Study on function of feline melanocortin 4 receptor and melanocortin 2 receptor accessory protein 2

Summary of Doctoral Thesis

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Melanocortin 4 receptor (MC4R), which is a member of the G protein-coupled receptor (GPCR) family, mediates regulation of energy homeostasis upon the binding of a-melanocyte-stimulating hormone (α-MSH) in the central nervous system (CNS). Melanocortin 2 receptor accessory protein 2 (MRAP2) is a membrane protein which modulates the function of MC4R. Deletion, mutations, or polymorphisms of MC4R and MRAP2 gene are associated with obesity in mice and humans. The aim of this study was to elucidate molecular mechanisms of feline obesity and establish genetic diagnosis of feline obesity. For this purpose we performed a series of experiments regarding MC4R and MRAP2.

In the first chapter, we performed cDNA cloning of cat MC4R and MRAP2 and characterized their mRNA expression profiles in order to obtain fundamental knowledge. cDNA cloning revealed the sequences of full-length cat MC4R and MRAP2 cDNA. The deduced amino acid sequences of cloned cat MC4R and MRAP2 displayed high overall sequence identity with other mammalian MC4R and MRAP2. Cat MC4R contained the motifs which are conserved among melanocortin receptor and G protein coupled receptor family. Moreover, the transmembrane regions (TM) of cat MC4R and MRAP2 displayed higher sequence identity with TM of other mammalian MC4R and MRAP2. These results suggest that cat MC4R and MRAP2 gene are conserved, and that cat MC4R and MRAP2 may act as receptor and membrane protein, respectively. On the other hand, cat MRAP2 contained 2 putative N-linked glycosylation sites in the N- (N9) and C-terminal domains (N175). While N9 was observed among many mammals, N175 was not observed in human, mouse, and rat MRAP2.This result suggests that cat MRAP2 might have different function compared to human, mouse, and rat MRAP2. Reverse transcription-polymerase chain reaction analysis revealed that cat MC4R and MRAP2 mRNA were expressed highly in the central nervous system. Similar expression profiles were reported in other mammals. Summarizing the above, cat MC4R may act as a neuronal mediator in the central nervous system and MRAP2 may act as a membrane protein modulating receptor function. Further investigations regarding functions of cat MC4R and MRAP2, their

interactions, and N-linked glycosylation of cat MRAP2 are needed to understand their role in energy homeostasis.

In the second chapter, we performed glycosylation analysis of cat MRAP2 and protein–protein interaction analysis as a preparation of functional analysis. Western blotting with MRAP2 expression construct harboring mutations at each putative N-linked glycosylation site ((N9Q, N175Q, and N9Q+ N175Q) revealed that both N9 and N175 residues are glycosylated in CHO-K1 cells. The glycosylation status at N9 is involved in MC2R signaling in humans, suggesting the glycosylation status at N9 and N175 may be involved in MRAP2 function in cats. In addition, MRAP1, an MRAP2 homolog, adopts reverse topology homodimeric structure. Similarly, it is assumed that MRAP2 also adopts reverse topology. Considering that N-linked glycosylation can take place only on the luminal side of rough-surfaced endoplasmic reticulum (RER) membrane, our results of the glycosylation in the N-terminus and C-terminus supports the reverse topology hypothesis. Protein–protein interaction analysis by co-immunoprecipitation and NanoBiT revealed that interaction dynamics between cat MC4R and MRAP2 in living cells for the first time. Cat MC4R and MRAP2 interact in the basal state, and stimulation with α-MSH increased their interactions slightly. The structural change of MC4R by binding α-MSH may affect the average level of interactions between MC4R and MRAP2 in whole cells. Summarizing the avobe, cat MRAP2 may modulate MC4R signaling near MC4R, and the glycosylation status may regulate its function. Further investigations regarding the role of MRAP2 in MC4R signaling are needed.

In the third chapter, we performed MC4R signaling analysis by luciferase reporter assay in order to know the functions of cat MC4R and MRAP2. Moreover, we performed MC4R homodimerization analysis by NanoBiT in order to clarify the mechanisms underlie the MC4R regulation by MRAP2. A luciferase reporter assay revealed that stimulation with α-MSH increased MC4R-mediated intracellular cAMP production in a dose-dependent manner in CHO-K1 cells transiently expressing MC4R. The EC_{50} values of cat MC4R were similar to that of human MC4R. The presence of MRAP2 increased MC4R-mediated intracellular cAMP production and decreased the

EC_{50} of MC4R. These alterations in the presence of MRAP2 were similar to results from MRAP2 and MC4R experiments in humans. Thus, our results indicate that MC4R can act as an α-MSH receptor in cats, and that MRAP2 may modulate the potency and efficacy of α-MSH toward MC4R in cats. The presence of N175Q, which is mutated MRAP2 construct prepared by replacing asparagine residues (N175) with glutamine, increased MC4R-mediated cAMP production compared to wild type, suggesting that the glycosylation status at N175 is involved in MRAP2 function. Furthermore, there might be a difference in MRAP2 function based on whether they have N175 or not in several species. NanoBiT revealed that cat MC4R can homodimerize in the basal state, and that MC4R homodimer was not affected by α-MSH stimulation in the short-term. Inhibition of MC4R homodimerization results in increased MC4R signaling. In addition, MRAP1 can inhibit MC5R homodimerization. Thus, we and Schonnop et al. hypothesized that dimer or oligomer separation of MC4R by MRAP2 interaction is related to MC4R signaling regulation of MRAP2. To verify the hypothesis, we performed competitive experiments with unfused MRAP2 expression constructs. However, the presence of MRAP2 did not inhibit MC4R homodimerization. Thus, MRAP2 may not alter MC4R homodimerization, suggesting that other mechanisms underlie the MC4R regulation by MRAP2. For example, it is known that MRAP1 modulate ligand binding of MCR. More detailed investigations are necessary to gain a further understanding of mechanisms of MC4R regulation by MRAP2.

In the fourth chapter, we performed SNP typing in cat MC4R and MRAP2 gene and case-control study between single nucleotide polymorphism (SNP) and body condition score in order to evaluate the possible relation between MC4R/MRAP2 genotypes and obesity in cats. The case-control study between SNP and BCS revealed that the genotype of c.*452C/T in the 3' untranslated region of MC4R and the genotype of c.*543T/G in the 3' untranslated region of MRAP2 were correlated with BCS. The frequencies for c.*452T>C and c.*543G>T of overweight cats were higher than that of normal cats. In this study, we did not evaluate the functional significance of these SNPs. However, 3'

untranslated regions are known to regulate mRNA stability, mRNA localization, and translation. c.*452T>C and c.*543G>T might have a functional significance regarding mRNA-based process. Further investigations such as expression analysis are needed to clarify their functional significance. Summarizing the above, although sample number is low, we identified 2 SNPs which is correlated with BCS, suggesting that MC4R and MRAP2 may play some role in weight regulation in cats. In addition, 2 SNPs may be a candidate marker for genetic diagnosis of feline obesity.

Genome-wide association studies (GWAS) for body mass index (BMI), waist-to-hip ratio and other adiposity traits have identified more than 300 SNPs in humans. Consequently, the understanding of molecular mechanisms underlying human obesity has developed rapidly. Among these obesity-related genes, MC4R is strongly related to obesity and has been studied especially. MRAP2 also has attracted a lot of attention as new weight regulation factor since it have become clear that MRAP2 interact with MC4R and is involved in weight regulation recently. However, the molecular mechanisms underlying obesity in cats are hardly understood. As with humans, obesity and following non-communicable diseases (NCDs) have become growing problems for domestic cats. Thus, it is an urgent task for veterinary medicine to develop treatment and prevention method for obesity. In this study, we clarified first structure of MC4R and MRAP2, their mRNA expression profile, their interactions, functions of N-linked glycosylation of MRAP2, MC4R homodimerization, and relation between their SNPs and BCS. Since MC4R has also attracted a lot of attention as drug discovery target, our findings can make a contribution to developing new treatment for obesity. In addition, further understanding of receptor regulation by MRAP2 may lead to find new mechanisms of receptor regulation. Two SNPs we identified in this study may be a candidate marker for genetic diagnosis of feline obesity. If genetic diagnosis of feline

obesity is established with other obesity-related gene, we can predict the obesity risk before onset, and can prevent obesity and NCDs by dietary and exercise management. Consequently, it is being expected to contribute to improving the quality of life of cats.