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Expression of Human Melanocortin 4 Receptor in *Saccharomyces cerevisiae*

Research Article

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Abstract: The melanocortin 4 receptor (MC4R) is involved in the regulation of energy homeostasis and is known as one of the major hypothalamic regulators of food intake. Several studies have shown that replacement of aspartic acid at position 126 of the MC4R abolishes the ligand binding. We used the modified yeast *Saccharomyces cerevisiae* strain MMY28 to functionally express the MC4R and characterise the importance of this amino acid for ligand based activation of the receptor. The efficiency of the functional expression system was estimated by activation with αMSH, ACTH and THIQ and compared with cAMP response in mammalian cells. We generated the library of MC4R mutants randomised at the amino acid position 126. Recombinant MC4R clones were screened for the αMSH induced activity in yeast. From 9 different amino acids obtained only the natural aspartic acid displayed the ligand dependent activity of MC4R. The MC4R variants with glutamic acid and leucine at position 126, however, displayed higher background activity than other amino acid substitutions. The results suggest that the yeast expression system is suitable for screening of the MC4R receptor ligands and that the substitution of aspartic acid at position 126 of MC4R by different amino acids functionally inactivates the receptor.

Keywords: *G-protein coupled receptors • Saccharomyces cerevisiae • Melanocortin receptors • Functional screens*

Abbreviations

ACTH	- adrenocorticotropin hormone;
AT	- 3-Amino–1,2,4-triazole;
GPCR	- G protein-coupled receptors;
MC4R	- melanocortin 4 receptor;
MSH	- melanocyte-stimulating-hormone;
THIQ	- tetrahydroisoquinoline.

1. Introduction

The melanocortin type 4 receptor (MC4R) is a member of the G- protein coupled receptor family. Five different melanocortin receptors have been discovered in humans [1-4]. Natural agonists of MCRs are specific peptides – melanocortins that are enzymatically cleaved from a single polypeptide precursor – proopiomelanocortin [5-7]. Different melanocortins, α-melanocyte-stimulating-hormone (αMSH), βMSH, adrenocorticotropic hormone (ACTH) and γMSH act as agonists on MC4R [3,4,8-11].

The MC4R is mostly expressed in the ventromedial, dorsomedial, paraventricular and arcuate hypothalamic nuclei regions of the central nervous system, controlling food intake [10,12-14]. It has been shown that MC4R knock-out mice have severe adiposity that is caused by a hyperphagic phenotype [15]. To a lesser extent, the MC4R is involved in the regulation of different autonomic, neuroendocrine, anti inflammatory, stress and sexual functions [12,16,17].

Since the main role of the MC4R in an organism is the control of feeding behaviour and energy homeostasis it has become a potential drug target for adiposity treatment. A number of novel agonists and antagonists activating the MC4R have been described, and different studies conducted to characterize MC4R receptor-ligand interactions [18-23].

So far the functional studies of the MC4R have revealed two conformationally important binding sites of the melanocortin pharmacophore His-Phe-Arg-Trp. The first binding site is the negatively charged conformation pocket of MC4R, formed by E100, D122

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and D126 [18,24], which is interacting with the amino group of arginine residue from the pharmacophore sequence. The second binding site is the hydrophobic cavity that is formed by F184, F202, F261, F262 and F284 residues of the MC4R [25] (Figure 1). It has been shown that phenylalanine and tryptophan residues of the pharmacophore sequence are involved in strong hydrophobic interaction with MC4R in this pocket [23].

Previously the importance of D126 residue in the MC4R has been shown by functional characterization of mutant human MC4R containing the substitutions with alanine and asparagine at this position, and mutant mouse MC4R containing the substitution of the corresponding amino-acid with lysine. All substitutions abolished the ligand binding properties of the receptor [18,25].

Expression of GPCRs in yeast *Saccharomyces cerevisiae,* which is engineered to couple with the mammalian GPCRs and requires activation of the receptor for growth, has been used to pharmacologically characterize a number of various receptors [26-31]. The advantages of such an approach in GPCR research include low cost propagation, automation of the screening and a low level of false positive results that are common in mammalian cells due to endogenous expression of ligands or related receptors. Another advantage of the growth dependant yeast screening system is the possibility to select the variants of the functional receptor from the randomized expression libraries [32,33]. So far functional expression of the melanocortin receptors in yeasts has not been described.

In this study we demonstrate the functional expression of MC4R in yeasts and perform the characterization of the library of MC4R constructs with randomized amino acid at position 126.

2. Experimental Procedures

2.1 Mammalian cell culture and cAMP assays

HEK 293 cells were grown in Dulbecco's Modified Eagle's medium (Sigma, USA). Human MC4R cloned in pcDNA3 vector was transfected using TurboFect™ *in vitro* transfection reagent (Fermentas, Lithuania) following the producer's protocol. The transfected HEK 293 cells were diluted and distributed in 384-well plates to concentration 1 x 10^4 cells/well. The cells were incubated with the serially diluted concentrations of αMSH and ACTH. Intracellular cAMP levels were measured using LANCE^{tm} cAMP 384 Kit (PerkinElmer, USA) following the recommendations of the manufacturer on Victor$^3_{TM}$V reader (PerkinElmer, USA). The cAMP concentrations were quantified by comparison to a standard curve of the known amounts of cAMP provided in the kit.

2.2 Cloning and construction of mutagenised MC4R library

A human MC4R gene was cloned from human genomic DNA using MC4R-Fw (ATTGGGAAGCTTATGGTGAACTCCACCCACCGT) and MC4R-Rs (AAGCTCGAGTTAATATCTGCTAGACAAGTCA)

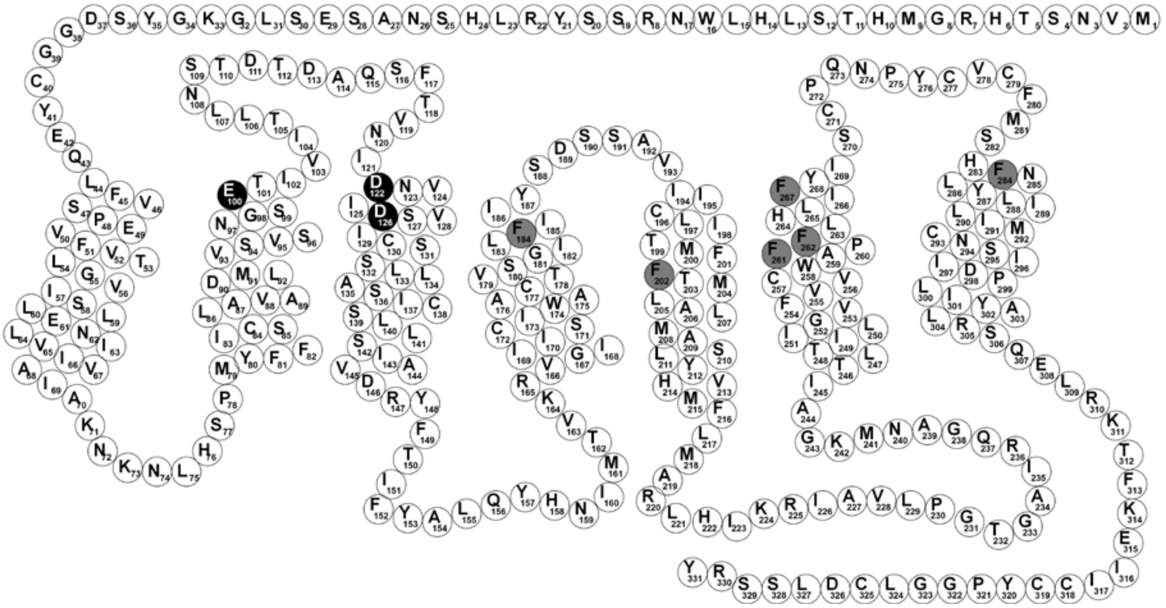

Figure 1. Residues of MC4R involved in receptor – melanocortin interaction. Residues shaded in black are amino-acids involved in formation of the acidic ligand binding pocket. Those shaded in grey are amino-acids involved in formation of the hydrophobic ligand binding pocket.

primers which contained *Hind*III and *Xho*I restriction sites respectively (underlined).

The mutagenised library of MC4R was constructed by PCR using *Pfu* polymerase (Fermentas, Lithuania) and oligonucleotides D126rand-Fw (CATTGATAATGTCATTNNNTCGGTGATCTGTAGC) and D126rand-Rs (GCTACAGATCACCGANNNAATGACATTATCAATG) (Operon, Germany) containing randomized trinucleotide (NNN) that corresponds to amino acid D126 of MC4R. The mutagenised PCR product was directly sequenced using primers MC4R-Fw and MC4R-Rs to confirm heterogeneity of randomization using 3130xl Genetic Analyser (Applied Biosystems, USA). The randomized library of the MC4R was cloned in *HindIII/XhoI* sites of the yeast shuttle vector p426-TEF that contains the URA gene for auxotrophic selection [34].

2.3 Yeast transformation

Yeast *Saccharomyces cerevisiae* strain MMY28 (MATa his3 leu2 trp1 ura3 can1 gpa1Δ: Gs far1Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU::FUS1-lacZ ste2Δ::G418^{R}) was kindly provided by Dr S.J. Dowell. The yeast strain expresses the chimeric yeast Gα/ human G$_{s}$α subunit, and has a receptor activation system fused with a specific signalling pathway to reporter genes enabling β-galactosidase production and histidine synthesis.

Yeast cells were grown overnight in 4 ml YPD medium (20 g/l bactotryptone, 10g/l yeast extract, both purchased from Difco Laboratories, USA). The transformation of yeasts with a randomized library was carried out in 100 mM lithium acetate (Sigma, Germany) and 0.2 mg/ml salmon sperm DNA (Sigma, USA), yeasts were heat shocked for 40 min and then harvested and transferred to Petri plates containing SC medium agar supplemented with 1.92 g/l yeast synthetic drop-out medium without uracil, 1.7 g/l yeast nitrogen base, 5 g/l ammonium sulfate, 2% glucose, 16.67 g/l agar (all purchased from Sigma, Germany).

2.4 Functional activation of receptor variants

After transformation, colonies from selective agar plates were harvested in 2 ml of liquid SC medium lacking uracil and grown overnight. Functional activation was carried out in 96-well plates (Sarstedt, USA) in 200 ml SC medium without histidine and uracil, but containing 2 mM AT (3-Amino–1,2,4-triazole by Sigma, Germany) which is a competitive inhibitor of imidazoleglycerol-phosphate dehydratase used to suppress the background synthesis of histidine, 10xBU salts (70 g/l Na2HPO4.7H2O, 30 g/l NaH$_{2}$PO$_{4}$ adjust to pH 7 with 2 M NaOH, all purchased from Реахим, Russia), 0.1 mg/ml chlorophenol - red-β-D-galactopyranoside (Roche, Germany) and various concentrations of αMSH, ACTH (PolyPeptide group, France) and THIQ (kindly provided by the Institute of Organic Synthesis of Latvia). Cells were diluted within an activation medium to a concentration of 250 cells/ml. Negative control of each clone was used containing activation medium without ligand to determine the background activity of receptor variants. The concentration of αMSH, ACTH and THIQ varied from 30 mM to 10 nM. Cells were grown at room temperature for 24 h. OD at 595 nm was measured using a Victor$^{3}_{TM}$V reader (PerkinElmer, USA). All activation assays were performed in duplicate and were repeated at least 3 times.

2.5 Data analysis

Data analysis was performed using GraphPad Prism software (GraphPad Software, USA). Data sets were normalized according to the highest and lowest values, and transformed using the function X=log(X). EC$_{50}$ values were calculated automatically.

3. Results

The human MC4R was expressed in modified *Saccharomyces cerevisiae* strain MMY28, which has a specific reporter system that upon activation of the expressed receptor allows the growth on medium lacking histidine. In addition, the β-galactosidase reporter gene permits the quantitative estimation of functional activity using the enzymatic colour reaction. In these yeast cells, agonist mediated MC4R activation induced the expression of the β-galactosidase gene in a dose-dependent manner. To validate the functional activity of MC4R and estimate the efficiency of the expression, yeast cells were activated by two endogenous ligands, αMSH, ACTH and a small molecule agonist THIQ (Figure 2a, Table 1).

	EC$_{50}$±SEM (nM)	
Ligand	*S. cerevisiae* ^{a}	HEK293 ^{b}
αMSH	269.0±28.0	5.81±1.9
ACTH	223.9±0.2	6.81±1.1
THIQ	490.4±11.8	nd

Table 1. Effects of αMSH, ACTH and THIQ on stimulation of wt MC4R activity.

nd – not detected
a- calculated from β-galactosidase production levels in Saccharomyces cerevisiae;
b- calculated from cAMP production levels in HEK293 cells

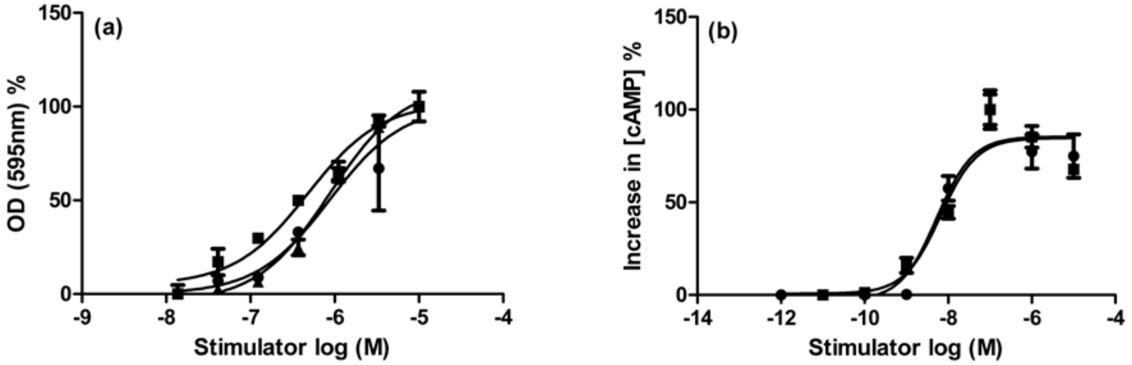

Figure 2. MC4R activation curves: (a) reporter gene expression in *Saccharomyces cerevisiae* strain MMY28 activated with αMSH (●), ACTH (■), THIQ (▲); (b) cAMP response curves in HEK293 cells activated with αMSH (●), ACTH (■). cAMP and OD are expressed as a percentage with the basal level set to 0% and the highest level in each data set to 100%.

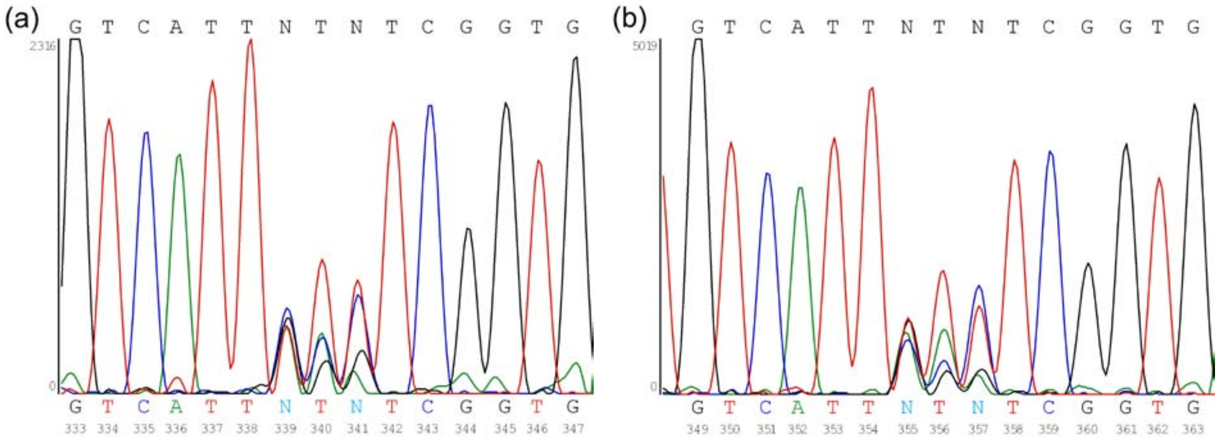

Figure 3. Sequence chromatograms representing the randomisation of MC4R at position 126: (a) sequence of PCR fragment; (b) sequence of the plasmid isolated from the pooled *E. coli* clones.

The ligand affinity curves and the obtained EC_{50} values show that in the yeast expression system the MC4R has a higher affinity for αMSH and ACTH as compared to THIQ. In order to compare the pharmacological properties of the MC4R expressed in the yeasts with those of mammalian cells, we performed a cAMP estimation in HEK293 cells transiently transfected with the MC4R upon incubation with αMSH and ACTH (Figure 2b). The basal level of cAMP was 0.34 pmol/10^4 cells while the maximal level of cAMP upon stimulation with the highest concentrations of αMSH and ACTH was 80 nmol/10^4 cells and 125 nmol/10^4 cells respectively.

The potency of MC4R to induce cAMP production upon activation in HEK293 cells was characterized by EC_{50} values that were similar to the previously published ones [35]. These values were 46 and 32 times lower than EC_{50} values of the αMSH and ACTH induced reporter gene expression in the yeast expression system respectively (Table 1), indicating that the yeast expression system has sufficient sensitivity for MC4R ligand testing.

The library of MC4R expression plasmids with various amino acid codons at position 126 was constructed by site directed mutagenesis using synthetic oligos with randomized nucleotides at specific positions. The heterogeneity of the obtained randomized library was confirmed by sequencing both: the PCR product before the cloning in yeast shuttle vector p426TEF and the plasmid isolated from the pooled *E. coli* cells after transformation (Figure 3).

Since we did not succeed in our initial attempt to perform functional selection of yeast clones containing the active receptor variants on ligand containing agar plates (data not shown), we continued with characterization of individual clones.

Fifty clones where selected and screened by direct sequencing. Twenty-four of all yeast clones analyzed contained more than one different plasmid as revealed from the presence of multiple sequencing chromatogram peaks at randomized positions. As many as 26 sequencing chromatograms contained no additional peaks at the corresponding positions, indicating the presence of plasmid with unique codon. In total, 14 different codons encoding 9 amino-acids were identified. Representation of these amino-acids in the pool of clones as well as the number of individual codons is shown in Table 2.

The obtained clones were further analysed by functional activation in the liquid yeast cultures supplemented with serial dilutions of αMSH and 2 mM AT for suppression of the background activation. All obtained wt MC4R clones were active upon stimulation and displayed similar EC_{50} values. At the same time none of the mutant MC4 receptors was able to induce the reporter gene expression (Figure 4, Table 3).

The same observation was made during the activation with THIQ (data not shown) indicating the importance of aspartic acid at position 126. Nevertheless, the glutamic acid and leucine variants displayed the increased levels of the basal reporter gene expression compared to other mutants. To confirm that this activity is not due to some physiological properties of the specific yeast cell clone or changes in plasmid sequence, we performed

Codon	Number of codons	Amino acid	Side chain characteristics
ATT	4	Asparagine	Polar, hydrophilic, neutral
GAC	5	Aspartic acid	Polar hydrophilic charged (-)
TGC	1	Cysteine	Polar, hydrophobic, neutral
GAA	1	Glutamic acid	Polar hydrophilic charged (-)
GGG	1	Glycine	Aliphatic, neutral
GGT	1	Glycine	
ATC	1	Isoleucine	Aliphatic, hydrophobic, neutral
ATA	1	Isoleucine	
CTA	2	Leucine	Aliphatic, hydrophobic, neutral
CTT	2	Leucine	
TTT	3	Phenylalanine	Aromatic hydrophobic neutral
TTC	1	Phenylalanine	
GTT	1	Valine	Aliphatic hydrophobic neutral
GTC	2	Valine	

Table 2. List of codons at amino-acid position 126 of MC4R identified from sequencing of individual clones.

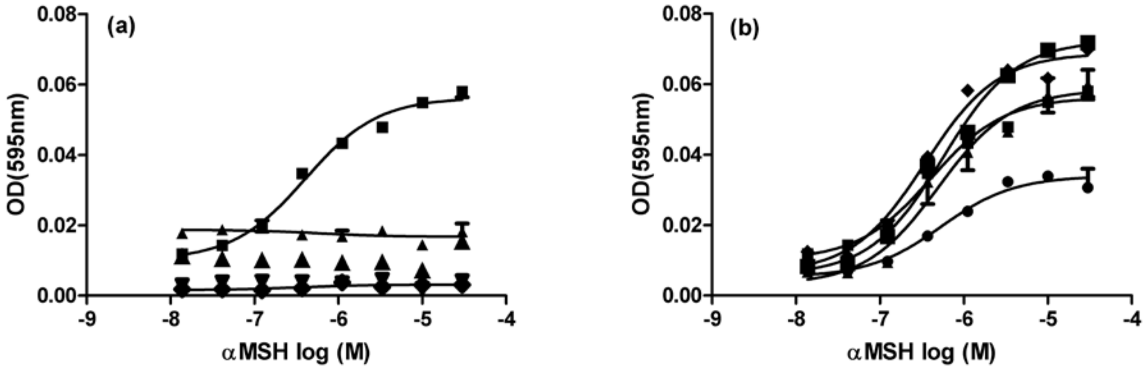

Figure 4. MC4R activation curves of individual MC4R codon variants: (a) wild-type (■) glutamic acid GAA (▲), leucine CTT (▲), phenylalanine TTT (▲) and phenylalanine TTC (♦); (b) several aspartic acid GAC (●), (▲), (▼), (■) variants.

the isolation of several recombinant MC4R constructs containing leucine and glutamic acid and retransformed them (as previously described by [27]). The same increase in reporter gene expression was observed for additional leucine and glutamic acid variants (Figure 5).

4. Discussion

In this study we demonstrate for the first time the functional expression of MC4R in yeasts. We used this expression system to explore the importance of amino acid residue at position 126 of human MC4R. Our results show that wt MC4R is activated in yeast cells by at least three different ligands: the short natural peptides αMSH and ACTH, and small molecule agonist THIQ. αMSH and ACTH displayed pharmacological properties comparable to MC4R expressed in mammalian cells. However, the potency of activation in yeasts was 30-40 times lower compared to mammalian cells (Table 1). A similar difference in ligand affinities between two expression systems has been observed also for adenosine A2b receptors [27,36]. One of the main differences between mammalian and yeast expression systems is the presence of the polysaccharide-rich yeast cell wall that has to be crossed by ligands in order to reach the receptors located in a plasma membrane. Our results show no relative difference in activation potency depending on the ligand size. THIQ, which is significantly smaller (Fw=589.169), actually has a lower affinity than peptide ligands and even the 39-amino acid peptide ACTH (Fw=4541.11) is capable of migrating through the yeast cell wall and activating the MC4R. It is reported that THIQ has approximately the same affinity for MC4R as αMSH [24,37]. The lower activity in yeasts may be explained by instability of THIQ in yeast culture where the activation process takes a significantly longer time when compared to the classical cAMP experiments. It cannot be excluded that THIQ may interact with the yeast polisaccharide-rich cell wall resulting in decreased affinity.

Yeast expression systems have been used to select the functionally active variants from G-protein coupled receptor libraries [26,38], or to isolate constitutively active receptors [39]. However, so far the GPCRs that bind small molecules as agonists have usually been explored [27,30,31]. For the analysis of GPCRs, which bind larger peptides, co-expression of ligand and receptor in the same yeast cells has been used [26,29]. The extracellular activation of the V2 vasopressin receptor with the 9-amino acid vasopressin peptide has been reported for the peptide binding GPCRs in yeast cells [28].

MC4R variant	EC$_{50}$±SEM (nM)^a
GAC wt	269.0±28.0
GAC c11*	535.2±15.3
GAC c18*	346.0±44.3
GAC c24*	497.4±12.1
GAC c27*	563.1±19.0

Table 3. β-galactosidase production in response to αMSH induced activation of different MC4R clones with aspartic acid at position 126.

**c11, c18, c24, c27 represent different clone id number*
a- calculated from β-galactosidase production levels in S. cerevisiae

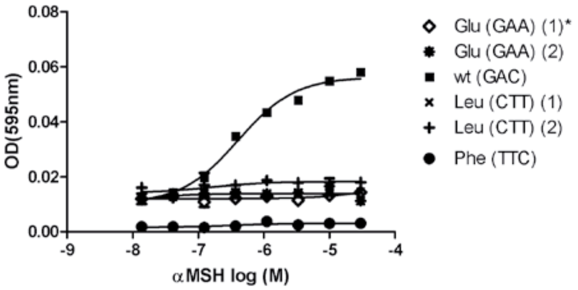

Figure 5. MC4R activation curves of individual MC4R codon variants after isolation and retransformation in *S. cerevisiae*. * - Amino acid (codon) (isolated clone No.).

Aspartic acid at position 126 is shown to be important for MC4R activity in a number of mutagenesis studies [18,24,25]. Typically, the neutral amino acid, e.g. alanine [19], is used for the substitution of the original amino acid during mutagenesis. Such an approach may lead to false results if the evaluation of the role of specific amino acid in the protein structure is considered. Aspartic acid at position 126 thus has been substituted with alanine and asparagine in the previously reported studies. The mutagenised libraries give an opportunity to increase the number of variants included in the functional analysis. In this study we were able to establish the functional consequences of 9 amino acids, representing residues with different chemical properties, at position 126 of the MC4 receptor.

During the functional activation of the obtained MC4R clones, only the variant with the aspartic acid displayed functional activity. Interestingly, the MC4R variant with the glutamic acid was functionally inactive. Both these amino acids have acidic chemical characteristics that are considered to be important for binding the pharmacophore of natural ligands at this position of the MC4R [23]. Although aspartic acid and glutamic acid are very similar structurally, glutamic acid has a longer carbohydrate tail with a –COOH terminus. We have shown that even a single –CH group, which distinguishes aspartic and glutamic acids, is sufficient to disarrange the binding pocket of the melanocortin pharmacophore and functionally inactivate the MC4R.

MC4R variants with glutamic acid and leucine at position 126 had a higher background activity (Figure 4a) than other mutants, even after repeated plasmid isolation and retransformation. It is possible that the receptor variants with neutral, aliphatic or acidic amino acid substitution have a higher constitutive activity, and D126 of the MC4R is not only crucial for the ligand binding but is also responsible for the background constitutive activity of the receptor. The constitutive activity of the MC4R has been previously demonstrated [40].

In conclusion, we have shown for the first time the functional expression of the MC4R in *Saccharomyces cerevisidae* and have used it for functional characterization of the randomized MC4R library. In addition to the previously known results on substitution of aspartic acid at position 126, we show that none of the other, previously not tested, amino acid substitutions obtained from the randomized MC4R gene library was functionally active, further confirming the crucial role of aspartic acid in this position for the MC4R activity.

Acknowledgements

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