locally and internationally. Even though the biochemical data were available for most patients, they were in different units and sometimes different normal ranges (especially for ACTH). This adds to the challenges of comparing different data. Furthermore, the different ethnic origin of the patients was not accounted for in the analysis, for example, comparing the height of an Irish Caucasian with a Turkish patient. Growth chart of each ethnic origin should be used for more accurate comparison.

Although the results suggest no difference in weight between FGD 1 and 2, the possibility cannot be excluded that there may be weight over-estimation for some taller patients in FGD type 1, since taller patients are heavier. As mentioned before, assessment of SDS for body mass index may provide further information for the true weight comparison.

4.6 Conclusion

In conclusion, the MC2R mutations are associated with a wide range of FGD phenotypes. Even though rare, it should still be considered in young patients who suffer from recurrent infections, seizure and hypoglycaemia when other causes have been excluded. Patients with FGD type 1 present later and have tall stature when compared to FGD type 2. This is consistent with the suggestion that prolonged ACTH excess or glucocorticoid deficiency increases linear growth. FGD type 2, in contrast, presents earlier and appropriate treatment may be sufficient to prevent the enhanced growth rate seen in FGD type 1. Currently, there was no evidence that MRAP deficiency had any influence on any other physiological functions beyond that seen with a defective MC2R. More clinical data is needed in order to compare the body weight differences between FGD type 1 and 2.

Chapter 5

Melanocortin 2 receptor dimerisation

5.1 Introduction

The idea that GPCRs might dimerise was first proposed in 1982 (Agnati *et al.* 1982), this idea did not gain much interest until a decade later. This idea came from the unexplained observation that larger than expected estimates of the size of receptor complexes were seen on gel filtration columns (Angers *et al.* 2002). Subsequently, many studies support the view that GPCRs can exist as dimers or higher-ordered oligomers (Terrillon & Bouvier 2004; Milligan & Bouvier 2005), however their functional importance remains controversial. There is a large body of evidence, with functional data to support homodimerisation and heterodimerisation of GPCRs such as chemokine receptors (Mellado *et al.* 2001), angiotensin receptors (Szidonya *et al.* 2007) and others. It would therefore be of interest to investigate if MC2R can homo or heterodimerise and if there is any functional consequence.

The first evidence of melanocortin receptor dimerisation was reported for MC4R. It was proposed that the dimerisation of MC4R may play a role in severe early-onset monogenic obesity (Biebermann *et al.* 2003). Inactivating mutations of the MC4R were found to be inherited in an autosomal-dominant manner. It was demonstrated that a heterozygous mutation D90N-MC4R mutant results in a nearly complete loss of function. Both the mutant and wild type MC4R (Wt-MC4R) demonstrated high expression at the cell surface but there was a poor signalling response in D90N. The co-expression of Wt-MC4R and D90N together gave rise to a defective signaling response. Using both ELISA and FRET, it was confirmed that MC4R forms homodimers and that Wt-MC4R and D90N heterodimerised, indicating that this protein-protein interaction resulted in a dominant-negative effect (Biebermann *et al.* 2003).

Similar findings were evident in MC1R mutants which exerted dominant-negative effects on the wild-type MC1R (Sanchez-Laorden *et al.* 2006). This group found that MC1R undergoes constitutive dimerisation in heterologus cells overexpressing the receptor and that MC1R dimerisation occurs in the ER. The mutant MC1R exerts a dominant-negative effect by trapping wild type MC1R intracellularly and results in loss in function (Sanchez-Laorden *et al.* 2006).

In contrast to MC1R and MC4R, there is no clinical data to suggest dominant negative effect of MC2R. The reason remains unclear. One possibility is that MC2Rs do not dimerise, or that they dimerise without functional consequences, hence the need for this study. In the phenotype-genotype characterisation for FGD type 1 (in Chapter 4), compound heterozygous patients were examined, whose individual mutations were both trafficking-defective. So far there has not been a report of a compound heterozygote with the combination of a ligand-binding defective (eg D103N) MC2R and a signalling defective (for example, R128C) MC2R, which are both trafficking-competent. It could be speculated that if there is cross talk by the receptors, such that a dimer between a ligand binding mutant and a signaling mutant created a functional receptor, this heterozygote may be a silent phenotype and hence un-diagnosed. If there is “transactivation” of the receptor, then when these two mutations are co-expressed, their function could theoretically be rescued. However, it may be such that a combination is merely a rare phenomenon. In principle the functional consequence of such a compound heterozygous mutation would be of interest.

There is evidence from RT-PCR that MC3R and MC4R are expressed in rat adrenal glands (Dhillo *et al.* 2003). It has been reported that α-MSH stimulates corticosterone release in rat adrenal cells (Oliver *et al.* 1994; Dhillo *et al.* 2005) and γ-MSH may be involve in the regulation of adrenal steroidogenesis (Harmer & Bicknell 2005). The

reason for this observation remains unclear, maybe MC3R and 4R expression result in heterodimerisation with MC2R in the adrenal and subsequently contribute in steroidogenesis.

5.2 Aims

The aim of this series of experiments was to: a) investigate if MC2R could homodimerise in the presence of MRAP; b) determine whether dimerisation is promoted or inhibited by MRAP or ACTH stimulation; c) investigate if there is any novel functional consequence of dimerisation which may rescue MC2R mutations; d) investigate if MC2R could heterodimerise with other melanocortin receptors (MCRs).

5.3 Materials and Methods

In order to conduct receptor co-immunoprecipitation, a different epitope tagged hMC2R is required. From chapter 3, 3XHA-hMC2R had been used and will also be used for the investigation of dimerisation. The 3XFlag-hMC2R was cloned to create a second epitope tagged hMC2R.

5.3.1 Cloning of 3XFlag-hMC2R

Materials:

Vector: p3XFLAG-CMV-10 expression vector (Sigma)

Untagged hMC2R (pcDNA3.1) (Missouri S&T cDNA Resource Center)

5.3.1.1 Identification of common cloning site for restriction digestion

For the insertion of untagged hMC2R into 3XFLAG-CMV vector, their common cloning sites were identified using vector maps. Vector maps from hMC2R vector identified *EcoR1* and *Xba1* to be common to the p3XFLAG expression vector (as seen in Figure 5.1 and 5.2).

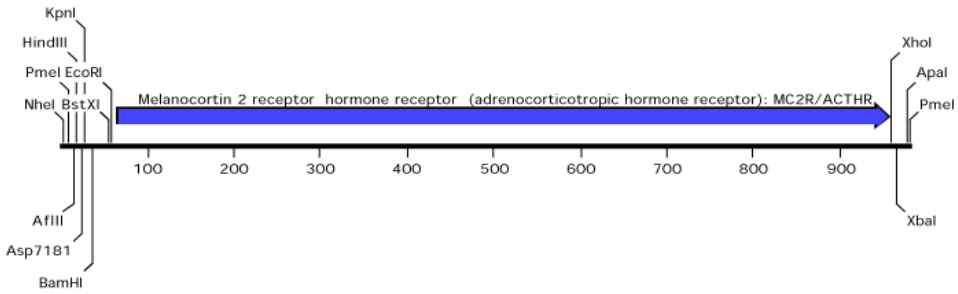

Figure 5.1: Vector map of human MC2R from cDNA.org. This illustrates the multiple cloning sites.

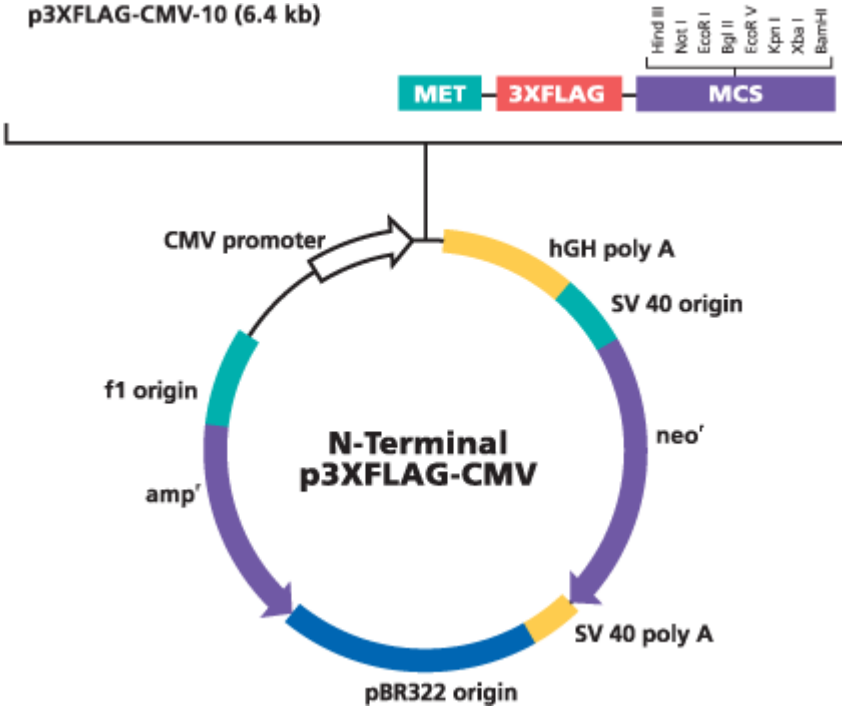

Figure 5.2: vector map of p3XFlag vector. MCS: multiple cloning sites are illustrated.

5.3.1.2 Restriction digestion

This has been described in section 2.4.3 under materials and methods.

EcoR1 and *Xba1* (New England Biolabs, NEB or Promega, UK) were used for double digests. The double enzyme digestion mixtures were prepared and incubated at 37ºC for 2 hours.

The digested DNAs were purified via agrose gel electrophoresis. This is followed by ligation of the insert and vector performed at 1:2, 1:3 and 1:4 ratios. The ligated mixtures were then transformed and colonies selected and sent for sequencing.

5.3.1.3 Site directed mutagenesis

The cloned 3XFLAG-hMC2R was confirmed to be out of frame after sequencing. Therefore site directed mutagenesis had to be performed for the insertion of an extra nucleotide.

The predicted out of frame sequence:

↓ Start of 3XFlag

ATG GAC TAC AAA GAC CAT GAC GGT GAT TAT AAA GAT CAT GAC ATC GAT TAC AAG GAT GAC GAT GAC AAG CTT GCG GCC GCG AAT TC ACC ATG AAG CAC ATT ATC

↑ Start of MC2R

With the insertion of C (grey) this put the sequence back into frame.

In frame sequence:

↓Start of 3XFlag

ATG GAC TAC AAA GAC CAT GAC GGT GAT TAT AAA GAT CAT GAC ATC GAT TAC AAG GAT GAC GAT GAC AAG CTT GCG GCC GCG AAT TCC ACC ATG AAG CAC ATT ATC

↑Start of MC2R

The forward primer used: TGCGGCCGCGAATTCCACCATGAAGCACA

The reverse primer used: TGTGCTTCATGGTGGAATTCGCGGCCGCA

PCR was performed with an annealing temperature of 65ºC for 12 cycles.

The new sequence was confirmed to be in frame via sequencing.

5.3.2 Co-immunoprecipitation (Co-IP)

To demonstrate MC2R dimerisation, HEK293 cells were transfected with 3XFlag-hMC2R and 3XHA-hMC2R. Immunoprecipitation of GPCRs was performed with anti-HA agarose (Sigma) and IB with anti-Flag M2 antibody (Sigma). Reciprocal Co-IP was performed with anti-Flag agarose (Sigma) and anti-HA antibody (Sigma).

To demonstrate heterodimerisation of the MC2R, Co-IP was performed on lysates from HEK293 cells co-transfected with all other HA-hMCRs (Missouri S&T cDNA Resource Centre) and 3XFlag-hMC2R.

The protocol was followed as described in section 2.5.7 (Materials and Methods), the only difference was that after the addition of β-mercaptoethanol, the samples were not denatured at 99ºC. The glycosylated MC2R can produce artificial aggregates during the boiling step, which makes the samples extremely difficult to load and give erroneous bands on SDS-PAGE. Instead, the samples were left to incubate at room temperature for 1 hour prior to loading. This step was extremely important for loading GPCRs.

5.4 Results

5.4.1 MC2R homodimerisation

HEK293 cells were transisently transfected with MRAP, 3XFlag-hMC2R and 3XHA-hMC2R. Co-IP showed that MC2R forms homodimers (Figure 5.3). The presence of MRAP does not seem to affect homodimerisation. Mock transfection and transfection of 3XFlag-hMC2R and 3XHA-hMC2R alone acted as controls.

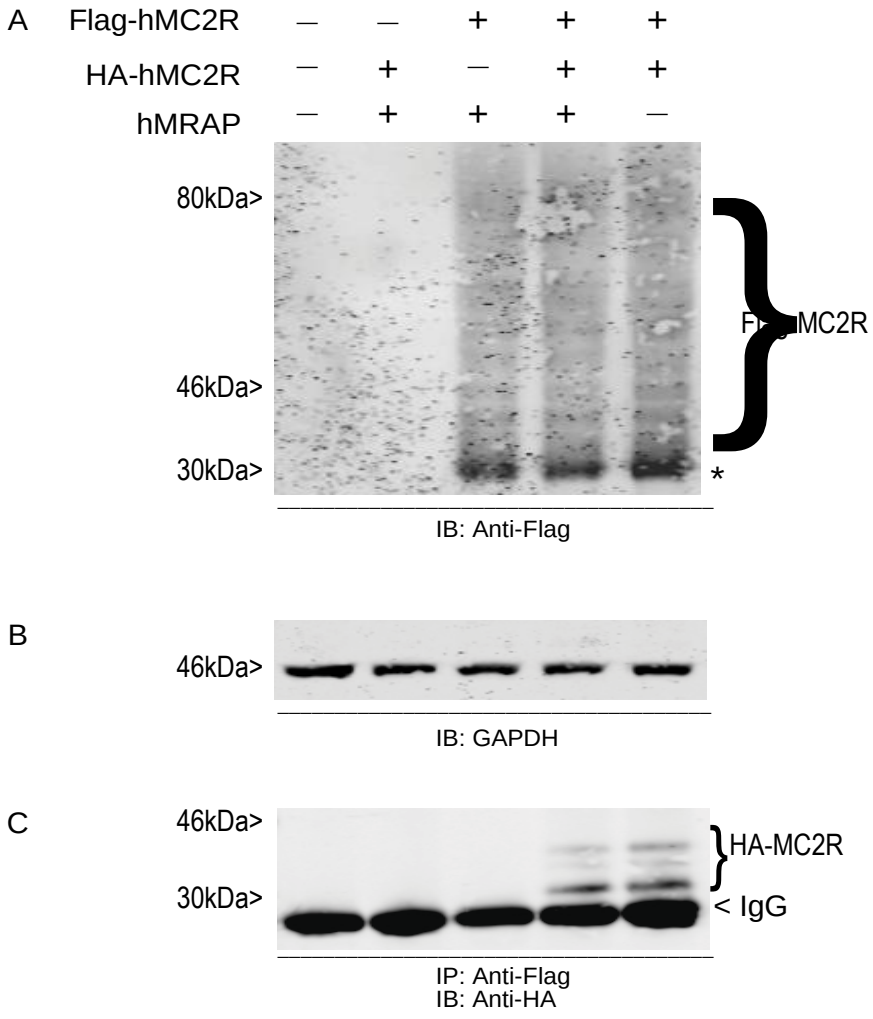

Figure 5.3: MC2R undergoes homodimerisation. Identical sample loading in individual lane for each of the blots and their common labels are indicated above. A: Immunoblot with anti-

Flag antibody in transfected HEK295 cells reveals equal protein expression of the Flag-MC2R. A protein at ~30kDa in size, indicated by the asterisk is suggestive of Flag-MC2R expression. B: Immunblot of same gel using anti-GAPDH antibody. C: Co-immunoprecipitation with anti-Flag agarose beads and immunoblotted with anti-HA, showing MC2R homodimerisation with or without MRAP. IgG light chain is indicated by the arrow.

The reciprocal experiment (ie immunoprecipitate with anti-HA and immunoblot with anti-Flag) was performed without MRAP (Figure 5.4). This confirmed that MC2R constitutively homodimerises. There were two bands, one at 30kDa and the other at 27kDa present in the Co-IPs (Figure 5.4B). To demonstrate that the lighter band is the light chain IgG, a repeat of the experiment with and without β-mercaptoethanol was performed. β-mercaptoethanol reduces disulphide-bonds and causes the dissociation of the antibody into light and heavy chains. In the absence of β-mercaptoethanol, the undissociated antibody runs at approximately 150kDa and is not seen in the image (Figure 5.4E).

hMRAP2-Flag (Chan *et al.* 2009c) was used in this experiment as a positive control for the Flag antibody. Human MRAP2 has a predicted molecular mass of 23.5kDa, MRAP2-Flag is seen in Figure 5.4A. Human MC2R is approximately 33.9kDa and when in association with 3XFlag this is predicted to be 36kDa. The band seen at 30kDa in Figure 5.4A and B indicates Flag-MC2R. The markers used are likely to be inaccurate, which would explain the difference in size.

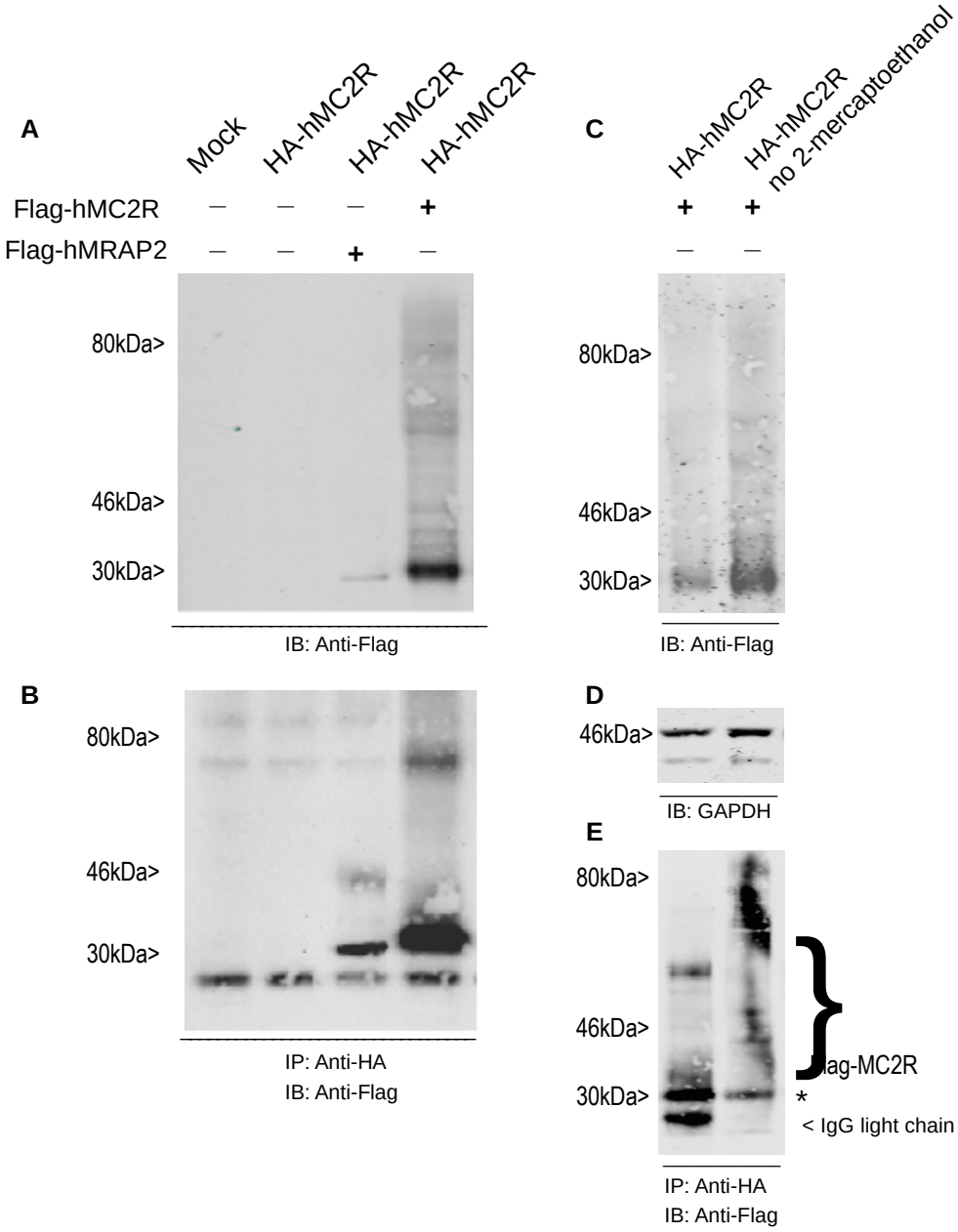

Figure 5.4: Immunoblot and co-immunoprecipitation assay showing MC2R homodimerisation. A: Immunoblot with anti-Flag in transfected HEK293 cells. Expression of Flag-MC2R or MRAP2-Flag is seen. B: Co-IP with anti-HA agarose beads and immunoblotted with anti-Flag showing dimerisation of MRAP2 with MC2R and MC2R homodimerisation. C: Immunoblot of the repeat experiment transfecting Flag-MC2R and HA-MC2R with or without β-mercaptoethanol. D: Blotting using anti-GAPDH antibody. E: Co-IP demonstrating the absence of heavy and light chain when β-mercaptoethanol is not used.

5.4.2 Homodimerisation in the presence of ACTH

The above data indicate that MC2R can homodimerise. An unanswered question is whether receptor activation by ACTH can promote or inhibit dimerisation. To investigate this, HEK cells were transiently transfected with epitope-tagged MC2Rs and untagged MRAP for 24 hours. Cells were then treated with ACTH 10^{-6}M for 20mins. Figure 5.5 demonstrates that homodimerisation of MC2R in the presence of ACTH remains unaltered.

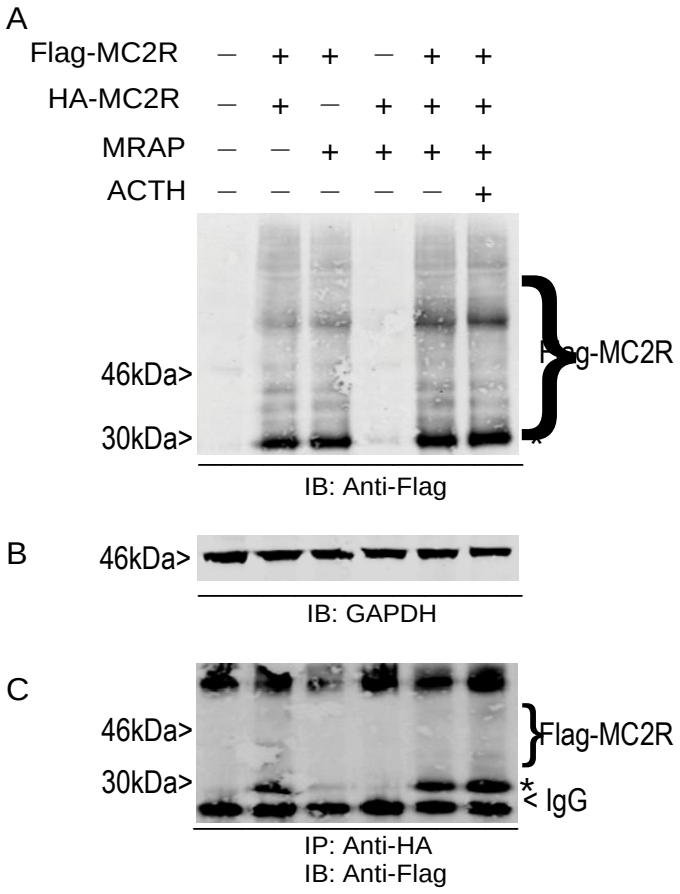

Figure 5.5: The effect of ACTH on MC2R dimerisation. Flag-MC2R, HA-MC2R and MRAP were co-expressed in HEK293 cells with and without 20 minutes of ACTH treatment. A: Immunoblot with anti-Flag demonstrating expression of Flag-MC2R (indicated by asterisk).

B: blotted with anti-GAPDH antibody showing equal loading. C: Co-IP with anti-HA agarose beads and immunoblotted with anti-flag.

5.4.3 Homodimerisation and MC2R signalling in trafficking-competent mutants

Although GPCRs have been demonstrated to signal as monomeric units (Ernst *et al.* 2007), there is evidence that dimerisation of GPCRs could be of functional importance, with respect to ligand binding and receptor activation (Hansen & Sheikh 2004; Szidonya *et al.* 2008). Receptor cross-talk has been demonstrated in AT1R receptors, supporting the concept of dimerisation (Szidonya *et al.* 2007), and therefore this phenomenon was tested in MC2R mutations. Two trafficking-competent MC2R mutations (already characterised in Chapter 3) and MRAP were cotransfected in HEK293 cells and then stimulated with ACTH. A cAMP responsive luciferase construct was used to detect cell signalling. D103N which is defective in ligand binding and R128C which is known to be defective in signalling were co-expressed. If receptor cross-talk takes place with the MC2R, then in theory dimerisation may allow ACTH to bind to R128C and signal through D103N and hence lead to the functional rescue of this mutant receptor combination.

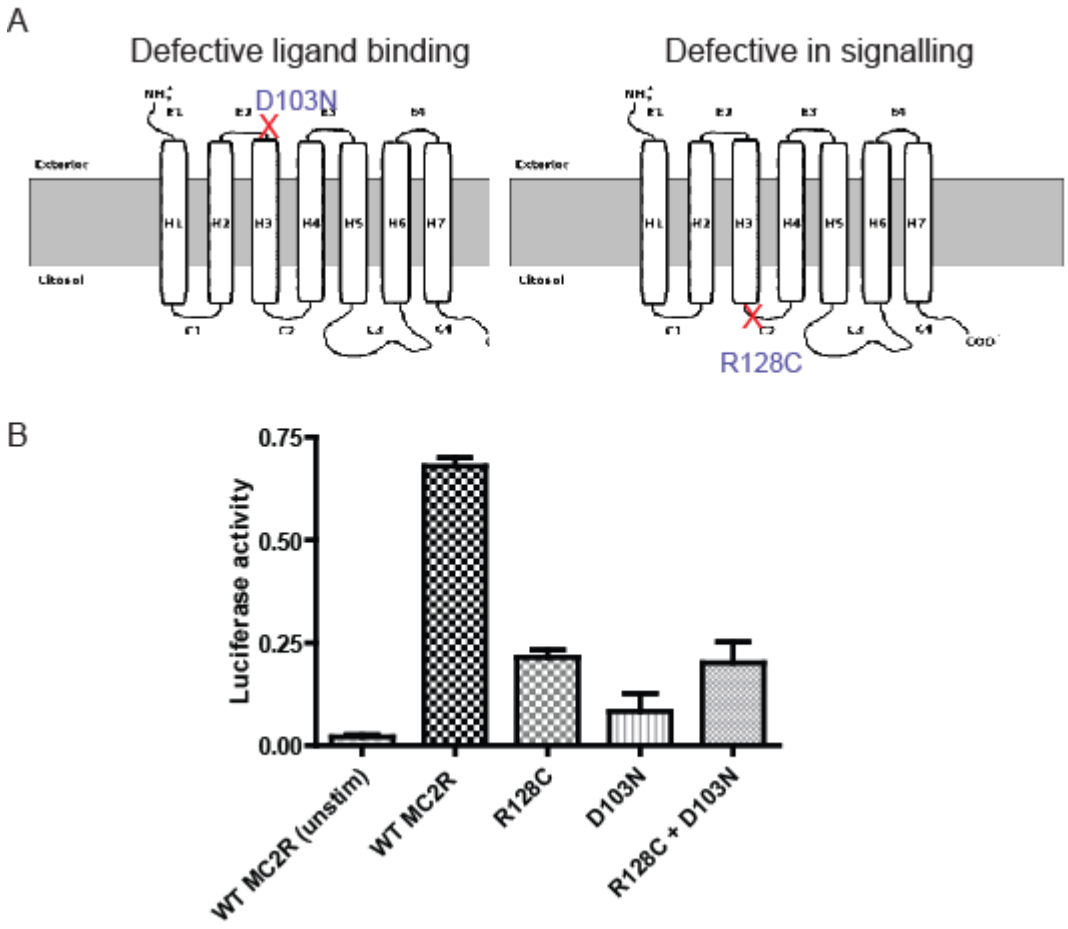

Figure 5.6: ACTH responsiveness in MC2R mutations. A: Schematic diagrams of D103N and R128C. B: Luciferase activity after stimulation with ACTH 10 $^{-7}$ M for 5 hours. Wild type MC2R stimulated and un-stimulated were used as controls. The experiments were independently repeated at least three times.

Wild type MC2R and MRAP were co-transfected into HEK293 cells as controls. Unstimulated cells showed a low basal luciferase activity, however, after stimulation with ACTH (10^{-7} M) the luciferase activity was significantly enhanced. When the signal defective mutation, R128C was stimulated after transfection, luciferase activity was detectable but at reduced levels compared to wild type. The signal in D103N after

stimulation with ACTH was markedly reduced. This is consistent with the data obtained in Chapter 3 (Figure 3.8). However, when the two mutations were co-expressed in the presence of MRAP, the luciferase response to ACTH stimulation was unchanged (Figure 5.6). This experiment suggests that there was no receptor cross-talk between these two mutations.

Even though there is evidence that MC2Rs could homodimerise on co-immunoprecipitation studies, the dimerisation of the two mutations did not enhance their function, suggesting that there is no transactivation of the receptors.

5.4.4 MC2R heterodimerises with other MCRs

In order to investigate whether MC2R heterodimerises with other MCRs, HEK293 cells were transfected with Flag-MC2R and HA-epitope tagged MC1R to 5Rs followed by Co-IP. HA-calcitonin receptor (Class B GPCR) was transfected to investigate the specificity of heterodimerisation. There is convincing evidence from Co-IP that MC2R appeared to heterodimerise with MC1R, MC3R and to a lesser extent MC4R and 5R (Figure 5.7). There is a 30kDa band seen in the IP (Figure 5.7C) for calcitonin receptor (last lane), however, the specific pattern of Flag-MC2R (as seen in other lanes) was not present. Therefore it is unclear if MC2R heterodimerises with calcitonin receptor or whether heterodimerisation with other MCRs is specific or not.

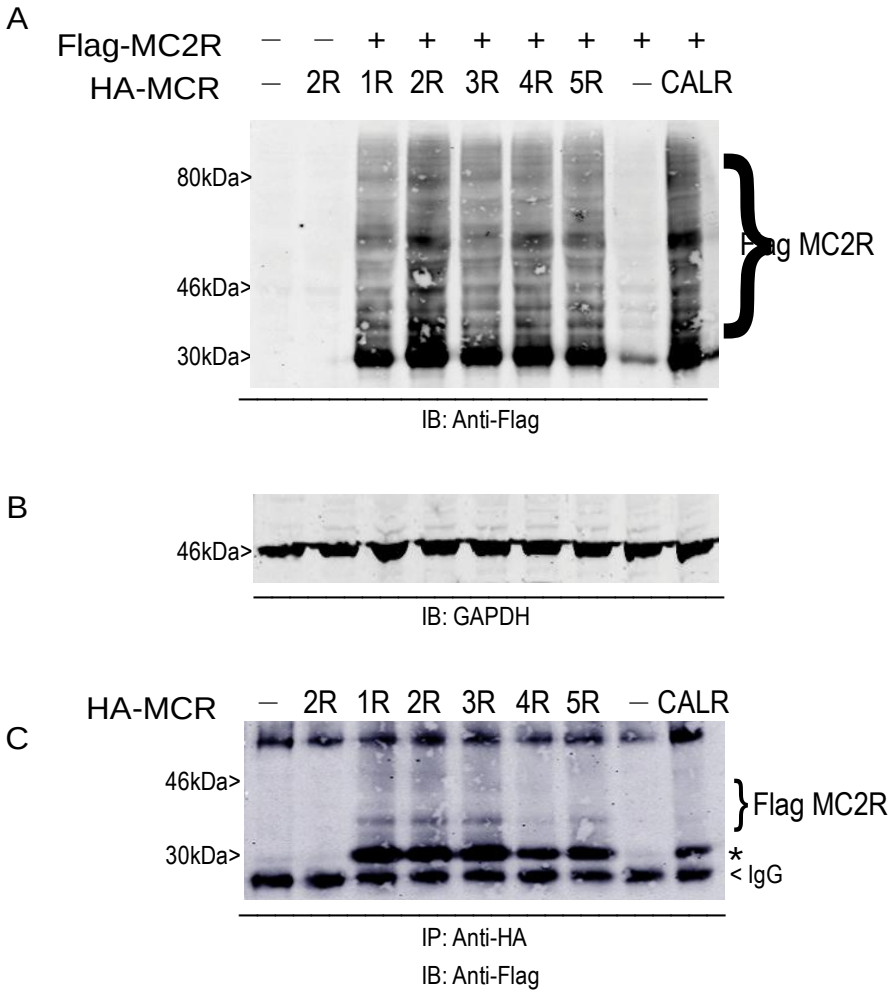

Figure 5.7: Heterodimerisation of MC2R with other MCRs. A: Immunoblotting by anti-Flag showing expression of Flag-MC2R. B: Immunoblotting with anti-GAPDH. C: Immunoprecipitation with anti-HA agarose beads and blotted with anti-Flag. IgG light chain is shown. The asterisk indicates the 30kDa band which indicates Flag-MC2R. The images shown are representative of two independent experiments.

5.5 Discussion

5.5.1 MC2R homodimerisation

The data shown in this chapter indicate that MC2R can homodimerise when overexpressed in HEK293 cells. The dimerisation does not seem to be affected by the presence of MRAP or ACTH.

Co-immunoprecipitation is the most frequently used method to detect the oligomerisation of GPCRs (Szidonya *et al.* 2008). This system uses the differentially epitope-tagged molecules coexpressed in heterologous cells. The receptor complex is electrophoresed, blotted and visualised using an antibody against the receptor of choice (Szidonya *et al.* 2008). Immunoprecipitation with the second antibody and immunoblotting with the first is performed to ensure specificity of the interaction.

The results of this chapter consistently showed that MC2R is seen as a particular molecular weight (approximately 30kDa) in either immunoblotting or immunoprecipitation. In addition, the presence of weaker bands at higher molecular weights is seen through out all the blots. This may be due to the various glycosylated forms of the receptor or just the nature of GPCRs (membrane soluble protein) which runs badly on SDS-PAGE. Flag-MC2R is represented as a 30kDa band. The 3XFlag epitope is only 22 amino acids long (approximately 2.2kDa). The naked MC2R is approximately 33.9kDa, therefore the approximate molecular weight of unglycoslyated 3XFlag-MC2R is 36kDa. The difference in molecular weight observed on the blots (ie 30kDa rather than 36kDa) suggests that the molecular weight marker used may be inaccurate. The different glycosylated form of HA-MC2R could also be seen in Figure 5.3c.

MC2R forms homodimers which were not affected by the presence of MRAP. This result is consistent with the theory that dimerisation occurs the ER. In systems without MRAP, MC2R are trapped inside the ER (Metherell *et al.* 2005; Cooray *et al.* 2008). MRAP interacts with MC2R and assists trafficking of the receptor. Furthermore, once at the cell surface, MC2R dimerisation was not influenced by ACTH. This indicates that ACTH binds to pre-existing MC2R dimers and induces subsequent conformational change and signal transduction without any change in dimerisation. Constitutive dimerisation has been shown to occur with other MCRs, for example, MC1R and MC3R have been shown to form homodimers and MC1R/MC3R heterodimers (Mandrika *et al.* 2005). It was found that there was no modulation of the constitutively formed dimers with either agonists or antagonists. The relevance of melanocortin receptor heterodimerisation remains unclear.

Studies on MC1R showed that dimerisation occurs early in the biosynthesis pathway (Sanchez-Laorden *et al.* 2006). This was evident by the finding of dimers of the deglycosylated “de novo” form of MC1R, when on immunoblotting the mobility of dimers was not affected by deglycosylation with endoglycosidase H. Dimerisation in intracellularly retained, loss of function mutants was also observed. Therefore the authors proposed that there are other factors apart from dimerisation which enables the receptor to traffic to the cell surface.

Biosynthesis of dimers in the ER have been reported in GPCRs such as β1-adrenergic (Hebert *et al.* 1996) and vasopressin receptors V1a and V2 (Terrillon *et al.* 2003), which form constitutive dimers (Hansen & Sheikh 2004). As discussed in Chapter 3, the ER quality-control system will only allow the correctly folded receptors to exit the ER and the misfolded or incompletely folded receptors will eventually degrade (Petaja-Repo *et al.* 2000). Studies using fluorescence or bioluminescence resonance energy transfer

(FRET and BRET respectively) have also shown that dimerisation takes place in the ER (Issafras *et al.* 2002; Overton & Blumer 2002; Floyd *et al.* 2003). The effect of dimerisation on some GPCRs such as rhodopsin mutants (Colley *et al.* 1995) and truncated vasopressin V2R (Zhu & Wess 1998) demonstrate that dimerisation enables dominant negative behaviour. The interaction of truncated mutants and wild type receptors prevented cell surface expression and subsequent retention of the receptor dimers in the ER. This dominant negative phenomenon does not exist in MC2R, as is evident by the observation that parents of FGD patients who have one normal allele and one mutant have normal adrenal function.

There is no consensus on the influence of ligand binding on dimerisation. Many studies are based on co-immunoprecipitation and resonance energy transfer techniques. Co-immunoprecipitation is semi-quantitative at best, and any change in the immunoreactivity could result from agonist-induced conformational changes that alter epitope recognition or may reflect a change in receptor dimers. Similarly, in BRET or FRET studies, the ligand-induced changes in energy transfer from donor to acceptor fluorophores could reflect conformational change of pre-existing dimers or change in receptor dimers. Therefore, a more defined experimental model is required to address the question whether ligand affects dimerisation. In co-immunoprecipitation of MC2R, ACTH did not promote or inhibit dimerisation. This data is in agreement with the BRET studies performed by another investigator in the lab. The BRET data (unpublished) showed that MC2R dimerises constitutively and that ACTH did not affect the dimerisation.

It has been seen in some GPCRs, such as MC1R, that dimerisation will allow the swapping of helices from each monomer and restores the function of two defective mutations (Sanchez-Laorden *et al.* 2006). This could only occur if the mutations on MC1R

are located in different domains. The authors managed to restore the function of mutations in the transmembrane fragments when co-expressed with a mutant deleted of five C-terminal amino acids.

A similar mechanism was investigated in MC2R mutations, with the analysis of the transactivation of the receptor complex formed by the potential dimerisation of two different mutations which traffic to the cell surface. The co-expression of R128C (signalling defective) and D103N (ligand binding defective) did not produce any signalling enhancement after stimulation with ACTH. The co-immunoprecipitations of MC2R were performed on wild type receptors only. Co-immunoprecipitation was not performed for these mutations, therefore raising the question of whether these mutants were capable of dimerisation. The alternative explanation is that there is no cross-talk between the receptors.

Most of the FGD 1 patients presenting with compound heterozygous mutations (see Section 4.4 and Table 4.1) are usually two separate trafficking-defective mutants. The exceptions are S74I/H170L and D103N/R137W, which is a combination of trafficking-defective (S74I and R137W) and trafficking-competent (D103N and H170L) mutations. D103N and H170H are thought to be ligand binding defective. These patients presented typically with ACTH insensitivity and phenotypic features of FGD. This implies that there is a loss of function and that there is no transactivation of receptors in S74I/H170L or D103N/R137W or, alternatively that the trafficking defective receptors are trapped inside the ER. However, if transactivation took place in a compound heterozygote with, for example, R128C and D103N, it would result in normal MC2R functions and therefore be a silent mutant for which the patient will not present with FGD.

5.5.2 MC2R Heterodimerises with other MCRs

MC2R appears to heterodimerise with MC1R, MC3R and to a lesser extent MC4R and 5R. It is unclear if these interactions are specific, as there is some evidence that MC2R dimerised with calcitonin receptor when receptors are overexpressed. Even though there is the presence of Flag-MC2R (indicated by asterisk in Figure 5.7), the lane does not display the broad range of glycosylated forms of MC2R as seen in the other lanes. Deglycosylation and proteolysis studies would better define this and additional experiments performed on dimerisation with other Class A GPCRs would also differentiate if these interactions with other melanocortin receptor are specific.

The biological significance of MC2R heterodimerisation could only be applied to receptors which are present in the same cells. MC5R (Chhajlani 1996) are present in the human adrenal cortex. There is also evidence from RT-PCR that MC3R and MC4R are present in rat adrenals (Dhillo *et al.* 2002). MC2R is present in the skin (Slominski *et al.* 1996), possibly in the same location as MC1R. Therefore, it would be of interest to investigate if the heterodimerisation of these receptors has any functional consequences. One way to test the transactivation theory is by stimulation with α-MSH. All MCRs except MC2R are responsive to α-MSH. Therefore, if a mutation at the highly conserved DRY sequence was created for all four MCRs, thereby creating signalling defective mutants and these were co-expressed with MC2R, cAMP responsive following stimulation with α-MSH would indicate cross-talk between the MC2R and other MCRs. If transactivation between MC2R and other MCRs occurred in vivo then further studies could explore if there is any physiological application.

A similar experimental approach was recently performed in AT1 angiotensin receptors (AT1R) by Szidonya *et al* (2007). They demonstrated that AT1Rs can "cross-inhibit" the G protein signalling. They created two AT1R mutants, firstly, AT1R-S109Y which is insensitive to binding of the AT1R-antagonist, candesartan, but has normal binding and signalling properties to angiotensin II (Ang II) which was co-expressed with a second mutant, the AT1R-DRY which is defective in G-protein activation yet preserved internalisation and ligand binding properties. They found that candesartan can inhibit Ang II induced G-protein activation, even though candesartan could only bind to the signalling deficient AT1R-DRY mutant. Therefore it was suggested that there is cross-inactivation of homo-oligomerised AT1Rs with candesartan (Szidonya *et al.* 2007; Karip *et al.* 2007).

Even more interesting is the therapeutic approach exploiting heterodimerisation which has been developed for treating pituitary tumours. It is known that somatostatin receptors (SSR) and D_2 dopamine (DR) receptors are significantly expressed in pituitary tumours (Stefaneanu *et al.* 2001; Moller *et al.* 2003; Saveanu *et al.* 2008). It has been shown that SSR does not just act as a monomer but can display a differential tendency to homo- and heterodimerise, depending on the subtype of receptor involved (Duran-Prado *et al.* 2008). The association of SSRs and DR in the majority of the pituitary tumours suggested the possibility of synthesising chimeric molecules containing the structural elements of both somatostatin and dopamine as a novel pharmacological approach (Figure 5.8), thereby targeting two families of GPCRs (Ferone *et al.* 2007; Saveanu *et al.* 2008). *In vitro* studies in somatotroph adenomas demonstrated that adenomas, which are partially responsive to a somatostatin analogue such as octreotide were found to have good response with this chimeric compound (Saveanu *et al.* 2006). This is another demonstration which highlights the possible biological significance of heterodimerisation.

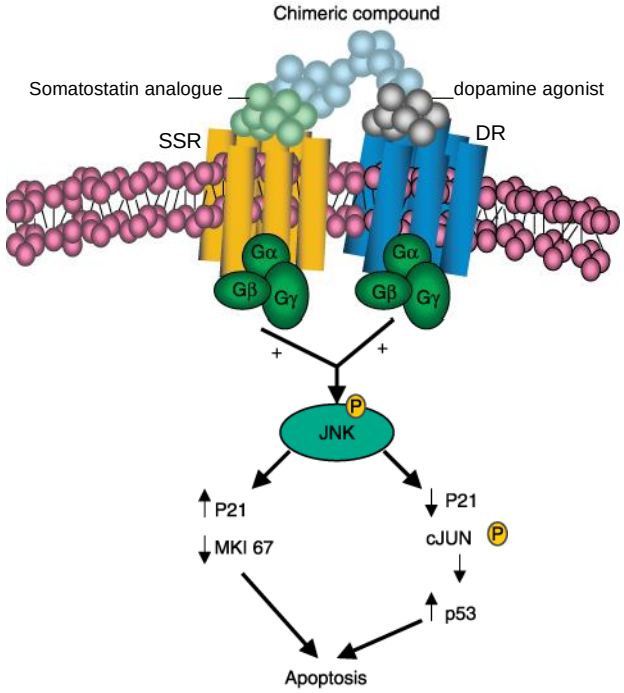

Figure 5.8: Somatostatin/dopamine chimera compound. This compound induces additive cytotoxicity via SSR and DR in neuroendocrine tumour cells. Diagram adapted from *Regulatory Peptide*s (Kidd *et al.* 2007).

5.5.3 Conclusion

In the HEK293 cells, co-immunopreicipitation of MC2R demonstrates the existence of homodimers which are independent of MRAP or ACTH. When a signalling defective mutant is co-expressed with a ligand binding mutant, there appears to be no transactivation of the receptors after stimulation with ACTH. MC2R can heterodimerise with other melanocortin receptors, and further investigation of this phenomenon would be required to define the specificity of the interaction and whether there were any functional consequences.

Chapter 6

Final Discussion

6.1 Summary of findings

The autosomal recessive disease of adrenal insufficiency known as Familial Glucocorticoid Deficiency (FGD) is characterised by isolated glucocorticoid deficiency. The resistance to the action of ACTH is due to genetic defects discovered so far, in the *MC2R*, *MRAP* and *STAR* genes, classified as FGD type 1, 2 or 3 respectively. These mutations account for approximately 50% of FGD patients (Metherell *et al.* 2009). The focus of this thesis is the mechanism of disease caused by *MC2R* mutations. Significant effort has previously been made to study this smallest member of the melanocortin receptor but this was previously hindered by difficulties in the functional expression in a system that is free of any endogenous MCR expression (Naville *et al.* 1996b; Noon *et al.* 2002; Rached *et al.* 2005). The characterisation of MC2R mutations in heterologuous expression systems is now possible with the discovery of the *MRAP* gene. This is the first comprehensive functional characterisation of MC2R using MRAP as a tool in a non-adrenal cell lines. In the presence of MRAP, MC2R is readily expressed and allows characterisation of mutant function and explore the existence and relevance of receptor dimerisation.

A total of twenty-four MC2R naturally occurring missense mutations found in FGD were characterised in the non-adrenal cell lines HEK 293 and CHO stably expressing MRAPα. The results of the cell surface assay and confocal studies in Chapter 3 concluded that majority of the MC2R mutants were non-functional as they failed to traffic and were trapped in the ER. The majority of the trafficking defective mutations were located in either the transmembrane domains or the intracellular loops, suggesting that their interaction with MRAP may be defective. Protein misfolding is the most common cause of defective GPCRs, for example in rhodopsin causing retinitis pigmentosa (Mendes

et al. 2005), vasopressin 2 receptor (V2R) in nephrogenic diabetes insipidus (Bernier *et al.* 2004) and others . Since MRAP has been identified to be an essential element for MC2R trafficking and ligand binding of ACTH (Metherell *et al.* 2005; Cooray *et al.* 2008; Sebag & Hinkle 2009b), the interaction with MRAPα was investigated with co-immunoprecipitation. Although all mutations interacted with the MRAP protein, this suggested that there may be multiple sites of interaction with MRAP and that a single point mutation was not enough to interfere with MRAP interaction but effectively disrupted folding of the receptor. But it is conceivable that the position of the mutations were not at the right site to disrupt the interaction with MRAP. It is also possible that the overexpression system used here may be too insensitive to pick up any defective interaction. Alternatively, there may be another closely associated protein yet to be defined, which assists in quality control and the correct folding of the receptor.

The trafficking competent mutations, all of which interacted with MRAP, failed to signal after stimulation with ACTH. Multiple attempts were made to investigate ligand binding but failed to provide adequate data to contribute to these findings. If binding results were available then it would be of interest to perform theoretical modelling to map out the possible ACTH binding site on the MC2R. Remarkably, the mutation R128C, which located within the highly conserved DRY sequence, trafficked efficiently to the cell surface and did not have complete loss of function as expected. In contrast, to this, the next mutation in Y129C, also part of the DRY sequence, failed to traffick to the cell surface, implying that the trafficking property of MC2R could be easily disrupted.

After the missense mutations were characterised, the genotype-phenotype correlation was analysed in Chapter 4. There were no phenotypic characteristics which could be related to the severity of the functional defect *in vitro*. Even with the most

common FGD mutation, S74I, there was a wide spectrum of presentation with respect to age of onset (Figure 4.1). This may be due to a variation of residual receptor functions, or that the normal adrenal function deteriorates with age. It is unclear why adrenal failure occurs later in life in some patients, it could be speculated that the misfolded receptor accumulates in the ER over a period of time overwhelms the degradation system and eventually kills off the zona fasciculata cells. In FGD type 2, in which patients had MRAP mutations, the age of onset of disease was earlier, indicating a more severe phenotype. Functional studies of these MRAP mutations would of interest. As previously reported (Elias *et al.* 2000), the FGD type 1 patients were significantly taller at presentation. The reason for this is yet to be determined but one hypothesis is that prolonged exposure to circulating high levels of plasma ACTH in the presence of defective MC2R could activate melanocortin receptors expressed in bone and growth plates, leading to accelerated growth.

The result of Chapter 4 found no weight differences between FGD 1 and 2, and the shortcomings of the study have been previously discussed. The BMI SDS of the patients would be a better marker for comparison. Given that MRAP has a negative effect on MC4R (Chan *et al.* 2009c), it would be logical to hypothesise that FGD type 2 patients would be underweight instead. However, it is yet to be determined whether MRAP is present in the hypothalamus in the same cell as the MC4R. MRAP appears to regulate other MCRs and in a recent study suggested mouse MRAP impaired MC5R dimer-oligomer formation, and that consequently MC5R was trapped in the ER and failed to reach the cell surface (Sebag & Hinkle 2009a). This interesting phenomenon raises the possibility of MRAP causing inhibition of dimerisation on other MCRs with potential functional consequence.

The lack of dominant negative effect seen in MC2R in FGD led to question if MC2R homodimerise or if dimerisation occurred if there is any physiological relevance. Therefore the final aim of the thesis was to investigate the dimerisation property of MC2R in the presence and absence of MRAP. Like other GPCRs, MC2R appears to homodimerise constitutively. This was not influenced by the presence of MRAP or ACTH stimulation. This suggests that dimerisation may be important in the formation of a functional receptor which occurs in the ER before interaction with MRAP. The pre-existing MC2R dimer traffics to the cell surface for ligand binding with ACTH, which induces conformational change or signal transduction without any change in dimerisation. The dimerisation of D103N and R128C did not influence the signalling property of the receptor after stimulation and therefore, at least in this instance, there does not appear to be transactivation between the receptors within the dimer formation. The functional relevance of MC2R dimerisation therefore remains unclear. Inhibition of MC2R homodimerisation by site directed mutagensis and studying receptor functionality may be one way to investigate this question.

MC2R appears to heterodimerise with other MCRs, especially with MC1R and MC5R and to a lesser extend MC3R and MC4R. The dimerisation may be specific but more experiments are needed with other classes of GPCR in order to further determine this and whether it has any physiological significance. If the MC2R heterodimerisation with other MCRs is specific then further investigation into whether there is any transactivation of the receptors would be of great interest.

6.2 Future Prospects

This is the first demonstration of the functional effect of MC2R mutations in the presence of MRAP. This work has provided a molecular explanation for the adrenal failure observed in FGD type 1 and also potentially highlights other areas for future investigation.

6.2.1 Identify the interaction site between MC2R and MRAP

Point mutations spread across the MC2R did not inhibit the interaction with MRAP. This suggests that there may be multiple sites of interaction between MC2R and MRAP. A systematic approach using a chimeric receptor, based, for example, on the β-adrenergic receptor, which does not interact with MRAP, and swapping domains with those from MC2R (Figure 6.1), thereby creating constructs with either transmembrane domains (TMD) (1-7), the intracellular loops (I to III), or the C-terminus, may allow mapping of the MRAP interaction domains in MC2R. A similar technique has previously been employed in the identification of the MRAP interaction site with the MC2R (Webb *et al.* 2009).

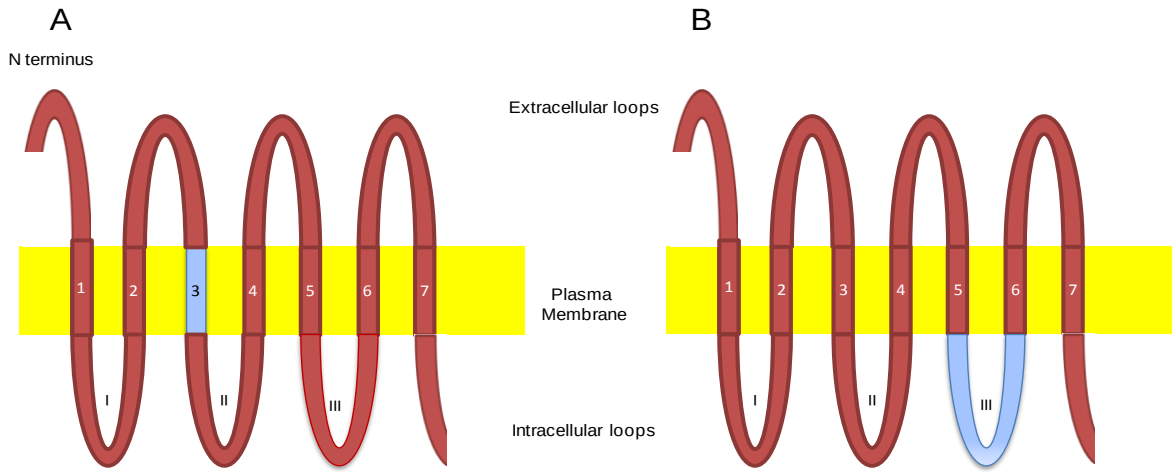

Figure 6.1: Schematic diagram of the possible chimeric receptor. A: insertion of the TMD 3 or B: intracellular loop III of the MC2R.

6.2.2 Rescue of the trafficking defective mutations

Multiple missense mutations of the MC2R led to failure to traffic and trapping in the ER probably due to misfolding of the receptor. It would be very interesting if small molecules could rescue the function of the misfolded receptor. This concept has been tested in other GPCRs such as V2 vasopressin receptor mutants found in diabetes inspidus (Morello *et al.* 2000), when the function of a trafficking defective mutant was restored after 16 hours of treatment with a cell permeant V2R antagonist. A similar advance has also been made by rescuing the activity of defective MC4R mutants with small molecule ligands *in vitro* (Fan & Tao 2009), which probably bind to stabilize the MC4R structure. Pharmacological rescue of naturally occurring GnRH mutations is also seen in patients with hypogonadotrophic hypogonadism (Janovick *et al.* 2002). Through this approach may offer some more insight into the basic biology of the receptor and may open up therapeutic advantage in the treatment of FGD.

6.2.3 Functional studies on MRAP mutations

FGD 2 patients with MRAP mutations had a more severe onset of disease. *In vitro* studies on the function of these MRAP mutations would be relevant. This work is currently been carried out by another researcher in the laboratory.

There has been much work on MRAP *in vitro* using heterologus expressing cells (Sebag & Hinkle 2007; Cooray *et al.* 2008; Sebag & Hinkle 2009b; Chan *et al.* 2009c). MRAP has been shown to modulate the expression and function of other MCRs (Chan *et al.* 2009c), MRAP interacted with other MCRs and significantly attenuated signalling in both MC4R and MC5R. Creation of MRAP knockout (MRAP-KO) mice may be informative

allowing the opportunity to further investigate whether MRAP has any influence on food intake, energy expenditure, or weight.

6.2.4 MC2R heterdimerisation with other MCRs and other GPCRs

As already discussed in Chapter 5, MC2R heterodimerised with other MCRs but it is unclear if this observation is specific. Therefore, it would be important to repeat these studies attempting to coimmunoprecipitate with other GPCRs of a similar class. If the interaction is specific then further exploration into the possible functional aspect would be exciting.

If there is MC2R heterodimerisation with other MCRs, one possibility is the study of transactivation of the receptors. By mutating the highly conserved DRY sequence for the four other MCRs and co-expressing each with Wt-MC2R, cAMP responsiveness following α-MSH stimulation would reveal whether cross-talk between receptors is possible. If there is indeed cross-talk then co-localisation of MC2R with the relevant MCRs in the same cells using *in situ* hybridization or co-immunohistochemical analysis would be desirable. However, the latter technique would depend on good quality MCRs antibodies being available. The relevance of any functional work is dependent on co-localisation of the receptors involved.

6.3 Conclusion

This thesis focused on identifying and characterising the molecular defects underlying FGD type 1. The functional defect of MC2R mutations have been examined in the presence of MRAP. The majority of MC2R mutations are due to trafficking defects, with the cause presumed to be the misfolding of the receptor and consequent retention in the ER. The degree of retention is variable such that there are some proteins with residual function and slow deterioration of adrenal cortex, perhaps explaining the later age of presentation compared to FGD type 2. MC2R was further found to constitutively homodimerise in a MRAP and ACTH independent manner, the functional importance of which is yet to be explored.

This thesis represents the first steps of MC2R characterisation enabled by the relatively recent discovery of MRAP. Further research into MC2R and MRAP interaction, ACTH induced conformational change of MC2R and homodimerisation and heterodimerisation with other MCRs could provide further insight into this important GPCR.

Chapter 7

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Chapter 8

Appendices

Appendix 8.1: Laboratory Equipment

Equipment	Manufacturer
Balances	
Mettler PM 300	Gallenkamp, London, UK
Can 28 automatic electrovalence	WT Avery Ltd, W. Mids, UK
Camera	
Kodak ID and ID Image Analysis Software	Kodak Ltd, Hemel Hempstead, Herts, UK
Centrifuges	
Sorvall OTD-55B Ultracentrifuge	Du Pont (UK) Ltd, Stevenage, Herts, UK
Sorvall RT 6000B	Du Pont Ltd, Herts, UK
Beckman J-6B	Beckman Instruments, CA, USA
Falcon 6/300	Sanyo Gallenkamp PLC, Leicester, UK
Microcentaur microfuge	MSC, UK
Electrophoresis tank	
BRL H5, H6, S2, V16.2	BRL, Paisley, UK
Liquid Nitrogen Storage	
Cryostat	Jencons, Bedfordshire, UK
Cryolab25	Statebourne Cryogenics, Tyne & Wear, UK
Microplate reader	
Lumistar Omega BMG Labtech.	BMG Labtech, Germany
Microscope	
Lieca DMIL	Leica Microsystem (UK) Ltd, Milton Keynes,

	UK
Fluorescence microscope	
Zeiss LSM 510 laser scanning focal microscope	Carl Zeiss Microimaging, NY, USA
PCR machines	
Hybaid Omnigene thermal cycler	Hybaid, Life Science Int., UK
GeneAmp PCR system 2400	PE Biosystems, Cheshire, UK
GeneAmp PCR system 9700	PE Biosystems, Cheshire, UK
Membrane and Plate Reader	
LI-COR® Odyssey plate reader	LI-COR® Odyssey, USA
pH meter	
EDT GP 353	Pentacourt Ltd, Haltead, Essex, UK
Power supply units	
LKB Bromma 2197	LKB Instruments Ltd, Croydon, Sussex, UK
GIBCO BRL 400L	Life Technologies Ltd, Paisley, UK
Atto AE3105	Genetic Research Instruments, Bishops Stortford, Herts, UK
Temperature controlled incubators	
Leec MK II	Luckhams, Sussex, UK
Orbital incubator: Innova 4300	New Brunswick Scientific, UK
Spectrophotometers	
GeneQuant RNA/DNA	Pharmacia Biotech, Cambridge, UK
Calculator spectrophotometer	

NanoDrop ND 1000	NanoDrop Technologies, USA
Semi Dry Blotter	
Transblot SD semi dry transfer cell	Biorad, Hemel Hempstead, Hertfordshire, UK
Ultraviolet Transilluminator	
UVP 20	Genetic Research Instruments, Herts, UK
Water baths	
Grant JB1 and SE 10	Chemlab Instruments, Hornchurch, Essex, UK

Appendix 8.2: Laboratory Chemicals and antibodies

Chemicals	Supplier
Deoxyribonucleotide	Promega, Southhampton, UK
Restriction and modifying enzymes	Promega, Southhampton, UK
Dpn 1 restriction enzyme	New England Biolabs, UK
Polymerase enzymes	Promega, Southhampton, UK
Advantage® HD Polymerase Mix	Clontech, CA, USA
Cell surface assay Blocking Buffer	LI-COR® Bioscience
S.O.C medium	Invitrogen, Renfrew Renfrewshire, UK
RNAsin	Promega, Southhampton, UK
DNA ladders	MBI, Fermentas
Loading dye	MBI, Fermentas
Kaleidoscope Prestained protein marker	Bio-rad laboratories, Hemel Hempstead, Hertfordshirem, UK
n-Dodecyl-β-D-maltoside	Sigma-Aldrich, Dorset, UK

16% Formaldehyde	TAAB, Bershire, UK
Oligonucleotides	Sigma-Genosys, Cambridge, UK
Protease inhibitor cocktail	Roche, cOmplete
MES or MOP SDS Running buffer	Invitrogen NuPAGE®, UK
10-12% Bis-Tri Gel	Invitrogen NuPAGE®, UK
Luciferase assay kit	Promega, Southhampton, UK
(^{125}I-iodotryosyl^{23})ACTH (1-39	Perkin Elmer, UK
Truncated ACTH	Bachem AGK, Switzerland
Plasmid vectors	
pcDNA 3.1	Invitrogen, Renfrew Renfrewshire, UK
pGEM T-Easy	Promega, Southhampton, UK
Bacterial strains	
JM109	Promega, Southhampton, UK
Tissue Culture reagents	
DMEM, DMEM+F12, F12	Sigma Reagents Ltd, Dorset, UK

Foetal calf serum	Sigma Reagents Ltd, Dorset, UK
Penicillin/Streptomycin	Gibco BRL, Paisley, UK
G418	Sigma Reagents Ltd, Dorset, UK
Trysin/EDTA	Gibco BRL, Paisley, UK

Transfection reagents

Lipofectamine 2000	Invitrogen, Renfrew Renfrewshire, UK
Opti-MEM® I reduced serum medium	Invitrogen, Renfrew Renfrewshire, UK

Primary Antibodies

Anti-Flag M2 mouse monolonial antibody (F1365)	Sigma-Aldrich, Dorset, UK
Anti-HA, mouse monoclonal, clonal HA-7 antibody (H9658)	Sigma-Aldrich, Dorset, UK
Anti-GAPDH antibody (sc-32233)	Santa Cruz biotechnology, USA

Secondary Antibodies

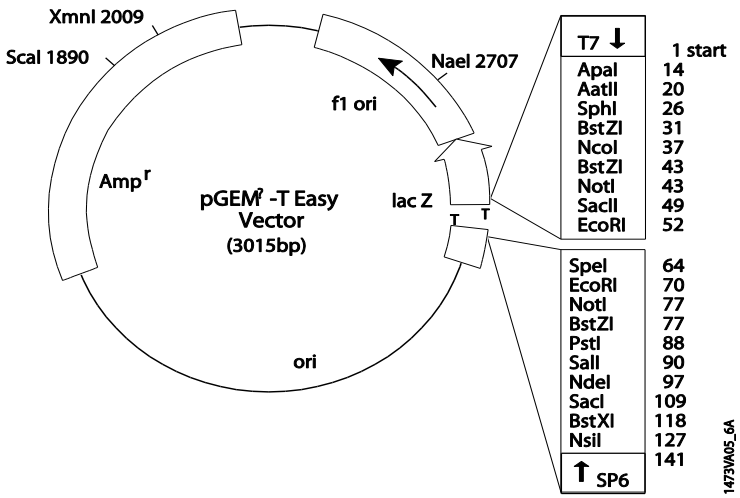
Cy2-conjugated donkey anti-rabbit (#711225-152)	Jackson ImmunoResearch, USA
Cy3-conjugated donkey anti-rabbit (#711165-152)	Jackson ImmunoResearch, USA
Cy2-conjugated donkey anti-mouse (#711225-150)	Jackson ImmunoResearch, USA
Cy3-conjugated donkey anti-rabbit (#711165-150)	Jackson ImmunoResearch, USA
IRDye®680CW Conjugated Goat Anti-Mouse IgG	LI-COR® Bioscience, USA
IRDye®800CW Conjugated Goat Anti-Mouse IgG	LI-COR® Bioscience, USA
IRDye®680CW Conjugated Goat Anti-Mouse IgG	LI-COR® Bioscience, USA

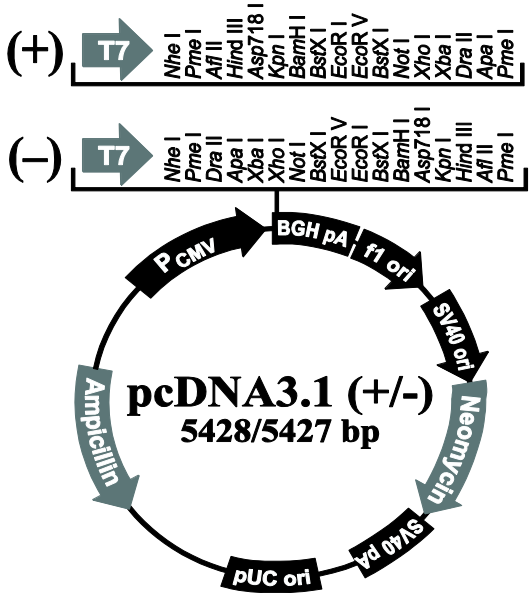
Plasmid Kits	
QIAGEN RNeasy® mini kit	QIAGEN, Crawley, UK
QIAGEN RNeasy® midi kit	QIAGEN, Crawley, UK

Appendix 8.3: List of 24 primers used for FGD type 1 mutations

Mutation	aa from	Change aa to	Primer sequencing forword	Reverse primer
D20N	aspartic	asparagine	AAGAAATAATTCCAACTGTCCTCGTGT	ACACGAGGACAGTTGGAATTATTTCTT
I44M	isoleucine	methionine	GGAGAATCTGATGGTCCTGCTGGCT	AGCCAGCAGGACCATCAGATTCTCC
L55P	leucine	proline	AGAATAAGAATCCCCAGGCACCCAT	ATGGGTGCCTGGGGATTCTTATTCT
S74I	serine	isoleucine	ATATGCTGGGCATCCTATATAAGAT	ATCTTATATAGGATGCCCAGCATAT
D103N	aspartic	asparagine	GAAACCACAGCCAATGACATCATCG	CGATGATGTCATTGGCTGTGGTTTC
D107N	aspartic	asparagine	GATGACATCATCAACTCCCTGTTTG	CAAACAGGGAGTTGATGATGTCATC
G116V	glycine	valine	TCTCCCTGCTTGTCTCCATCTTCAG	CTGAAGATGGAGACAAGCAGGGAGA
S120R	serine	arginine	GGCTCCATCTTCCGCCTGTCTGTGA	TCACAGACAGGCGGAAGATGGAGCC
R128C	arginine	cysteine	ATTGCTGCGGACTGCTACATCACCA	TGGTGATGTAGCAGTCCGCAGCAAT
Y129C	tyrosine	cysteine	CTGCGGACCGCTGCATCACCATCTT	AAGATGGTGATGCAGCGGTCCGCAG
I130N	isoleucine	asparagine	CGGACCGCTACAACACCATCTTCCA	TGGAAGATGGTGTTGTAGCGGTCCG
R137W	arginine	tryptophan	TTCCACGCACTGTGGTACCACAGCA	TGCTGTGGTACCACAGTGCGTGGAA
H139Y	histidine	tryosine	GCACTGCGGTACTACAGCATCGTGA	TCACGATGCTGTAGTACCGCAGTGC
R146H	arginine	histidine	TGACCATGCGCCACACTGTGGTGGT	ACCACCACAGTGTGGCGCATGGTCA
T152K	threonine	lysine	TGGTGGTGCTTAAGGTCATCTGGAC	GTCCAGATGACCTTAAGCACCACCA
T159K	threonine	lysine	GGACGTTCTGCAAGGGGACTGGCAT	ATGCCAGTCCCCTTGCAGAACGTCC
H170L	histidine	leucine	TGATCTTCTCCCTTCATGTGCCCAC	GTGGGCACATGAAGGGAGAAGATCA
L198P	leucine	proline	TGCACATGTTCCCGCTGGCTCGATC	GATCGAGCCAGCGGGAACATGTGCA
G226R	glycine	arginine	ACCATCCTGCTCAGGGTCTTCATCT	AGATGAAGACCCTGAGCAGGATGGT
A233P	alanine	proline	ATCTTCTGCTGGCCCCCCTTTGTGC	GCACAAAGGGGGGCCAGCAGAAGAT
C251F	cysteine	phenylalanine	GTAACCCCTACTTCGCCTGCTACAT	ATGTAGCAGGCGAAGTAGGGGTTAC
Y254C	tyrosine	cysteine	ACTGCGCCTGCTGCATGTCTCTCTT	AAGAGAGACATGCAGCAGGCGCAGT
S256F	serine	phenylalanine	CCTGCTACATGTTTCTCTTCCAGGT	ACCTGGAAGAGAAACATGTAGCAGG
P273H	proline	histidine	CCGTCATTGACCACTTCATATATGC	GCATATATGAAGTGGTCAATGACGG

Appendix 8.4: Vector map of pGEM®T-easy and pcDNA3.1 (+/-).

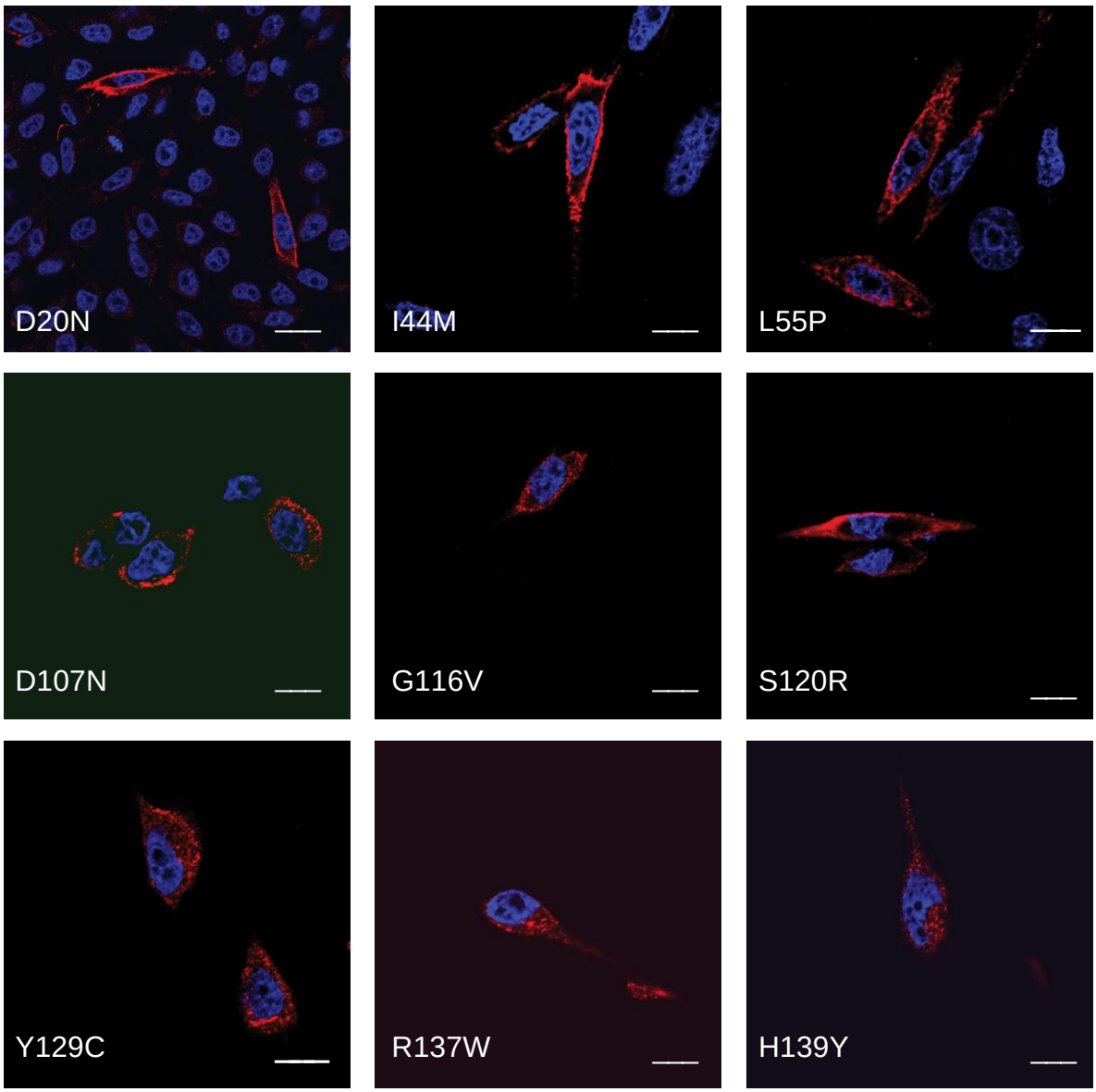




Comments for pcDNA3.1 (+)
5428 nucleotides

Appendix 8.5A: Confocal microscopy of MC2R mutations.

The scale bar represents 10 µm.



Appendix 8.5B: Confocal microscopy of MC2R mutations.

The scale bar represents 10 µm.