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Stable expression of human melanocortin 3 receptor fused to EGFP in the HEK293 cells

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Abstract

Among the melanocortins α-MSH is known to be involved in feeding behavior. These hormones mediate their effects through G protein-coupled receptors by stimulating adenylate cyclase. In this study, we have developed an in vitro expression model for human melanocortin 3 receptor (hMC3R) tagged at its C terminus with EGFP. The corresponding chimeric cDNA was stably expressed in HEK293 cells. The selected clones expressing the hMC3R–EGFP exhibited cell surface fluorescence and responded to NDP-MSH stimulation by producing cAMP in a dose-dependent manner (EC_{50}: 0.3 nM). Binding studies revealed a single class of binding sites with a K_D of 2.24 nM. Moreover, Agouti-related protein was also demonstrated to be an antagonist of the hMC3R–EGFP. Thus, the hMC3R tagged with EGFP stably expressed in HEK293 cells, exhibiting the same characteristics than the wild-type hMC3R, is the only model of expression of this receptor allowing its direct localization inside living cells.

Keywords: Melanocortin 3 receptors; Enhanced green fluorescent protein; Chimeric protein; Receptor binding; Confocal microscopy; cAMP

The melanocortin receptor family belongs to the G-protein coupled receptor (GPCR) superfamily and consists of five isoforms (MC1R–MC5R) identified to date [1–6]. The melanocortin peptides (α, β, and γ melanocyte stimulating hormones and ACTH) are the endogenous agonist ligands for the melanocortin receptors and are derived by post-translational processing of the proopiomelanocortin (POMC). The five melanocortin receptors exhibit distinct physiological functions and tissue-specific expression. MC1R is involved in pigmentation, MC2R is the ACTH receptor which mediates the corticotrophic effect of ACTH, MC3R and MC4R play a role in feeding behavior, and MC5R seems ubiquitous and its exact role is unknown.

The human MC3R was the third member of the melanocortin receptor family to be cloned using the polymerase chain reaction primed with oligonucleotides based on the homologous transmembrane regions of the GPCR [3]. The cloning of the rat, mouse, and chicken MC3R has also been reported [4,7,8].

The MC3R is predominantly expressed in the brain, but it was also found to be expressed in placenta by Northern blot analysis and in stomach, duodenum, pancreas [3], heart, and testis [9] by RT-PCR. Although both MC3R and MC4R are expressed in the brain, they serve non-redundant roles in the regulation of energy homeostasis as demonstrated by respective inactivation of the mouse MC3R and MC4R [10–12]. In contrast to MC4R which mainly regulates food intake, MC3R influences feed efficiency. To date, only one group reported an MC3R gene mutation associated with human obesity, but functional analysis of this MC3R mutation has not been reported [13]. At that time no appropriate cell culture model expressing the human MC3R and no specific antibodies directed against the MC3R were available. Thus, we have developed an in vitro expression model for the MC3R in human embryonic kidney (HEK293) cells. The melanocortin receptor was tagged at its C terminus with the enhanced green fluorescent protein (EGFP) allowing the direct visualization of the receptor by

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fluorescence microscopy. This model will be a perfect tool to study the relationships between the structure and the function of the MC3R, the characterization of naturally occurring MC3R mutations, and the subcellular distribution of the MC3R. It could also be used for comparative pharmacological studies between new agonists or antagonists of the MC3R.

Materials and methods

Materials. Fetal calf serum (FCS), Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (DMEM/F12 1:1), Lipofectamine Plus reagent, L-glutamine, and restriction enzymes were purchased from Life Technologies (Cergy Pontoise, France); geneticin (G418), 3-isobutyl-1-methyl-xanthine (IBMX), and poly-D-lysine hydrobromide from Sigma–Aldrich (St. Quentin Fallavier, France); human Agouti-related protein (hAGRP) (83–132) from Phoenix Pharmaceuticals (USA); ACTH (1–24) and (Nle^4, D-Phe^7) α-MSH (NDP-MSH) from Bachem (Voisins le Bretonneux, France).

Construction of the chimeric cDNA. A chimeric DNA encoding the human melanocortin receptor type 3 (hMC3R) with EGFP at its C terminus was constructed by PCR. The entire coding sequence of the hMC3R gene was amplified by PCR from human genomic DNA using the specific primers MC3EcoS (5′-CGCTTCGAATTCATGAGCAT CCAAAAGAAG-3′) and MC3BamAS (5′-CAACGGATCCCTCCC AAGTTCATGCCGTT-3′). The MC3EcoS primer introduced an *Eco*RI site in 5′ of the hMC3R coding region and the MC3BamAS primer replaces the stop codon with a glycine codon introducing a *Bam*HI site. The PCR conditions were 94 °C, 1 min for 1 cycle; 94 °C, 30 s; 60 °C, 30 s; and 68 °C, 1 min for 30 cycles followed by 68 °C for 10 min. This PCR product was purified (NucleoSpin Extract, Macherey-Nagel, Hoerdt, France) and subcloned into *Bam*HI and *Eco*RI sites of the pEGFP-N2 vector (Clontech Laboratories, Palo Alto, USA), which contained the neomycin resistance gene for selection of stably transfected clones. The hMC3R–EGFP vector was subsequently checked by dideoxynucleotide sequencing using [α-^{33}P]deoxyATP and the T7 Sequencing Kit (Amersham Pharmacia Biotech, Orsay, France) to insure that no errors were induced by PCR.

Cell culture and transfection. HEK293 (human embryonic kidney) cells were grown in DMEM/F12 supplemented with 7.5% FCS and 2 mM glutamine. Stable transfections were performed in serum-free DMEM/F12 using the Lipofectamine Plus reagent according to the manufacturer's protocol as described previously [14]. Individual geneticin resistant clones were selected with 800 μg/ml geneticin and cultured in complete medium containing 200 μg/ml geneticin.

Fluorescence microscopy. The clones selected with geneticin were observed on fluorescence confocal microscopy (Leica Microsystems, Rueil Malmaison, France) after stimulation with UV (488 nm).

Northern blot analysis. The expression of the chimeric construction MC3R-EGFP was evaluated by Northern blot analysis after extraction of total RNA from selected transfected HEK293 cells as previously described [15]. Hybridizations were performed using the coding sequence of the EGFP as a probe, labeled in the presence of [α-^{32}P]deoxyCTP using the Rediprime DNA labelling system (Amersham Pharmacia Biotech, Orsay, France). Several clones expressing a transcript at the expected size and having almost a similar MC3R-EGFP expression were used for further characterization.

Cyclic AMP assay. To study the coupling of the chimeric protein MC3R-EGFP to adenylate cyclase in the stably transfected HEK293 cells, NDP-MSH and ACTH dose responses were performed. For this assay, cells were plated on 12-well dishes, pretreated with poly-D-lysine (0.1 mg/ml), at 0.2×10^6 cells/well, in DMEM/F12 supplemented with 7.5% FCS. After 24-h plating, cells were incubated for 20 min at 37 °C in media containing various concentrations of NDP-MSH or ACTH in the presence of 1 mM IBMX to inhibit phosphodiesterases. The intracellular accumulation of cAMP was measured using a radioimmunological assay using ^{125}I-labeled cAMP (Beckman Coulter, Roissy, France) [16].

Binding studies. Iodinated NDP-MSH, [^{125}I](Lys^{11})(Nle^4-D-Phe^7) α-MSH (SA: 2000 Ci/mmol, Amersham Pharmacia Biotech, France) was used to assess agonist binding affinity. Stably transfected HEK 293 cells were grown in complete medium on poly-D-lysine coated 12-well plates at 0.5×10^6 cells/well. After 24-h plating, the medium was removed and cells were incubated for 2 h at room temperature with 0.15 nM [^{125}I]NDP-MSH and various concentrations of cold NDP-MSH in DMEM/F12 containing 0.5% bovine serum albumin (BSA) and 0.1% bacitracin. Binding reactions were stopped on ice by removing the media and washing the cells quickly three times with 0.9% NaCl. Then, the cells were solubilized in 0.5 ml of 0.5 M NaOH and 0.4% sodium deoxycholate [17]. Radioactivity in the lysate was quantified in a Packard γ counter (Perkin–Elmer). Specific binding was determined by subtracting from the total binding the radioactivity associated with the cells in the presence of 10^{-6} M cold NDP-MSH. This non-specific binding accounted for almost 2% of the total binding.

Results and discussion

An MC3R-EGFP chimera was constructed by linking the EGFP-cDNA in frame to the 3′ end of the human MC3R coding sequence resulting in an MC3R fused to the EGFP on its carboxy-terminal tail. This chimeric receptor was stably expressed in HEK293 cells. The selected clones were observed on a fluorescence confocal microscope under UV illumination. Whereas, parental HEK293 cells did not exhibit any significant fluorescence level (data not shown), cell surface fluorescence as well as perinuclear and intracellular fluorescence were observed in the clones expressing the hMC3R–EGFP protein (Fig. 1A). This observation suggests that the protein was correctly folded and that the presence of EGFP at the C-terminus of the protein did not interfere with its targeting to the cell membrane. Therefore, this is the first hMC3R expression model allowing the direct localization of the receptor inside living cells. Moreover, after addition of the agonist NDP-MSH (10 nM) on the clones for 10 min at 37 °C, the fluorescence at the plasma membrane completely disappeared and numerous fluorescent vesicles were observed intracellularly (Fig. 1B). Thus, like other GPCRs, the hMC3R–EGFP was internalized after ligand binding, which suggests that the fusion protein was in an active conformation before endocytosis. Another member of the melanocortin receptor family, MC2R, internalizes in the presence of ACTH in Y1 cells via a clathrin-mediated G protein receptor kinase-dependent mechanism [18].

Functional analysis of the clones expressing the hMC3R–EGFP chimera was performed by measuring the intracellular cAMP levels after stimulation by increasing concentrations of the agonist NDP-MSH, the most universally used agonist of α-MSH which exhibits prolonged biological activity [19] (Fig. 2). The maximal

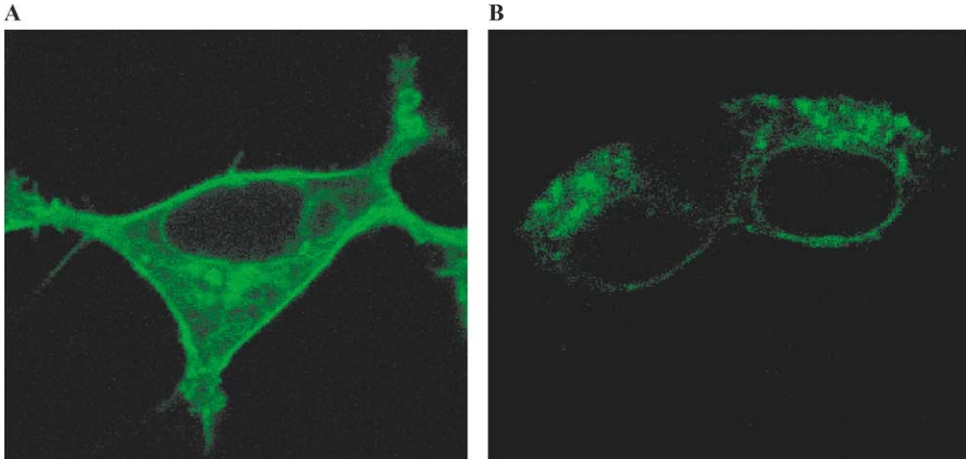

Fig. 1. Fluorescent confocal microscope images of HEK293 cells stably expressing hMC3R–EGFP after incubation for 10 min at 37 °C without (A) or with (B) 10 nM NDP-MSH.

cAMP production was obtained with 10 nM NDP-MSH corresponding to an 85-fold increase above the levels found in unstimulated cells. The parental HEK293 cells did not respond to stimulation by the same peptide which rules out the possibility of the presence of endogenous melanocortin receptors in the HEK293 cells used for transfection. The cAMP response curves obtained under stimulation of the clones with NDP-MSH or ACTH were exactly similar (Fig. 2), which confirms that the human MC3R recognizes both melanocortin peptides equally well [3]. The different clones tested gave similar results and the EC_{50} values calculated from the dose–response curves were highly reproducible and were 0.3 ± 0.04 nM ($n = 6$) and 0.15 ± 0.05 nM ($n = 3$) for NDP-MSH and ACTH, respectively. Since there are no cell lines expressing endogenous MC3R, all the pharmacological characteristics of the MC3R have been obtained from heterologous expression systems. The present results that we obtained with a human MC3R-EGFP fusion protein appear quite similar to those already published with the MC3R alone from different species expressed in different cell lines [3,4,7]. Moreover, the EC_{50} for the human MC3R-EGFP is in good agreement with those published for the human MC3R alone stably expressed in HEK293 cells (0.87 ± 0.2 nM) [20].

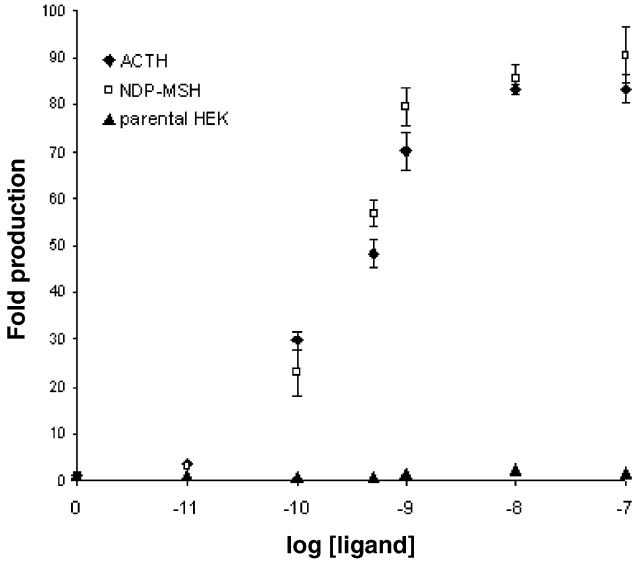

Fig. 2. Intracellular cAMP production in HEK293 cells stably expressing hMC3R–EGFP in response to increasing concentrations of NDP-MSH or ACTH. The response of the parental HEK293 is also shown. Each point represents the mean ± SD of one experiment performed in triplicate. The experiment was repeated at least three times. Basal level of cAMP production in the absence of agonist stimulation was between 3.7 and 9.3 pmol/10^6 cells depending on the experiments.

Further characterization of the stable clones expressing the MC3R-EGFP chimera was performed by NDP-MSH binding studies. No specific binding was observed on HEK parental cells. Fig. 3 showed a dose-dependent inhibition of the binding of [^{125}I]NDP-MSH to the selected clones by increasing concentrations of unlabeled NDP-MSH. Scatchard analysis of the binding data indicated a single class of binding sites with a dissociation constant K_D of 2.24 ± 0.5 nM and $3.74 \pm 0.32 \times 10^5$ sites per cell ($n = 3$). Since the K_D was 10 times higher than the EC_{50} obtained for cAMP production, these results suggest that activation of a small percentage of the receptors present at the plasma membrane is sufficient to fully activate adenylate cyclase, as it has been reported for the mouse MC3R expressed in CHO-K1 cells [7]. This is the only report on binding studies with a human MC3R tagged with EGFP stably expressed in HEK293 cells. These results are quite similar to those obtained with human MC3R transiently expressed in COS cells, but no data on the number of binding sites were available [21].

We have also studied the activity of AGRP, a natural antagonist of the MC3 and MC4 receptors [22,23], in one of the clones expressing the construct MC3R-EGFP. We used AGRP (83–132), a synthetic fragment

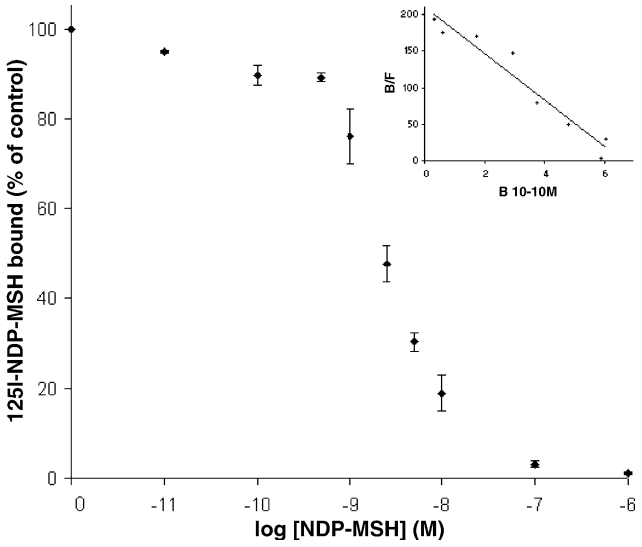

Fig. 3. Inhibition of binding of [^{125}I]NDP-MSH to HEK293 cells stably expressing hMC3R–EGFP by increasing concentrations of unlabeled NDP-MSH. Each point represents the mean ± SD of at least three independent experiments performed in triplicate. (Inset) Scatchard analysis of the binding data.

of human AGRP lacking the N terminus of AGRP and consisting of only the cysteine-rich carboxyl-terminal region of AGRP which retains the biological activity of the full-length AGRP [24]. As shown in Fig. 4, the production of cAMP in HEK293 cells stably expressing the MC3R-EGFP stimulated by 0.5 or 1 nM NDP-MSH was completely abolished by AGRP (83–132) at concentrations 100-fold higher than those of NDP-MSH. Thus, AGRP (83–132) is also a functional antagonist of the hMC3R–EGFP fusion protein stably expressed in HEK293 cells as it has been reported for the wild-type hMC3R [22,23,25]. Moreover, the basal production of cAMP by these clones incubated in the presence of AGRP (83–132) alone was decreased by 35% when

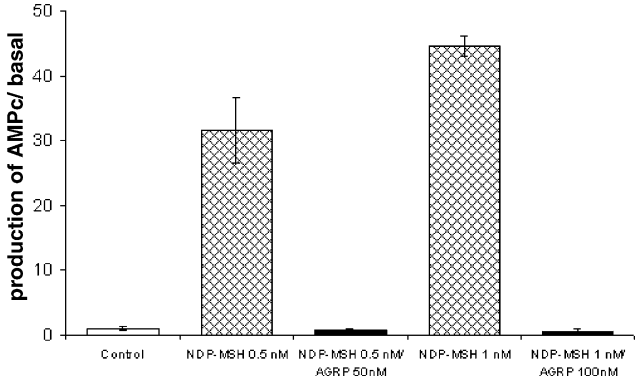

Fig. 4. Intracellular cAMP production in HEK293 cells stably expressing hMC3R–EGFP in response to 0.5 and 1 nM NDP-MSH in the presence or absence of 50 and 100 nM AGRP, respectively. Each point represents the mean ± SD of one experiment performed in triplicate. The experiment was repeated at least three times.

compared to unstimulated cells (3.1 ± 0.28 versus 4.76 ± 0.6 pmol cAMP/10^6 cells, $n = 3$, for 100 nM AGRP and basal, respectively). These results demonstrate that AGRP (83–132) acts as an inverse agonist on the hMC3R–EGFP as on the hMC3R alone [26]. This inverse agonist effect can be seen because of the high level of expression of the hMC3R–EGFP in these clones.

All these data indicate that the hMC3R–EGFP fusion protein stably expressed in HEK293 cells is functional, since it was expressed at the plasma membrane and its binding and signaling potencies were comparable to those of the wild-type hMC3R. This in vitro model of expression for hMC3R tagged with EGFP at its C-terminus would be a perfect tool for conducting structure–function studies of this receptor, since it allows the direct localization of the receptor by fluorescence microscopy. This model could also be used for comparative pharmacological studies between new agonists or antagonists and also for characterization of naturally occurring mutations.

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