We constructed several mutant human MC4R cDNAs by site directed mutagenesis and expressed these receptors in COS-1 cells. The conserved DRY motif among GPCRs was mutated to generate eight mutants. While no MC4R ligand binding was detected in any of the mutants, one mutant, D146A, resulted in higher cAMP production in cells than the wild-type receptor without ligand stimulation.

Key words: melanocortin type 4 receptor; site directed mutagenesis; cAMP; G-protein coupled receptor; DRY motif

The involvement of melanocortins in anxiogenic-like behavior in experimental animals has been reported. Specifically, melanocortins have been reported to stimulate the stress mediated response via the HPA axis.1–3) Five types of melanocortin receptor have been reported, from MC1R to MC5R. MC2R is an ACTH receptor. Rat MC4R cDNA was cloned and characterized as a seven transmembrane type (7TM) receptor.4) The MC4R is distributed throughout the brain, including the limbic system, and is involved in the regulation of emotional response and behavior.5,6) The MC4R cascade in animals is involved in the stress response, as well as in the induction of obesity.7)

Recently, a nonpeptide MC4 receptor antagonist was reported to exhibit antidepressant-like activity in rodent models.8) Evaluating the interaction of MC4R with a compound (an antagonist or an inverse agonist) requires the generation of constitutively activated MC4R. Some receptors were constitutively activated by introducing mutations for DRY motif, and expressed on CHO cells, it demonstrated the capacity to be constitutively active, and the mutation of D115 resulted in a receptor that was highly constitutively active.14) A constitutively active mouse MC4R was recently constructed by site directed mutagenesis, and an agouti-related protein was confirmed to function as an inverse agonist.15,16) Twenty-three mutants were generated. Among them, M192F mutant resulted in constitutive activity.16)

Since the conserved DRY motif in the 7TM receptor was reported as G-protein coupling, and given numerous reports of the construction of constitutively active receptors, we focused on D146, R147, and Y148 residues in constructing human MC4R mutants. It is known that MC4R is linked to Gs, and that its second messenger is cAMP. These mutated receptors were expressed, and their ligand binding and the second messenger levels were determined, in order to obtain a constitutively active human MC4R as well as to determine how DRY conserved motif relates to receptor activation.

Mutations for DRY motif were introduced using the QuickChange™ site-directed mutagenesis kit (Stratagene, California, U.S.A.), using a recombinant human MC4R cDNA17) as a template. The introduction of mutations was determined by Sanger’s chain termination method. COS-1 cells were transfected with these mutant MC4Rs. For receptor binding, ten μg of the membrane fraction was incubated with 0.2 nM of [125I]NDP-α-MSH (74TBq/mmol, Amersham Biosciences, Japan). One millimolar NDP-α-MSH was used to determine non-specific binding. The cAMP concentration of the transfected cells was measured using a cAMP EIA assay system (Amersham Biosciences, Japan).

Each amino acid was replaced with alanine and the acidic residue was replaced with a basic residue, and vice versa, or aromatic (Y148) to aromatic (F148), according to reported mutagenesis studies. Since MC4R is Gs protein-coupled, we determined cAMP production for each cell, transfected with mutant cDNA. Figure 1
was about 2 nM, while that of the D146A mutant was

vector transfection. The Kd value of wild-type MC4R showed only basal binding levels compared to the mock changed receptor conformation. The D146A mutant shows higher cAMP levels in the cytoplasm without a negative charge at each side chain, the introduction of Since both aspartic acid and glutamic acid have one retained about 20% of the binding of the wild type.

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Another receptor. 3) Figure 2 shows the specific binding was in agreement with a reported mutagenesis study for the mutation is assumed not to have dramatically changed receptor conformation. The D146A mutant showed only basal binding levels compared to the mock (vector) transfection. The Kd value of wild-type MC4R was about 2 nM, while that of the D146A mutant was undetectable (data not shown).

In conclusion, we produced a human MC4R, that shows higher cAMP levels in the cytoplasm without a ligand stimulation by introducing a D146A mutation. This mutant construct might prove to be a promising tool for assessment of inverse agonists of MC4R.


