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Expression, Purification and NMR Studies on MC4R-TM2 Mutant

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Abstract: Melanocortin-4 receptor (MC4R) subtype is associated with obese humans. Especially, in a patient with severe early-onset obesity, novel heterozygous mutation in the MC4R gene was detected, resulting in an exchange of aspartic acid to asparagine in 90^{th} amino acid residue located in the predicted second trans-membrane domain (TM2). Mutations in the melanocortin-4 receptor (MC4R) gene are the most frequent monogenic causes of severe obesity which have been described as heterozygous with loss of function. In order to compare structure difference between MC4R wild type (MC4R-TM2-wt) and mutant (MC4R-TM2-D90N), we designed both MC4R-TM2-wt and MC4R-TM2- D90N construct in pET 21b vector. In this study, we optimized high-yield purification procedure for recombinant TM2-D90N.Eluted recombinant protein was resolubilized under urea condition for thrombin cleavage reaction and we conducted the high-performance liquid chromatography (HPLC) with reverse phase column under 1% acetonitrile, 0.01% TFA buffer solution. The molecular size of purified target peptide was confirmed by Tricine-SDS page analysis. To characterize MC4R-TM2-D90N, we have performed ^{15}N-isotope labeling of peptide using M9 media and purified labeled target peptide for hetero-nuclear NMR spectroscopy.

Keyword: MC4R, Tans-membrane, 6M Guanidium HCl, NMR, High performance liquid chromatography, Cloning, Purification

INTRODUCTION

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The melanocortin receptors are members of the G protein-coupled receptor (GPCR) super family that have seven trans-membrane (TM) domains[1]. Five melanocortin receptors are involved in a multitude of physiological functions including thermoregulation, obesity, learning, memory and other biological functions. Two melanocortin receptors, MC3R and MC4R have been especially studied as modulators of feeding and obesity[2-4], however, the functional roles of both MC3R and MC4R and ligand binding mode are still not clear. The melanocortin-4 receptor (MC4R) subtype has been identified recently by genetic studies in obese humans. Especially, in a patient with severe early-onset obesity, novel heterozygous mutation in the MC4R gene was detected, resulting in an exchange of aspartic acid to asparagine in 90^{th} residue located in the second trans-membrane domain (TM2). Mutations in the melanocortin-4 receptor (MC4R) gene are the most frequent monogenic causes of severe obesity which have been described as heterozygous with loss of function[5-8]. Therefore, we initiated the structural and functional studies on MC4R TM2 mutant (MC4R-TM2-D90N) using the nuclear magnetic resonance (NMR) spectroscopy[10-12]. The purified peptide has been confirmed and characterized by Tricine-SDS-PAGE and HPLC. NMR spectroscopy has revealed that it forms a stable helix in the presence of detergent.

EXPERIMENTAL

Cloning and expression of MC4R-TM2 D90N mutant

The MC4R TM2 domain was cloned in the expression vector, pET 21b containing histidine tag at N-terminal. PCR was used to amplify the MC4R TM2 domain from full length MR4R with BamHI/XhoI restriction site and thrombin recognition site (LVPRGS) was added at sense primer to cleave tag of the fusion protein. Sense primer was used as a 5' - CGC GGA TCC CTG GTG CCA CGC GGT TCT AGC CCG ATG TAC TTT - 3' and anti-sense primer 5' - CCG CTC GAG TCA CTT TTT CTT TTT GCC ACC CAG GGT AAT CAC GAT GGT – 3'. Similarly, point mutation was made by another primer. Plasmid containing MC4R-TM2-D90N was over- expressed in *Escherichia coli* strain. BL21 (DE3) and the LB (Lauria-Bertani) plates were incubated overnight at 37˚C with 0.1mg/ml Ampicillin. Positive colonies were grown in LB media (small scale) at 37˚C. Well grown cells were transformed into large scale until the $O.D_{600}$ reached 0.5, at this O.D induction was done by adding 1mM IPTG and cultured at 25˚C in 180 rpm until $O.D_{600}$ of 1.5. Protein expression was confirmed using Tricine-SDS-PAGE analysis.

Isotope (^{15}N) labeling of MC4R-TM2-D90N

For hetero-nuclear NMR experiment, we prepared isotope-labeled form of MC4R-TM2-D90N. Cells were cultured in M9 minimal media containing ^{15}N-labeled NH_4Cl (^{15}N, 99%, Cambridge Isotope Laboratory, Inc.) at 37˚C. Protein expression was induced by 1mM IPTG (isopropyl-beta-D-

thiogalactopyranoside) when the cell density reached $O.D_{600}$ of 0.6, then the cells were cultured at 25˚C for 18hours till $O.D_{600}$ reached to 1.5.

Purification of MC4R-TM2-D90N

The cells containing fusion proteins (His_6-Thrombin-Target protein), were sonicated in a lysis buffer (20mM Tris-HCl, 200mM NaCl, 5% Glycerol, 5mM ß-mercaptoethanol, pH 7.2). Protease inhibition cocktail (Roche) of 50μl was added to inhibit protease activity. The cell lysate was centrifuged at 14000rpm at 4˚C for 30min. The inclusion body was denatured using by binding buffer (20mM Tris-HCl, 200mM NaCl, 5mM Imidazole, 6M Guanidine HCl, pH 8.0) overnight, room temperature, with stirring. The denatured inclusion body was centrifuged same condition, after finished centrifuge clear supernatant was loaded in Ni-NTA open column (affinity chromatography). Fusion proteins were eluted with 350mM imidazole in elution buffer and immediately transferred to 1 kDa membrane dialysis bag in order to remove the guanidine HCl and dialyzed 1% ACN, 0.01% TFA buffer 3 times for 3hour each time. After dialysis, fusion proteins were removed guanidine HCl, salt, imidazole and dissolved in dialysis buffer. The soluble fusion peptide was collected and lyophilized. The lyophilized fusion peptide was dissolved in 8M urea, 50mM Tris HCl pH 7.6 buffer and urea concentration was decreased until 200mM for Thrombin cleavage. Fusion peptide was incubated with 0.1X Thrombin protease at 37˚C for 4 hours and the products after cleavage were dialyzed against 1% ACN, 0.01% TFA buffer at room temperature in order to remove the urea. The

dialyzed solution was lyophilized and confirmed by Tricine-SDS-PAGE. To achieve high purity and size confirm of the target peptide, we performed the HPLC purification. MC4R-TM2-D90N peptides were purified using HPLC C8 column in a linear gradient of acetonitrile (ACN, 10–80%), 0.1% trifluoroacetic acid (TFA) at a flow rate of 1ml/min. Absorbance was monitored at both 220 nm and 280 nm using a PDA detector.

NMR spectroscopy

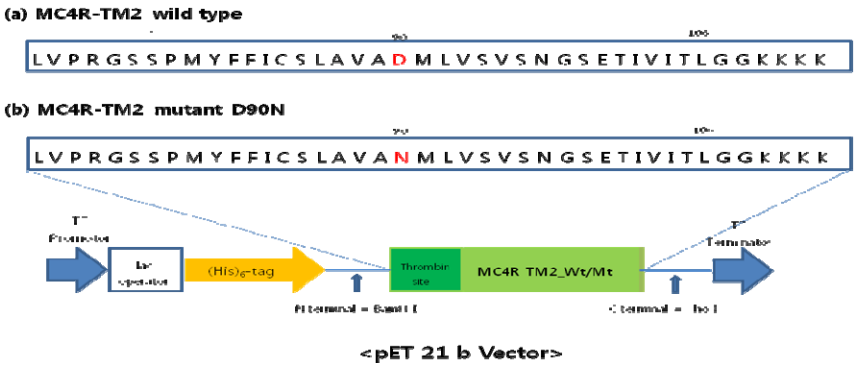
NMR experiments were performed on a Bruker DRX-500 spectrometer in quadrature detection mode using a triple-resonance probe equipped with an actively shielded pulsed field gradient (PFG) coil. Pulsed-field gradient techniques were used for all experiments to suppress the strong solvent signal[9]. The purified MC4R TM2 mutant D90N powder sample was dissolved in 200mM d-SDS, 20mM Tris buffer for NMR experiments. The protein concentration was about 0.3mM. To identify optimum condition for structural study, we executed ^{1}H-^{15}N 2D-HSQC experiment[10-12] in 3 different pH conditions (pH 4.0, 6.0, 7.0). All NMR data were processed using NMRPipe/NMRDraw (Biosym/Molecular Simulations, Inc.) or XWIN-NMR (Bruker Instruments) software on a Linux workstation. The proton chemical shifts were referenced with internal sodium 2, 2 -dimethyl-2-silapentane 1-sulfonate (DSS).

RESULTS AND DISCUSSION

Domain construction of MC4R-TM2-D90N

We designed MC4R-TM2-D90N ranged from 77 to 106 based on secondary structure prediction by molecular modeling method. MC4R-TM2-D90N has been cloned into the *E. coli* over expression vector pET21b and expressed as His_6-Thrombin cleavage site fusion protein (Fig. 1A). Sequence alignment for MC4R-TM2 region is shown in (Fig. 1B). All sequences of MCRs showed high degree of sequence homology. Secondary structure prediction suggests that MC4R TM2 consisted of α-helix and turn structure (Fig. 1B).

A.



B.

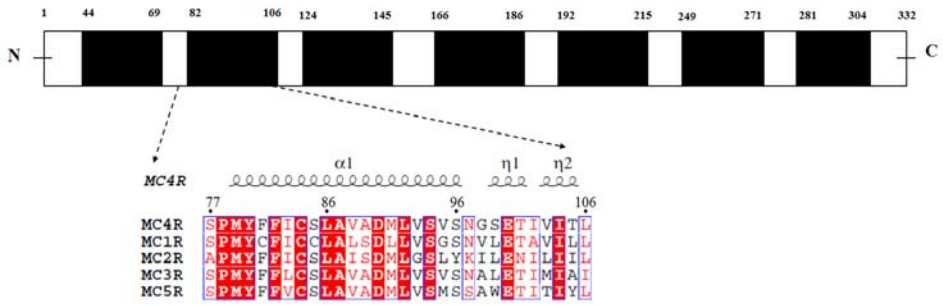


Figure 1. Vector map of MC4R-TM2-D90N for cloning and sequence alignments for five MCRs. (A) MC4R-TM2-D90N construct with amino acid sequence from 77 to 106 with an extra sequence, GGKKK. (B) Amino acid sequences for five MCR are aligned, showing a high sequence homology among five MCRs.

Purification and characterization of MC4R-TM2-D90N

E. coli cells cloned with MC4R TM2 mutant D90N domain were cultured in M9 minimal medium. At $O.D_{600}$ of 0.6, protein induction was done by 1 mM IPTG and cultured at 25 ˚C for adequate induction and harvested after 18 hours. The cells were sonicated to obtain the fusion protein (His_6-Thrombin-Target protein). After sonication, insoluble fusion proteins were denatured by 6M guanidine HCl and purified using Ni-NTA affinity chromatography. In order to remove guanidine HCl, the solution was dialyzed 1% ACN, 0.01% TFA buffer 3 times for 3hour each time. The fusion proteins were dissolved in guanidine HCl, salt, imidazole dialysis buffer, and were finally collected and lyophilized. The lyophilized fusion protein was dissolved in 8M urea, 50mM Tris HCl at pH 7.6 buffer solution and decreased concentration of urea up to 200mM urea for effective cleavage of the fusion-tag. We confirmed target proteins size using Tricine-SDS-PAGE after cleaving the fusion tag (Fig. 2A). The final peptide was collected from HPLC (High-performance liquid chromatography) analysis. A reverse phase HPLC chromatograms of MC4R-TM2-D90N showed that the peptide was eluted at 20% ACN concentration. The purified target peptide was confirmed by Tricine-SDS-PAGE

(Fig. 2A), gel filtration HPLC (Fig. 2B) and one-dimensional ^{1}H NMR spectrum (Fig. 2C). Final yield of the MC4R-TM2-D90N peptide is calculated as 3mg/500ml in M9 minimal medium.

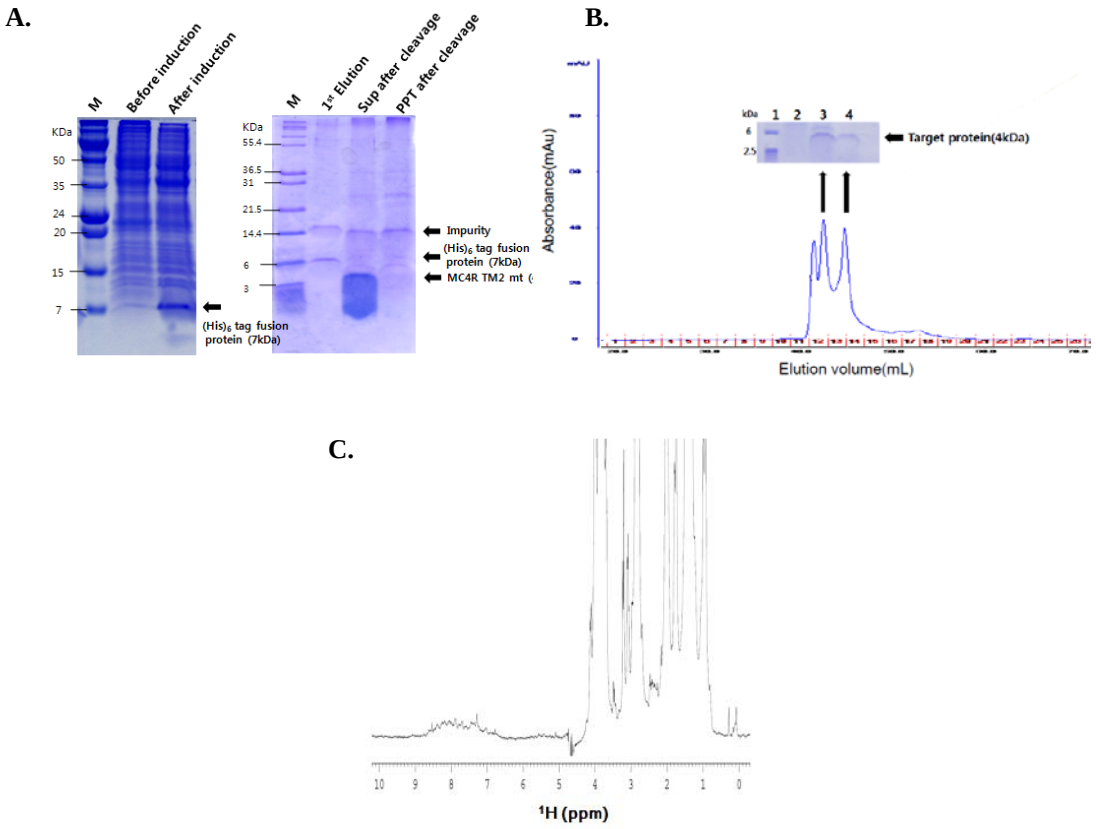


Figure 2. Tricine-SDS-PAGE, Reverse phase HPLC profile and NMR spectrum of MC4R-TM2-D90N. (A) Tricine-SDS-PAGE of the MC4R-TM2-D90N shows the expression pattern and existence of the fusion proteins from Ni-NTA open column. Fusion protein eluted was cleaved using thrombin. (B) The peptide has been identified as a monomer in 20% ACN concentration. (C) ^{1}H NMR spectrum

was recorded in 200mM d-SDS, 20mM Tris buffer, at 313K using on a Bruker DRX 500 MHz spectrometer.

pH dependence of MC4R-TM2-D90N

We performed a series of ^{1}H- ^{15}N HSQC experiments with different pH conditions, looking for the optimum experimental condition (Fig. 3). ^{1}H- ^{15}N HSQC spectrum at pH 7.0 provides most of resonance peaks with strong intensities, implying that the peptide could be stable at this experimental condition (Fig. 3C). Fig. 3D shows spectral comparison of wild type with MC4R-TM2-D90N at pH 7.0. Preliminary back-bone resonance assignment of MC4R-TM2 suggests that two spectra are mostly identical except resonance peak of D90, which is indicative of D90 mutation. The spectra suggest that MC4R-TM2-D90N exists as a monomer in 200mM SDS micelle.

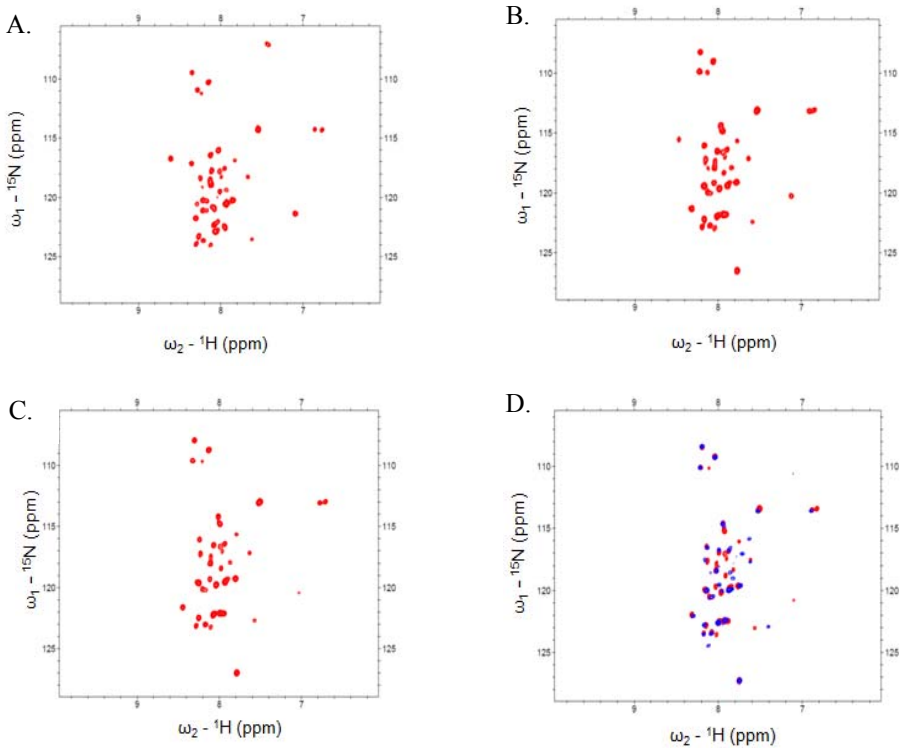


Figure 3. ^{1}H-^{15}N 2D HSQC spectra of MC4R-TM2-D90N at different pH conditions. The concentration of the peptide is 0.3mM dissolved in 200mM d-SDS, 20mM Tris buffer, 10% D_2O at 313K. ^{1}H- ^{15}N 2D-HSQC experiment were performed at pH values of 4.0 (A), 6.0 (B), 7.0 (C) on Bruker DRX 500MHz equipped with Cryoprobe. (D) ^{1}H- ^{15}N 2D-HSQC spectrum of MC4R-TM2 wild type (blue) is compared with that of mutant (red) at pH 7.0, respectively.

CONCLUSION

Mutant peptide of MC4R-TM2 at D90 has been successfully cloned and purified using *E. coli* expression vector containing thrombin cleavage site. MC4R-TM2-D90N was dissolved as a denatured form using 6M guanidine HCl and further refolded. Purified peptide after HPLC was size confirmed by Tricine-SDS-PAGE, one-dimensional ^{1}H NMR and 2D ^{1}H- ^{15}N 2D-HSQC spectra in SDS micelle environment. Final yield of the MC4R-TM2-D90N peptide is calculated as 3mg/500ml in M9 minimal medium. Data from two dimentional ^{1}H- ^{15}N 2D-HSQC provided an optimum condition for further structural study of MC4R-TM2-D90N.

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