Deletions of the N-terminal regions of the human melanocortin receptors

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Abstract The non-homologous N-terminal regions of four human melanocortin (MC) receptors were truncated in order to investigate their putative participation in ligand binding. Eleven constructs were made, where different numbers of residues from the N terminus were deleted. These constructs were used for transient expression experiments in COS cells and analysed by ligand binding. The results show that 27, 25, 28, and 20 amino acids could be deleted from the N terminus of the human MC1, MC3, MC4 and MC5 receptors, respectively, including all potential N-terminal glycosylation sites in the MC1 and the MC4 receptors, without affecting ligand binding or expression levels. The results indicate that the N-terminal regions of the human MC1, MC3, MC4 and MC5 receptors, do not play an important role for the ligand binding properties of these receptors.

Key words: Melanocortin (MC) receptor; MSH; Ligand binding; N terminus; Truncation

1. Introduction

Pro-opiomelanocortin (POMC) is mainly expressed in the pituitary, hypothalamus and brain stem, and is post-translationally processed into the melanocortins, i.e. α-MSH (melanocyte stimulating hormone), β-MSH, γ-MSH and ACTH (adrenocorticotropic hormone). Besides the well-known effects of ACTH on steroid production in the adrenal gland and α-MSH on pigmentation, the melanocortins display a broad array of additional physiological actions including effects on behaviour, memory, thermoregulation, pain perception, nerve regeneration, inflammation and blood pressure [1,2].

Five melanocortin receptor subtypes have been cloned [3–7]. The MC1 receptor is expressed in melanocytes and has high affinity for α-MSH. The MC2 receptor is the ACTH receptor in the adrenal gland. The MC2 receptor has a distinct pharmacology from the other MC receptors as it binds ACTH with high affinity but not the MSH peptides [8]. The human MC3 and especially the human MC4 and MC5 receptors bind the MSH peptides with lower affinity than the human MC1 receptor [9,10]. The physiological roles of these receptors are much less established compared to the MC1 and the MC2 receptors. The MC3 receptor is expressed in the brain and also in the periphery where it has been found in the placenta, gut tissues and the heart [5,11]. The MC4 is predominantly found in the brain where it is widely distributed in almost every brain region [6,12]. Recently, it has been shown that disruption of the MC4 receptor results in obesity [13]. The MC5 receptor is also found in the brain, but is more abundantly expressed in peripheral tissues [7,14,15].

Very little is known about how the MSH peptides bind to the MC receptors. By use of site directed mutagenesis amino acids in the N terminal, extracellular loops (EL) [16] and in the transmembrane region (TM) 3 and TM6 [17] in the MC1 receptor have been identified, which may participate in the ligand binding. Multiple mutations in TM4, EL2 and TM5 in the MC3 receptor indicate that these regions may not influence specific ligand binding [18]. Two quite different 3D models of the MC1 receptor which were based on bacteriorhodopsin homology modelling and ligand docking in between the TM regions have been published [19,20]. The sequences of the MC receptor subtypes are highly conserved, particularly in the TM regions whereas the N-terminal regions varies quite a lot between the different subtypes.

The aim of this study was to investigate a putative participation of N-terminal regions of the human MC1, MC3, MC4 and MC5 receptors in ligand binding.

2. Materials and methods

2.1. Chemicals

[Nle^4, D-Phe^7]α-MSH (NDP-MSH), α-MSH and β-MSH were purchased from Saxon Biochemicals, Germany, or Bachem, Switzerland. NDP-MSH was radioiodinated by the Chloramine T method and purified by HPLC.

2.2. Generation of truncated clones

The truncated clones were made by PCR. Fragments of the wild type MC receptor clones were amplified by PCR, using downstream primers carrying a *Hin*dIII linker and a ATG start codon for various positions in the N-terminal region of the MC receptors and an upstream primer with a *Xba*I linker. These PCR products were purified and cloned into *Hin*dIII and *Xba*I sites of the pRc/CMV vector (Invitrogen) (or the pcDNA3.1/Zeo vector (Invitrogen), see Section 3), and the inserts and adjacent sequences of the vector DNA used for transfection experiments were confirmed by sequencing. The 3′ end primer used for the MC1 clone was: GAC GTC TAG ATT CAC CAG GAG CAT GTC A (all primers are written in 5′ to 3′ direction), for the MC3 clones: GGT CTA GAC TAT CCC AAG TTC ATG CCG, for the MC4 clones: GAC GTC TAG ATT CAA TAT CTG CTA GAC AAG GTC, for the MC5 clones: GAC GTC TAG ATT CAA TCC CTT CTG GGA AAG CT. The 5′ end primer used for the MC1^{ATG28} clone was: GGG AAG CTT CAC ATA TGC AGA CAG GAG CCC GGT, for the MC3^{ATG44} clone: GGA AGC TTG AAT GCC CTC TGT TCA GCC AAC A, for the MC3^{ATG51} clone: GGA AGC TTG AAT GCC TAA TGG CTC GGA GCA C, for the MC3^{ATG63} clone: GGG AAG CTT CAC ATA TGA GCA ACC AGA GCA GCA GCG CCT, for the MC3^{ATG69} clone: GGA AGC TTG AAT GAA TGC TTC GTG CTG C, for the MC4^{ATG24} clone: GGG AAG CTT CAC ATA TGA GCA ATG CCA GTG AGT, for the MC4^{ATG29} clone: GGG AAG CTT CAC ATA TGT CCC TTG GAA AAG GCT A, for the MC4^{ATG35} clone: GGG AAG CTT CTC ATA TGT CTG ATG GAG GGT GCT A, for the MC5^{ATG14} clone: GGG AAG CTT CAC ATA TGA ATG CCA

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Receptor	N-terminus	TM1	2)
MC1 (X65634)	M A V Q G S Q R R L L G S L [N] S T P T A I P Q L G L A A [N] Q T G A R C L E V S	I S D G L F ...	+
MC1-ATG28	M Q T G A R C L E V S	I S D G L F ...	+
MC3 (I06155)	1) M N A S C C L P S V Q P T L P [N] G S E H L Q A P F F S [N] Q S S S A F C E Q V F	I K P E I F ...	+
MC3-ATG44	M P S V Q P T L P [N] G S E H L Q A P F F S [N] Q S S S A F C E Q V F	I K P E I F ...	+
MC3-ATG51	M P [N] G S E H L Q A P F F S [N] Q S S S A F C E Q V F	I K P E I F ...	+
MC3-ATG63	M S [N] Q S S S A F C E Q V F	I K P E I F ...	+
MC3-ATG69	M A F C E Q V F	I K P E I F ...	-
MC4 (I08603)	M V [N] S T H R G M H T S L H L W [N] R S S Y R L H S [N] A S E S L G K G Y S D G G C Y E Q L F	V S P E V F ...	+
MC4-ATG24	M S [N] A S E S L G K G Y S D G G C Y E Q L F	V S P E V F ...	+
MC4-ATG29	M S L G K G Y S D G G C Y E Q L F	V S P E V F ...	+
MC4-ATG35	M D G G C Y E Q L F	V S P E V F ...	-
MC5 (I27080)	M [N] S S F H L H F L D L N L [N] A T E G [N] L S G P [N] V K [N] K S S P C P C E D	M G I A V F ...	+
MC5-ATG14	M [N] A T E G [N] L S G P [N] V K [N] K S S P C P C E D	M G I A V F ...	+
MC5-ATG21	M S G P [N] V K [N] K S S P C P C E D	M G I A V F ...	+
MC5-ATG28	M [N] K S S P C P C E D	M G I A V F ...	-

Fig. 1. Alignment of the N-terminal amino acid sequences of the cloned human MC receptors (MC2 not included) with the mutant clones described in the text. Numbers in parentheses denote the accession numbers in EMBL and GenBank Data Libraries. Borders between the N-terminal region and TM1 are as proposed [19]. Potential N-terminal glycosylation sites are boxed. (1) The human MC3 receptor is displayed from the Met^{38} as the preceding sequence may not be expressed [21]. (2) Constructs which did not give rise to specific binding after transfection are denoted by (−) and the others by (+).

CAG AGG GCA, for the $MC5^{ATG21}$ clone: GGG AAG CTT CAC ATA TGT CAG GAC CAA ATG TCA and for the $MC5^{ATG28}$ clone: GGG AAG CTT CAC ATA TGA AGT CTT CAC CAT GTG A.

2.3. Cells and expression

The wild type human MC1 and human MC5 receptor [3,7] were cloned into the expression vector pRc/CMV (Invitrogen). The wild type human MC3 and human MC4 receptor DNA, cloned into the expression vector pCMV/neo, were a gift from Dr. Ira Gantz [5,6]. For receptor expression, COS-1 cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum free medium (for details see [10]). After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 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 [9] and distributed into 96-well micro titer plates (approximately 40 000 cells/well). The cells were then incubated for 2 h at 37°C with 0.05 ml binding buffer in each well, containing the same concentration of [^{125}I]NDP-MSH and appropriate concentrations of an unlabelled ligand. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 M NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with a software package for radioligand binding analyses (Wan System, Umeå, Sweden). Data were analysed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling. For saturation analysis, 12 concentrations of [^{125}I]NDP-MSH in the range of 0.02 up to 20 nM were used. Non-specific binding was determined in the presence of 3 μM NDP-MSH. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I]NDP-MSH.

3. Results

Eleven truncated clones of the human MC1, MC3, MC4 and MC5 receptors were created in order to investigate the putative participation of the N terminus in ligand binding (see schematic representation of the clones in Fig. 1). The human wild type MC receptors and truncated clones were used for expression experiments in COS-1 cells which were subsequently tested in binding assays using [^{125}I]NDP-MSH as radioligand. The truncated clones expressed at levels similar to the wild type clones, except the $MC3^{ATG69}$, $MC4^{ATG35}$ and $MC5^{ATG28}$ which did not show any specific binding in the COS-1 cells after transfection (data not shown). Saturation curves for the wild type and the $MC1^{ATG28}$, $MC3^{ATG63}$, $MC4^{ATG29}$ and $MC5^{ATG21}$ clones, are shown in Fig. 2. Competition curves of different MSH-peptides for the same receptors are shown in Fig. 3. In Table 1, the K_i values obtained from the computer analysis of these data are shown together with the K_d values for [^{125}I]NDP-MSH obtained from the saturation curves.

We have earlier shown that the comparatively long cDNA derived N-terminal sequence of the human MC3 receptor up to ATG^{38} does not participate in ligand binding [21]. Further truncation of the human MC3, as well as the human MC4 and human MC5 receptors resulted in clones termed $MC3^{ATG44}$, $MC3^{ATG51}$, $MC4^{ATG24}$ and $MC5^{ATG14}$. When these constructs were expressed they bound NDP-MSH (α-MSH) in similar fashion as respective wild type receptor (see Table 1). Deletion of additional amino acids from these receptors and the MC1 receptor, resulted in clones termed $MC1^{ATG28}$, $MC3^{ATG63}$, $MC4^{ATG29}$ and $MC5^{ATG21}$. These clones were expressed and fully characterised by ligand binding using both saturation analysis ([^{125}I]NDP-MSH) and competition analysis (NDP-MSH, α-MSH and β-MSH). The results show (Table 1) that the binding constants of the MSH peptides for these truncated receptors are indistinguishable from those of the corresponding wild type receptors. Moreover, the binding constants presented in Table 1 are in close agreement to those found earlier for the wild type MC1, MC3, MC4 and MC5 receptors [9,10,22].

Additional constructs, termed $MC3^{ATG69}$, $MC4^{ATG35}$ and $MC5^{ATG28}$ did not show any specific binding to labelled NDP-MSH when transfected to COS-1 cells. We made two clones of each of these constructs, and we also cloned the $MC4^{ATG35}$ and the $MC5^{ATG28}$ into the pcDNA3.1/Zeo vector (in addition to the pRc/CMV vector) without creating any specific binding in COS-1 cells after transfection.

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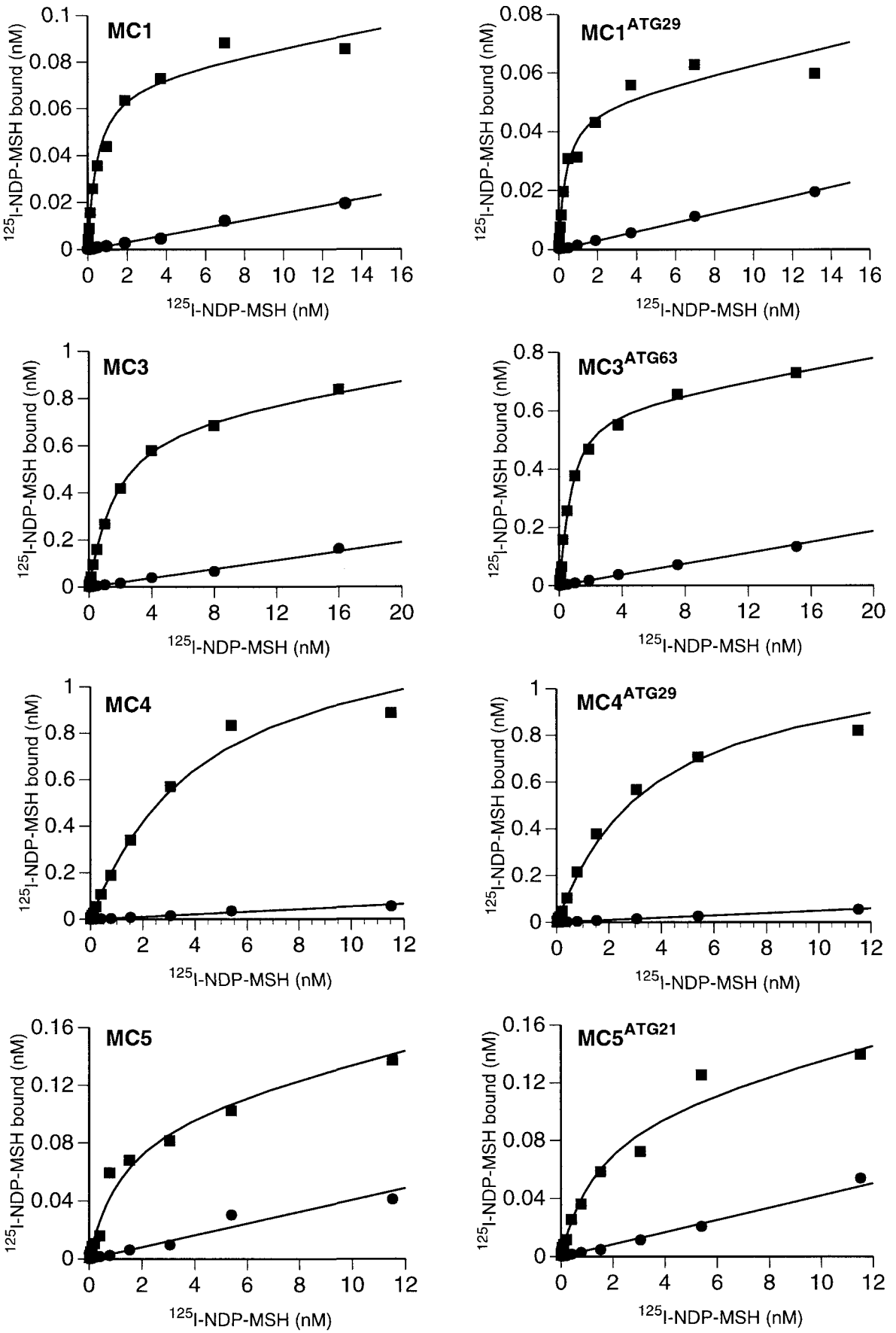
Fig. 2. Saturation curves of [^{125}I]NDP-MSH obtained from transfected COS cells. The figures show total binding (■) and binding in the presence of 3 μM unlabeled NDP-MSH (●) for the wild type and $MC1^{ATG28}$, $MC3^{ATG63}$, $MC4^{ATG29}$, $MC5^{ATG21}$ clones. Lines represent the computer modelled best fit of the data using an one-site model.


MC1
MC1ATG29
MC3
MC3ATG63
MC4
MC4ATG29
MC5
MC5ATG21
125I-NDP-MSH bound (nM)
125I-NDP-MSH (nM)

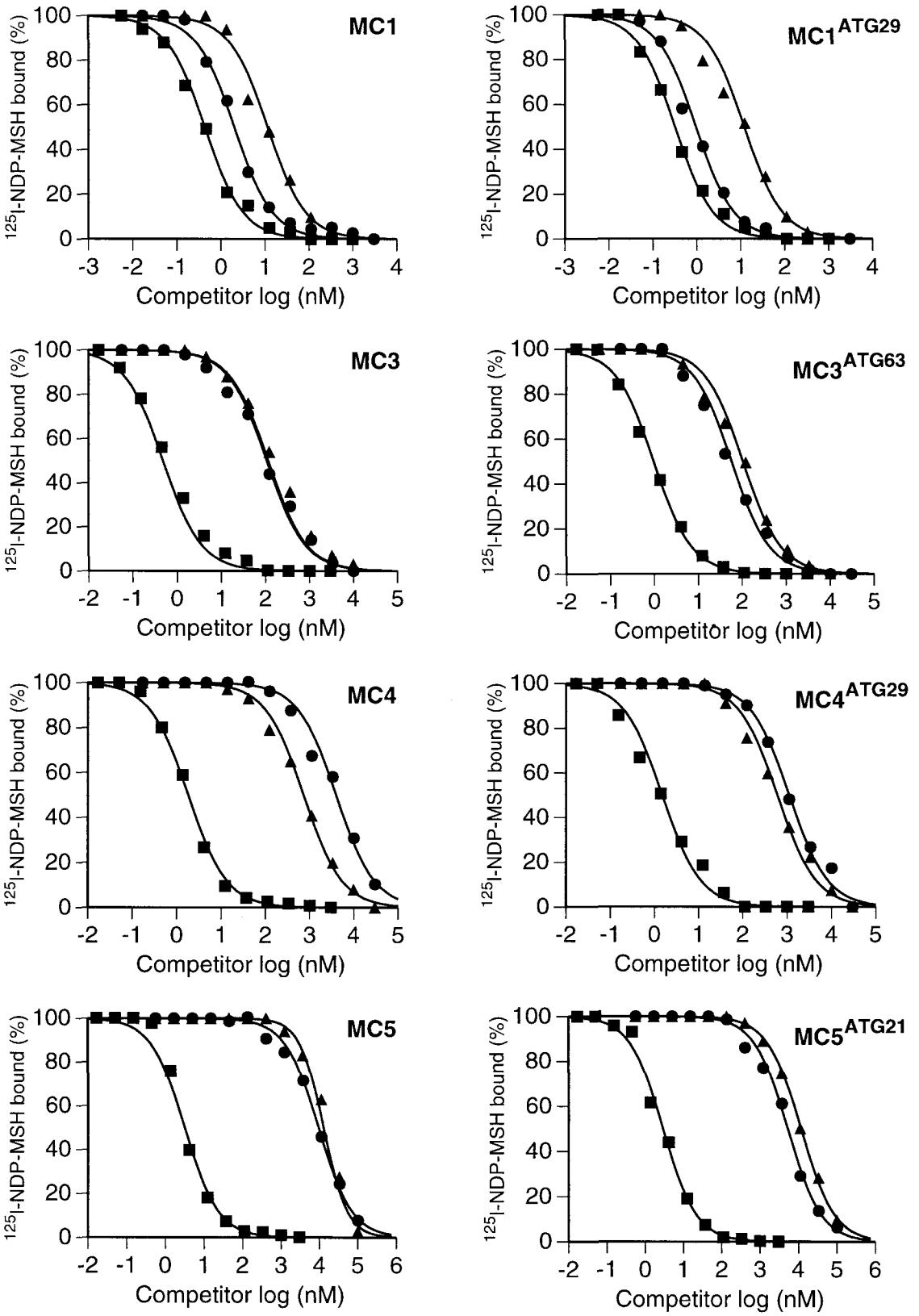


Fig. 3. Competition curves of NDP-MSH (■), α-MSH (●), β-MSH (▲) obtained on transfected COS cells using a fixed concentration of ~2 nM [^{125}I]NDP-MSH for the different MC receptor clones.

Table 1
K_i and K_d (nmol/l) values (mean ± S.E.M) obtained from competition and saturation curves using [^{125}I]NDP-MSH and different MSH analogues on transfected COS-1 cells

Receptor^{Ligand}	K_d	K_i		
	[^{125}I]NDP-MSH	NDP-MSH	α-MSH	β-MSH
MC1	0.109 ± 0.062	0.046 ± 0.011	0.210 ± 0.089	2.53 ± 0.93
MC1^{ATG29}	0.144 ± 0.027	0.050 ± 0.015	0.141 ± 0.090	2.75 ± 0.66
MC3	0.394 ± 0.061	0.493 ± 0.035	53.2 ± 9.62	57.0 ± 12.0
MC3^{ATG44}	nd	0.534 ± 0.092	nd	nd
MC3^{ATG51}	nd	0.413 ± 0.038	31.2 ± 4.2	nd
MC3^{ATG63}	0.435 ± 0.036	0.593 ± 0.044	34.4 ± 5.8	64.8 ± 7.8
MC4	2.29 ± 0.27	1.86 ± 0.35	2030 ± 410	642 ± 150
MC4^{ATG24}	nd	1.05 ± 0.10	1730 ± 630	nd
MC4^{ATG29}	2.59 ± 0.22	1.28 ± 0.28	957 ± 319	513 ± 150
MC5	2.86 ± 0.71	2.65 ± 0.37	4990 ± 800	13 900 ± 5 600
MC5^{ATG14}	nd	1.09 ± 0.21	5570 ± 1730	nd
MC5^{ATG21}	1.44 ± 0.23	2.15 ± 0.20	3270 ± 1080	10 100 ± 1 700

nd, not determined.

4. Discussion

For most G-protein coupled receptors, in particular those serving as recognition sites for low molecular weight compounds like adrenaline, the ligand binding pocket is thought to be localised between the TM regions, which are predicted to form α-helices analogous to the extensively characterised bacteriorhodopsin. For other G-protein coupled receptors that bind small peptides, like neurokinins [23], enkephalins [24] or secretin and vasoactive intestinal polypeptide [25] the N-terminal region is known to play an role for the ligand binding. For glycoprotein hormone receptors [26] the N-terminal region can be even more important, such as for the lutropin/chorigonadotropin receptor, where the N-terminal domain alone is sufficient for high affinity hormone binding [27]. Truncations of G-protein coupled receptors have been shown to both increase or decrease ligand affinities [28]. The melanocortin receptor family has the shortest amino acid sequence among the superfamily of G-protein coupled receptors. The human MC1, MC2, MC3, MC4 and MC5 receptors are 317, 297, 361 (or 324 [21]), 333, 325 amino acids long, respectively. They are characterised by having short N- (ca. 25–39 amino acids) and C-terminal regions (ca. 17–21 amino acids) as well as a very small second extracellular loop (ca. 9 amino acids).

Our present results show that 27 amino acids of the human MC1, 25 amino acids of the human MC3 receptor, 28 amino acids of the human MC4 receptor and 20 amino acids from the human MC5 receptor could be deleted from the N termini without affecting the expression or binding of [^{125}I]NDP-MSH, NDP-MSH, α-MSH or β-MSH. Further deletions of the N termini resulted in 'total loss of binding', which may have been caused by misfolding or problems with the membrane integration of the receptor.

Our results show that all potential N-terminal glycosylation sites can be removed from the MC1 and MC4 receptors (MC1Asn^{15}, MC1Asn^{29}, MC4Asn^{3}, MC4Asn^{17} and MC4Asn^{26}) as well as two potential glycosylation sites (Asn^{53} and Asn^{39}) from the MC3 receptor and three potential sites from the MC5 receptor (Asn^{2}, Asn^{15} and Asn^{20}) without causing any effects on ligand binding or expression. Our results thus indicate that N-terminal glycosylation is unlikely to play an important role in the folding and membrane targeting of the MC receptors.

It remains unclear whether the non-binding clones (MC3^{ATG69}, MC4^{ATG35} and MC5^{ATG28}) were expressed and inserted into the cell membrane. It is unlikely that the complete absence of specific ligand binding for these constructs was caused by deletion of residues crucial for ligand binding while all other deletions did not even change the ligand binding properties at all. Structural or expression complications, or influence on the integration of the receptors to the cell membrane are more probable explanations.

The precise border between the extracellular N-terminal part and TM1 is not known. Amino acids proposed to precede TM1, like the conserved Glu (MC1Glu^{36}) and positive charges may be important for the structure, folding or ligand binding. Moreover, the conserved Cys (MC1Cys^{34}) which may form a disulphide bridge with a corresponding Cys residue in extracellular loop 3 (or even elsewhere in the receptor) may also be important for receptor structure. The observation that the MC1^{ATG28} is giving binding, but not the equally truncated MC5^{ATG28}, may indicate important role for amino acids preceding the TM1. A recent analysis of G-protein coupled receptors revealed, that those lacking a leader sequence, like the MC receptors, have a high preference for a positive charge in the N terminus close to TM1[29]. This is in contrast to the more general 'positive inside' rule developed earlier [30] and may indicate a specific role for Lys^{32} and Lys^{28} in the MC4 and the MC5 receptors, respectively. Thus, the loss of a positive charge may play some role in the loss of binding observed, but no such positive charge exists in the MC3 receptor. In the latter case one might speculate that a lack of hydrophilic residues in the N-terminal region, besides the earlier mentioned conserved Glu, might cause problems in membrane insertion and orientation of TM1.

All the MC receptors have a Ser residue close to the N-terminal end (MC1Ser^{6}, MC3Ser^{4}, MC4Ser^{4} and MC5Ser^{4}). This Ser was recently proposed to participate in ligand binding based on the finding that a Ser/Ala exchange in the MC1 receptor resulted in loss in affinity to NDP-MSH and α-MSH [16]. Our data contradict those previous results but at present we do not have any rational explanation to this discrepancy.

In summary, our results indicate that neither the N-terminal regions nor carbohydrates added by glycosylation of these are likely to be essential for ligand binding for the human MC receptors or for their structural fidelity.

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References

[1] O'Donahue, T.L. and Dorsa, D.M. (1982) Peptides 3, 353–395.
[2] Eberle, A.N. (1988) The Melanotropins: Chemistry, Physiology and Mechanisms of Action, pp. 210–319, Karger, Basel, Switzerland.
[3] Chhajlani, V. and Wikberg, J.E.S. (1992) FEBS Lett. 309, 417–420.
[4] Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. and Cone, R.D. (1992) Science 257, 1248–1251.
[5] Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S.J., DelValle, J. and Yamada, T. (1993) J. Biol. Chem. 268, 8246–8250.
[6] Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S.J., DelValle, J. and Yamada, T. (1993) J. Biol. Chem. 268, 15174–15179.
[7] Chhajlani, V., Muceniece, R. and Wikberg, J.E.S. (1993) Biochem. Biophys. Res. Commun. 195, 866–873.
[8] Schiöth, H.B., Chhajlani, V., Muceniece, R., Klusa, V. and Wikberg, J.E.S. (1996) Life Sci. 59, 797–801.
[9] Schiöth, H.B., Muceniece, R., Wikberg, J.E.S. and Chhajlani, V. (1995) Eur. J. Pharmacol., Mol. Pharm. Sect. 288, 311–317.
[10] Schiöth, H.B., Muceniece, R. and Wikberg, J.E.S. (1996) Pharmacol. Toxicol. 79, 161–165.
[11] Chhajlani, V. (1996) Biochem. Mol. Biol. Int. 38, 73–80.
[12] Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B. and Cone, R.D. (1994) Mol. Endocrinol. 8, 1298–1308.
[13] Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D., Smith, F.J., Campfield, L.A., Burn, P. and Lee, F. (1997) Cell 88, 131–141.
[14] Labbé, O., Desarnaud, F., Eggerickx, D., Vassart, G. and Parmentier, M. (1994) Biochemistry 33, 4543–4549.
[15] Fathi, Z., Iben, L.G. and Parker, E.M. (1995) Neurochem. Res. 20, 107–113.
[16] Chhajlani, V., Xu, X., Blauw, J. and Sudarshi, S. (1996) Biochem. Biophys. Res. Commun. 219, 521–525.
[17] Frändberg, P.-A., Muceniece, R., Prusis, P., Wikberg, J.E.S. and Chhajlani, V. (1994) Biochem. Biophys. Res. Commun. 202, 1266–1271.
[18] Schiöth, H.B., Muceniece, R., Szardenings, M., Prusis, P. and Wikberg, J.E.S. (1996) Biochem. Biophys. Res. Commun. 229, 687–692.
[19] Prusis, P., Frändberg, P.-A., Muceniece, R., Kalvinsh, I. and Wikberg, J.E.S. (1995) Biochem. Biophys. Res. Commun. 210, 205–210.
[20] Haskell-Luevano, C., Sawyer, T.K., Trumpp-Kallmayer, S., Bibber, J.A., Humlet, C., Gantz, I. and Hruby, V.J. (1996) Drug Design and Discovery 14, 197–211.
[21] Schiöth, H.B., Muceniece, R., Wikberg, J.E.S. and Szardenings, M. (1996) Eur. J. Pharmacol. 314, 381–384.
[22] Schiöth, H.B., Kuusinen, A., Muceniece, R., Szardenings, M., Keinänen, K. and Wikberg, J.E.S. (1996) Biochem. Biophys. Res. Commun. 221, 807–814.
[23] Huang, R.-R.C., Vicario, P.P., Strader, C.D. and Fong, T.M. (1995) Biochemistry 34, 10048–10055.
[24] Wang, W.W., Shahrestanifar, M., Jin, J. and Howells, R.D. (1995) Proc. Natl. Acad. Sci. USA 92, 12436–12440.
[25] Holtman, M.H., Hadac, E.M. and Miller, L.J. (1995) J. Biol. Chem. 270, 14394–14398.
[26] Reichert, L.E., Dattatreyamurty Jr., B., Grasso, P. and Santa-Coloma, T.A. (1991) Trends Pharmacol. Sci. 12, 199–203.
[27] Xie, Y.-B., Wang, H. and Segaloff, D.L. (1990) J. Biol. Chem. 265, 21411–21414.
[28] Kenakin, T. (1996) Pharmacol. Rev. 48, 413–451.
[29] Wallin, E. and von Heijne, G. (1995) Protein Eng. 8, 693–698.
[30] von Heinje, G. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 167–192.