ELSEVIER

European Journal of Pharmacology 314 (1996) 381–384

ejp

Alternative translation initiation codon for the human melanocortin MC_3 receptor does not affect the ligand binding

Helgi B. Schiöth [a], Ruta Muceniece [b], Jarl E.S. Wikberg [a,c,*], Michael Szardenings [a]

[a] *Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, Uppsala University, S-75 124 Uppsala, Sweden*
[b] *Laboratory of Pharmacology, Institute of Organic Synthesis Riga, Latvia*
[c] *Wa Pharm, Umeå, Sweden*

Received 6 May 1996; revised 4 July 1996; accepted 16 July 1996

Abstract

The genomic DNA for the human melanocortin MC_3 receptor indicates an unusually long N-terminus. Two possible translation initiation sites, the one originally proposed and one alternate 111 bp downstream, were mutated. For a third mutant the DNA between these initiation sites was deleted. All mutants were expressed in COS (*C*V-1 *O*rigin, *S*V40) cells in the same level, and they bound peptide hormones in the same fashion, as did the wild type clone. The data obtained indicate that both sites can function as the sole translation initiation sites of the human clone and that the proposed N-terminus of the human melanocortin MC_3 receptor is not important for the ligand binding of the receptor.

Keywords: Melanocortin MC_3 receptor; MSH (melanocyte-stimulating hormone); Ligand binding; Mutagenesis

1. Introduction

The melanocortin hormones originate from proopiomelanocortin, which is proteolytically cleaved into three biologically active families of peptides, the adrenocorticotropins, melanocortins and endorphins. Adrenocorticotropin (ACTH), α-MSH (melanocyte-stimulating hormone) and β-endorphin have a well-known role in adrenal cortical function, pigmentation and analgesia. The melanocortins have, additionally to their involvement in pigmentation, a broad array of other physiological functions, which underlying mechanisms are not understood. In the CNS, melanocortins have been shown to influence behaviour, memory, thermoregulation, analgesia and control of cardiovascular systems (Eberle, 1988).

Molecular cloning has identified five melanocortin receptor subtypes, known as MC_1–MC_5 receptors (Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993a,b; Mountjoy et al., 1992). The melanocortin MC receptors form a family of their own with distinct differences to other G-protein-coupled receptors. Besides other characteristic features, the MC receptors are the smallest G-protein-coupled receptors showing short N and C terminal sequences.

The melanocortin MC_1 receptor has high affinity to α-MSH. It has been detected in human melanocytes and in small numbers in the brains of rats and humans. The melanocortin MC_2 receptor is expressed in the adrenal gland (Mountjoy et al., 1992). The melanocortin MC_3 receptor is expressed in the brain, i.e. predominantly in the arcuate nucleus and in few regions of the brain stem, in addition to placenta and gut tissues (Gantz et al., 1993a). The melanocortin MC_4 receptor is expressed in the brain and the melanocortin MC_5 receptor has, in addition to its expression in the brain, a wide peripheral distribution (for review see Siegrist and Eberle, 1995).

The genomic DNA sequence for the human melanocortin MC_3 receptor revealed a 361 amino acids long open reading frame, which allowed for the assignment of an unusually long N-terminal sequence. Comparison of the human melanocortin MC receptor subtypes shows that methionine in position 38 (Met^{38}) of the melanocortin MC_3 receptors aligns with the Met^1 in the human melanocortin MC_1 receptor. The cloning of the homologous receptors from rat (Roselli-Rehfuss et al., 1993) and mouse (Desarnaud et al., 1994) revealed reading frames of only 323 amino acids for the receptors of both

* Corresponding author. Fax: (46-18) 559-718.

PII S0014-2999(96)00566-3

species. The rat and mouse sequences are lacking the first ATG corresponding to the proposed translation initiation codon of the human melanocortin MC_3 receptor, but they both have translation initiation codons corresponding to Met^{38} of the human clone. The N-terminus of these proposed receptor proteins is therefore 37 amino acids shorter than the human clone. It has been suggested that the start codon of the human melanocortin MC_3 receptor should be assumed to be identical to these other receptors.

Mutagenesis studies (Frändberg et al., 1994) and modelling (Prusis et al., 1995) of the MC-receptors are presently carried out in our laboratory, and we need to know which parts of the receptor proteins are expressed and which are of importance for ligand binding. The length of the N-terminal sequence is also of importance for making specific antibodies against the melanocortin MC_3 receptor. The aim of the present study was therefore to investigate by mutagenesis and binding which parts of the N-terminal sequence of the human melanocortin MC_3 receptor are really essential for the expression and hormone binding of the protein.

2. Materials and methods

2.1. Chemicals

The [Nle^4,D-Phe^7]α-MSH, α-MSH, β-MSH and γ_1-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2) were purchased from Saxon Biochemicals, Germany. [Nle^4,D-Phe^7]α-MSH was radioiodinated by the Chloramine-T method and purified by HPLC (high-pressure liquid chromatography).

2.2. Mutated and truncated melanocortin MC_3 receptor clones

The mutated and truncated melanocortin MC_3 clones were created with PCR (polymerase chain reaction). The following primers were used in combination with 5′*Hin*dIII linker and 3′*Xba*I linker (mutated positions in bold, the ATG corresponding to Met^1 is underlined). The 5′primer for MC_3-ATG1 was; GGAAGCTT GA**CTG**AGCATC-CAAAAGAAGTATCTGG (the mutation is shown in bold and the position of the first ATG codon is underlined). The 5′primer for MC_3-ATG2 was; GGAAGCTTGA*ATG*AGCATCCAAAAGAAGTATCTG-GAGGGAGATTTTGTCTTTCCTGTGAGCAGCAGCA-GCTTCCTACGGACCCTGCTGGAGCCCCAGCTCGG-ATCAGCCCTTCTGACAGCACTGAATG. The 5′primer for MC_3-MtoM was; GGAAGCTTGAATGAAT-GCTTCGTGCTGC. The 3′primer used for all the clones were; GGTCTAGACTATCCCAAGTTCATGCCG. (All primers are written in 5′ to 3′direction). The PCR products were cloned into the *Hin*dIII and *Xba*I sites of the pRc/CMV vector (Invitrogen) and the inserts and adjacent sequences of the vector DNA used for transfection experiments were confirmed by sequencing.

2.3. Expression of receptor clones

The human melanocortin MC_3 receptor DNA, cloned into the expression vector CMV/neo, was a gift from Dr Ira Gantz (Gantz et al., 1993a). For receptor expression, COS (*C*V-1 *O*rigin, *S*V40) cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty per cent confluent cultures were transfected with the DNA mixed with liposomes in serum free medium. The liposomes were the commercially available lipofectin (BRL, USA) or produced according to Campbell (1995). After transfection the serum-free medium was replaced with the serum containing medium and the cells were cultivated for ca. 48 h. Cells were then scraped off, centrifuged and used for radioligand binding.

2.4. Binding studies

The transfected cells were washed with binding buffer (Minimum Essential Medium with Earle's salts, 25 mM Hepes, pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg/l leupeptin and 200 mg/l bacitracin) and distributed into 96 well plates. The cells were then incubated for 2 h at 37°C with 0.1 ml binding buffer in each well, containing a constant concentration of [^{125}I][Nle^4,D-Phe^7]α-MSH and appropriate concentrations of an unlabelled ligand. After incubation, the plates were put on ice, the cells washed with 0.1 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with the BindAid software (Wan System AB, Umeå, Sweden). Data were either analysed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling, or by fitting to the four parameter logistic function. K_i values were calculated by using the Cheng and Prusoff equation. The binding assays were performed in duplicate wells and repeated three times.

3. Results

Three mutants of the human melanocortin MC_3 receptor were created in order to investigate whether the proposed extracellular N-terminal domain of the human melanocortin MC_3 receptor is essential for its hormone binding activity. The ATG codons, corresponding to the first and second methionine in the original sequence (Gantz et al., 1993a), were mutated to CTG resulting in the clones MC_3-ATG1 and MC_3-ATG2, respectively. In a third clone the entire sequence between the first and the second ATG was deleted (MC_3-MtoM). (See schematic presentation of the clones in Fig. 1).

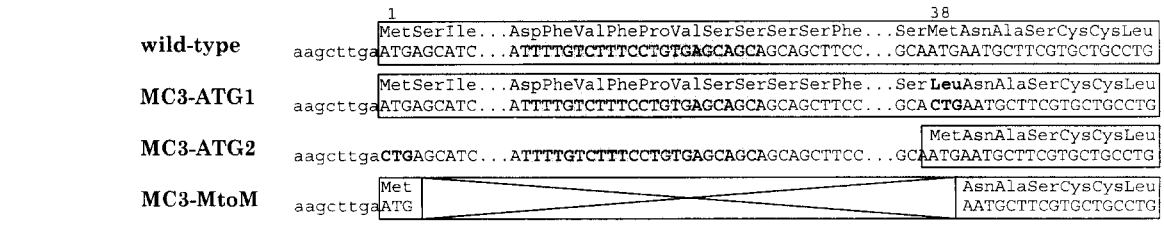

Fig. 1. Comparison of the wild type sequence with the mutant clones described in the text. The 5'-DNA sequence starting from the *Hind*III cloning site in the vector pRc/CMV and the likely translated proteins are shown. The sequence of the proposed intron/exon boarder (Desarnaud et al., 1994) is shaded in grey.

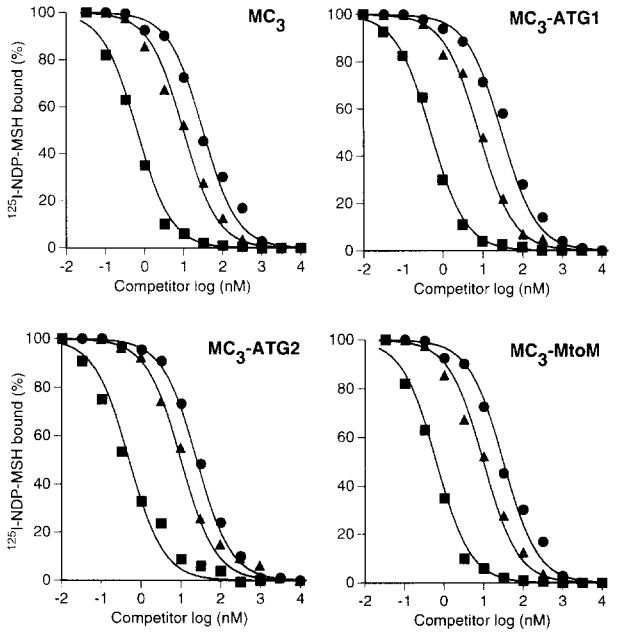

Fig. 2. Competition curves of [Nle^4,D-Phe^7]α-MSH (■), α-MSH (●), and γ_1-MSH (♦) obtained on transfected COS cell using a fixed concentration of ~2 nM [^{125}I][Nle^4,D-Phe^7]α-MSH for the different melanocortin MC$_3$ receptor clones.

COS cells were transfected with the wild type melanocortin MC$_3$ receptor and the three mutants. The binding properties of [^{125}I][Nle^4,D-Phe^7]α-MSH for the mutated and the truncated clone were indistinguishable from that of the wild type MC$_3$ clone. The K_i values are in close agreement with results obtained earlier in our laboratory (Schiöth et al., 1995). The competition curves for [Nle^4,D-Phe^7]α-MSH, α-MSH and γ_1-MSH on transfected COS cells are shown in Fig. 2 for the wild type MC$_3$, the MC$_3$-ATG1, MC$_3$-ATG2 and the MC$_3$-MtoM, respectively. The K_i values for [Nle^4,D-Phe^7]α-MSH, α-MSH, β-MSH and γ_1-MSH are presented in Table 1. As can be seen from the table the K_i values for different mutants and the wild type receptors were almost the same, and well within the range of the normal variability of radioligand assay used. Moreover, the level of expression indicated by the data analysis was similar for all the four clones investigated (data not shown).

4. Discussion

The alignment of all known melanocortin receptors has already indicated that the proposed N-terminus of the human melanocortin MC$_3$ receptor and a murine melanocortin MC$_5$ receptor (Fathi et al., 1995) are unusually long. Both clones contain a possible alternative translation initiation codon, which can be aligned to the initiation codon in clones of the same melanocortin receptor subtype of other species. Recent data also indicate that amino acid residues in the N-terminal loop of the melanocortin MC$_1$ receptor might be important for the ligand binding (Chhajlani et al., 1996).

Comparison of the sequences surrounding both translation initiation codons reveals strong deviation from what is believed to be a standard eukaryotic translation initiation site (Kozak, 1984). Data from a more recent statistical analysis of known translated sequences (Cavener and Ray, 1991) indicate that the first site (GCAAUGA) occurs in fewer vertebrate sequences than the second (TGAAUGA). Both sequences are underrepresented and this has made it impossible to draw direct conclusions.

In order to investigate the importance of the putative extra long N-terminal chain of the human melanocortin MC$_3$ receptor we made three mutant clones. The proposed

Table 1
K_i values (means ± S.E.M), obtained from competition curves for MSH peptides on melanocortin MC$_3$, MC$_3$-ATG1, MC$_3$-ATG2 and MC$_3$-MtoM

Ligand	MC$_3$ (nmol/l)	MC$_3$-ATG1 (nmol/l)	MC$_3$-ATG2 (nmol/l)	MC$_3$-MtoM (nmol/l)
[Nle^4,D-Phe^7]α-MSH	0.347 ± 0.032	0.289 ± 0.041	0.279 ± 0.075	0.316 ± 0.023
α-MSH	23.1 ± 3.2	35.2 ± 4.9	28.5 ± 4.3	25.8 ± 5.5
β-MSH	32.8 ± 4.7	18.8 ± 1.4	16.6 ± 1.8	31.1 ± 3.5
γ_1-MSH	7.52 ± 0.91	8.68 ± 2.1	12.8 ± 3.7	9.19 ± 3.02

translation initiation codon and the alternate translation initiation codon 111 bp downstream were independently mutated by a point mutation ATG → CTG (MC_3-ATG1 and MC_3-ATG2), while the rest of the sequence was not changed to keep proposed intron/exon borders (Desarnaud et al., 1994). In a third mutant the 111 bp between the first and the second ATG were deleted (MC_3-MtoM). All three mutants were easily expressed in COS cells in similiar levels and readily characterized by radioligand binding. All three clones showed binding properties similiar to those of the original wild type melanocortin MC_3 clone. The data obtained for the MC_3-ATG1 and the MC_3-MtoM mutant is therefore in agreement with previously published data for melanocortin MC_3 receptors from other species. It is thus conceivable that Met^{38} can indeed function as the sole translation initiation site of the human clone. More surprising is the fact that even the MC_3-ATG2 mutant, which does not contain the Met^{38} codon, could be expressed successfully and in amounts similiar to those obtained with the other constructs. The proposed splicing site (Desarnaud et al., 1994) between the first and the second ATG might therefore not be functional for the human melanocortin MC_3 clone, at least in the vector system used for this study. It remains unclear whether both translational products are present when the receptor is expressed in tissues in vivo. Still the similarity of the binding properties suggests that this would have no effect on the pharmacology of this receptor. Our data shows, that the proposed N-terminus of the human melanocortin MC_3 receptor is not important for the ligand binding of the receptor; at least for modelling and mutagenesis studies, only the sequence from Met^{38} needs to be taken into account.

Acknowledgements

This study was supported by grants from the Swedish MRC (04X-05957), CEC (ERBCHRXCT 940505), the Swedish CFN and the Howard Hughes Medical Institute (HHMI 75195-548501).

References

Campbell, M.J., 1995, Lipofectin reagents prepared by a simple ethanol injection technique, BioTechniques 18, 1027.

Cavener, D.R. and S.C. Ray, 1991, Eurkaryotic start and stop translation sites, Nucleic Acids Res. 19, 3185.

Chhajlani, V. and J.E.S. Wikberg, 1992, Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA, FEBS Lett. 309, 417.

Chhajlani, V., R. Muceniece and J.E.S. Wikberg, 1993, Molecular cloning of a novel human melanocortin receptor, Biochem. Biophys. Res. Commun. 195, 866.

Chhajlani, V., X. Xu, J. Blauw and S. Sudarshi, 1996, Identification of ligand binding residues in extracellular loops of the melanocortin 1 receptor, Biochem. Biophys. Res. Commun. 219, 521.

Desarnaud, F., O. Labbé, D. Eggerickx, G. Vassart and M. Parmentier, 1994, Molecular cloning, functional expression and pharmacological characterization of a mouse melanocortin receptor gene, Biochem. J. 299, 367.

Eberle, A.N., 1988, The Melanotropins; Chemistry, Physiology and Mechanisms of Action (Karger, Basel, Switzerland) p. 252.

Fathi, Z., L.G. Iben and E.M. Parker, 1995, Cloning, expression, and tissue distribution of a fifth melanocortin receptor subtype, Neurochem Res. 20, 107.

Frändberg, P.A., R. Muceniece, P. Prusis, J.E.S. Wikberg and V. Chhajlani, 1994, Evidence for alternate points of attachment for α-MSH and its steroisomer NDP-MSH at the melanocortin 1 receptor, Biochem. Biophys. Res. Commun. 202, 1266.

Gantz, I., Y. Konda, T. Tashiro, Y. Shimoto, H. Miwa, G. Munzert, S.J. Watson, J. DelValle and T. Yamada, 1993a, Molecular cloning of a novel melanocortin receptor, J. Biol. Chem. 268, 8246.

Gantz, I., H. Miwa, Y. Konda, Y. Shimoto, T. Tashiro, S.J. Watson, J. DelValle and T. Yamada, 1993b, Molecular cloning, expression, and gene localization of a fourth melanocortin receptor, J. Biol. Chem. 268, 15174.

Kozak, M., 1984, Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs, Nucleic Acids Res. 12, 857.

Mountjoy, K.G., L.S. Robbins, M.T. Mortrud and R.D. Cone, 1992, The cloning of a family of genes that encode the melanocortin receptors, Science 257, 1248.

Prusis, P., P.A. Frändberg, R. Muceniece, I. Kalvinsh and J.E.S. Wikberg, 1995, A three dimentional model for the interaction of MSH with the melanocortin 1 receptor, Biochem. Biophys. Res. Commun. 210, 205.

Roselli-Rehfuss, L., K.G. Mountjoy, L.S. Robbins, M.T. Mortrud, M.J. Low, J.B. Tatro, M.L. Entwistle, R.B. Simerly and R.D. Cone, 1993, Identification of a receptor for γ melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system, Proc. Natl. Acad. Sci. USA 90, 8856.

Schiöth, H.B., R. Muceniece, J.E.S. Wikberg and V. Chhajlani, 1995, Characterisation of melanocortin receptor subtypes by radioligand binding analysis, Eur. J. Pharmacol. Mol. Pharmacol. Sect. 288, 311.

Siegrist W. and A.N. Eberle, 1995, Melanocortins and their implications in melanoma, Trends Endocrinol. Metab. 6, 115.