Molecular cloning, functional expression and pharmacological characterization of a mouse melanocortin receptor gene

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We describe the cloning of the mouse *HGMP01A* gene that encodes a melanocortin receptor functionally distinct from the adrenal cortex corticotropin (adrenocorticotrophic hormone; ACTH) receptor and the melanocyte-stimulating hormone (MSH) receptor expressed in melanoma. The gene encodes a protein of 323 amino acids with a calculated molecular mass of 35800 Da, displaying potential sites for N-linked glycosylation and phosphorylation by protein kinase C. An RNAase protection assay detected weak expression in the brain, but not in adrenal gland, skin, or any of the other tissues tested. Stable CHO cell lines expressing over 100000 receptors per cell were generated. The recombinant receptor binds iodinated [Nle^4,D-Phe^7]α-MSH (NDP-MSH) with an apparent K_d of 700 pM. Displacement of the ligand by a variety of pro-opiomelanocortin-derived peptides revealed a pharmacological profile distinct from that of the classical ACTH and MSH receptors. NDP-MSH was the most powerful competitor (IC_{50} 1.4 nM), followed by γ-MSH (IC_{50} 7 nM). α-MSH, β-MSH and ACTH-(1–39) were significantly less potent, with IC_{50} values of 30, 19 and 21 nM respectively. ACTH-(4–10) was poorly active (IC_{50} 2.4 μM), while corticotropin-like intermediate lobe peptide (CLIP) and β-endorphin were totally ineffective. The recombinant receptor was found to stimulate adenylate cyclase. The potency order of the agonists in this assay was consistent with that of the binding displacement assays. This receptor represents the orthologue of the human melanocortin 3 receptor reported recently. The growing family of melanocortin receptors constitute the molecular basis for the variety of actions of melanocortins that have been described over the years. The availability of functionally expressed receptors from the melanocortin family will allow the development of a specific pharmacology, and a better understanding of the function of the pro-opiomelanocortin-derived peptides.

INTRODUCTION

Melanocortins are derived through a series of ordered proteolytic cleavages from the precursor peptide pro-opiomelanocortin (POMC); other peptides, including endorphins, are also produced in this way. POMC is processed differently in the anterior pituitary, where corticotropin (adrenocorticotrophic hormone; ACTH) and β-lipotropin are the main products, and in the intermediate lobe, which essentially releases α-melanocyte-stimulatory hormone (α-MSH) and β-endorphin. Expression of the POMC gene is also detected in numerous non-pituitary tissues, including hypothalamus, testis, ovary, placenta, duodenum, liver, kidney, lung, thymus and lymphocytes (Pintar et al., 1984; Jingami et al., 1984; Chen et al., 1986; Debold et al., 1988; Buzzetti et al., 1989). POMC peptides have been detected by immunohistochemistry and radioimmunoassay in many human and animal tissues (Debold et al., 1988). Whether these peptides are effectively secreted and endowed with physiological functions is not proven in all cases.

ACTH was discovered as having a stimulatory function in corticoadrenal steroidogenesis, while the most clearly established function for α-MSH is the stimulation of melanogenesis in melanocytes. A number of additional functions for melanocortins have, however, been proposed over the years, in direct correlation with the extra-pituitary localization of POMC peptides. The action of melanocortins in the brain is well established. ACTH and MSH both have potent neurotrophic properties during development and neuronal regeneration (Scott, 1991; Strand et al., 1991; Zohar and Salomon, 1992; McCaig and Stewart, 1992); they also have profound effects on the behaviour of mammals and on memory (De Wied and Jolles, 1982; De Wied and Croiset, 1991). α-MSH was also reported to have anti-inflammatory properties, antagonizing the action of interleukin 1 (Lipton et al., 1991; Hiltz et al., 1991, 1992; Weiss et al., 1991). It is a potent antipyretic agent and inhibits neutrophil chemotaxis, among other effects (Villar et al., 1991; Ehymayed and Jansky, 1992). It has also been suggested that brain γ-MSH is involved in the central control of blood pressure (Gruber and Callahan, 1989).

Recently the mouse ACTH receptor and the human and mouse MSH receptors have been cloned and characterized functionally (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992). The identity between the mouse MSH receptor locus and the extension locus responsible for the agouti character was further demonstrated, together with the involvement of point mutations in the MSH receptor genes in a number of coat colour variants in other mammals (Robbins et al., 1993). A related receptor, the human melanocortin 3 receptor, was cloned recently and characterized by cyclic AMP accumulation assays (Gantz et al., 1993). We describe here the independent cloning of the mouse melanocortin 3 receptor gene, and the pharmacological and functional characterization of the recombinant receptor expressed in eukaryotic cells in culture.

EXPERIMENTAL

Ligands

ACTH, α-MSH, β-MSH, γ_2-MSH, [Nle^4,D-Phe^7]α-MSH (NDP-MSH), ACTH-(4–10), corticotropin-like intermediate lobe peptide (CLIP) and β-endorphin were purchased from Peninsula Laboratories.

Cloning and sequencing

A mouse genomic DNA library (Stratagene) constructed in λ FIXII was screened at low stringency (Sambrook et al., 1989) with the HGMP01 probe, a 600 bp PCR fragment amplified from genomic DNA by low-stringency PCR (Libert et al., 1989; Parmentier et al., 1989). The restriction map of the genomic clone was determined and a relevant XbaI–HindIII fragment of 1700 bp was subcloned in pBluescript SK$^+$ (Stratagene). Sequencing was performed on both strands after subcloning in M13mp derivatives, using fluorescent primers and an automated DNA sequencer (Applied Biosystem 370A). Sequence handling and data analysis was carried out using DNASIS/PROSIS software (Hitachi) and the GCG/VMS software package (Genetic Computer Group, Wisconsin).

Expression in cell lines

An XbaI–BstYI 1242 bp fragment containing the entire coding region was cloned in the eukaryotic expression vector pSVL (Pharmacia). The resulting construct (pSVL-HGMP01A) was transfected in COS-7 cells as described previously (Gérard et al., 1991). CHO-K1 cells were co-transfected with pSVL-HGMP01A and pSV2Neo as described (Perret et al., 1990), with the exception that no carrier DNA was added. After 2 days, selection for transfectants was initiated by the addition of 400 μg/ml G418 (Gibco), and resistant clones were isolated at day 10. Cos-7 and CHO-K1 cells were cultured using Dulbecco's modified Eagle's medium and Ham's F12 medium respectively, as previously described (Perret et al., 1990).

Preparation of radioiodinated NDP-MSH

NDP-MSH was radioiodinated using the chloramine T method. Briefly, NDP-MSH (2.5 μg) was incubated in presence of 1 mCi of Na^{125}I (2175 Ci/mmol; Amersham) and chloramine T (10 μg) for 40 s in a total volume of 35 μl. The reaction was stopped by the addition of 0.6 ml of sodium phosphate buffer, pH 7.4, containing 0.25% BSA and 0.1% β-mercaptoethanol. Mono-iodinated NDP-MSH was purified according to Siegrist et al. (1988), with the exception that the pre-purification step was carried out on a C$_{18}$ Sep-Pak cartridge (Waters). The specific radioactivity of the purified radioiodinated compound was 2175 Ci/mmol.

Binding assays

All assays were carried out on whole cells in 0.5 ml polypropylene microcentrifuge tubes in a final volume of 200 μl, and incubated for 40 min under constant shaking at room temperature. Cos-7 cells (3 days after transfection) or stably transfected CHO-K1 cells at confluence were detached from culture dishes by incubation in PBS supplemented with 1 mM EDTA. The cell suspension was collected and counted in a Neubauer cell. A total of 1×10^5 cells were incubated in culture medium, supplemented with 1 mM 1,10-phenanthroline, 0.5 μg/ml leupeptin and 200 μg/ml bacitracin as protease inhibitors, in the presence of radioiodinated NDP-MSH and competing agonists. The concentration of labelled ligand in displacement experiments ranged from 0.1 to 0.2 nM (85000 to 170000 c.p.m.). Non-specific binding was determined by adding an excess of unlabelled NDP (1 μM). The incubation medium was further layered on to a 10% (w/v) sucrose cushion (200 μl) in PBS, kept at 0 °C in 0.5 ml microcentrifuge tubes, and the cell-bound ligand was separated by centrifugation (13000 g, 0 °C, 10 min) through the cushion. The tubes were further frozen in liquid nitrogen, and the bottom of each tube containing the cell pellet was cut with a blade and radioactivity was counted in a γ-radiation counter.

Cyclic AMP accumulation assays

Sterile glass tubes were seeded with 200 μl of culture medium containing 5×10^4 transfected CHO-K1 cells. The next day, the cells were washed once with 1 ml of Krebs–Ringer Hepes buffer (KRH; 25 mM Hepes, pH 7.4, 124 mM NaCl, 5 mM KCl, 1.25 mM KH$_2$PO$_4$, 1.5 mM CaCl$_2$, 1.25 mM MgSO$_4$, 8 mM glucose). Ligands (10^{-12} to 10^{-5} M) were added in 200 μl of KRH supplemented with 0.5% BSA, 1 mM 1,10-phenanthroline, 0.5 μg/ml leupeptin and 200 μg/ml bacitracin as protease inhibitors, and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) as an inhibitor of phosphodiesterases. Controls were performed on untransfected CHO-K1 cells, and on transfected cells incubated in the absence of ligands or in the presence of 500 nM forskolin. After 2 h at 37 °C, cyclic AMP accumulation was stopped by the addition of 500 μl of boiling water, and tubes were further kept in a boiling water bath for 10 min. Samples were vacuum-dried overnight, and cyclic AMP was measured by radioreceptor assays following the kit manufacturer's instructions (Amersham).

RNAase protection assays

RNAase protection assays were performed as described (Sambrook et al., 1989). Briefly, a 210 bp PstI–KpnI fragment of HGMP01A was subcloned in pBluescript SK+ (Stratagene) and used as template for the synthesis of an antisense RNA probe in the presence of [α-^{32}P]UTP (800 Ci/mmol; Amersham). Total RNA was prepared from various mouse tissues using the guanidinium thiocyanate/caesium chloride gradient method (Sambrook et al., 1989). RNA samples (10 μg) were incubated overnight at 45 °C with 5000 c.p.m. of the labelled antisense probe. After digestion with RNAases A (2 μg/ml) and T1 (40 μg/ml), samples were analysed by electrophoresis on a 6% poly-acrylamide denaturing gel and autoradiographed. RNA extracted from CHO-K1 cells transfected with pSVL-HGMP01A was used as a positive control.

RESULTS AND DISCUSSION

Cloning and structural analysis

The sequence similarity that characterizes genes encoding G-protein-coupled receptors has allowed the cloning by low-stringency PCR of new members of this gene family (Libert et al., 1989; Parmentier et al., 1989). One of the PCR clones (HGMP01) amplified from human genomic DNA was used to screen a mouse genomic library and 14 clones were isolated, corresponding to two loci. Clones from one locus hybridized strongly with the probe, and a representative clone was named HGMP01A. A 1700 bp XbaI–HindIII fragment of HGMP01A was subcloned in pBluescript SK+ and the sequence revealed a 91% match with the human probe, suggesting that HGMP01A encoded the mouse orthologue. The clones corresponding to the second locus hybridized weakly to the human probe. One of

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                                                                    - 119 TCTAGACTGGACAGCATCC
- 90 ACAAGAGAAGCACCTAGAAGGAGAATTTTCCCCAGCAGCTTGCTCAGGACCCTGCAGGAGCCGCAGCTGGGACTGGACCTGCTGTTAACC
   1 ATGAACTCTTCCTGCTGCCTGTCTTCTGTTTCTCCGATGCTGCCTAACCTCTCTGAGCACCCTGCAGCCCCTCCTGCCAGCAACCGGAGC
   1  M  N  S  S  C  C  L  S  S  V  S  P  M  L  P  N  L  S  E  H  P  A  A  P  P  A  S  N  R  S

  91 GGCAGTGGGTTCTGTGAGCAGGTCTTCATCAAGCCGGAGGTCTTCCTGGCTCTGGGCATCGTCAGTCTGATGGAAACATCCTGGTGATC
  31  G  S  G  F  C  E  Q  V  F  I  K  P  E  V  F  L  A  L  G  I  V  S  L  M  E  N  I  L  V  I

 181 CTGGCTGTGGTCAGGAATGGCAACCTGCACTCTCCCATGTACTTCTTCCTGTGCAGCCTGGCTGCAGCCGACATGCTGGTGAGCCTGTCC
  61  L  A  V  V  R  N  G  N  L  H  S  P  M  Y  F  F  L  C  S  L  A  A  A  D  M  L  V  S  L  S

 271 AACTCCCTGGAGACCATCATGATCGCCGTGATCAACAGCGACTCCCTGACCTTGGAGGACCAGTTTATCCAGCACATGGATAATATCTTC
  91  N  S  L  E  T  I  M  I  A  V  I  N  S  D  S  L  T  L  E  D  Q  F  I  Q  H  M  D  N  I  F

 361 GACTCTATGATTTGCATCTCCCTGGTGGCCTCCATCTGCAACCTCCTGGCCATTGCCATCGACAGGTACGTCACCATCTTCTATGCCCTT
 121  D  S  M  I  C  I  S  L  V  A  S  I  C  N  L  L  A  I  A  I  D  R  Y  V  T  I  F  Y  A  L

 451 CGGTACCACAGCATCATGACAGTTAGGAAAGCCCTCACCTTGATCGGGGTCATCTGGGTCTGCTGCGGCATCTGCGGCGTGATGTTCATC
 151  R  Y  H  S  I  M  T  V  R  K  A  L  T  L  I  G  V  I  W  V  C  C  G  I  C  G  V  M  F  I

 541 ATCTACTCCGAGAGCAAGATGGTCATCGTGTGTCTCATCACCATGTTCTTCGCCATGGTGCTCCTCATGGGCACCCTATATATCCACATG
 181  I  Y  S  E  S  K  M  V  I  V  C  L  I  T  M  F  F  A  M  V  L  L  M  G  T  L  Y  I  H  M

 631 TTCCTCTTCGCCAGGCTCCACGTCCAGCGCATCGCAGTGCTGCCCCCTGCTGGCGTGGTGGCCCCACAGCAGCACTCCTGCATGAAGGGG
 211  F  L  F  A  R  L  H  V  Q  R  I  A  V  L  P  P  A  G  V  V  A  P  Q  Q  H  S  C  M  K  G

 721 GCTGTCACCATCACTATCCTGCTGGGTGTTTTCATCTTCTGCTGGGCGCCTTTCTTCCTCCACCTGGTCCTCATCATCACCTGCCCCACC
 241  A  V  T  I  T  I  L  L  G  V  F  I  F  C  W  A  P  F  F  L  H  L  V  L  I  I  T  C  P  T

 811 AATCCCTACTGCATCTGCTACACGGCCCATTTCAACACCTACCTGGTTCTCATCATGTGCAACTCCGTCATCGACCCCCTCATCTACGCC
 271  N  P  Y  C  I  C  Y  T  A  H  F  N  T  Y  L  V  L  I  M  C  N  S  V  I  D  P  L  I  Y  A

 901 TTCCGCAGCCTGGAGCTGCGCAACACGTTCAAGGAGATTCTCTGCGGCTGCAACAGCATGAACTTGGGCTAGGATGCCCGTGGAGGTGTT
 301  F  R  S  L  E  L  R  N  T  F  K  E  I  L  C  G  C  N  S  M  N  L  G  *

 991 CCACATCCAGCCAAGAGACAAAAACAACGCTCAGACGGGACGTAAAAGGGTGTTAGGAGCTGGAACTGTGCTTGGCTTCGTCTGTAAGCT
1081 CGTGGCCCTTTGCAGACGGGACACGGCGTAGGATGGGCTGTCTGTGAGGATCTGTGTGTGGGTAAGTCAGTTTGATCTAGCACATAGCCT
1171 GGAAGAATCAGGCAAAGCAGCCCTGAGTGTCATCTGTGTTCATTGCTAGGCACCCAGGGTTTGTGGCCCCTGCCTGCTTATTGGCTTTGT
1261 ACCAGTAACTGTGCTTCAAGCCAACCAGACCGGAGGGCTCTCGTGAGCAGAAAGAGTGCTTAGACTTCCGGCAAGCATCCTGGCTCACAG
1351 CGGCCACCTCCTGACCACTACCGGGAGAGCTTTGCACATATTCTGTGGGAGATTGAGTGAAGCCCTGAAAACAATGTGATATTTGCTGCT
1441 CCCTTCCAGAAACTTACATCTGTGCCAGCCTCCCCGAACCCCTGCACAGAGACATGACCCCCTTCTCCCTGTGCCGTTGTCATGGTTGTTA
1531 TTATTGTTGGAGTTTTGTTCGTTAAAATCTAAGCTT 1566
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Figure 1   Nucleotide and deduced amino acid sequences of the mouse HGMP01A receptor gene

Numbering is relative to the putative initiation codon. A dot tags every tenth residue.

these clones, named HGMP01B, was analysed, and was found to encode a receptor related to HGMP01A that is presently being characterized (Labbe et al., 1994).

The sequence of HGMP01A revealed a single open reading frame of 323 codons (Figure 1) encoding a protein of 35 800 Da. The sequence surrounding the proposed initiation codon is in good agreement with the consensus as described by Kozak (1989). The hydropathy profile (not shown) of the deduced amino acid sequence is consistent with the existence of seven transmembrane segments. Alignment with known receptors revealed 46 % identity with the reported ACTH (Mountjoy et al., 1992) and MSH (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992) receptors (Figure 2). There is also 88 % identity with the recently reported sequence for the human melanocortin 3 receptor (Gantz et al., 1993), which is identical to our human HGMP01 PCR probe. These two receptors therefore represent species homologues. The main difference between our mouse HGMP01A sequence and the human melanocortin 3 receptor is the length of the N-terminal extracellular domain (Figure 2). The reported human sequence is 37 amino acids longer. Nevertheless, the identity between human and mouse sequences falls ahead of the conserved methionine that we consider as the translation initiation codon, and the mouse sequence does not contain the in-phase ATG codon corresponding to the first methionine codon of the human sequence. The existence in both sequences (cloned from genomic DNA) of segments compatible with the consensus sequence [(C/T)$_n$N(C/T)AG|G] proposed for intron/exon borders (Mount, 1982) at the upper limit of sequence identity (−50) further suggests that the first part of these sequences is

intronic material that would not be present in the processed message. These putative intron/exon borders are TTTT-CCCCAG|C and TTTTGTCTTTCCTGTGAGCAG|C for the mouse and human sequences respectively. The human coding region would therefore start at the second methionine of the open reading frame reported by Gantz et al. (1993).

The dendrogram representing similarities between presently known melanocortin receptors is displayed in Figure 2. Three potential sites for N-linked glycosylation are found in the N-terminal extracellular domain of HGMP01A, and are conserved in the melanocortin 3 receptor. Two potential sites for phosphorylation by protein kinase C are present in the second intracellular loop and the C-terminal domain of the receptor (Figure 2). One of these potential phosphorylation sites is shared by the ACTH receptor, the other by the MSH receptor. These sites could be involved in the regulation of receptor function by protein kinase C (Dohlman et al., 1991). HGMP01A does not contain sites that could be phosphorylated by the cyclic AMP-dependent protein kinase, in contrast with the mouse MSH receptor and the ACTH receptors. By analogy with the β-adrenergic receptor, a conserved cysteine located in the C-terminal segment (Figure 2) could be palmitoylated and thus anchor the C-terminal segment of the receptors in the plasma membrane (O'Dowd et al., 1989).

Expression in CHO-K1 cells

The coding region of HGMP01A was subcloned as an XbaI–BstYI fragment in the expression vector pSVL. The resulting
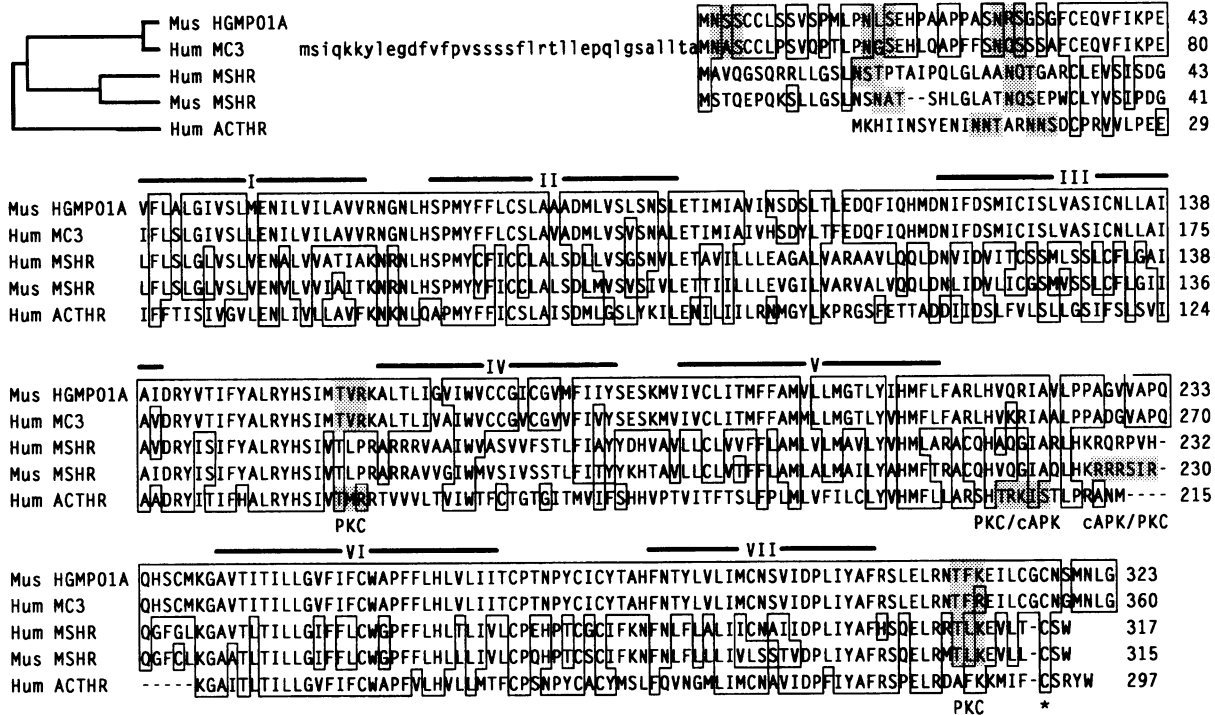
Figure 2   Alignment and dendrogram representing sequence similarities between melanocortin receptors

Sequences of mouse (HGMP01A) and human (MC3) melanocortin 3 receptors were aligned with the human (Hum) and mouse (Mus) MSH and human ACTH receptors, and a dendrogram representing sequence similarities was generated using the Clustal software (Higgins and Sharp, 1988). Identities with HGMP01A are boxed. The N-terminal extension of the human MC3 sequence (lowercase characters) could be translated partly from an intron. Putative transmembrane segments are indicated by the roman numbers I–VII. Potential N-linked glycosylation sites in the N-terminal extracellular segment are shaded, as are potential phosphorylation sites by protein kinase C (PKC) or cyclic AMP-dependent protein kinase (cAPK), located in the second and third intracellular loops and in the C-terminal domain. The conserved cysteine possibly involved in palmitoylation is indicated by *.
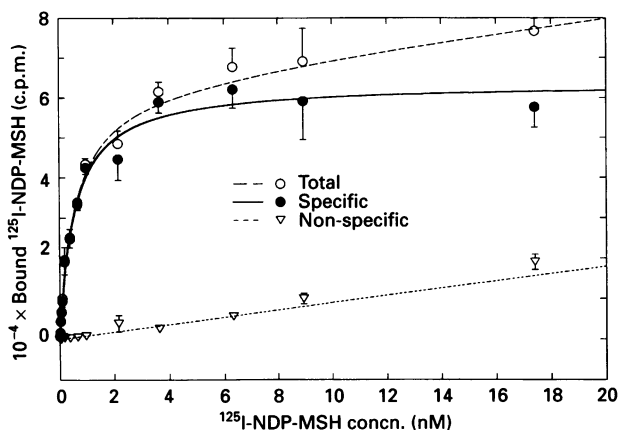

Figure 3   Saturation binding assay

Saturation binding experiments were performed using ^{125}I-NDP-MSH and a stable CHO cell line (clone #1) expressing the mouse recombinant HGMP01A receptor. Total, specific and non-specific binding are represented. Results represent means ± S.D of triplicate experimental values. Curve fitting using a non-linear regression algorithm and a one-site model yielded an apparent K_d of 0.6 nM.

pSVL-HGMP01A construct was first transfected in COS-7 cells, and binding of the analogue NDP-MSH was assayed both on membranes prepared from the transfected cells and on intact

cells in culture dishes. Binding did not occur with membrane preparations, while specific binding could be detected using whole cells, despite a high degree of non-specific binding (results not shown). It thus appeared that HGMP01A encoded a receptor of the melanocortin family, but that the receptor was unstable during classical membrane preparation procedures. This situation is similar to what has been reported for the classical MSH receptor. The binding assay was further optimized in order to reduce the non-specific binding to a minimum. The ideal approach, as described in the Experimental section, was found to involve intact cells dissociated in EDTA-containing medium, and a separation step through a sucrose cushion. Under these conditions the non-specific binding represented around 3 % of the total binding for a ligand concentration equal to the K_d. Equilibrium was found to be reached as early as 5 min after addition of membranes, and to be stable for several hours (results not shown). The standard incubation time was set to 40 min. Accumulation of cyclic AMP was also measured in transfected COS-7 cells after addition of NDP, α-MSH and ACTH (1 μM). The elevated cyclic AMP levels obtained in these preliminary experiments (results not shown) demonstrated that the HGMP01A receptor was positively coupled to adenylate cyclase and responded similarly to all three ligands.

The same pSVL-HGMP01A construct was co-transfected with the pSV2Neo plasmid in CHO-K1 cells, and stably transfected lines were generated. Individual clones and control cells were tested for their response to NDP-MSH in a cyclic AMP assay, and for their binding properties. Out of the 30 clones tested, 22 responded maximally to NDP-MSH in the cyclic AMP assay,
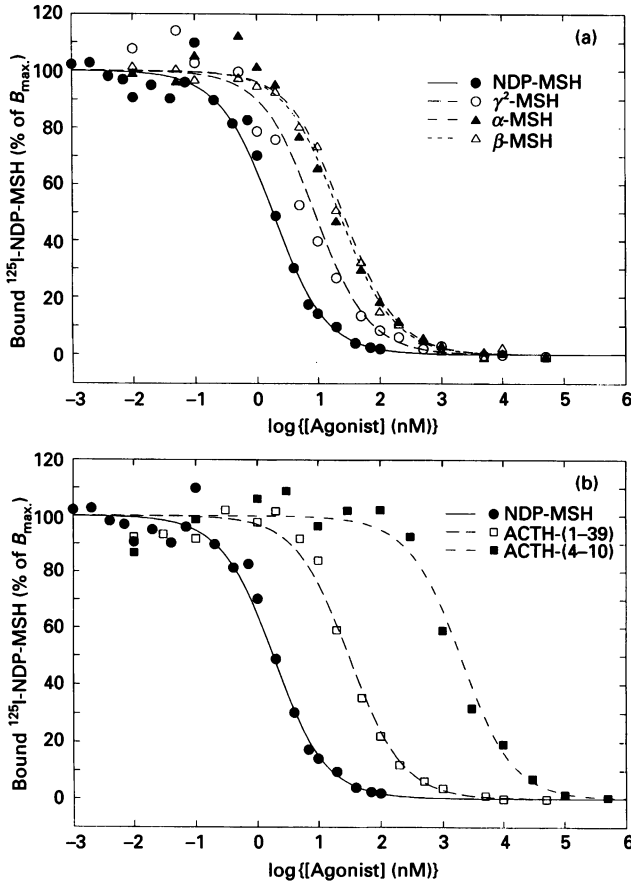
Figure 4 Pharmacological characterization of the mouse HGMP01A receptor

Displacement curves were obtained for a variety of analogues reported as agonists of the classical ACTH and MSH receptors. The ordinate is expressed as percentage of the total specific binding (B_{max}). Each point is the mean of triplicate experimental values. The relative S.D. calculated for each point was on average 8%. Each curve is representative of three independent experiments. The IC_{50} values for the displayed curves are 1.8 nM (NDP-MSH), 9 nM (γ_2-MSH), 22 nM (β-MSH), 26 nM (α-MSH), 12 nM [ACTH-(1–39)] and 2 μM [ACTH-(4–10)].
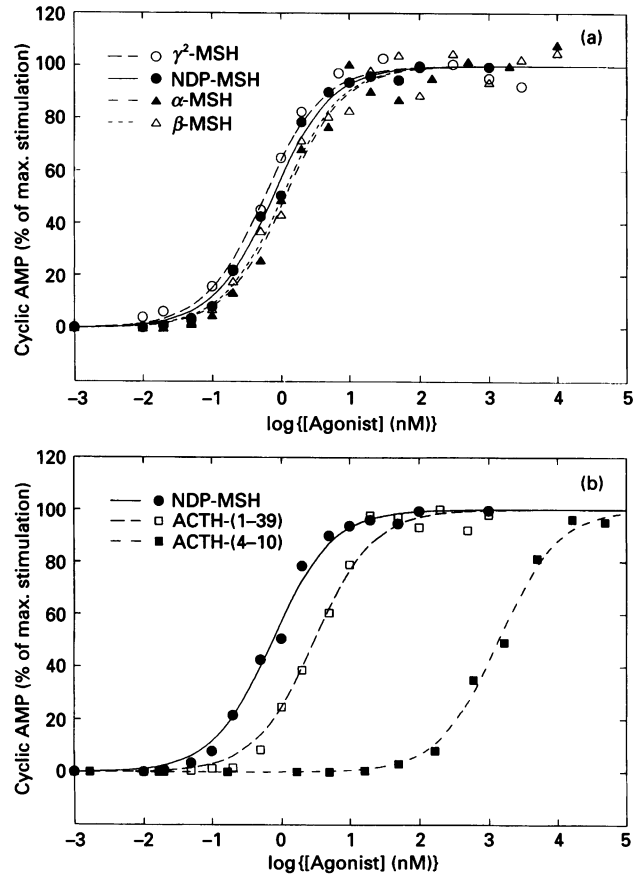
Figure 5 Functional coupling of HGMP01A in CHO-K1 cells

A CHO clonal cell line stably transfected with pSVL-HGMP01A (clone #1) was assayed for cyclic AMP accumulation in response to NDP-MSH and other agonists derived from POMC. The ordinate is expressed as percentage of the maximal cyclic AMP level obtained for each curve. Each point represents the mean of triplicate experimental values. The relative S.D. calculated for each point was on average 9%. Each curve is representative of three independent experiments. The EC_{50} values for the displayed curves are 0.58 nM (NDP), 0.56 nM (γ_2-MSH), 1.04 nM (β-MSH), 1.15 nM (α-MSH), 3.05 nM [ACTH-(1–39)] and 1.38 μM [ACTH-(4–10)].

whereas all control cells were negative (results not shown). In binding experiments, however, binding capacity was distributed over a wider range, and one clone (#1) was selected for its high expression level (results not shown). This clone was used for all subsequent cyclic AMP and binding assays. The lack of discrimination of the cyclic AMP accumulation assay is attributed to the saturation of adenylate cyclase activity in the presence of large numbers of stimulated receptors. Saturation binding curves with iodinated NDP-MSH as ligand were consistent with a single binding site characterized by an apparent K_d of 0.6 nM (Figure 3). From the B_{max} value it was estimated that clone #1 contains approx. 130000 binding sites per cell. Displacement curves were obtained for a variety of peptides derived from POMC (Figure 4). NDP-MSH itself had the lowest IC_{50} value (1.43 ± 0.47 nM). γ-MSH (IC_{50} 6.7 ± 1.6 nM) was the most powerful natural competitor, followed by β-MSH (19.4 ± 2.6 nM), ACTH (21.1 ± 7.1 nM) and α-MSH (30.2 ± 3.3 nM). The truncated peptide ACTH-(4–10), corresponding to the heptapeptide sequence common to ACTH, α-MSH and β-MSH, was about 100-fold less potent (IC_{50} 2.4 ± 0.4 μM) than ACTH-(1–39). Other POMC-derived peptides, CLIP and β-endorphin, were totally ineffective up to micromolar concentrations (results not shown).

Cyclic AMP accumulation was assayed in clone #1 in response to various agonists (Figure 5). Cyclic AMP levels in unstimulated cells were below the limit of detection of the assay (1 pM per tube). Maximal stimulation levels ranged from 60 to 220 pmol of cyclic AMP per tube, depending on the experiment. In each experiment, similar maximal levels were obtained for all agonists. NDP-MSH concentrations as low as 20 pM were able to elevate cyclic AMP concentrations within the assayable range (Figure 5). The maximal effect was obtained above 10 nM, and the half-maximal effect at 0.43 ± 0.11 nM (EC_{50}), a concentration somewhat lower than the apparent K_d value. The effects of other POMC-derived agonists are depicted in Figure 5. Again, NDP-MSH was the most active compound, together with γ-MSH (EC_{50} 0.64 ± 0.31 nM), followed by β-MSH (EC_{50} 1.1 ± 0.40 nM), α-MSH (EC_{50} 1.21 ± 0.21 nM) and ACTH (EC_{50} 3.3 ± 1.0 nM). ACTH-(4–10) was poorly active (EC_{50} = 1120 ± 320 nM), and β-endorphin and CLIP were totally ineffective (results not shown). The potency order of POMC agonists therefore appears similar in both the binding displacement assay and the functional cyclic AMP accumulation assay. The functional EC_{50} values are lower than the binding IC_{50} values, suggesting that activation of a small percentage of the receptors present at the cell surface

HGMP01A CHO cells

Whole brain

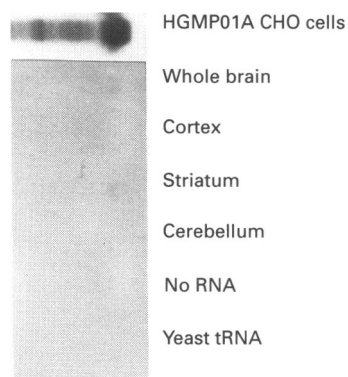Cortex

Striatum

Cerebellum

No RNA

Yeast tRNA

Figure 6   Tissue distribution of HGMP01A transcripts

The tissular distribution of HGMP01A expression was investigated by RNAase protection assay, and protected fragments were separated on polyacrylamide gel. RNA extracted from transfected CHO-K1 cells was used as positive control (3 days autoradiography). Negative controls included yeast tRNA and omission of RNA. A faint positive signal (15 days autoradiography) was obtained only in total brain RNA and in RNA extracted from the basal region of the brain (hypothalamus, thalamus and striatum). Negative results obtained for other tissues are not shown.

is sufficient to fully activate G_s proteins and/or adenylate cyclase.

The pharmacological profile of the HGMP01A receptor is quite different from those of the classical ACTH and MSH receptors cloned recently. With the exception of minor differences, Mountjoy et al. (1992), Chhajlani and Wikberg (1992) and Gantz et al. (1993) reported the cloning of the mouse MSH receptor which discriminates clearly between α-MSH, the most active natural peptide, the moderately active ACTH and β-MSH peptides, and the poorly active γ-MSH. ACTH-(4–10) was almost inactive with this receptor, and other POMC-derived peptides were without effect. The pharmacological profile of the cloned ACTH receptor was not reported (Mountjoy et al., 1992), but the natural corticoadrenal receptor shows a marked specificity towards ACTH with little or no activity with the MSH peptides. From structural and functional points of view, the HGMP01A receptor appears therefore to be significantly different from both the classical MSH and ACTH receptors. Our cyclic AMP accumulation assays are in good general agreement with the profile described for the human melanocortin 3 receptor (Gantz et al., 1993). While these workers reported similar EC_{50} values for all active ligands (NDP-MSH, ACTH, α-, β- and γ-MSH), we found a somewhat stronger activity for γ-MSH, in both binding and cyclic AMP assays.

Tissue distribution of HGMP01A transcripts

The human HGMP01 probe was used on Northern blots of RNA extracted from nine dog tissues (brain, liver, kidney, testis, lung, heart, thyroid, stomach and spleen). No signal could be detected (results not shown). When the mouse HGMP01A clone became available, RNAase protection assays were performed on a larger panel of mouse tissues (Figure 6). Despite the RNAase protection assay sensitivity and the strong signal obtained for the cell line expressing the HGMP01A receptor, a faint signal could only be obtained with brain RNA and with RNA extracted from the basal brain regions (hypothalamus, thalamus and striatum). No signal could be obtained from the adrenal gland or skin, nor from any of the other tissues tested (pituitary, lung, thymus, heart muscle, skeletal muscle, spleen, kidney, oesophagus,

stomach, duodenum, jejunum, ileum, colon, liver, pancreas, uterus, ovary, placenta, testis, prostate and seminal vesicle; results not shown).

Conclusion

We report here the cloning of the HGMP01A gene, which encodes a mouse melanocortin 3 receptor. The most powerful natural agonist appears to be γ-MSH, which is almost inactive with the classical ACTH and MSH receptors. The HGMP01A receptor was found to be weakly expressed in mouse brain, and transcripts were undetectable in other tissues. This receptor could therefore mediate some of the central effects of POMC-derived peptides, including those attributed to γ-MSH. The availability of the cloned receptor will allow the development of a specific pharmacology of central melanocortin receptors.

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