Vol. 200, No. 3, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 16, 1994 Pages 1214-1220

MOLECULAR CLONING, EXPRESSION, AND CHARACTERIZATION OF A FIFTH MELANOCORTIN RECEPTOR

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Received March 21, 1994

SUMMARY: We report the isolation of a gene encoding a novel member of the family of melanocortin receptors. The mouse melanocortin-5 receptor (mMC5R) responds to melanocortins with an increase in intracellular cyclic 3', 5' - adenosine monophosphate (cAMP) concentrations. Stimulation of the mMC5R by the melanocortins revealed a hierarchy of potency in which α-melanocyte stimulating hormone (α-MSH) > β-melanocyte stimulating hormone (β-MSH) > adrenocorticotropic hormone (ACTH) > γ- melanocyte stimulating hormone (γ-MSH). Further structure-activity studies indicated that amino- and carboxyl-terminal portions of α-MSH appear to be key determinants in the activation of mMC5R whereas the melanocortin core heptapeptide sequence is devoid of pharmacological activity. Northern blot analysis demonstrated the expression of mMC5R mRNA in mouse skeletal muscle, lung, spleen, and brain.

The melanocortins such as adrenocorticotropic hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH) are products of pro-opiomelanocortin (POMC) post-translational processing (1). In addition to their familiar functions in the regulation of adrenal corticosteroidogenesis and melanocyte pigmentation, the melanocortins have been implicated as mediators in a broad array of physiological functions including immunomodulation, musculoskeletal action, and parturition (2-4). In the central nervous system, melanocortins have been shown to influence attention span, behavior, learning, memory, thermoregulation, control of the cardiovascular system, release of other neurohumoral agents, and analgesia (5,6). Such diverse actions by a small group of peptides with overlapping functional specifications implies that there must be a variety of receptors with distinct functional and pharmacological properties to mediate these actions. Indeed, four different members of the melanocortin receptor (MCR) family have been identified and characterized on the basis of the genes encoding them (7-10). Although all of the melanocortin receptors thus far identified are similar in that they belong to the seven transmembrane G-protein linked receptor classification and induce intracellular cyclic 3', 5'-adenosine monophosphate (cAMP) generation in response to stimulation, there are unique characteristics of each receptor that distinguish them from each other. MC1R is

0006-291X/94 $5.00

found on melanocytes and appears to be the α-MSH receptor. MC2R is localized to adrenocortical cells and, thus, is thought to be the ACTH receptor. MC3R and MC4R receptors are localized primarily to sites in the brain. Each receptor has an unique pattern of affinity for the various melanocortin ligands. In the present studies, we report the molecular cloning of a novel gene encoding a fifth member of the MCR family.

MATERIALS AND METHODS

Polymerase Chain Reaction. To clone the mMC5R, we utilized mouse genomic DNA (Clontech, Palo Alto, CA) as template for the polymerase chain reaction (PCR). Highly conserved sequences in the second intracytoplasmic loop and the seventh transmembrane domain of the melanocortin receptors already cloned served as the basis for designing primers (5' PCR primer TACGCA/GCTG/CCGCTACCACAGCATC and 3' PCR primer GAAG/AGCA/GTAT/GATGA A/GG/TGGGTCA/GAT) for the reaction. The conditions for the PCR as well as the techniques for DNA isolation, subcloning into M13 and dideoxynucleotide sequencing were as previously described (9,10).

Genomic Cloning. The partial length PCR product derived as described above was random primed with ^{32}P and used as probe to screen a mouse EMBL3 phage library (Clontech, Palo Alto, CA). A single clone was isolated, its phage DNA prepared (11), and the insert mapped by restriction endonucleases and Southern blot analysis. Dideoxy-nucleotide sequencing of fragments containing the receptor coding region was then performed in M13.

Receptor Expression. The coding region of the novel receptor was subcloned into the eukaryotic expression vector CMVneo (12) using PCR according to methods previously described (13). L-cells (a mouse fibroblast-like cell line) were transfected by the calcium phosphate co-precipitation method (14) and permanently transfected L-cells were selected by resistance to the neomycin analogue G418.

Northern Blotting. For surgical specimens, total RNA was extracted from cell lines and tissues using the acid guanidinium thiocyanate-phenol-chloroform method (15). RNA was transferred to nitrocellulose and hybridized in 50% formamide, 5X SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 0.005 M Na_2EDTA pH 7.4), 10X Denhardt's solution, 100 ug/ml salmon sperm DNA and 2% sodium dodecyl sulfate for 18 hours according to standard methods (16). Commercially available human and rat/mouse multiple tissue mRNA blots (Clontech, Palo Alto, CA) were also examined. Northern blot analysis was performed using a random primed ^{32}P labeled murine probe corresponding to the 975 bp coding region of the mMC5R. The mouse RNA blot was hybridized at 42°C and the human RNA blot at a lower temperature (40°C) to try to compensate for cross-species sequence mismatches.

cAMP Assays. For our studies, we measured intracellular cAMP using an assay kit (Amersham, TRK 432, Arlington Heights, IL). Cells transfected with the novel receptor gene were grown to confluence in 12-well (2.4 x 1.7 cm) tissue culture plates. Varying concentrations of agonist were added and the cells were incubated for 30 min at 37 °C in the presence of 2 x 10^{-2} M isobutylmethylxanthine. The following peptides obtained from Peninsula Laboratories, Inc (Belmont, CA) were used as agonists for our studies: human and rat ACTH (1-39), human ACTH (4-10), ACTH (1-10), α-MSH, β-MSH, γ-MSH, β-endorphin, [Met]enkephalin, and porcine β-lipotropin. ACTH (4-13) was synthesized by the University of Michigan Protein and Carbohydrate Structure Facility on an Applied Biosystems Model 431 peptide synthesizer using standard FMOC chemistry. Ice cold 30% trichloracetic acid (500 ul/well) was added to stop the reaction and precipitate cellular protein. The cells were scraped and transferred to 16 x 150 mm glass tubes then placed on ice for 30 min. The precipitate was then centrifuged for 10 min at 1,900 x g and the supernatant was ether extracted, lyophilized, and resuspended in 50 mM Tris, 2 mM EDTA (pH 7.5). cAMP content was then measured by competitive binding assay according to the assay kit instructions.

Binding Assays. After removal of media the cells were washed twice with Earle's balanced salt solution (EBSS, Gibco, Grand Island, NY) and incubated for 1h with 36nCi (1Ci = 37GBq) of the ^{125}I-labeled [Nle^4, D-Phe^7]α-MSH ([^{125}I]NDP-MSH) which was prepared according to the protocol of Tatro and Reichlin (17). Binding reactions were terminated by removing the media and washing the cells twice with 0.2 M sodium phosphate/0.15 M NaCl (pH 7.4). The cells were lysed with 1% Triton X-100 and the radioactivity in the lysate was quantified in a gammer counter. Nonspecific binding was determined by measuring the amount of [^{125}I]NDP-MSH remaining bound in the presence of 10^{-5} M unlabeled NDP-MSH and specific binding was calculated by subtracting nonspecifically bound radioactivity from total bound radioactivity.

RESULTS AND DISCUSSION

PCR reactions primed with the oligonucleotide primers described above using mouse genomic DNA as template resulted in the isolation of four DNA fragments with homology to the melanocortin receptor gene family. One 459 bp fragment had 98.4% nucleotide sequence homology to the mouse α-MSH receptor cDNA (mMC1R) previously described by Mountjoy et al. (7) and, therefore, most likely represented the same clone. Two other fragments of 461 bp and 456 bp showed 87.2% and 87.7% nucleotide sequence homology, respectively, to the human hMC3R and hMC4R indicating that they probably represented the mouse counterparts of these human melanocortin receptor genes. The fourth mouse DNA fragment of 456 bp shared less than 70% nucleotide sequence homology with any of the published human or rodent melanocortin receptors. This fragment was used as a probe to obtain a full length mouse genomic clone (mMC5R) which contained an open reading frame of 975 bp (325 amino acids). The remarkable homology of the amino acid sequence to other known melanocortin receptors identified it to be a member of the family of melanocortin receptors (Figure 1). The highest overall homology of the new receptor, which we called the mouse melanocortin-5 receptor (mMC5R), is to the human melanocortin-4 receptor (hMC4R) with which it shares 63% nucleotide sequence identity, 60% amino acid identity, and 77.6% amino acid similarity. Nevertheless, a mouse counterpart for the hMC4R was identified in another of our PCR fragments (see above) thus mMC5R was clearly distinguishable from this receptor gene. Deduced amino acid sequences of rodent melanocortin receptors have been published only for the mouse melanocortin-1 receptor (mMC1R or murine α-MSH receptor) and the rat melanocortin-3 receptor (rMC3R) (18). By comparison to their human melanocortin receptor counterparts, these mouse receptors share considerably greater sequence homology within a single receptor subtype than they do to each other. Although we considered the possibility that mMC5R was the murine counterpart of one of the human melanocortin receptors already cloned, its pharmacological characteristics and tissue distribution, coupled with the DNA sequence comparison as noted, identified it as a new member of the MCR family.

The tissue distribution of mMC5R mRNA further distinguishes it as a gene encoding a novel melanocortin receptor. By Northern blot analysis we detected high levels of mMC5R expression in skeletal muscle and lesser levels of expression in lung, spleen,

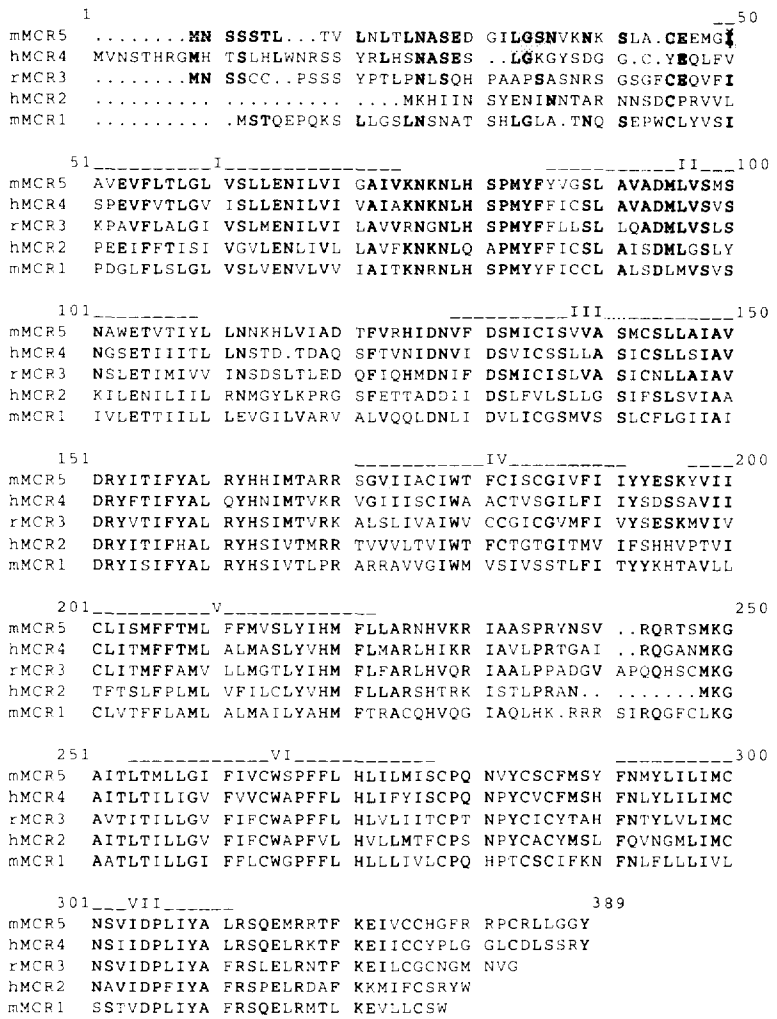

Figure 1. Deduced amino acid sequences of the melanocortin receptor subtypes. The shading denotes amino acids present in the mMC5R which are shared by the other melanocortin receptor subtypes. The putative transmembrane domains are denoted by over-lines and Roman numerals. The GenBank accession number for the mMC5R is L22527. The sequences of the other melanocortin receptors are derived from references 7, 9, 10, and 18.

and brain (Figure 2). Utilizing the same mMC5R probe under conditions of reduced stringency to accommodate cross-species differences, no expression was observed in the human A375 melanoma cell line, which expresses hMC1R, in normal and malignant human adrenocortical tissues which express hMC2R, or in placenta, a tissue which expresses hMC3R (9,10).

The pharmacological profile of mMC5R is unique in that α-MSH is clearly more potent than ACTH in stimulating the production of intracellular cAMP in L-cells expressing the receptor (Figure 3). Rat ACTH, which differs from human ACTH in two amino acids

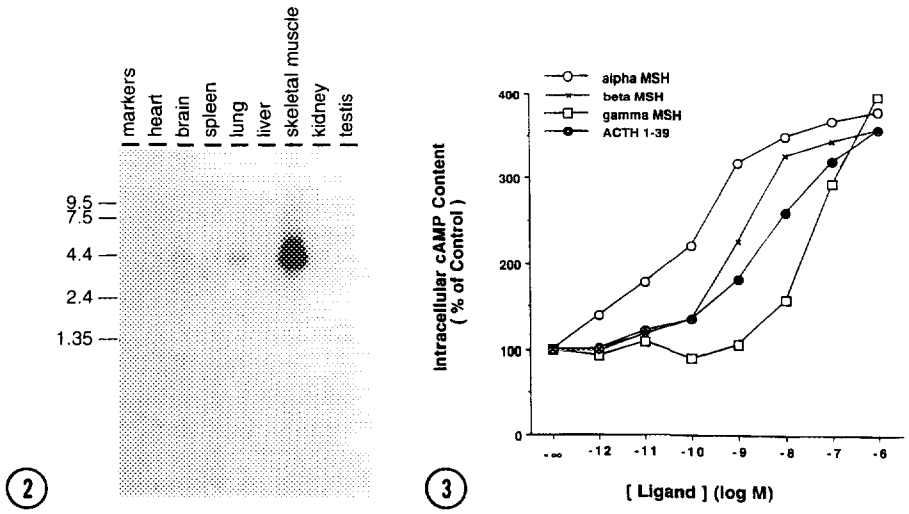

Figure 2. Rat/mouse multiple tissue Northern blot analysis demonstrating the pattern of organ expression of mMC5R mRNA. Each lane represents 2 μg of poly A+ RNA.

Figure 3. Generation of cAMP in L-cells transfected with the mMC5R. Responses to α-, β-, and γ-MSH, and ACTH (1-39) are depicted. Each point represents the average of the three separate experiments. Standard errors were less than 10% for each point.

produced the same results. Those data contrast with our previous studies demonstrating that α-MSH and ACTH are equipotent in stimulating cAMP production by other human melanocortin receptors (hMC1R, hMC3R, and hMC4R) expressed in the same cell line (9,10). Mountjoy et al. (7) have reported, however, that α-MSH is more potent than ACTH in stimulating cAMP production via mMC1R. It is possible that the ability to differentiate between α-MSH and ACTH is a property unique to murine melanocortin receptors.

We examined the possibility that the ability of mMC5R to distinguish between α-MSH and ACTH (1-39) might be dependent on the presence or absence of the amino terminal acetyl moiety. As depicted in Figure 4, however, we observed no difference between non-acetylated α-MSH [(ACTH (1-13)] and α-MSH in stimulating cAMP production in our transfected cells. Furthermore, as shown in Figure 5, the binding affinity of mMC5R for α-MSH and ACTH (1-13) appeared to be three times greater than that for ACTH (1-39). These data imply that the carboxyl terminal extension of ACTH (1-39) [(ACTH (14-39)] must be the determinant of its diminished potency relative to α-MSH or ACTH (1-13).

Further studies were performed to characterize the ligand specificity of mMC5R. As shown in Figure 3, both β- and γ-MSH were full agonists, but were one and three orders of magnitude lower in potency, respectively, than α-MSH. The central core heptapeptide [(ACTH (4-10)] common to all melanocortins had essentially no biological activity (Figure 4). Neither were the other products of pro-opiomelanocortin processing, β-

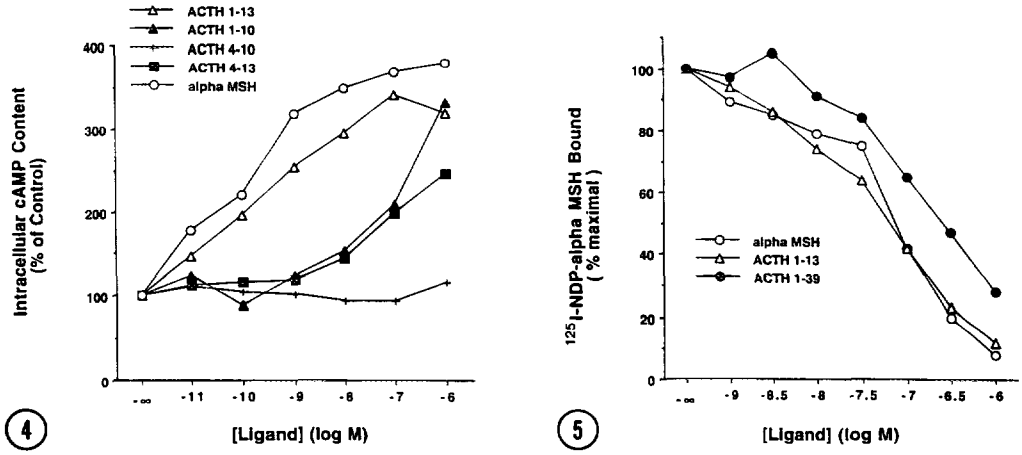

Figure 4. Comparison of the cAMP response produced by L-cells transfected with the mMC5R to α-MSH and the truncated peptides ACTH (4-10), ACTH (1-10), ACTH (4-13), and ACTH (1-13). Each point represents the average of the three separate experiments. Standard errors were less than 10% for each point.

Figure 5. Inhibition by α-MSH, ACTH (1-13) and ACTH (1-39) of [^{125}I] NDP-MSH binding to L-cells transfected with the mMC5R. Binding is depicted as a percentage of total specific binding. Non-specific binding was less than 10% of total binding

endorphin, met-enkephlin, or porcine β-lipotropin active on mMC5R (data not shown). However, truncated α-MSH peptides which contain an amino [ACTH (1-10)] or carboxyl [ACTH (4-13)] terminal extension had full efficacy albeit with greatly diminished potency relative to α-MSH in inducing cAMP production in L-cells transfected with mMC5R (Figure 4). Our structure-function studies indicate that amino acids in both the carboxyl and amino terminal extention regions (relative to the heptapeptide core) of α-MSH are critical determinants of agonist activity at mMC5R. A comparison of the amino acid sequences of various ligands tested in conjunction with our data suggests the possibility that Tyr^2 and Pro^{12} of α-MSH are particularly important in this regard, but further studies are required for confirmation of this hypothesis. The lack of activity of ACTH (4-10) and the full efficacy of α-MSH imply that the core heptapeptide may be important as a spacer between amino and carboxyl terminal extensions, but the specific sequences may be a less important factor in the binding of melanocortins to mMC5R. In this regard, mMC5R resembles hMC4R more closely than the other members of the melanocortin receptor family (10).

The pharmacological potency of α-MSH as well as the unique nucleotide sequence and pattern of tissue expression indicate that the mMC5R is a peripheral melanocortin receptor distinct from the α-MSH receptor present on the melanocyte. Its expression in skeletal muscle identifies it to be the putative melanocortin receptor previously described in rodent neuromuscular tissue (19) and implicated to be responsible for the mitogenic effects of ACTH and α-MSH (20). During the course of our studies Chhajlani et al. (21) published the nucleotide sequence of a human melanocortin receptor gene derived by PCR

of brain tissue with 81.3% nucleotide sequence homology to mMC5R. Although neither the tissue specific expression of the receptor nor the pharmacology of this receptor were reported on the basis of sequence homology it may represent the human equivalent of mMC5R. Further studies are required to establish the functional significance of this receptor.

ACKNOWLEDGMENTS

This work was supported by USPHS grants RO1-DK34306 and RO1-DK33000 and funds from the University of Michigan Gastrointestinal Peptide Research Center (USPHS grant P30-DK34933). Dr. Gantz is a recipient of a Veterans Administration Research Associate Award, Dr. Dickinson is a recipient of an NIH Clinical Investigator Award (USPHS grant K08-DK01823 and NIH K08-DK01913). We thank the Biochemistry and Histochemistry Cores of the University of Michigan Gastrointestinal Peptide Research Center for their assistance in this work. I.G. and Y.S. contributed equally to this paper.

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