Molecular Cloning of a Mouse Melanocortin 5 Receptor Gene Widely Expressed in Peripheral Tissues[†,‡]

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ABSTRACT: A mouse genomic clone named HGMP01B has been isolated by homology screening with a probe representing part of the human melanocortin 3 receptor gene. HGMP01B was found to encode a 325 amino acid protein with all the landmarks of G-protein-coupled receptors and belonging to the growing melanocortin receptor family. This receptor displays four potential sites for N-linked glycosylation and five potential sites of phosphorylation by protein kinase C. The HGMP01B gene was found to be expressed in many tissues, including skin, adrenal gland, skeletal muscle, bone marrow, spleen, thymus, gonads, uterus, and brain. A stable Chinese hamster ovary (CHO) cell line expressing approximately 10 000 receptors per cell was established. This cell line displayed a saturable binding capacity for the radioiodinated α-melanocyte-stimulating hormone (α-MSH) analog [Nle^4,D-Phe^7]-α-MSH (NDP-MSH) with an apparent K_d of 1.47 ± 0.15 nM. Binding of the labeled ligand was competed for by all melanocortin peptides, except β-endorphin or corticotropin-like intermediate lobe peptide (CLIP). NDP-MSH was the most powerful competitor, followed by α-MSH, adrenocorticotropic hormone (ACTH), β-MSH, the γ-MSHs, and ACTH 4-10. Functional assays confirmed that HGMP01B, like other melanocortin receptors, stimulated adenylyl cyclase. The potency order obtained in these cyclic adenosine monophosphate (cAMP) accumulation assays was consistent with that of the binding studies. HGMP01B therefore appears as a fifth melanocortin receptor (MC5), responding mainly to α-MSH (EC_{50} = 1.07 ± 0.13 nM) and endowed with a pharmacological profile similar to that of the melanocyte MSH (MC1) receptor, but characterized by a broad tissue distribution. The expression of MC5 in lymphoid organs suggests that this receptor could be implicated in the reported antiinflammatory action of melanocortins.

Melanocortin peptides are generated from a common precursor glycoprotein, the proopiomelanocortin (POMC[1]), by posttranslational processing [reviewed by Krieger (1983)]. These peptides include adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormones (α-, β-, γ-MSH), endorphins, and β-lipotropin. The POMC gene is expressed not only in pituitary but also in many non-pituitary tissues, including hypothalamus and other brain regions, skin, testis, ovary, gastrointestinal tract, pancreas, placenta, lung, kidney, thyroid, and the immune system (Krieger, 1983; Pintar et al., 1983; Saito et al., 1983; Debold et al., 1988; Fenger, 1990). Similarly, melanocortin binding sites have been described as largely distributed in tissues such as the lacrimal and submandibular glands, pancreas, adipose tissue, bladder, duodenum, spleen, and brain (Tatro & Reichlin, 1987). Brain receptors were further reported to bind both corticotropin and α-MSH with a similar affinity (Tatro, 1990). An increasing number of functions has been assigned to melanocortins over the years. The stimulatory action of ACTH on corticoadrenal steroidogenesis (Krieger, 1983) and that of α-MSH on melanogenesis in mice and humans are well-established (Burchill et al., 1992; De Luca et al., 1993).

Melanocortins appear to play a major role in the control of behavior, memory, learning, and motivation [reviewed by De Wied and Jolles (1982)]. They have marked neurotrophic properties, both during development and during the recovery process following nerve injury (Zohar & Salomon, 1992; Strand et al., 1991; McCaig & Stewart, 1992). They also have potent antipyretic and antiinflammatory properties (Nordlund, 1991; Hiltz et al., 1991) antagonizing the action of cytokines such as interleukin 1, interleukin 6, and TNFα (Martin et al., 1991). α-MSH was described as a mammotrophic factor released after estrogen treatment (Ellerkmann et al., 1992). Muscle fibers were found to respond to ACTH, as well as to α-, β-, and γ-MSHs (Strand et al., 1993). Four melanocortin receptors have been characterized to date. These include the melanocyte-specific MSH receptor (also called MC1) and the corticoadrenal-specific ACTH receptor (Mountjoy et al., 1992; Chhajlani & Wikberg, 1992) and the melanocortin 3 (Gantz et al., 1993a; Desarnaud et al., 1994) and melanocortin 4 (Gantz et al., 1993b) receptors, both present in the brain. Many of the proposed actions of melanocortins on peripheral tissues therefore cannot be

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[‡] The nucleotide sequence encoding the HGMP01B receptor has been deposited with the Genbank/EMBL data libraries under accession number X76295.

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[1] Abbreviations: ACTH, adrenocorticotropic hormone; bp, base pair; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; cAPK, cAMP-dependent protein kinase; CHO, Chinese hamster ovary; CLIP, corticotropin-like intermediate lobe peptide; KRH, Krebs–Ringer Hepes buffer; MC, melanocortin; MSH, melanocyte-stimulating hormone; NDP-MSH, [Nle^4,D-Phe^7]-α-MSH; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; POMC, proopiomelanocortin.

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explained. We describe here the cloning of a mouse melanocortin 5 receptor characterized by a broad tissue distribution, which could mediate at least some of these peripheral melanocortin actions, including their antiinflammatory properties. The cloning of a human receptor was reported (Chhajlani et al., 1993) after the completion of the present work. Sharing 78% identity with HGMP01B, this receptor could possibly represent the human homolog of our mouse receptor.

MATERIALS AND METHODS

Ligands. Rat ACTH, α-MSH, human β-MSH, human γ1-, γ2-, and γ3-MSHs, [Nle^4,D-Phe^7]-α-MSH (NDP-MSH), ACTH 4-10, human CLIP, human β-endorphin, and porcine β-lipotropin were purchased from Peninsula Laboratories (Belmont, CA).

Cloning and Sequencing. A mouse genomic clone was obtained as described (Desarnaud et al., 1993) from a λ FIXII library (Stratagene), using a probe encoding the human melanocortin 3 receptor. A 2179-bp *Eco*RI fragment was subcloned in pBluescript SK+ (Stratagene) and sequenced on both strands by the combination of forced cloning in M13mp vectors and exonuclease III deletion (Henikoff, 1984), using fluorescent primers and an automated DNA sequencer (Applied Biosystems 370A). Sequence analysis was carried out using DNASIS/PROSIS software (Hitachi) and the GCG/VMS software package (Genetic Computer Group, Madison, WI).

Expression in Cell Lines. The entire coding region was amplified by PCR as a 1047-bp fragment, using primers including, respectively, the *Xba*I and *Bam*HI recognition sequences, and cloned after restriction into the corresponding sites of the eukaryotic expression vector pSVL (Pharmacia). The resulting construct was verified by sequencing and cotransfected with the antibiotic resistance plasmid pSV2Neo in CHO-K1 cells. Transfected clones were selected and isolated as described (Perret et al., 1990; Desarnaud et al., 1993).

Binding Assays. NDP-MSH (2.5 μg) was radioiodinated using the chloramine T method as previously described (Desarnaud et al., 1993), using 1 mCi of Na^{125}I (2175 Ci/mmol, Amersham). Monoiodinated NDP-MSH was purified according to Siegrist et al. (1988) after prepurification on a C_{18} Sep-Pak cartridge (Waters). The specific activity of the purified, labeled peptide was 2175 Ci/mmol. Binding assays were performed on stably transfected CHO-K1 cells. Whole-cell suspensions were obtained by incubation in 1 mM EDTA supplemented phosphate-buffered saline (PBS). A total of 100 000 cells was incubated for 40 min under constant shaking in 200 μL of MEM medium (Gibco) supplemented with 1 mM 1,10-phenanthroline, 200 μg/mL bacitracin, and 0.5 μg/mL leupeptin, and in the presence of labeled (and competing) peptides. The concentration of iodinated ligand in displacement experiments was 0.1 nM (85 000 cpm). Nonspecific binding was estimated by adding an excess of unlabeled NDP-MSH (1 μM). Cells were separated from free ligands by centrifugation through a 10% sucrose cushion, as described (Desarnaud et al., 1994). The binding procedure had been set up within the framework of the analysis of the MC3 receptor (Desarnaud et al., 1994) and was used without modification. Care was taken to verify that, in these conditions, the specific binding obtained with the MC5 receptor was reversible and that binding equilibrium was attained well before the end of the incubation period. Binding data were analyzed by a nonlinear regression algorithm (Marquart, 1963) using a single-site model and locally designed programs (S. Swillens, unpublished results).

cAMP Accumulation Assays. Sterile glass tubes were seeded with 5×10^4 cells in 200 μL of Ham's F12 medium. The next day, the cells were rinsed 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_2PO_4, 1.5 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 8 mM glucose). Ligands (10^{-5}–10^{-12} M) were added in 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 included wild-type CHO-K1 cells and incubation in the presence of 10 mM forskolin. After an incubation of 2 h at 37 °C, the cAMP accumulation was stopped by the addition of 1 mL of boiling water, and the tubes were kept in boiling water for an additional 10 min. Samples were vacuum-dried overnight, and cAMP was measured by radioreceptor assay (TRK432 kit, Amersham).

RNAase Protection Assays. A 241-bp fragment corresponding to the end of the open reading frame of HGMP01B (+807 to +1047 relative to the translation initiation codon) was amplified by PCR and cloned through synthetic *Xba*I and *Bam*HI restriction sites in pBluescript SK+ (Stratagene). The resulting plasmid was used as a template for the synthesis of an antisense ^{32}P-labeled probe ([α-^{32}P]UTP, 800 Ci/mmol, Amersham). Mouse RNAs were prepared with the guanidium thiocyanate/cesium chloride gradient method, and RNAse protection assays were performed as described (Sambrook et al., 1989). RNA samples (30 μg) were denatured (85 °C, 10 min) and further hybridized with the probe (6000 cpm) overnight at 45 °C. The next day, samples were digested by RNAases A (40 μg/mL) and T1 (2 μg/mL), separated by electrophoresis on a 6% polyacrylamide gel, and autoradiographed. RNA extracted from the HGMP01B-transfected CHO-K1 cell line was used as a positive control and tRNA as negative control. Since HGMP01B is an intronless gene, protected bands theoretically could result from the hybridization of the labeled probe with genomic DNA contaminating the RNA samples. All samples therefore were hybridized in parallel experiments with a probe for the other intronless gene MC3. With this probe, a signal was obtained exclusively in the expected location (brain), demonstrating the absence of significant genomic DNA contamination.

RESULTS

Cloning and Structural Analysis. Starting from a partial clone (HGMP01) amplified by low-stringency PCR from genomic DNA (Libert et al., 1989, Parmentier et al., 1989), we cloned, from a mouse genomic DNA library, two related genes belonging to the melanocortin receptor family. One of these genes (HGMP01A) was characterized recently as encoding the mouse melanocortin 3 receptor (MC3; Desarnaud et al., 1994). A genomic clone corresponding to the second locus, named HGMP01B, was analyzed by Southern blotting, and a 2179-bp *Eco*RI restriction fragment, containing the full coding region, was subcloned and sequenced. A single open reading frame of 325 codons was found encoding a protein of 36 950 Da. The putative transmembrane organization of HGMP01B, on the basis of its hydropathy profile and its similarities with other G-protein-coupled receptors, is displayed in Figure 1. The alignment of HGMP01B with the other members of the melanocortin receptor family is represented

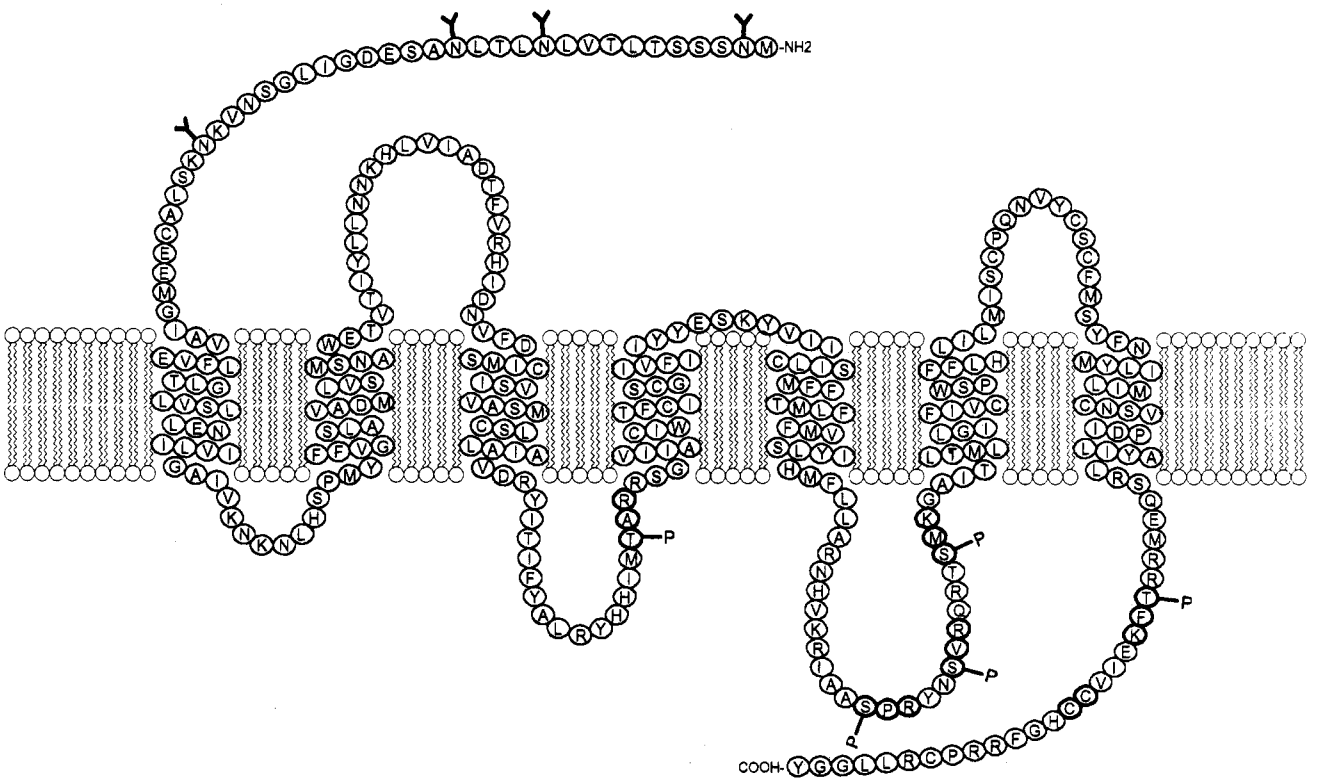
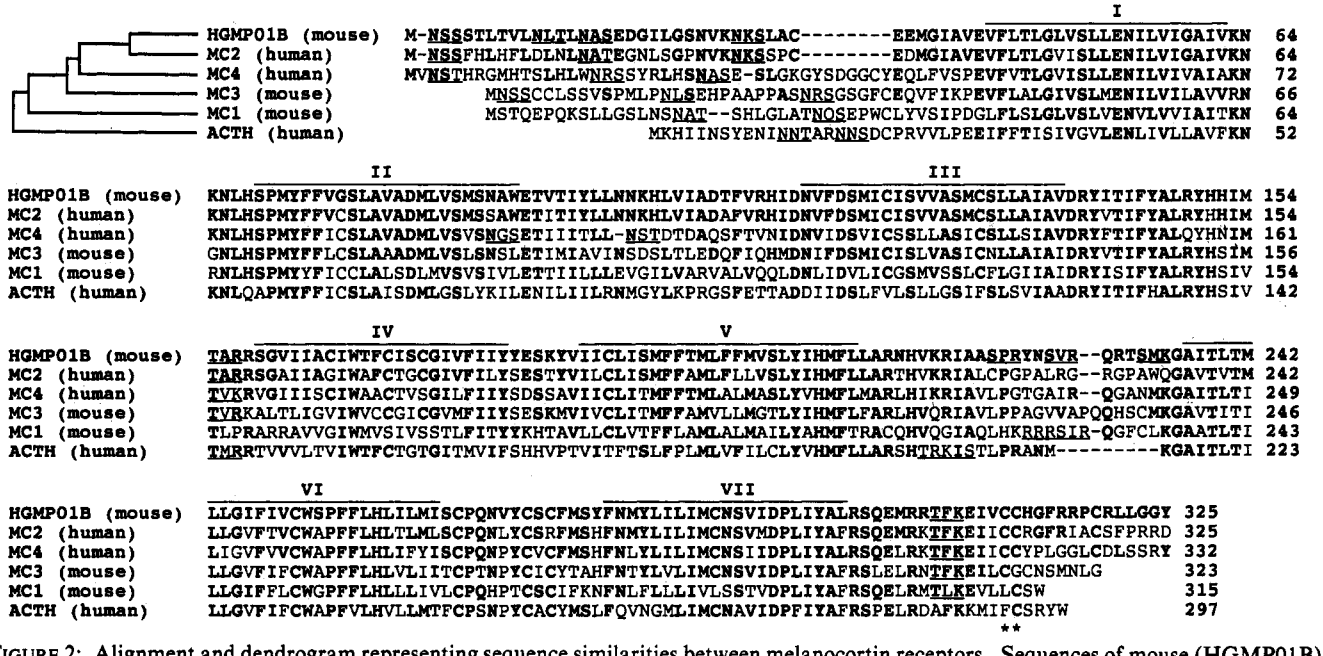


FIGURE 1: Putative organization of the HGMP01B receptor in the plasma membrane. The limits of transmembrane segments were determined on the basis of the hydropathy profile and by analogy with the other members of the G-protein-coupled receptor family. The four potential sites for N-linked glycosylation located in the N-terminal extracellular domain are indicated, as well as the five consensus recognition sequences for protein kinase C. The two cysteines potentially palmitoylated are represented with bold circles. The nucleotide sequence encoding the HGMP01B receptor has been deposited with the Genbank/EMBL data libraries.



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                                                                                I
HGMP01B (mouse)  M-NSSSTLTVLNLTLNASEDGILGSNVKNKSLAC--------EEMGIAVEVFLTLGLVSLLENILVIGAIVKN  64
MC2 (human)      M-NSSFHLHFLDLNLNATEGNLSGPNVKNKSSPC--------EDMGIAVEVFLTLGVISLLENILVIGAIVKN  64
MC4 (human)      MVNSTHRGMHTSLHLWNRSSYRLHSNASE-SLGKGYSDGGCYEQLFVSPEVFVTLGVISLLENILVIVAIAKN  72
MC3 (mouse)            MNSSCCLSSVSPMLPNLSEHPAAPPASNRSGSGFCEQVFIKPEVFLALGIVSLMENILVILAVVRN  66
MC1 (mouse)            MSTQEPQKSLLGSLNSNAT--SHLGLATNQSEPWCLYVSIPDGLFLSLGLVSLVENVLVVIAITKN  64
ACTH (human)                         MKHIINSYENINNTARNNSDCPRVVLPEEIFFTISIVGVLENLIVLLAVFKN  52

                    II                                                  III
HGMP01B (mouse)  KNLHSPMYFFVGSLAVADMLVSMSNAWETVTIYLLNNKHLVIADTFVRHIDNVFDSMICISVVASMCSLLAIAVDRYITIFYALRYHHIM 154
MC2 (human)      KNLHSPMYFFVCSLAVADMLVSMSSAWETITIYLLNNKHLVIADAFVRHIDNVFDSMICISVVASMCSLLAIAVDRYVTIFYALRYHHIM 154
MC4 (human)      KNLHSPMYFFICSLAVADMLVSVSNGSETIIITLL-NSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYALQYHNIM 161
MC3 (mouse)      GNLHSPMYFFLCSLAAADMLVSLSNSLETIMIAVINSDSLTLEDQFIQHMDNIFDSMICISLVASICNLLAIAIDRYVTIFYALRYHSIM 156
MC1 (mouse)      RNLHSPMYYFICCLALSDLMVSVSIVLETTIILLLEVGILVARVALVQQLDNLIDVLICGSMVSSLCFLGIIAIDRYISIFYALRYHSIV 154
ACTH (human)     KNLQAPMYFFICSLAISDMLGSLYKILENILIILRNMGYLKPRGSFETTADDIIDSLFVLSLLGSIFSLSVIAADRYITIFHALRYHSIV 142

                        IV                                  V
HGMP01B (mouse)  TARRSGVIIACIWTFCISCGIVFIIYYESKYVIICLISMFFTMLFFMVSLYIHMFLLARNHVKRIAASPRYNSVR--QRTSMKGAITLTM 242
MC2 (human)      TARRSGAIIAGIWAFCTGCGIVFILYSESTYVILCLISMFFAMLFLLVSLYIHMFLLARTHVKRIALCPGPALRG--RGPAWQGAVTVTM 242
MC4 (human)      TVKRVGIIISCIWAACTVSGILFIIYSDSSAVIICLITMFFTMLALMASLYVHMFLMARLHIKRIAVLPGTGAIR--QGANMKGAITLTI 249
MC3 (mouse)      TVRKALTLIGVIWVCCGICGVMFIIYSESKMVIVCLITMFFAMVLLMGTLYIHMFLFARLHVQRIAVLPPAGVVAPQQHSCMKGAVTITI 246
MC1 (mouse)      TLPRARRAVVGIWMVSIVSSTLFITYYKHTAVLLCLVTFFLAMLALMAILYAHMFTRACQHVQGIAQLHKRRRSIR-QGFCLKGAATLTI 243
ACTH (human)     TMRRTVVVLTVIWTFCTGTGITMVIFSHHVPTVITFTSLFPLMLVFILCLYVHMFLLARSHTRKISTLPRANM---------KGAITLTI 223

                    VI                                  VII
HGMP01B (mouse)  LLGIFIVCWSPFFLHLILMISCPQNVYCSCFMSYFNMYLILIMCNSVIDPLIYALRSQEMRRTFKEIVCCHGFRRPCRLLGGY 325
MC2 (human)      LLGVFTVCWAPFFLHLTLMLSCPQNLYCSRFMSHFNMYLILIMCNSVMDPLIYAFRSQEMRKTFKEIICCRGFRIACSFPRRD 325
MC4 (human)      LIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMSHFNLYLILIMCNSIIDPLIYALRSQELRKTFKEIICCYPLGGLCDLSSRY 332
MC3 (mouse)      LLGVFIFCWAPFFLHLVLIITCPTNPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFKEILCGCNSMNLG       323
MC1 (mouse)      LLGIFFLCWGPFFLHLLLIVLCPQHPTCSCIFKNFNLFLLLIVLSSTVDPLIYAFRSQELRMTLKEVLLCSW           315
ACTH (human)     LLGVFIFCWAPFVLHVLLMTFCPSNPYCACYMSLFQVNGMLIMCNAVIDPFIYAFRSPELRDAFKKMIFCSRYW         297
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FIGURE 2: Alignment and dendrogram representing sequence similarities between melanocortin receptors. Sequences of mouse (HGMP01B) melanocortin 5 receptors are aligned with the other melanocortin receptors, and a dendrogram representing sequence similarities was generated using the Clustal software (Higgins & Sharp 1988). Identities with HGMP01B are represented as bold characters. Putative transmembrane segments are indicated by the roman numerals I–VII. Potential N-linked glycosylation sites in the N-terminal extracellular segment are underlined, as well as potential phosphorylation sites by protein kinase C (PKC) or cAMP-dependent protein kinase (cAPK), located in the second and third intracellular loops and in the C-terminal domain. The conserved cysteines possibly involved in palmitoylation are indicated by *. The sequences of the human MC2, human MC4, and mouse MC3 receptors are, respectively, from Chhajlani et al. (1993), Gantz et al. (1993b), and Desarnaud et al. (1993). Sequences of the mouse MC1 and human ACTH receptors are from Mountjoy et al. (1992).

in Figure 2. HGMP01B shares 48% and 46% identity with the MSH (also named MC1) receptor (Mountjoy et al., 1992; Chhajlani & Wikberg, 1992) and the ACTH (Mountjoy et al., 1992; Chhajlani & Wikberg, 1992) and the ACTH (Mountjoy et al., 1992) receptor, respectively. There is also 56% identity with the mouse and human MC3 receptors (Desarnaud et al., 1994; Gantz et al., 1993a), 61% with the MC4 receptor (Gantz et al., 1993b), and 78% with a human melanocortin receptor reported after completion of the present work by Chhajlani et al. (1993). This receptor was reported

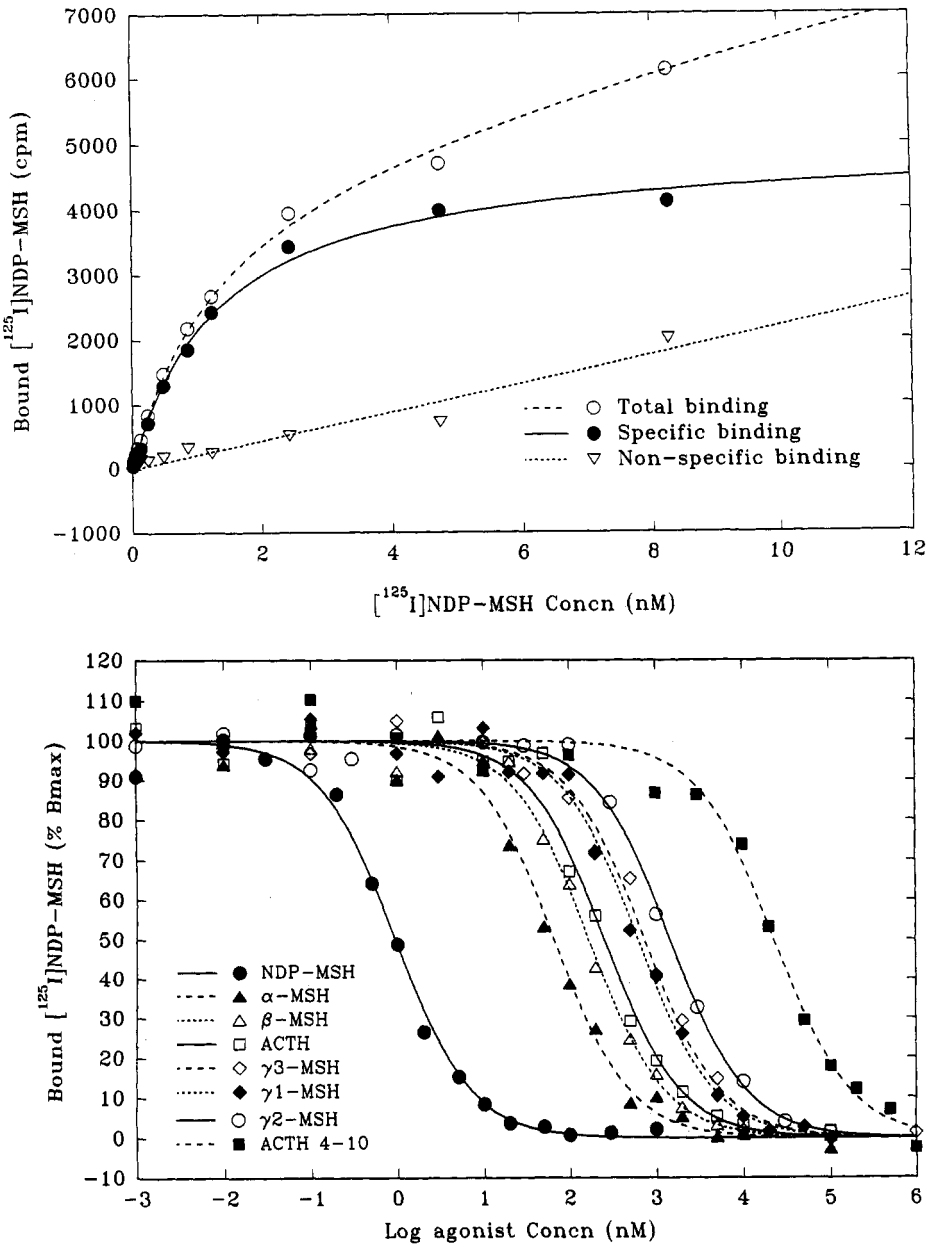

FIGURE 3: Binding assays. (a, top) Saturation binding curve representative of two independent experiments, using [^{125}I]NDP-MSH and CHO cells expressing the mouse recombinant HGMP01B receptor. Total, specific, and nonspecific binding are represented. Each point represents the mean of triplicate experimental values. Curve fitting using a nonlinear regression algorithm and a one-site model yielded an apparent K_d of 1.335 nM. (b, bottom) Competition binding curves representative of the 3–4 independent experiments that were performed for each of the peptides. The ordinate is expressed as a percentage of the total specific binding (B_{max}). Each point is the mean of triplicate experimental values. The IC_{50} values for the displayed curves are 0.941 nM (NDP), 65.4 nM (α-MSH), 166 nM (β-MSH), 252 nM (ACTH 1-39), 616 nM (γ1-MSH), 735 nM (γ3-MSH), 1.45 μM (γ2-MSH), and 22 μM (ACTH 4-10).

as MC2 by Chhajlani et al., but this name is rather ambiguous since the ACTH receptor was already named MC2 by Gantz et al. (1993a,b).

The N-terminal extracellular domain of HGMP01B contains four putative sites of N-linked glycosylation (Figures 1 and 2). Motifs possibly involved in the regulation of receptor function include five potential sites of phosphorylation by protein kinase C (Woodget et al., 1986), located in the second and third intracellular loops and in the C-terminal domain. No phosphorylation sites for the cAMP-dependent protein kinase (Glass et al., 1986) can be found. The C-terminal intracellular domain of the receptor is not enriched in serine and threonine residues, which could constitute targets for the family of G-protein-coupled receptor kinases. Conserved cysteines located in this region could, by analogy with the adrenergic receptors (O'Dowd et al., 1989), be palmitoylated and anchor the C-terminal segment of the receptors in the plasma membrane, delimiting a fourth intracellular loop. Palmitoylation of adrenergic receptors was shown, however, not to be required for receptor function (O'Dowd et al., 1989).

Expression in CHO-K1 Cells. The HGMP01B coding sequence was subcloned in the expression vector pSVL, and stably transfected CHO cell lines were established. The cell line displaying the highest expression level was selected by binding assay and subsequently used to determine the pharmacological profile of the receptor, using binding and cAMP accumulation assays. The binding conditions, using iodinated NDP-MSH as a tracer, had been set up for the HGMP01A (melanocortin 3) receptor (Desarnaud et al., 1994) and proved to be as efficient for HGMP01B. Saturation binding experiments were consistent with the existence of a single binding site, characterized by an apparent K_d of 1.47

Table 1: Binding and Functional Parameters of Various Melanocortin Peptides for the Recombinant Mouse MC5 and MC3 Receptors Expressed in CHO-K1 Cell Lines[a]

	MC5 receptor (HGMPO1B)		MC3 receptor (HGMPO1A)	
agonist	IC_{50} (nM) mean ± SD (*N*)	EC_{50} (nM) mean ± SD (*N*)	IC_{50} (nM) mean ± SD (*N*)	EC_{50} (nM) mean ± SD (*N*)
NDP-MSH	1.1 ± 0.2 (3)	0.050 ± 0.002 (2)	1.43 ± 0.47 (3)	0.43 ± 0.11 (3)
α-MSH	62.5 ± 5.5 (3)	1.07 ± 0.13 (3)	30.2 ± 3.3 (3)	1.21 ± 0.21 (3)
β-MSH	212 ± 36 (3)	6.5 ± 1.0 (3)	19.4 ± 2.6 (3)	1.10 ± 0.40 (3)
γ1-MSH	736 ± 106 (3)	9.2 ± 1.8 (3)	nd	nd
γ2-MSH	1270 ± 190 (3)	42.9 ± 1.8 (3)	6.7 ± 1.6 (3)	0.64 ± 0.31 (3)
γ3-MSH	603 ± 125 (3)	68.7 ± 7.2 (3)	nd	nd
ACTH 1–39	236 ± 41 (3)	6.02 ± 0.17 (3)	21.1 ± 7.1 (3)	3.3 ± 1.0 (3)
ACTH 4–10	125000 ± 80000 (3)	nd	2400 ± 400 (3)	1120 ± 320 (3)

[a] IC_{50} and EC_{50} represent the concentrations producing half-displacement in competition binding assays and half-maximal stimulation in cAMP accumulation assays, respectively. Values are means and standard deviations (SD) for *N* independent experiments. Binding and functional parameters for the mouse MC3 receptor are from Desarnaud et al. (1993). nd indicates values not determined.

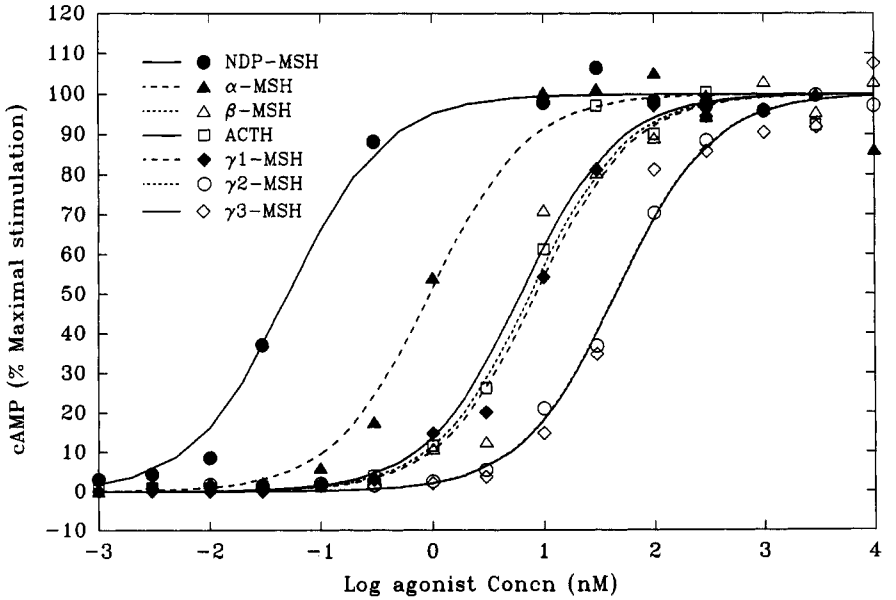

FIGURE 4: Functional coupling of HGMP01B in CHO-K1 cells. A CHO clonal cell line stably transfected with pSVL-HGMP01B was assayed for cAMP accumulation in response to NDP-MSH and other agonists derived from proopiomelanocortin. The ordinate is expressed as a percentage of the maximal cAMP level obtained for each curve. Each point represents the mean of triplicate experimental values. Each curve is representative of three independent experiments. The EC_{50} values for the displayed curves are 51 pM (NDP), 0.94 nM (α-MSH), 7.54 nM (β-MSH), 6.18 nM (ACTH 1-39), 8.42 nM (γ1-MSH), 44.7 nM (γ2-MSH), and 43.9 nM (γ3-MSH).

± 0.15 nM. A representative experiment is displayed in Figure 3a. From the B_{max} values, the density of binding sites was estimated to be 10 000 sites per cell. Under the same experimental conditions, no specific binding was obtained on the wild-type CHO cell line (not shown). Competition binding curves were obtained for a number of peptides derived from proopiomelanocortin (Figure 3b). By close to 2 orders of magnitude, NDP-MSH was the most powerful competitor with an IC_{50} value of 1.15 ± 0.23 nM. It was followed by α-MSH, β-MSH and ACTH, γ3-MSH, and γ1-MSH and γ2-MSH. IC_{50} values for each peptide are given in Table 1. ACTH 4-10 was poorly active, while no competition was observed with other POMC-derived peptides, such as CLIP and β-endorphin (data not shown).

The ability of melanocortins to stimulate the intracellular accumulation of cAMP was assayed on the CHO cell line expressing HGMP01B. cAMP levels in unstimulated cells were generally at or below the lower limit of detection of the assay (1 pmol of cAMP per tube). One curve representative of each of the tested peptides is displayed in Figure 4. Results are expressed as the percentage of the maximal stimulation level for the sake of clarity. This maximal cAMP level was found to be variable (70–300 pmol of cAMP per tube) from one experiment to another, but within experiments, maximal levels were similar for all agonists. Melanocortins were unable to stimulate cAMP production in untransfected CHO cells. In this functional assay, NDP-MSH also emerged as the most powerful agonist, with a concentration inducing half-maximal stimulation (EC_{50}) of 50 ± 2 pM. The potency order of the other peptides is almost superimposable with what was obtained in binding assays (see Table 1): α-MSH > ACTH = β-MSH > γ1-MSH > γ2-MSH = γ1-MSH. β-Endorphin, CLIP, and β-lipotropin were totally ineffective (not shown).

Tissue Distribution. A mouse HGMP01B gene fragment was used as the probe in RNase protection assays, in order to determine the tissue distribution of transcripts encoding the receptor (Figure 5). RNA extracted from the CHO cell line expressing the receptor was used as a positive control. A strong signal was found with RNA extracted from skin and skeletal muscle. Moderate signals were obtained from spleen, thymus, and bone marrow, while low levels of expression could be detected in adrenal gland, brain (in cortex and cerebellum, but not in striatum and hypothalamus), testis, ovary, and uterus. Organs where no expression of HGMP01B could be found included pituitary, lung, heart muscle, kidney, esophagus, stomach, duodenum, jejunum, ileum, caecum, colon, liver, pancreas, placenta, prostate, and seminal vesicle (Figure 5 and not shown).

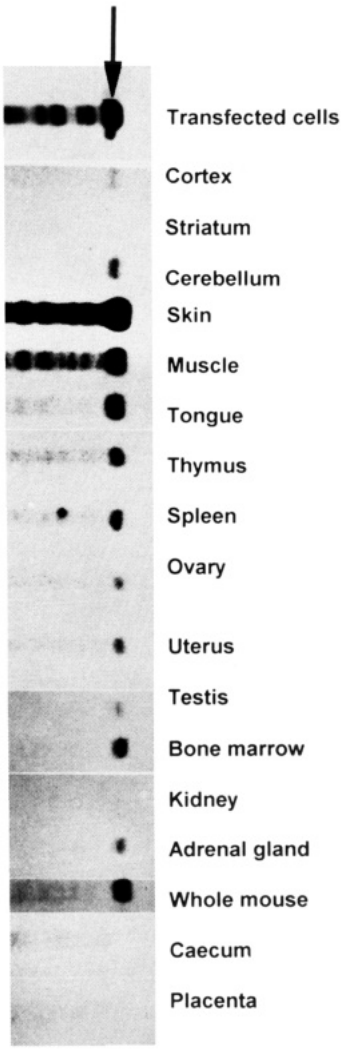

FIGURE 5: Tissue distribution of HGMP01B transcripts. The tissue distribution of HGMP01B expression was investigated by the RNAase protection assay, and protected fragments were separated on a polyacrylamide gel. RNA extracted from transfected CHO-K1 cells was used as a positive control (3 days of autoradiography). Positive signals (15 days of autoradiography) were obtained in a wide variety of tissues. Negative controls included yeast tRNA and omission of RNA (not shown). Other negative tissues (not shown) included heart muscle, lung, pituitary, esophagus, stomach, duodenum, jejunum, ileum, colon, liver, pancreas, prostate, seminal vesicle, and eye.

DISCUSSION

Melanocortin receptors constitute a subfamily fo G-protein-coupled receptors characterized by short N- and C-terminal domains. Like other melanocortin receptors, HGMP01B lacks the cysteines in the first and second extracellular loops that are believed to form a disulfide bridge in most of the G-protein-coupled receptors (Dohlman et al., 1991). HGMP01B also lacks cAMP-dependent protein kinase phosphorylation sites (Glass et al., 1986) and a C-terminal domain rich in serines and threonines that could be the target for G-protein-coupled receptor kinases (Dohlman et al., 1991). The function of this receptor therefore is not expected to be regulated by mechanisms of homologous or heterologous desensitization relying on these sites (Dohlman et al., 1991). The absence of a serine- and threonine-rich C-terminal domain is common to other melanocortin receptors (Mountjoy et al., 1992; Gantz et al., 1993a,b). However, the human ACTH and mouse melanocyte MSH (MC1) receptors do contain cAMP-dependent protein kinase recognition sites (Mountjoy et al., 1992). The presence of four putative phosphorylation sites by protein kinase C (Woodget et al., 1986) suggests that the activation of phospholipase C in the target cells could possibly alter their response to melanocortins.

The pharmacological profile of HGMP01B was determined from binding and functional assays (cAMP accumulation) on a CHO cell line expressing the recombinant receptor. The apparent K_d for the radioiodinated analog NDP-MSH (1.47 nM) was in the same range as that found for the related MC3 receptor (0.7 nM). In displacement experiments, IC_{50} values (Table 1) for natural melanocortins were, however, found to be significantly higher than that obtained for the mouse MC3 receptor (Desarnaud et al., 1993). In contrast to this latter receptor, which discriminates poorly between ACTH and α-, β-, and γ-MSHs, HGMP01B has a marked preference for α-MSH and a low affinity for γ2-MSH. Both receptors share a low affinity for ACTH 4-10, which constitutes the core heptapeptide common to ACTH, α-MSH, and β-MSH. cAMP accumulation assays confirmed the potency order of peptides for HGMP01B, with NDP-MSH being the most active peptide and α-MSH the most potent natural agonist (Figure 4 and Table 1). We noted a significant difference between the functional EC_{50} and the binding IC_{50} values (Table 1). As an example, α-MSH, which has a moderate affinity for the receptor (IC_{50} = 62.5 nM), is functionally active at much lower concentrations (EC_{50} = 1.07 nM). Binding conditions were identical for the analysis of the MC3 and MC5 receptors (with the exception that the MC3 receptor was expressed at higher levels). Under these conditions, the concentration of labeled tracer is 0.1 nM, while the K_d for NDP-MSH is 1.45 nM for the MC5 receptor (0.7 nM for the MC3 receptor), and there is no significant depletion of ligand. Following Cheng and Prussof (1973), IC_{50} values therefore represent overestimates of the dissociation constants by a factor of 1.14 for MC3 and 1.07 for MC5. Functionally, HGMP01B appears to have a profile similar to those of the MC1 and MC4 receptors. The difference between IC_{50} and EC_{50} suggests efficient coupling of the activated MC5 receptor to the G-protein. This hypothesis will have to be studied in more detail in the future.

The tissue distribution of HGMP01B gene expression is probably the most interesting characteristic of the MC5 receptor. The other melanocortin receptors have been reported to exhibit a specific and restricted tissue distribution: MC1 and ACTH receptors are restricted to melanocytes and corticoadrenal tissue, respectively, and MC4 is brain-specific (Gantz et al., 1993b). Mouse MC3 was found to be brain-specific as well (Desarnaud et al., 1994), while human MC3 was found to be expressed in brain and placenta by Northern blotting and additionally in gut by PCR (Gantz et al., 1993a). In contrast, transcripts encoding mouse MC5 are detected by RNase protection assay in skin, adrenal tissue, and brain and also in a wide variety of peripheral tissues including gonads, muscle, and organs of the immune system. Nevertheless, the distribution of the MC1, ACTH, and MC4 receptors was determined by Northern blotting only; more sensitive methods of transcript detection could uncover additional sites of expression. It should be noted in this context that no signal was obtained for the MC5 receptor in organs where melanocortin binding sites had previously been described, such as duodenum and pancreas (Reichlin & Tatro, 1987), suggesting that other melanocortin receptor subtypes are present in these tissues.

From the distribution of MC5, we hypothesize that HGMP01B is possibly responsible for the various effects attributed to melanocortins on peripheral tissues. One of these

main actions includes the strong antiinflammatory action of α-MSH, which antagonizes the action of various cytokines such as IL1, IL6, and TNFα (Martin et al., 1991). It has been shown that the active site of α-MSH in this context is localized in the C-terminal tripeptide and that proline-12 is essential (Hiltz et al., 1991). The respective actions of the peptides tested up to now are consistent with this structure–function relationship. Future work will focus on the detailed distribution of the HGMP01B transcripts in the various organs by *in situ* hybridization. The presence of MC5 receptors in cell types involved in host defense mechanisms in tissues such as spleen, thymus, or skin would considerably reinforce the hypothesis that this receptor is a possible mediator of the immunomodulation properties of melanocortins.

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