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# Characterization of Cell Lines Stably Expressing Human Normal or Mutated EGFP-Tagged MC4R

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**The melanocortin receptor type 4 (MC4-R) is involved in food intake and represents a potential target for the treatment of some forms of obesity. The fluorescent protein EGFP was fused to the wild-type or mutated coding sequence of the human MC4-R. After transfection in HEK 293, clones stably expressing hMC4-R-EGFP were selected. Wild-type chimeric hMC4-R was well addressed to the cell membrane as demonstrated using confocal microscopy and displayed the same pharmacological characteristics as native hMC4R. NDP-αMSH induced a time-dependent internalization of MC4-R that was partially prevented by AgRP. The two mutated chimeric receptors studied here (CTCT-deleted and C271A) showed a high alteration of their response to ligand and were retained inside the cells. In conclusion, we have developed a model of clones stably expressing EGFP-tagged-hMC4-R. This is the only such model available to date and it provides a useful tool to follow the trafficking of MC4-R inside living cells.**

**Key words: confocal microscopy, EGFP, melanocortin receptor type 4, stable expression.**

Abbreviations: EGFP, Enhanced green fluorescent protein; MC4-R, melanocortin receptor type 4.

Melanocortin receptors belong to the G-protein coupled receptors (GPCRs) family and signal through the activation of the adenylate cyclase pathway (*1*). To date, five melanocortin receptors (MCRs) have been cloned. MC1-R, MC2-R and MC5-R are expressed in the skin (*2*), adrenal cortex (*2*), and exocrine glands and cerebral cortex (*3*), respectively. MC3-R and MC4-R are found primarily in the brain and particularly in the hypothalamic paraventricular nucleus, which has been demonstrated to be strongly involved in the central control of food intake (*4*). Both MC3-R and MC4-R display a similar affinity for α-melanocyte-stimulating hormone (α-MSH), a cleavage product of pro-opiomelanocortin precursor that is expressed by neurons of the hypothalamic arcuate nucleus (*2*). They also exhibit a similar affinity for potent agonists NDP-αMSH and MTII or antagonists Agouti-Related Peptide (AgRP) and SHU9119 (*2*). Pharmacological studies using α-MSH, alone or in the presence of SHU9119, have clearly shown that feeding behaviour in rodents is controlled by MC4-R (*5*). A specific role for MC4-R is supported by genetic evidence. In humans, a severe obesity has been associated with several different mutations inside the coding sequence of MC4-R (*6*, *7*). Homozygous deficient transgenic mice develop a late-onset obesity syndrome (*8*). Interestingly, human obese individuals are heterozygous for the MC4-R mutations, and heterozygous +/– mice present an intermediate average increase in weight as compared to homozygous –/– mice (*8*). Thus, the obese phenotype has been speculated to be the result of haploinsufficiency and mechanisms controlling the number of MC4-Rs available for hormone binding, and signal transduction is an important aspect of the function of these receptors.

The melanocortin receptors are small GPCRs with short amino- and carboxy-terminal domains and short extracellular loops, which makes it difficult to prepare antibodies recognizing specifically the native receptors and precludes further analyses of the wild-type or mutant receptors in their unbound state, in particular regarding the trafficking in the intracellular compartments. As an alternative to immunological detection of MC4-R, we have generated a fusion protein between the human MC4-R and the enhanced green fluorescent protein (EGFP) derived from Aqueora Victoria stably expressed in HEK-293 cells. Our objective was to establish and characterize HEK 293 cell lines stably expressing wild-type or mutated MC4-R fused to EGFP, since the other proposed models of tagged MC4-R used transiently transfected cells (*9*, *10*). This approach has been successfully used for several other GPCRs such as GnRH receptor (*11*) and LH receptor (*12*) and allows one to follow the behaviour of the fusion protein in a real-time imaging and to observe regulation and trafficking in living cells.

In this report, we show that the wild-type hMC4R-EGFP fusion protein stably expressed in HEK 293 cells is appropriately addressed to the plasma membrane and retains the physiological characteristics of native MC4-R. Moreover, we demonstrate that this model is appropriate to follow the wild-type receptor upon activation by agonist and to localize mutant receptors.

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## MATERIALS AND METHODS

*Materials*—Penicillin, streptomycin, fetal calf serum (FCS), Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture (DMEM/F-12, 15 mM Hepes) and restriction enzymes were purchased from Invitrogen (Cergy-Pontoise, France); [$^{125}$I]cAMP from Immunotech (Beckman, Marseille, France) and Taq polymerase from Eurobio (Les Ulis, France). Oligonucleotides were prepared by Sigma-Genosys (Cambridge, UK).

*Expression Vector Construction*—A chimeric cDNA encoding the human melanocortin receptor type 4 (hMC4R) fused to the Enhanced Green Fluorescent Protein (EGFP) at the C terminus was constructed by PCR. The entire coding sequence of the human MC4 receptor gene was amplified by PCR using the oligonucleotides:

- sense CTTCGAATTCATGGTGAACTCCACCCACCG
- antisense CAACGGATCCCATATCTGCTAGACAAGTCA

as primers and a human genomic DNA as template. The 5′ primer contains an *Eco*RI site upstream of the ATG codon of hMC4R, and the 3′ primer has the stop codon replaced by a codon corresponding to a glycine and contains a *Bam*HI site. The PCR conditions were 2 min at 94°C; then 30 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 68°C; and finally 68°C for 10 min.

This PCR product was purified and inserted into a *Bam*HI/*Eco*RI cleaved pEGFP-N2 vector (Clontech, Ozyme, Montigny-le-Bretonneux), which contains the neomycin-resistance gene for selection of stably transfected clones. This construct corresponds to the wild-type hMC4R and was called phMC4R-EGFP-N2. The sequence of the insert was confirmed by sequencing using a T7 sequencing kit (Amersham France, Saclay).

To obtain vectors containing a mutated hMC4R, we performed site-directed mutagenesis using mutated oligonucleotides and Pfu polymerase (Quick-Change, Stratagene). The mutations studied were cysteine 271 to alanine (the mutation C271Y has been described in Ref. (*13*) and the deletion CTCT described by Yeo *et al.* (*7*). For this mutation, a second step was necessary as it induced premature stop codon. This stop codon was replaced by a glycine codon by PCR as described above to give a truncated hMC4R fused to EGFP.

*Cell Culture and Transfections*—The day before transfection, 293 Human Embryonal Kidney (HEK) cells were plated at $10^6$ cells per 100-mm Petri dish in DMEM-F12 supplemented with 7.5% FCS, 0.5 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Transfections were performed in serum free DMEM/F12 without antibiotics using the Lipofectamine PLUS reagent (Invitrogen) and 12 μg of hpMC4R-EGFP-N2 following the method suggested by the manufacturer. The cells were cultured for 2 days. Cell lines stably expressing wild-type or mutated hMC4R-EGFP were then selected for resistance to 800 μg/ml G418 (Sigma France, Saint-Quentin Fallavier), cloned and maintained in 200 μg/ml G418.

*Confocal Microscopy*—Cells were observed using a TCS SP2 confocal laser microscope (Leica Microsystemes SA, Rueil-Malmaison, France) and a 63× water-immersion objective. EGFP fluorescence was detected at 543 nm after excitation at 488 nm. Images were recorded on a cross-section through the cells.

*Cyclic AMP Assay*—Intracellular cyclic 3′-5′ adenosine monophosphate (cAMP) was measured by a radioimmunological assay (RIA).

For this assay, cells were plated on 12-well dishes and washed once with serum-free medium. The cells were incubated for 5 min at 37°C in media containing 1 mM 3-isobutyl-1methyl-xanthine (IBMX, Sigma) and then treated for 20 min in the presence of IBMX with various concentrations of NDP-αMSH, a potent agonist of MC4R (Bachem, Voisins-le-Bretonneux, France) in the presence or not of AgRP 83-132 (Phoenix, Karlsruhe, Germany). Ice-cold 60% ethanol (500 μl/well) was added to stop the reaction and precipitate cellular proteins. Cells were scraped free, transferred to a 1.5-ml tube and centrifuged for 5 min at 12,000 ×*g* to remove insoluble material. The supernatant was evaporated, and the pellet was dissolved in 1 ml of RIA buffer.

Each experiment was performed three times in triplicate.

Statistical analysis was performed using one-way ANOVA analysis followed by *post-hoc* testing with Fisher's protected least square difference (PLSD). Differences were considered significant when $P < 0.05$.

*Binding Studies*—An iodinated NDP-α MSH (Amersham France, Saclay) labelled on Lys 11 was used to measure the agonist binding affinity. Stably transfected HEK 293 cells were grown in poly-D lysine coated 12-well plates (Sigma). Before binding, the medium was removed and replaced by medium without serum. Cells were kept at 37°C for at least 2 h in this medium. Cells were then incubated for 1 h at room temperature in the presence of $3 \times 10^{-10}$ M [$^{125}$I]NDP-α MSH and various concentrations of unlabelled NDP-α MSH diluted in F12-DMEM containing 0.5% bovine serum albumin (BSA) and 0.1% bacitracin. Binding reactions were terminated on ice by removing the media and washing the cells once with 0.9% NaCl containing 1% BSA and three times with 0.9% NaCl. The cells were detached from the plates with 0.4% sodium deoxycholate diluted in 0.5 M NaOH. Radioactivity of the lysate was quantified in a γ counter. Nonspecific binding was determined by measuring the amount of $^{125}$I-labelled NDP-α MSH bound in the presence of $10^{-6}$ M unlabelled NDP-α MSH.

## RESULTS AND DISCUSSION

About 30–40 clones of HEK 293 cells stably transfected with either wild-type or mutated phMC4R-EGFP-N2 were isolated. The expression of the integrated receptor was confirmed both by Northern blot analysis using hMC4R as probe and by epifluorescence (data not shown).

*Coupling to Adenylate Cyclase Studies*—Intracellular cAMP was measured in the presence of various amounts of NDP-αMSH. Non-transfected HEK 293 cells did not respond to increasing concentrations of NDP-αMSH (data not shown), indicating the absence of functional endogenous MCR. For clones stably expressing the wild-type hMC4R-EGFP (Fig. 1A), we observed a dose-dependent increase of the intracellular cAMP secretion with a maximal increase over control at $10^{-7}$ M NDP-αMSH. This increase varied between the different clones used (with a minimum of 17-fold increase) as illustrated in Fig. 1A, depending on the degree of hMC4R-EGFP over-

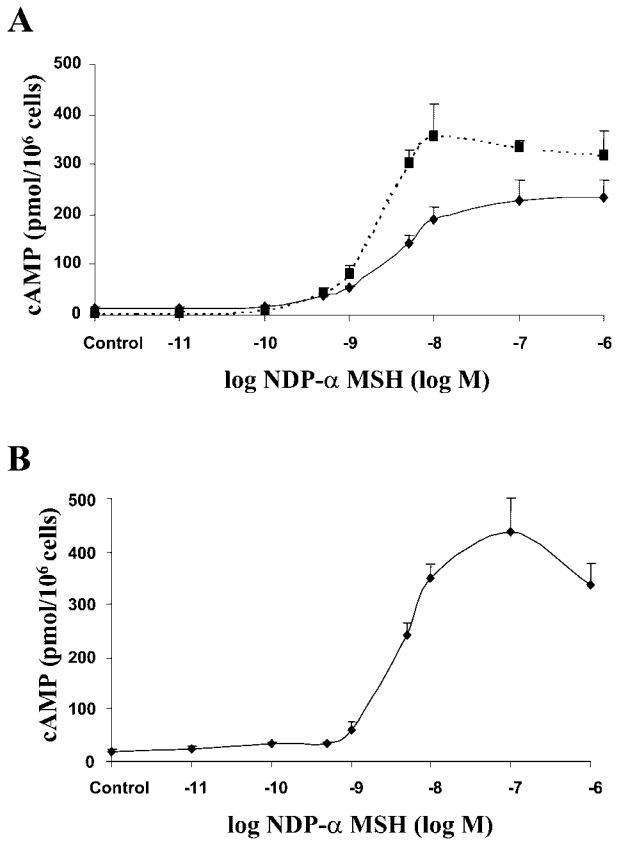


Fig. 1. **Dose-response curve of intracellular cAMP stimulation by various concentrations of NDP-αMSH obtained for wild-type hMC4R.** HEK 293 cells stably expressing the wild-type hMC4R fused to EGFP (A) or transiently transfected with the native hMC4R (B) were incubated in the presence of NDP-αMSH at different concentrations for 20 min and intracellular cAMP was measured. Results are expressed as pmoles of cAMP secreted by $10^6$ cells and represent mean ± SEM of three separate experiments performed in quadruplicate. For the clones stably expressing the wild-type MC4R-EGFP, results presented here were obtained using two different clones (clone 6 in dotted line and clone 28 in solid line). Similar results were obtained for two other individual clones.

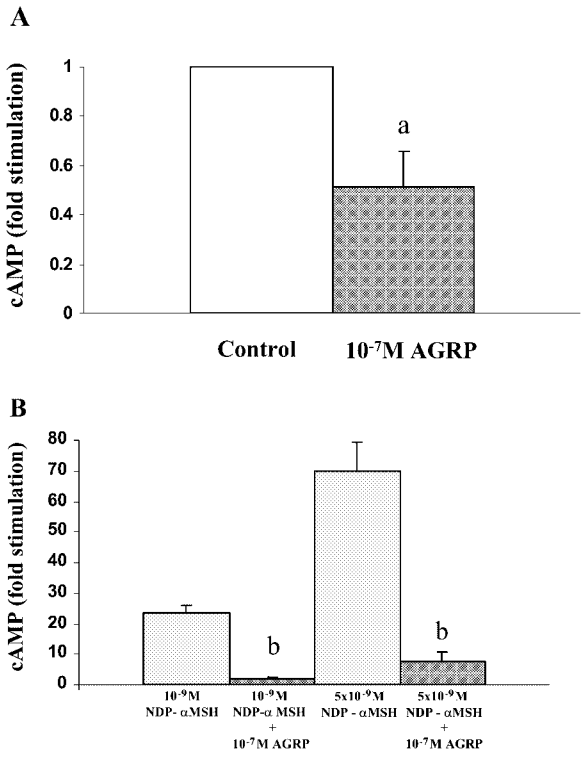


Fig. 2. **Inhibition of NDP-αMSH-induced intracellular cAMP stimulation by AgRP.** HEK 293 cells stably expressing the wild-type hMC4R fused to EGFP were incubated for 20 min in the presence or not of $10^{-7}$ M AgRP alone (A) or in the additional presence of NDP-αMSH at two different concentrations (B), and intracellular cAMP was measured. Results are expressed as fold stimulation over the control (without NDP-αMSH) and represent the mean ± SEM of three different experiments performed in quadruplicate. Statistical analysis was performed using ANOVA. a: $p < 0.03$ compared with control. b: $p < 0.02$ compared with corresponding NDP-αMSH-treated cells.

expression. Basal concentrations of cAMP were relatively high: around 14 pmoles per $10^6$ cells. The $ED_{50}$ was calculated in six different experiments and found to be equal to $2.95 \pm 0.46 \times 10^{-9}$M, which is similar to the $ED_{50}$ obtained for wild-type hMC4R not fused to EGFP: $5.16 \pm 0.62 \times 10^{-9}$M after transient transfection in HEK 293 cells (Fig. 1B and Refs. *6*, *13–15*). In these transiently transfected cells, the maximal cAMP secretion (24-fold increase over control) was obtained with $10^{-7}$ M NDP-αMSH.

To further characterize the wild-type hMC4-R fused to EGFP in the stably transfected HEK 293 cells, we studied the effect of AgRP 83-132 on basal or NDP-αMSH-induced cAMP secretion. When treated with $10^{-7}$ M AgRP alone, the cells showed a decreased cAMP secretion compared to control by about 50% (Fig. 2A). The same concentration of AgRP completely abolished the cAMP secretion induced by $10^{-9}$ M NDP-αMSH and reduced by more than 90% the cAMP secretion induced by $5 \times 10^{-9}$ M NDP-αMSH (Fig. 2B). In HEK 293 cells transiently transfected with the native wild-type hMC4R, the same concentration of AgRP also reduced the basal cAMP production by more than 50%. It also reduced the NDP-αMSH-stimulated cAMP production, although the maximal inhibition (60%) was less pronounced than when stable clones were used (*16*). This may be due to the heterogeneity of the transiently transfected cell population. These results were compatible with an inverse agonist/antagonist effect of AgRP on hMC4R. This inverse agonist effect of AgRP has been described for native hMC4R and hMC3R by Nijenhuis *et al.* (*17*) on clones expressing a high level of either one of these receptors. In these cell lines, the efficacy of AgRP to decrease the basal cAMP secretion is well correlated with the expression level of the receptor. Then, AgRP partly suppressed the constitutive activity of both native hMC4R and hMC4R fused to EGFP at the C-terminus. It has also been described as a strong antagonist of the native hMC4R in brain (*18*). In our study, this peptide suppressed the stimulating effect of NDP-αMSH-induced cAMP secretion by cells stably expressing hMC4R-EGFP.

*Binding Studies*—NDP-αMSH binding studies were also performed in the presence of $3 \times 10^{-10}$ M labelled compound. Figure 3A shows the inhibition of binding of [$^{125}$I] NDP-αMSH by increasing concentrations of unlabelled NDP-αMSH. Non-specific binding was determined

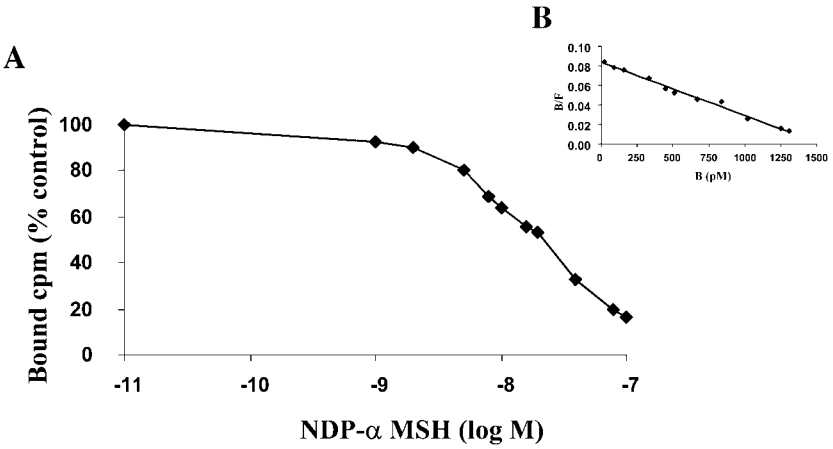


Fig. 3. **Inhibition of binding of $^{125}$I-labelled human [Lys$^{11}$]NDP-αMSH to a clone stably expressing normal hMC4R-EGFP by increasing concentrations of unlabelled NDP-αMSH.** Representative curve corresponding to one experiment is shown here. Four other experiments gave similar results using two different clones. A: Control (in the absence of unlabelled NDP-αMSH) was taken as 100% of binding, and the percentage of specifically bound labelled NDP-αMSH relative to the control is plotted against the concentration of unlabelled NDP-αMSH. B: Scatchard analysis of the binding data.

using $10^{-6}$ M unlabelled NDP-αMSH. Scatchard analysis of the binding data (Fig. 3B) revealed the presence of only one type of binding site with a $K_d$ of $1.58 \pm 0.11 \times 10^{-8}$ M and a number of about 273,000 ± 35,000 sites/cell (mean of 5 different experiments). Thus, the presence of EGFP at the C-terminus of the receptor did not modify its affinity for the ligand, as this calculated $K_d$ was similar to that reported by several authors (*6*, *19*).

From these results we can conclude that the modification induced by the presence of EGFP on wild-type hMC4R did not affect the biochemical characteristics of hMC4R as compared to the native receptor. In particular, the responses to agonist and antagonist were comparable.

*Confocal Microscopy Study*—The observation of cells from clones stably expressing hMC4R-EGFP by confocal microscopy showed that the fluorescence was maximal at the cell membrane (Fig. 4A). The hMC4R fused to EGFP was then correctly addressed to the plasma membrane. In the presence of $10^{-9}$ M NDP-αMSH, MC4 receptors were internalized in a time-dependent manner (Fig. 4, B and C). After 10 min, the fluorescence was present both at the cell membrane and in cytoplasmic vesicles. But

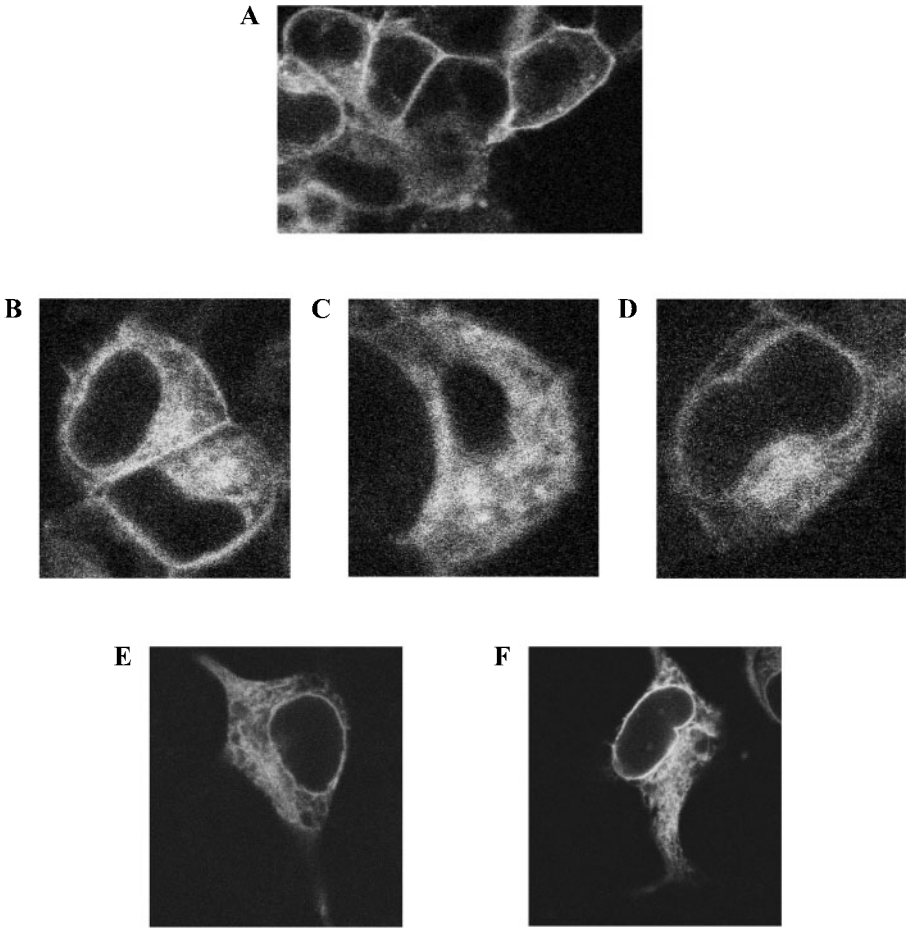


Fig. 4. **Localization of wild-type or mutated hMC4R-EGFP using confocal microscopy.** HEK 293 cells expressing wild-type hMC4R-EGFP in the absence of ligand (A), after 10 min (B) and 30 min (C) in the presence of $10^{-9}$ M NDP-αMSH or after 30 min in the presence of both $10^{-9}$ M NDP-αMSH and $10^{-7}$ M Agrp (D). HEK 293 cells expressing CTCT-deleted (E) or C271A-mutated (F) hMC4R-EGFP. Cells were examined by confocal microscopy directly on 60-mm Petri dishes.

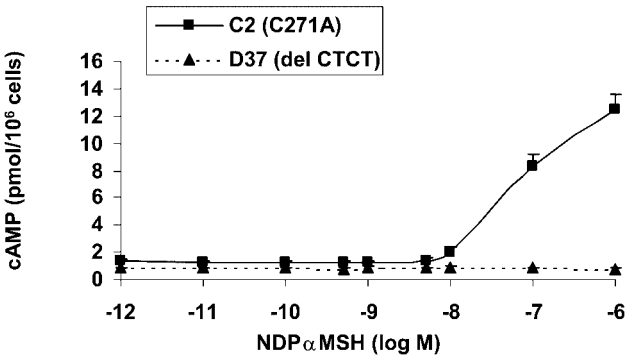


Fig. 5. **Dose-response curve of intracellular cAMP stimulation by various concentrations of NDP-αMSH obtained for C271A and CTCT-deleted hMC4R-EGFP.** HEK 293 cells stably expressing either the mutated C271A (clone C2) or the CTCT-deleted (clone D37) hMC4R fused to EGFP were incubated in the presence of NDP-αMSH at different concentrations for 20 min, and intracellular cAMP was measured. Results are expressed as pmoles of cAMP secreted by $10^6$ cells and represent mean ± SEM of three separate experiments performed in quadruplicate.

after 30 min, the fluorescence was totally restricted to the cytoplasm. These results were compatible with a desensitization phenomenon induced after activation of receptors by their agonist (*10*, *20*). Internalization of the receptors in the presence of α-MSH was partially prevented by additional exposure to $10^{-7}$ M AgRP (Fig. 4D). Indeed, after 30 min of treatment in the presence of both NDP-αMSH and AgRP, the fluorescence was displayed both at the cell membrane and inside cytoplasmic vesicles. AgRP might block the MC4 receptors in an inactive form, possibly by preventing the binding of αMSH to its receptors and thus their activation prior to their internalization. This hypothesis is strengthened by the recent observation by Shinyama *et al.* (*10*), showing that AgRP pretreatement of cells maintained the MC4-R in an inactive form resulting in an increase of cAMP production upon subsequent NDP-αMSH activation compared to cells that were not pretreated.

*Utilization of This Model for the Study of Mutant EGFP-MC4-R*—As shown above, the wild-type hMC4R-EGFP was mostly addressed to the cell membrane, contrarily to the two studied mutated receptors (Fig. 4, E and F). Indeed, the majority of the fluorescence was present inside the cells and mostly at the periphery of the nucleus in the clones expressing CTCT-deleted or C271A-mutated hMC4R EGFP, with no visualization of the cell outlines. Abnormal receptors might be trapped in cytosolic compartments, possibly due to defective folding. Consistent with this finding that mutated hMC4R-EGFP had an impaired addressing to the cell membrane, the cellular cAMP response to various concentrations of NDP-αMSH was largely altered (Fig. 5). There was no response even to high levels of NDP-αMSH for clones expressing CTCT-deleted hMC4R-EGFP. Clones expressing the C271A mutated hMC4R-EGFP displayed no response to concentrations of NDP-αMSH lower than $10^{-8}$ M, with a dose-response curve completely shifted to the right and showing no saturation at $10^{-6}$ M, which indicated an ED50 increased by at least 100-fold. The mutation C271Y, which is associated with morbid obesity, has been described by Farooqi *et al.* (*13*) and also induces a decreased response to α-MSH. We report that the distribution of the receptor inside the cells expressing the mutant receptor C271A-hMC4-R-EGFP, as shown by confocal microscopy, was very similar to that of cells expressing the CTCT-deleted hMC4R-EGFP. All these data obtained using stable clones are in accordance with previous studies on deleted or mutated receptors, performed using models of transiently transfected cells (*9*, *19*, *21*). Thus intracellular immobilization of hMC4R seems to be a common feature of naturally mutated hMC4R (*9*, *22*). The advantage of a stably expressed receptor tagged with EGFP is that we can follow the traffic of this tagged receptor in well-characterized living cells and perform dynamic studies. Thus this model is suitable to better characterize the defects of mutant receptors.

In conclusion, we developed here cell lines stably expressing the fused hMC4R-EGFP. This established and well-characterized model will be useful for further studies, to control the addressing of modified hMC4R to the cell membrane and to visualize wild-type or modified receptors in living cells in different conditions of activation. In particular, these cells will make possible pharmacological studies using new agonists or antagonists.

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