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Functions of acidic transmembrane residues in human melanocortin-3 receptor binding and activation

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ABSTRACT

The melanocortin-3 receptor (MC3R) is an important regulator of energy homeostasis, inflammation, and cardiovascular function. Inactivating mutations in *MC3R* gene are associated with childhood obesity. How MC3R binds to its ligands has rarely been studied. In the present study, we systematically mutated all ten acidic residues in transmembrane (TM) domains and measured the cell surface expression levels as well as ligand binding and signaling properties of these mutants. Our results showed that of the 19 mutants stably expressed in HEK293 cells, all were expressed on the cell surface, although some mutants had decreased levels of cell surface expression. We showed that with the superpotent analog [Nle^4, D-Phe^7]-α-melanocyte stimulating hormone (MSH), E92, E131, D154, D158, D178, and D332 are important for ligand binding. D121 and D332 are important for binding and signaling. Further experiments using other ligands such as D-Trp^8-γ-MSH, α-MSH and γ-MSH showed that different ligands induce or select different conformations. In summary, we showed that acidic residues in TMs 1 and 3 are important for ligand binding whereas the acidic residues in TMs 2 and 7 are important for both ligand binding and signaling.

1. Introduction

The melanocortin-3 receptor (MC3R) is a member of the superfamily of G protein-coupled receptors (GPCRs). It was originally cloned independently by Cone and Gantz from rat and human, respectively [1,2]. It is expressed in several brain regions, including the arcuate nucleus and ventromedial nuclei of the hypothalamus and limbic system [2]. It is also expressed in a variety of peripheral tissues, including the placenta, heart, and the gut [1], as well as in immune cells such as macrophages [3,4].

During the past few years, the MC3R was increasingly recognized as an important regulator of energy homeostasis, especially fat metabolism. In gene targeting studies, MC3R and melanocortin-4 receptor (MC4R) were shown to have non-redundant roles in regulating energy homeostasis. Whereas the MC4R regulates both food intake and energy expenditure, the MC3R was shown not to affect food intake or energy expenditure [5–7]. Despite normal or decreased food intake and normal energy expenditure, MC3R knockout (KO) mice exhibited increased fat mass (approximately twice that of wild type (WT) littermates) due to increased feed efficiency [6,7]. Mice lacking both the MC3R and MC4R showed exacerbated obesity compared to MC3R or MC4R single gene KO mice, suggesting that the two neural MCRs regulate different aspects of energy homeostasis [6]. These studies were done in C57BL/6J mice. Recently, Gettys and co-workers generated KO mice in Black Swiss; 129, a mouse strain resistant to obesity. This study showed that MC3R KO produced a comparable degree of increased adiposity as the MC4R KO [8]. Recent studies showed

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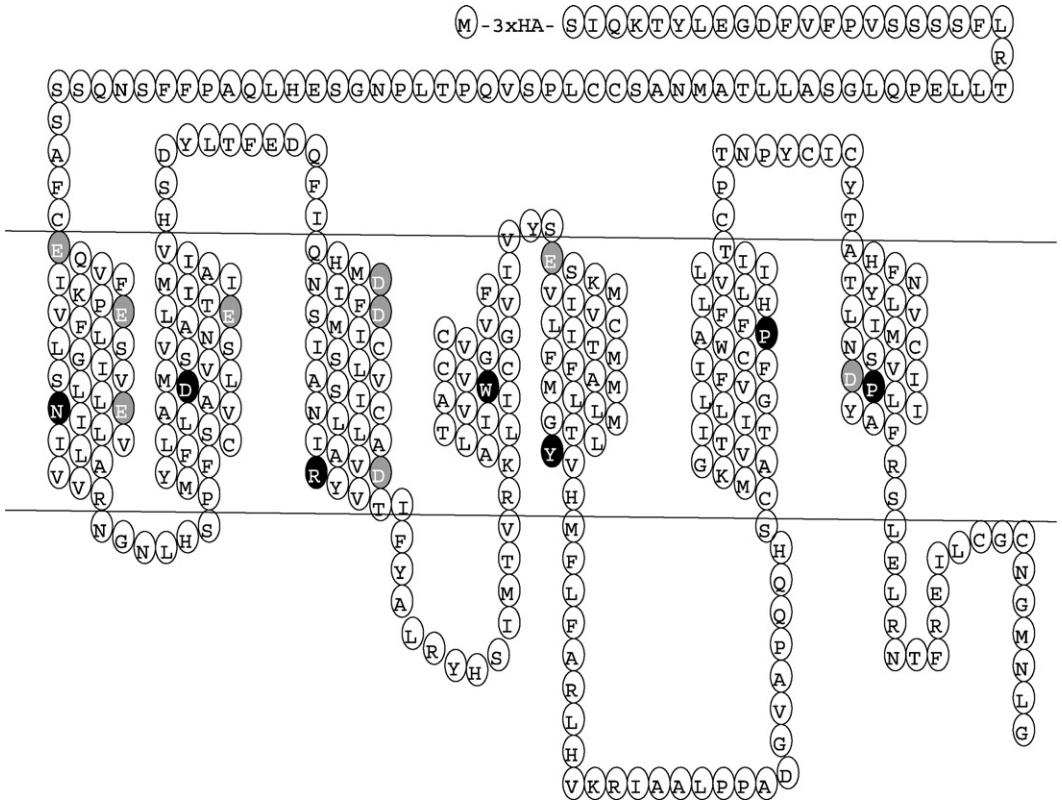

Fig. 1 – Serpentine model of the human MC3R. The most highly conserved residues in each TM are indicated by the white letter on dark background. The residues mutated in this study are highlighted in white letter on gray background except D121 that is also the most highly conserved residue in TM2.

that injections of a selective MC3R agonist D-Trp^{8}-γ-MSH increase food intake in mice [9,10], suggesting that MC3R is also involved in regulating food intake.

Naturally occurring mutations and polymorphisms in the *MC3R* gene have been identified from obese patients. So far, eight *MC3R* variants have been reported, including T6K, A70T, V81I, M134I, I183N, A293T, I335S, and X361S [11–15]. Functional studies revealed a number of defects, including decreased total expression, intracellular retention, and defects in receptor activation [12,14–18]. Clinical studies showed that there is a gene–diet interaction in weight-loss program in carriers of the common polymorphic variants T6K/V81I [19], probably due to defect in substrate oxidation [20], consistent with data obtained in MC3R KO mice [21].

In addition to its role in regulating energy homeostasis, MC3R is also involved in direct regulation of the cardiovascular system, including the heart and blood pressure [22–24] and inflammation [25].

As is typical of GPCRs, the MC3R consists of the typical heptahelical structure, with an extracellular NH_2 terminus and intracellular COOH terminus (schematically depicted in Fig. 1). Previous studies with the MC4R have identified several residues critical for binding to the superpotent analog of α-melanocyte stimulating hormone (MSH), [Nle^{4}, D-Phe^{7}]-α-MSH (NDP-MSH) [26,27]. These include acidic residues D122 and D126 in transmembrane (TM) domain 3 as well as other residues such as hydrophobic residues in TM5 and TM6. Modeling studies (not based on rhodopsin crystal structure) suggested that the hydrophobic pocket of the ligand binding site are different between the MC3R and MC4R, whereas charge–charge interactions between the ligand and the receptor are similar in the two melanocortin receptors (MCRs) [28]. This prediction has not been verified experimentally. Since few studies have been performed to investigate the possible ligand interaction sites on the MC3R, we systematically investigated the functions of ten acidic residues in the TMs of human MC3R (hMC3R) in ligand binding and receptor activation.

In addition to D154 (3.25) and D158 (3.29) in TM3 (corresponding to D122 and D126 in the MC4R), there are eight additional acidic residues that can potentially form a salt bridge with Arg in the pharmacaphore (HFRW) of the ligands [29]. These include: E73 (1.30), E80 (1.37), E92 (1.49), D121 (2.50), E131 (2.60), D178 (3.49), E221 (5.27), and D332 (7.49) Fig. 1 (The numbers in the brackets are numbering based on the Ballesteros and Weinstein system, with the first number representing the helix, and the second number representing the residue's relative position to the most highly conserved residue in that TM designated as 50, decreasing towards the amino terminus, and increasing towards the carboxyl terminus). These ten acidic residues were mutated to investigate their possible roles in ligand binding and signaling.