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Clinical, Genetic, and Functional Characterization of Adrenocorticotropin Receptor Mutations Using a Novel Receptor Assay

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The ACTH receptor (MC2R) is expressed predominantly in the adrenal cortex, but is one of five G protein-coupled, seven-transmembrane melanocortin receptors (MCRs), all of which bind ACTH to some degree. Testing of MC2R activity is difficult because most cells express endogenous MCRs; hence, ACTH will elicit background activation of assayable reporter systems. Inactivating mutations of MC2R lead to hereditary unresponsiveness to ACTH, also known as familial glucocorticoid deficiency (FGD). These patients are usually seen in early childhood with very low cortisol concentrations, normal mineralocorticoids, hyperpigmentation, and increased bodily growth. Several MC2R mutations have been reported in FGD, but assays of the activities of these mutants are cumbersome. We saw two patients with typical clinical findings of FGD. Genetic analysis showed that patient 1 was homozygous for the mutation R137W, and patient 2 was a compound heterozygote for S74I and Y254C. We tested the activity of these mutations in OS-3 cells, which are unresponsive to ACTH but have intact downstream cAMP signal transduction. OS-3 cells transfected with a cAMP-responsive luciferase reporter plasmid (pCREluc) were unresponsive to ACTH, but cotransfection with a vector expressing human MC2R increased luciferase activity more than 40-fold. Addition of ACTH to cells cotransfected with the pCREluc reporter and wild-type MC2R activated luciferase expression with a 50% effective concentration of 5.5×10^{-9} M ACTH, which is similar to previously reported values. By contrast, the MC2R mutant R137W had low activity, and the S74I or Y254C mutants elicited no measurable response. This assay provides excellent sensitivity in an easily assayed transient transfection system, providing a more rapid and efficient measurement of ACTH receptor activity. (*J Clin Endocrinol Metab* 87: 4318–4323, 2002)

FAMILIAL GLUCOCORTICOID deficiency (FGD), also known as hereditary unresponsiveness to ACTH, is a rare autosomal recessive disorder in which unresponsiveness to ACTH leads to deficient secretion of cortisol and adrenal C_{19} androgen precursors; by contrast, mineralocorticoid production, regulated by the renin-angiotensin system, is normal (1, 2). These patients are usually seen in early childhood with hyperpigmentation, recurrent hypoglycemic episodes, and seizures due to severe glucocorticoid deficiency (2). Lack of adrenal C_{19} steroid production may result in poor development of pubic hair in successfully treated adult female patients (3, 4). Excessive growth has been described in several patients, but remains unexplained (5, 6). Patients with familial glucocorticoid deficiency who have mutations in the ACTH receptor are said to have type 1 FGD; patients in whom no such mutations are found have type 2 FGD (7). For example, patients with Algrove syndrome (8), also known as triple A syndrome and more recently termed ALADIN syndrome (ACTH unresponsiveness, alacrima, achalasia, and neurological disorders), are ACTH resistant, but have mutations in the *AAAS* gene, encoding a member of the WD-repeat family of regulatory proteins (9).

The ACTH receptor is a member of the melanocortin receptor family, consisting of five closely related genes that encode seven-transmembrane G protein-coupled receptors (10). All five of these receptors can bind ACTH to some extent, but MC2R binds ACTH at the highest affinity, is expressed almost exclusively in the adrenal cortex, and hence is the physiological ACTH receptor (11). Shortly after the MC2R gene was cloned (10), point mutations were described causing FGD (12–17). However, studies of MC2R and its mutations have been hampered by relatively poor systems for assaying its activity. Measurement of the activities of normal and mutant human MC2R in COS-7 cells (18) and in mouse Cloudman M3 melanoma cells (16) was confounded by the presence of endogenous melanocortin receptors (MCRs), which contribute background activity. Better results were obtained with mouse adrenal Y6 cells, which express no other MCRs; however, this assay is cumbersome, as it used stable transfections and an immunoassay for generated cAMP (19). We now describe two new families with hereditary unresponsiveness to ACTH, identify the responsible MC2R mutations, and describe a quick and easy assay for MC2R activity.

Abbreviations: EC_{50}, 50% Effective concentraion; FGD, familial glucocorticoid deficiency; MCR, melanocortin receptor; PRA, plasma renin activity.

Case Reports

Patient 1

A male child of consanguineous Hispanic parents was born at 41 wk gestation with a birth weight of 3475 g (50% percentile), a length of 53.5 cm (90% percentile), and a head circumference of 35 cm (50% percentile). He had recurrent pneumonia during his first 2 yr of life, but sweat chloride testing and immunological and metabolic evaluations were negative, and he was treated with albuterol and beclomethasone beginning at 4 months of age. Febrile seizures occurred at 6.5 months of age; the electroencephalogram and brain imaging studies were normal, but his motor and language developments were delayed at 2 yr of age. During a hospitalization at 2 3/12 yr of age, one of us (W.L.M.) was asked to evaluate apparent hyperglycemia, which was a laboratory error. His weight was 15.8 kg (+3.7 SD score), height was 95.4 cm (+2.0 SD score),

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and head circumference was 52 cm (+2.1 SD score); his physical examination was otherwise unremarkable, except for generalized hyperpigmentation. His morning plasma cortisol was less than 27 nmol/liter (<1 μg/dl) and did not rise in response to iv administration of 250 μg synthetic ACTH-(1–24). Baseline plasma ACTH was markedly elevated on two separate occasions at 252 and 142 pmol/liter (1146 and 645 ng/liter; normal range, 2–11 pmol/liter); plasma renin activity (PRA), aldosterone, and electrolytes were normal. The patient did well on glucocorticoid replacement therapy.

Patient 2

A male child of unrelated Caucasian parents was born at 40 wk gestation with a birth weight of 3200 g (25% percentile) and a length of 53.5 cm (90% percentile). Except for 1 month of mild jaundice, his neonatal course was benign. Hyperpigmentation was noted at 2 months of age, but his electrolytes were normal. Growth was at the 95th percentile for height and weight. Several upper respiratory infections and roseola with temperature above 40 C occurred without incident. At 19 months of age, he had nausea, vomiting, dehydration, hyponatremia, hypoglycemia, and a grand mal seizure; he responded to iv saline, glucose and hydrocortisone. Plasma ACTH was more than 330 pmol/liter (>1500 ng/liter), and cortisol was low on two occasions at 27 and 55 nmol/liter (1 and 2 μg/dl). T_4, TSH, and PRL were normal, and he was discharged on hydrocortisone (~13 mg/m^2·d). When first seen by one of us (F.A.C.) at 4 3/12 yr of age, his height was 115 cm (+2.0 SD score), and his weight was 23.1 kg. He had mild generalized hyperpigmentation, but his physical examination was otherwise unremarkable. His plasma ACTH was 330 pmol/liter (1500 ng/liter) 6 h after his dose of hydrocortisone, cortisol was less than 27 nmol/liter (<1 μg/dl), and thyroid function tests, electrolytes, PRA, and long chain fatty acids were normal. His growth and development continue to be normal with hydrocortisone replacement. A male sibling born in 2001 was hyperpigmented at birth with basal ACTH greater than 330 pmol/liter (>1500 ng/liter). In response to 15 μg/kg synthetic ACTH-(1–24), his cortisol rose minimally from 27 to 55 nmol/liter (1 to 2 μg/dl). Electrolytes and PRA were normal. He has done well on replacement doses of hydrocortisone, with stress doses at the time of illness.

Materials and Methods

DNA preparation and analysis

Leukocyte genomic DNA was prepared as previously described (20). The entire coding sequence of the ACTH receptor was amplified by PCR, using oligonucleotide primers 1S and 2A (12). Amplification with a 20:1 mixture of *Taq* and *Pfu* polymerases was performed for 30 cycles of 1 min at 95 C, 1 min at 60 C, and 1 min at 72 C, except that the first denaturation was for 5 min (95 C), and the last extension step was for 5 min (72 C) (20). The PCR-amplified products were purified and subjected to automated direct sequencing with two pairs of primers (1S/1A and 2S/2A) (12). Restriction endonuclease digestion of PCR products with *Hpy*CH4III and *Fnu*4HI was performed under conditions recommended by the supplier (New England Biolabs, Inc., Beverly, MA).

Construction of ACTH receptor expression vectors

Mutant MC2R cDNA expression vectors were generated by PCR-based, site-directed mutagenesis (21) of wild-type cDNA in pcDNA1.1neo (10) using the primers shown in Table 1, except that we used 750 μM deoxy-NTP and only 12 cycles of PCR. The methylated parental wild-type cDNA was digested with 10 U *Dpn*I at 37 C for 90 min, and the remaining unmethylated mutagenized cDNA plasmid was used to transform *Escherichia coli* DH5α cells. The resulting cDNA was subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA), and the mutagenized MC2R cDNAs were sequenced to confirm the mutations.

Cell culture and transient transfection

OS3 cells were cultured as previously described (22). Cells were divided into 2-cm, six-well plates (Falcon 3046, BD Biosciences, Lincoln Park, NJ) 24 h before transfection at approximately 50% confluence. For transfection, cells were transferred to αMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with serum and antibiotics; cells were incubated overnight with calcium phosphate precipitates (total DNA content, 2.5 μg/well) of vectors expressing wild-type or mutant MC2R cDNA plus a cAMP-responsive luciferase reporter plasmid (pCREluc) that contained 16 cAMP response element units (23). At the end of the incubation, the calcium phosphate precipitates were removed, and cells were incubated in fresh medium for 36 h to allow for gene expression before stimulation with synthetic ACTH-(1–24).

ACTH stimulation and dual luciferase assay

Two days after transfection, cells were incubated with 10^{-12}–10^{-6} M ACTH-(1–24) (Sigma, St. Louis, MO) for 18 h at 37 C in 5% CO_2. Cells were then lysed and assayed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega Corp., Madison, WI); cotransfection of 100 ng/well *Renilla* luciferase reporter vector (pRL-CMV, Promega Corp.) was used as a control for transfection efficiency, and the results were expressed as relative luciferase activity. Data represent the mean ± SEM of three independent experiments, each performed in triplicate. The analysis of the sigmoid dose-response curve for wild-type MC2R and the calculation of its 50% effective concentraion (EC_{50}) were performed by personal computer using PRISM 3.02 (GraphPad Software, Inc., San Diego CA).

Results

Mutation analysis

Both patients came to medical attention in the first 2 yr of life with hypoglycemia, hyperpigmentation, and macrosomia. Both had unmeasurably low concentrations of cortisol and grossly elevated concentrations of ACTH without evidence of mineralocorticoid deficiency, and both did well with physiological replacement doses of cortisol, suggesting a diagnosis of hereditary unresponsiveness to ACTH. Examination of the sequence of MC2R from leukocyte genomic DNA is facilitated by the fact that the entire MC2R protein-coding region is contained in exon 2 of the MC2R gene. Patient 1 was homozygous for the nucleotide mutation 409 C→T, resulting in the mutation R137W (arginine at the position 137 changed to tryptophan) in MC2R. The 409 C→T mutation changes the sequence CTGCGG to CTGTGG, creating a new restriction site for the endonuclease *Hyp*CH4III, which recognizes the sequence ACNGT. Because there are many sites that can be cleaved by *Hyp*CH4III in MC2R exon 2, we used oligonucleotides 2S and 1A to amplify a 178-bp product that contains the site of this mutation (Fig. 1, A and

TABLE 1. Sequences of oligonucleotide primers used for site-directed mutagenesis

Nuc Δ	Prot Δ	
221G→T	S74I	Sense: 5′-CTGATATGCTGGGCATCCTATATAAGATCTTGG-3′ Antisense: 5′-CCAAGATCTTATATAGGATGCCCAGCATATCAG-3′
409C→T	R137W	Sense: 5′-CATCTTCCACGCACTGTGGTACCACAGCATCG-3′ Antisense: 5′-CGATGCTGTGGTACCACAGTGCGTGGAAGATG-3′
761A→G	Y254C	Sense: 5′-CCTACTGCGCCTGCTGCATGTCTCTCTTCC-3′ Antisense: 5′-GGAAGAGAGACATGCAGCAGGCGCAGTAGG-3′

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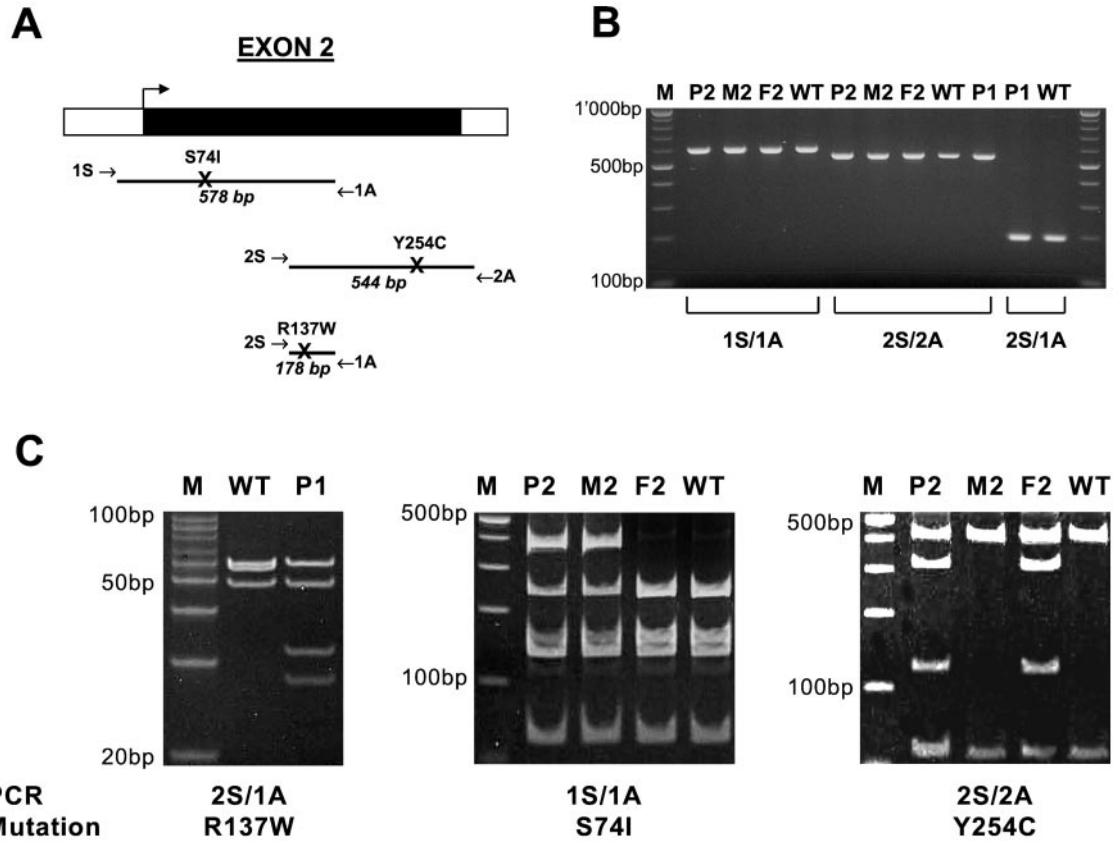

FIG. 1. Genetic analysis of the MC2R gene in patients with FGD. A, Diagram of exon two of the MC2R gene (■, protein-coding region) and the primers used for PCR. The locations of the MC2R mutations found in our patients are indicated by X. B, PCR-amplified DNA sequences before restriction endonuclease digestion. C: *Left panel*, *Hpy*CH4III digest of the PCR product 2S/1A, separated on a 20% polyacrylamide gel. The R137W mutation creates an additional restriction site producing bands of 34 and 29 bp. *Middle panel*, *Fnu*4HI digest of the PCR product 1S/1A, separated on a 10% polyacrylamide gel. The S74I mutation disrupts a restriction site, giving rise to the 397-bp band found in the patient (P2) and the mother (M2), but not in the father (F2) or the wild type (WT). *Right panel*, *Fnu*4HI digest of the PCR product 2S/2A, separated on a 10% polyacrylamide gel. An additional restriction site is created by the Y254C mutation, seen as bands at 324 and 163 bp in P2 and F2, but not in M2 or the WT.

B) and only two *Hyp*CH4III sites in the normal sequence. Cleavage of this 178-bp product from patient 1 yielded bands of 34 and 29 bp, but cleavage of the wild-type sequence showed a band at 63 bp, and the 66- and 49-bp bands were shared in both; confirming the presence of the 409 C→T mutation encoding R137W (Fig. 1C, *left panel*).

Patient 2 was a compound heterozygote; examination of the parents' DNA showed that he received the mutation 221 G→T encoding S74I from his mother and the mutation 761 A→G encoding Y254C from his father. The 221 G→T mutation changes the sequence GGCAGCCTA to GGCATCCTA destroying a restriction site for *Fnu*4HI, which recognizes the sequence GCNGC. When the 578-bp 1S/1A PCR product of exon 2 from the father or from the wild type is digested with *Fnu*4HI, four bands of 242, 155, 120, and 61 bp are seen, but when the DNA from the mother or patient was digested, a new band of 397 bp was seen, corresponding to the combination of the 242- and 155-bp fragments due to the loss of a *Fnu*4HI site in one allele (Fig. 1C, *middle panel*). Similarly, the 761 A→G mutation changes the sequence TGCTACATG to TGCTGCATG, creating an additional *Fnu*4HI site. Digestion of the 544-bp 2S/2A PCR product of exon 2 from the patient and his father showed the presence of the additional 324- and 161-bp bands, whereas digestion of the DNA from the mother or from a control did not (Fig. 1C, *right panel*).

Characterization of the ACTH receptor assay

To evaluate the activities of the MC2R mutations found in our patients, we used OS-3 cells, which are derived from mouse adrenocortical Y1 cells and are unresponsive to ACTH, but have an intact downstream cAMP signal transduction pathway (22, 24). We expressed the wild-type MC2R in OS-3 cells and examined their ability to respond to synthetic ACTH-(1–24) by measuring the activity of a cAMP-dependent reporter that reflects the activation of G protein-coupled receptors. For this purpose we chose a luciferase reporter fused to 16 copies of the consensus cAMP response element, as this reporter has been used to assay the activity of a closely related receptor, MC4R (23), thus permitting a simple photometric readout of receptor activity. OS-3 cells transiently expressing pCREluc readily respond to 1 mM 8-bromo-cAMP regardless of whether the cells are cotransfected with empty pcDNA3 vector or one expressing MC2R, showing that this reporter is activated by cAMP (Fig. 2A). OS-3 cells cotransfected with pCREluc and the vector for

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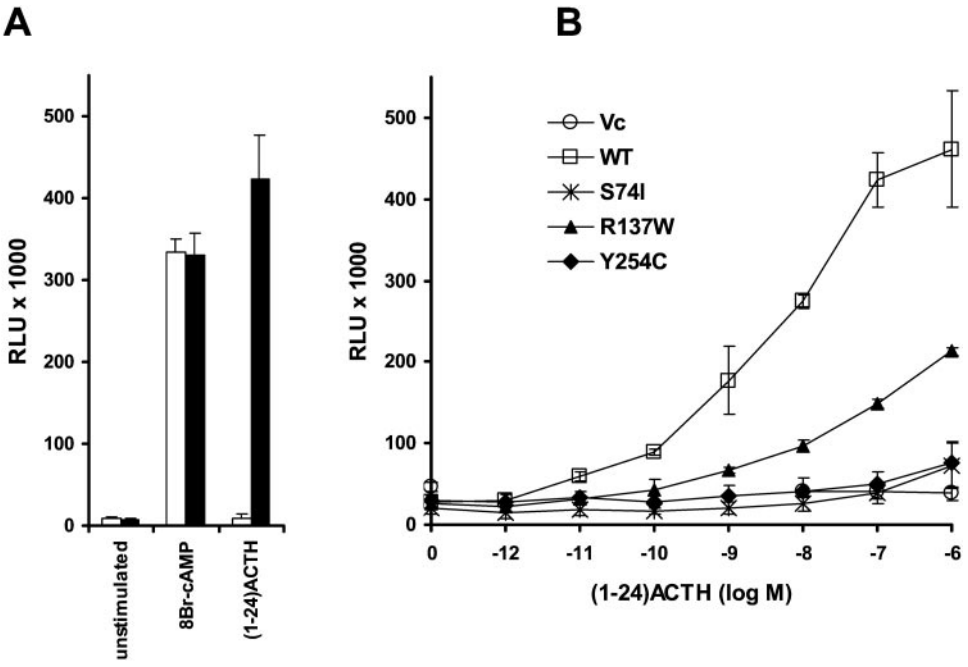

FIG. 2. Assay of the activities of the mutations. A, OS-3 cells were transfected with the reporter plasmid pCREluc and cotransfected with the pcDNA3 vector expressing the wild-type MC2R cDNA (■) or with an equal mass of empty pcDNA3 (□). Relative luciferase activity (RLU) was measured in unstimulated cells or after stimulation with 1 mM 8-bromo-cAMP or 10^{-7} M ACTH-(1–24) overnight. Data are the mean ± SEM of three independent experiments, each performed in triplicate. B, Dose dependence of ACTH activation of wild-type (WT) and mutant MC2Rs. Cells transfected with the empty pcDNA3 expression vector (Vc) served as the control. Values are expressed as the mean ± SEM of three independent transfection experiments, each performed in triplicate.

MC2R had a robust response to 10^{-7} M ACTH-(1–24), but cells cotransfected with pCREluc and an empty pcDNA3 vector had no response (Fig. 2A). Thus, the OS-3 cells expressing pCREluc are not stimulated by high doses of ACTH, but expression of MC2R in these cells yields a response equivalent to that elicited by 1 mM 8-bromo-cAMP. The EC_{50} of our system to ACTH occurred at 5.5×10^{-9} M (Fig. 2B), which is similar to the value of 6.8×10^{-9} M reported with stably transfected Y6 cells (19). However, the reported EC_{50} values for MC2R assays must be viewed with caution when plateau values for the ACTH response are not reported. Thus, our assay is equally sensitive but more rapid than the stable transfection assay.

Activities of the ACTH receptor mutants

The three mutants found in our patients were tested using ACTH concentrations from 10^{-12}–10^{-6} M (Fig. 2B). The S74I mutant had minimal activity only with 10^{-6} M ACTH-(1–24), consistent with previous data showing that this mutant has a very low affinity for ACTH in ligand binding studies (19). The Y254C mutant also had little, if any, activity, and the R137W mutation had low activity when stimulated with very high doses of ACTH-(1–24).

Discussion

At least 21 different mutations have been reported in the MC2R gene that cause FGD (Table 2). Two of the mutations found in our patients were described previously, but their activities were not tested. The S74I mutation, located in the second transmembrane domain of the receptor, resulted in a nearly complete lack of ACTH response as described previously (19), further validating our assay. The Y254C mutation in the third extracellular loop of the receptor had virtually no activity. This mutation has been reported previously and appears to disrupt the tertiary structure of MC2R, disturbing ligand binding and/or G protein coupling by introducing an extra cysteine (15); however, the activity of this mutant was not examined previously. The R137W mutation responded slightly to very high concentrations of ACTH. R137W and the previously described R128C and R146H mutations lie in the second intracellular loop and may impair G protein coupling, although R128C and R146H also impair ligand binding (19).

Most of the clinical features in FGD can be explained by the MC2R gene mutations. Because MC2R mediates both the acute and chronic steroidogenic responses to ACTH, a receptor defect will cause Addison's disease. Cortisol deficiency contributes to diminished steroidal feedback on the anterior pituitary and hypothalamus and promotes increased ACTH secretion, which overstimulates MC1R in melanocytes and causes hyperpigmentation. However, the mechanism underlying the increased growth reported in several patients with MC2R mutations is unclear and is not associated with abnormalities in the GH/IGF-I axis (5, 6). Tall stature is specifically associated with MC2R mutations and is not found in other forms of Addison's disease (25). However, tall stature is also found in patients with MC4R mutations, typically presenting with severe obesity (body mass index >40 kg/m^2) and normal adrenal function (26).

MC2R is a 297-amino acid protein, encoded by a gene on chromosome 18p11.2 (10). The five MCRs are the smallest G protein-coupled receptors and share substantial sequence similarity except in the carboxyl-terminal domain, the first extracellular loop, and the third intracellular loop (27). MC2R shares 50% amino acid sequence identity with MC4R, a 333-amino acid protein encoded by a gene on chromosome 18q21.3; 46% identity with MC5R, a 325-amino acid protein encoded by a gene on chromosome 18p11.2; 45% identity with MC3R, a 361-amino acid protein encoded by a gene on chromosome 20q13.2; and 39% identity with MC1R, a 317-amino acid protein encoded by a gene on chromosome

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TABLE 2. MC2R mutations causing FGD

Mutation	Function		Effect of mutation	Ref.
	Binding assay	Activity		
P27R	ND	ND	Polymorphism	34
I44M	4.4×10^{-7} M[a]	ND	Loss of ligand affinity	14, 19
V45I	ND	$8.8 \pm 1.2 \times 10^{-10}$ M[b]	Polymorphism	16
S74I	1.9×10^{-7} M[a]	6.7×10^{-8} M[c]	Loss of ligand affinity	0, 12, 14, 18
		Not measurable[d]		
D103N	10.6×10^{-7} M[a]	ND	Loss of ligand affinity	4, 19
D107N	$\sim 10^{-7}$ M[e]	$3.0 \pm 0.9 \times 10^{-9}$ M[b]	Loss of ligand affinity	16, 33
		$6.4 \pm 1.3 \times 10^{-9}$ M[f]		
I118 fs[7]	ND	ND	Truncated receptor	19
F119 fs	ND	ND	Truncated receptor	35
S120R	ND	ND	?	13
R128C	4.8×10^{-7} M[a]	ND	Loss of ligand affinity	14, 19
R137W	ND	Not measurable[d]	Loss of signal transduction	0, 4
V142L	ND	ND	?	36
R146H	1.7×10^{-7} M[a]	ND	Loss of ligand affinity	14, 19
T159K	0.5×10^{-7} M[a]	ND	Loss of ligand affinity	19
L192 fs	ND	ND	Truncated receptor	14
R201X	ND	ND	Truncated receptor	13
G217 fs		$4.8 \pm 0.9 \times 10^{-9}$ M[b]	Truncated receptor	16
A233P	ND	ND	?	37
C251F	$\sim 5 \times 10^{-7}$ M[e]	$3.5 \pm 0.5 \times 10^{-9}$ M[b]	Structural disruption	16, 33
		$4.1 \pm 0.9 \times 10^{-9}$ M[f]		
Y254C	ND	Not measurable[d]	Loss of signal transduction	0, 15
P273H	ND	Severely impaired[g]	Loss of signal transduction	38
Wild type	$\sim 7 \times 10^{-9}$ M[e]	5.5×10^{-9} M[c]		18
	1.1×10^{-7} M[a]	5.1×10^{-10} M[b]		16
		2.4×10^{-10} M[f]		33
		6.8×10^{-9} M[h]		19

ND, Not determined; fs, frameshift.

[a] Concentration given 50% inhibition (IC_{50}) in binding assays performed in mouse Y6 adrenocorticocarcinoma cells.

[b] Concentration given 50% effective concentration (EC_{50}) using a transient transfection assay based on mouse Cloudman M3 melanoma cells and cAMP measurement by RIA.

[c] EC_{50} in COS-7 cells transiently transfected; cAMP measurement by RIA.

[d] Concentration given as EC_{50} after transient transfection of mouse OS-3 adrenocorticocarcinoma cells and a cAMP luciferase reporter system.

[e] IC_{50} in binding assays performed in stable transfected M3 cells.

[f] EC_{50} in M3 cells stable transfected; cAMP measurement by RIA.

[g] Transient transfection in M3 cells; cAMP measurement by RIA; activity not quantified as EC_{50}.

[h] EC_{50} in Y6 cells stable transfected; cAMP measurement by RIA.

16q24.3. The five MCRs differ from one another in their tissue distribution and binding affinity for ACTH and the various melanocortins (α-, β-, and γMSH) (28), all of which derive from proopiomelanocortin (29, 30). In contrast to the other four MCRs, MC2R is only activated by ACTH, whereas the other MCRs can be activated by both ACTH and MSH (11, 28). Although the three-dimensional structure of MC2R has not been determined, modeling of MC1R suggests that the binding pocket for the ligand is located between the second, third, and sixth transmembrane domains, with several points of interactions between receptor and ligand (27). Binding of ACTH activates MC2R to form a heterotrimeric G protein complex that activates adenylase cyclase to form cAMP, which stimulates steroidogenesis acutely through the action of the steroidogenic acute regulatory protein (31) and chronically through transcriptionally induced accumulation of mRNAs for steroidogenic enzymes (32).

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