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Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA

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Melanocytes and melanoma cells are known to possess receptors for melanocyte stimulating hormone (MSH). A cDNA clone, designated 11D, has been isolated from human melanoma cells and encodes a MSH receptor. The cloned cDNA encodes a 317 amino acid protein with transmembrane topography characteristics of a G-protein-coupled receptor, but it does not show striking similarity to already published sequences of other G-protein-coupled receptors. When 11D cDNA is expressed in COS-7 cells, it binds an ^{125}I-labelled MSH analogue (NDP-MSH) in a specific manner. The bound ligand could be displaced by melanotropic peptides, α-MSH, β-MSH, γ-MSH and ACTH (adrenocorticotropic hormone), but not by the non-melanotropic peptide, β-endorphine. This is the first report of the cloning of a receptor gene of the melanotropin receptor family.

Melanocyte stimulating hormone; Receptor; cDNA; Expression

1. INTRODUCTION

Melanocyte stimulating hormone (MSH) is a strong stimulator of pigment cells, modulating the colour change in animals [1]. In humans injections of α-MSH induces darkening of the skin through melanogenesis [2]. Many cultured melanoma cell lines undertake melanogenesis through the formation of cyclic AMP [3], which in turn activates tyrosinase, the rate-limiting enzyme of melanin formation [4]. Besides the pigmenting effect, MSH has also been shown to act as a neurotransmitter in the central nervous system [5], as an endocrine stimulant [6] and as a modulator of immune inflammatory responses [7]. The hormone is considered a potential tool in the diagnosis and therapy of melanoma as it has been used in conjugation with cytotoxin [8] and cytotoxic T-cells [9] for killing melanoma cells by recognizing their MSH receptors. Recently it has been shown that MSH can increase intracellular calcium and activate protein kinase C [10]. Two other G-protein-coupled receptors, namely the α_{1b}-adrenergic receptor and serotonin receptor, which are also coupled through the above mentioned second messenger system, have been shown to be protooncogenic [11,12]. In the following report we describe for the first time the primary structure of the MSH receptor.

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2. MATERIALS AND METHODS

2.1. *Polymerase chain reaction*

The oligonucleotide primers were designed by careful examination of the sequence homologies in membrane-spanning segments, 3 and 6, of the earlier cloned G-protein-coupled receptors. The design of the primers was not inclined towards any one receptor or any one class of the receptors. The primers were degenerate (redundancy = 8 for the primer from segment 3 and 32 for the primer from segment 6), and also had an inert nucleotide, deoxyinosine, at places which otherwise would have become highly degenerate. Primers were synthesized with *Eco*RI (segment 3) and *Bam*HI (segment 6) linkers at the 5′ ends to facilitate the cloning. The primer sequences were: (i) 5′-GGGAATTCTGTG(TC)G(TC)IATCICI(GC)TGGACCGGTA (from segment 3); (ii) 5′-GGGGATCCGAAGAAGGGI(AC)(GA)-CCAGCAGA(GC)I(AG)(CT)GAA (from segment 6)

1 μg of human genomic DNA was subjected to PCR using the above described primers. The PCR was done in a final volume of 50 μl and contained 1 μg of human genomic DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each deoxynucleotide, 1 μM of each primer and 1 U of the enzyme, *Taq* DNA polymerase (Perkin Elmer Cetus, USA). The PCR thermal profile used was 93°C for 60 s, 55°C for 40 s and 72°C for 60 s for a total of 25 cycles, using a thermal Cycler (Hybaid, UK).

10% of the reaction was analysed by agarose gel electrophoresis. One product was observed at the 372 bp position which was cloned into the pGEM7zf(+) vector (Promega Corp., USA) and the resulting plasmid, termed GE4, was sequenced using the chain-termination method [13].

2.2. *Northern blot analysis of the GE4 mRNA*

Poly(A)$^+$ RNA was prepared from the following tissues: brain, thymus, parathyroid gland, parotid gland, salivary gland, adrenal gland, testis, liver, lung, heart, spleen, skeletal muscle, intestine, colon and WM 266-4 human melanoma cells (ATCC # CRL 1676). 10 μg of poly(A)$^+$ RNA was subjected to electrophoresis through a 0.8% agarose–formaldehyde gel. The RNA was then blotted onto a GeneScreen membrane (New England Nuclear, USA). The hybridization was done in a solution containing 50% formamide, 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, 10 mM sodium phosphate, pH 7.0,

10 mM EDTA, 0.1 mg/ml denatured calf thymus DNA and ^{32}P-labelled GE4 DNA probe. The GE4 DNA was labelled with ^{32}P using a commercial multiprime kit (Amersham, UK). The membrane was left in the hybridization solution for 12 h at 42°C. The membrane was then washed in a solution of 0.1 × SSC and 0.1% SDS at 60°C for 30 min, air dried and then exposed to autoradiographic film for 16 h (see Fig. 1).

2.3. *Construction and screening of the cDNA library from the human melanoma cells*

The WM 266-4 cells were obtained from ATCC, Bethesda, USA, and grown in the medium as advised by ATCC. Poly(A)$^+$ RNA from these cells was made using the fast-track mRNA isolation kit (Invitrogen Corp., USA). A random primed cDNA library was constructed in lambda gt11 vector (Amersham, UK) from poly(A)$^+$ RNA of WM 266-4 melanoma cells. Approximately, 7×10^5 plaques from the unamplified library were screened with ^{32}P-labelled GE4 probe. Hybridization was done in a buffer (6 × SSC, 5 × Denhardt's solution, 10 mM sodium phosphate pH 7.0, 1 mM EDTA, 0.5% SDS and 0.1 mg/ml denatured salmon testis DNA) at 60°C for 12 h. Filters were then washed at 65°C in a solution of 0.1 × SSC and 0.1% SDS for 20 min, and exposed to X-ray film for 24 h. Positive plaques were picked and after repeating the screening two more times a positive plaque, designated 11D, was isolated. Both strands of the entire coding region were sequenced by the chain-termination method [13].

2.4. *Functional expression of the 11D cDNA*

The cDNA from clone 11D was inserted between *Eco*RI and *Nsi*I sites of pcDNA1 vector (Invitrogen, USA). COS-7 cells were grown in Dulbecco's modified Eagle's medium with 8% foetal calf serum and non-essential amino acids. 80% confluent cultures were transfected with 1 μg DNA and 40 μg lipofectin (BRL, USA) in serum-free medium. 5 h after transfection, the serum-containing medium was replaced, and cells were cultivated for 20 h. Cells were then scraped off, centrifuged, resuspended in serum-containing medium, plated on 48 well plates, and allowed to grow for 24 h. The cells were then washed with 0.3 ml of binding buffer (minimum essential medium with Earle's salts, 25 mM HEPES, pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg per litre leupeptin and 200 mg per litre bacitracin) and then incubated at 37°C for 2 h with 0.3 ml binding buffer containing 24,000 cpm of [^{125}I]NDP-MSH and the appropriate concentration of unlabelled peptides. NDP-MSH was labelled with ^{125}I and purified as described before [14], to a specific activity of 8.6×10^4 Ci per mol. The plates were then put on ice, the cells washed with 0.3 ml of ice-cold binding buffer, and detached from plates with 0.3 ml of 0.1 N NaOH. Radioactivity was counted and data analysed by a iterative, non-linear curve fitting programme suitable for radioligand binding analysis.

3. RESULTS AND DISCUSSION

PCR primers were designed based on the consensus sequences of transmembrane segments 3 and 6 of known G-protein-coupled receptors. Primers were used to perform PCR on human genomic DNA, as many G-protein-coupled receptors are known to be intronless. One product, designated GE4, was obtained which was cloned into pGEM7Zf(+) vector and sequenced to completion. It was found to contain a 372 nucleotide-long sequence, showing G-protein coupled-receptor characteristics. This DNA was then used as a probe to screen tissues for its expression using Northern blot analysis (Fig. 1). A specific RNA band was seen only in the melanoma cell line, WM 266-4, poly(A)$^+$ sample. This prompted us to screen a cDNA library from a WM

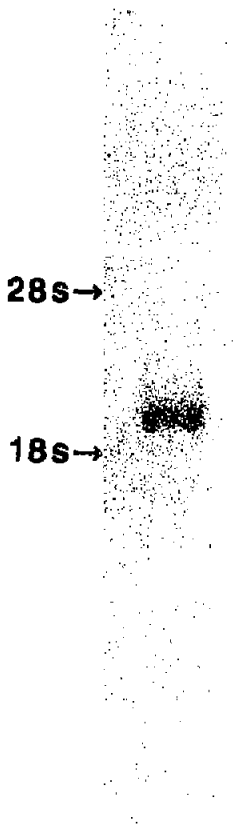

Fig. 1. Northern blot analysis of the tissue distribution of GE4 mRNA. Poly(A)$^+$ RNA (10 μg) from different tissues, namely, brain, thymus, parathyroid gland, parotid gland, salivary gland, adrenal gland, testis, liver, lung, heart, spleen, skeletal muscle, intestine, colon and WM 266-4 melanoma cells was subjected to electrophoresis through a 0.8% agarose–formaldehyde gel, blotted onto GeneScreen (New England Nuclear, USA) and hybridized to a ^{32}P-labeled GE4 probe. Here, only the WM 266-4 lane is shown as all the other tissues were completely negative. Positions for 28 S and 18 S ribosomal RNA are marked with arrows.

266-4 cell line with the GE4 probe. One clone, designated 11D, was isolated with an insert of 1,800 bp, containing a long open reading frame and an entire coding sequence (Fig. 2). The MSH receptor contained seven hydrophobic segments of amino acids, a characteristic of G-protein-coupled receptors, which are predicted to span the cell membrane seven times.

The 951 bp coding sequence begins with ACTATGG which is in agreement with the Kozak consensus sequence for translation initiation sites [15]. The entire protein consists of 317 amino acids with a total molecular weight of 34,795 Da, without considering any post-translational modifications. The N-terminal region contains two potential *N*-glycosylation sites at amino acid positions 15 and 29. The second cytoplasmic loop and COOH-terminal region (amino acids 142–145, 151–154 and 306–308) contain consensus sequences for cAMP-dependent protein kinase recognition [16]. The third cytoplasmic loop consists of 30 amino acids, which is similar in size to other G-protein-coupled peptide recep-

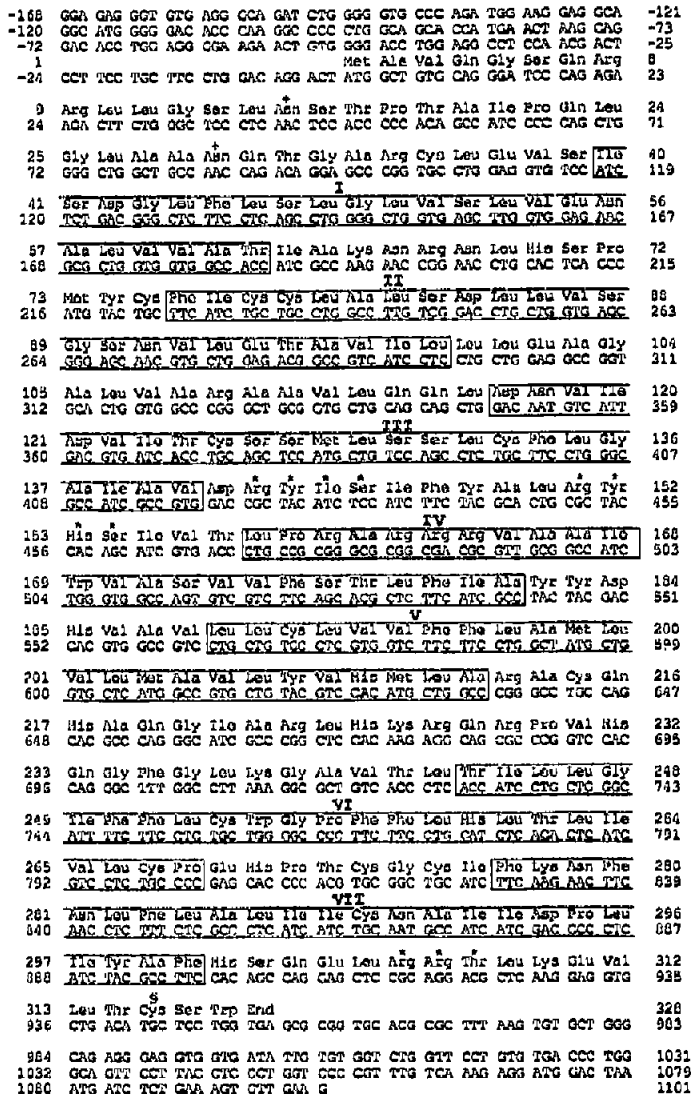

Fig. 2. Nucleotide and predicted amino acid sequence of human MSH receptor. Transmembrane segments (enclosed within boxes) were determined by hydropathy analysis according to Kyte and Doolittle [22]. The glycosylation sites (+), possible phosphorylation sites (*) and possible palmitylation site (§) are marked.

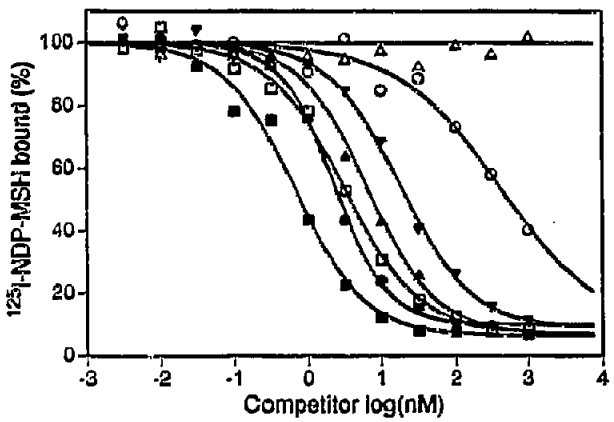

Fig. 3. Relative potencies of melanotropins for inhibiting [^{125}I]NDP-MSH binding to COS-7 cells transfected with MSH receptor cDNA. Shown are competition curves for non-labelled NDP-MSH (■), α-MSH (●), ACTH (1-39) (□), β-MSH (▲), γ-MSH (▼), ACTH (4-10) (○) and β-endorphin (△). Each point represents the mean of quadruplicate determinations. Non-specific binding was less than 8% of the total binding.

tors. Previously it has been shown that the MSH receptor is positively coupled to adenylate cyclase [3], an effect likely to be mediated by the stimulatory guanine nucleotide regulatory proteins, G_s. Amino acid cysteine, corresponding to the Cys-315 in the COOH-terminal region of the cloned MSH receptor, is conserved in other G_s-coupled receptors, where it is implicated to have a role in the covalent attachment to palmitic acid [17,18].

It is possible to classify G-protein-coupled receptors, based on similar amino acid sequences, into subfamilies, such as into the muscarinic, adrenergic, dopaminergic, histaminergic, serotoninergic and tachykinin receptor families [19]. Different receptor molecules in an individual subfamily have similar primary structure, showing more than 60–75% homology in the seven transmembrane segments. The cloned MSH receptor nucleotide sequence and the predicted amino acid sequence did not show significant homology to any of the other sequences in the EMBL and SwissProt databases. The MSH receptor thus seems to belong to a new subfamily among the G-protein-coupled receptors.

To prove that the clone, 11D, codes for human MSH receptor, it was inserted into a pcDNA1 vector and expressed in COS-7 cells. Transfected COS-7 cells were then analysed by radioligand binding. An MSH analogue, NDP-MSH, was chosen as the radioligand because of its resistance to chemical and enzymatic degradation and because of its reported high affinity for native MSH receptors [3,14]. A series of POMC (proopiomelanocortin)-derived peptides showed differential potencies in inhibiting [^{125}I]NDP-MSH binding to 11D-transfected COS-7 cells (Fig. 3). The potency order was NDP-MSH (K_i = 23 ± 0.5 pM) > α-MSH (K_i = 92 ± 19 pM) > ACTH(1–39) (K_i = 170 ± 37 pM) > β-MSH (K_i = 449 ± 74 pM) > γ-MSH (K_i = 1,010 ± 200 pM). ACTH (4–10) (adrenocorticotropic hormone) showed very low binding affinity (K_i = 22,400 ± 7,200 pM), whereas the non-melanotropic POMC peptide, β-endorphin, showed no affinity for the expressed MSH receptor. These data are in close agreement with the affinities reported earlier for MSH receptors on several melanoma cell lines [3,20]. The high affinity of ACTH(1–39) for the MSH receptor can be attributed to the fact that the first 13 amino acids in the ACTH(1–39) molecule makes up the α-MSH molecule [21], as they are produced from the same POMC polypeptide but by alternative cleavage [21]. It has also been shown that the MSH receptor on melanoma cells recognizes different

forms of the ACTH molecule with high affinity, in some cases an even higher affinity than α-MSH itself [20].

In summary, we have cloned a human MSH receptor cDNA which shows structural properties of a G-protein-coupled receptor. When the MSH receptor cDNA is expressed in an eukaryotic system, it displays binding properties for melanotropic hormones as expected for a native MSH receptor.

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