Induction of constitutive melanogenesis in amelanotic mouse melanoma cells by transfection of the human melanocortin-1 receptor gene

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SUMMARY

The human melanocortin-1 (MC1) receptor was stably expressed in the amelanotic mouse melanoma cell clone B16-G4F which does not express its own (mouse) MC1 receptor and hence is unresponsive to α melanocyte stimulating hormone (αMSH). From several stable transfectant cell lines expressing the human MC1 receptor in relatively high numbers, three melanin producing clones (G4F-12, 14, and 15) and one amelanotic clone (G4F-7) were further analyzed in competition binding experiments and in cAMP and melanin assays. The dissociation constants (KD) for [Nle4, D-Phe7]-αMSH in all four clones ranged from 0.187 to 0.705 nmol/l, thus corresponding to the KD observed with the different human melanoma cell lines so far studied. Intracellular cAMP content was 3- to 5-fold higher than that of control cells, and αMSH induced an additional 1.5- to 1.7-fold increase. G4F-15 cells secreted melanin into the medium whereas the other clones did not secrete melanin. The extent of melanin secretion was similar to that of fully αMSH-stimulated B16-F1 mouse melanoma cells but the onset of secretion was delayed. αMSH induced an additional dose-related increase (up to 1.3-fold) in melanin production which could be suppressed by the addition of specific αMSH antibodies without altering the constitutive part of melanogenesis. Human and mouse agouti proteins, which inhibit basal and αMSH-induced melanogenesis in B16-F1 cells, both reduced αMSH-induced melanin production in G4F-15 cells but did not affect the constitutive melanogenesis. These results indicate that human MC1 receptor expressed in mouse B16-G4F cells induces constitutive activation of the signalling pathway controlling melanogenesis, most likely by tightly coupling to Gαi, in a similar manner to that reported for constitutively active receptor mutants in other systems.

Key words: α Melanocyte stimulating hormone (αMSH), Melanoma, Melanin, Receptor, Transfection, Constitutive activation, Agouti

INTRODUCTION

Melanogenesis in mammalian melanocytes and melanoma cells is stimulated in vitro and in vivo by α melanocyte stimulating hormone (αMSH) which binds to the melanocortin-1 (MC1) receptor and induces activation of tyrosinase through stimulation of adenylate cyclase and protein kinase A (Eberle, 1988a; Cone et al., 1993). In humans, injection of αMSH was shown to result in a marked increase in skin pigmentation (Lerner, 1993) but the implication of αMSH for normal skin or hair colour and its variations between different skin areas or during UV-induced skin tanning is not yet clear. In vitro, αMSH induces melanin formation in human melanocytes (Hunt et al., 1994a,b) and also affects their proliferation (De Luca et al., 1993). Human melanoma cells, however, frequently do not respond to αMSH, neither with altered melanogenesis nor growth (Halaban, 1993), although they express receptors for αMSH (Siegrist et al., 1989). In some of these cell lines, αMSH induces MC1 receptor up-regulation (Siegrist et al., 1994). On the other hand, in mouse B16-F1 melanoma cells, αMSH leads to a massive increase in melanin production and concomitantly to MC1 receptor down-regulation and inhibition of cell growth (Siegrist et al., 1996a).
ferences in the biological response of mouse and human melanoma cells following MC1 receptor stimulation by αMSH. To address these questions, mutagenized mouse or human MC1 receptors can be studied in cellular expression systems such as COS or HEK-293 cells, which have become indispensable tools to analyze receptor binding and cAMP formation by αMSH and its synthetic analogues (Chhajlani and Wikberg, 1992; Robbins et al., 1993; Hruby et al., 1995). However, in view of the complex signalling of MC1 receptors in melanocytes and melanoma cells, the expression of MC1 receptors in their original environment may provide more reliable results since the studies of receptor function and coupling can be extended to the analysis of the final response of the cells, i.e. melanogenesis. In addition, the rate of receptor expression may also be regulated differently in the cells of origin of the receptor as compared to the commonly used cellular expression systems, as e.g. shown with transfection studies of the serotonin 5-HT1A receptor in neuronal and non-neuronal cell lines (Banerjee et al., 1993).

These considerations initiated experiments in which the gene of the human MC1 receptor was transfected into B16-G4F cells (Chluba-de Tapia et al., 1995), an amelanotic B16 mouse melanoma line which had previously been shown to be deficient of MC1 receptors, both at the protein and mRNA level (Solca et al., 1993), and hence to be unresponsive to αMSH. We have now analyzed the characteristics of four of the stably transfected clones with respect to αMSH binding, cAMP formation and melanin production and also with respect to interaction with agouti protein, an MC1 receptor antagonist and inhibitor of cAMP production and melanin formation in murine melanoma cells (Lu et al., 1994; Willard et al., 1995; Siegrist et al., 1996a).

MATERIALS AND METHODS

Cell culture and transfection

B16-G4F mouse melanoma cells (Solca et al., 1993) and B16-F1 cells (Fidler, 1973), were grown in MEM with Earle’s salts (Gibco, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum, 2 mm L-glutamine, 1% MEM non-essential amino acids (×100; Gibco), 1.5% MEM vitamin solution (×100; Gibco) and ciprofloxin (0.005%; Gibco) or penicillin (50 units/ml) and streptomycin (50 μg/ml; Gibco). The cells were free of mycoplasma contamination. A full-length coding fragment of the pE-11D clone, originally constructed by Chhajlani and Wikberg (1992), was subcloned into the HindIII site of the expression vector pRC/CMV (Invitrogen, San Diego, CA). The construct was then transfected into B16-G4F cells by a modified calcium phosphate procedure (Davis et al., 1986) and populations of stably transfected cells were selected by growth in 1.5 μg/ml of the neomycin analogue G418 (Gibco). After six weeks of selection, the cells were subcloned and total mRNA was isolated from the different cell clones using the guanidium thiocyanate method (Chirgwin et al., 1979). mRNA (10 μg) was subjected to electrophoresis through a 1% formaldehyde agarose gel and blotted onto Zetabind (Cuno Inc. Meriden, CT). Hybridization with the randomly primed 32P-labelled insert of pE-11D was carried out at 65°C for 16 hours in 0.5 M Na-phosphate, pH 7.2, containing 7% SDS, 1% BSA and 10 mM EDTA. The membrane was then washed in 2× SSC buffer containing 0.1% SDS for 30 minutes (SSC: 150 mM NaCl + 15 mM Na-citrate) at 65°C, followed by a 30-minute wash in 0.1x SSC containing 0.1% SDS, again at 65°C. Finally, the dry membrane was exposed to X-ray film for 12 days.

Peptides, reagents and radioligand

αMSH was kindly supplied by Ciba-Geigy AG (Basel, Switzerland). [Nle5, D-Phe6]-αMSH was obtained from Bachem AG (Bubendorf, Switzerland). Mouse and human agouti proteins were kindly provided by Glaxo Inc. (Research Triangle Park, NC). Forskolin and cholera toxin (CT) were purchased from Calbiochem (San Diego, CA). Polyclonal antibodies to αMSH were raised in mini-guinea pigs, resulting in antiserum of very high titer (>1×109), high affinity and specificity; details for antiserum 118/36 are found in Eberle (1988b). Bovine serum albumin (BSA) and all other reagents were obtained from Sigma (St Louis, MO) or Fluka (Buchs, Switzerland).

[Nle5, D-Phe6]-αMSH was labelled with Na125I (NEN/DuPont, Wilmington, DE) using Enzymobeads (Bio-Rad, Richmond, CA) as described previously (Eberle et al., 1991). The iodination product was incubated in the presence of 0.1 M dithiothreitol for 12 hours at room temperature and then purified in a 1 ml syringe packed with 0.3 cm3 Spherosorb ODS/10 μm RP-silica (Phase Separations Inc., Norwalk, CT). The peptide was freshly iodinated every 4 weeks and additionally purified by reverse-phase HPLC before each experiment.

Binding assay and bioassays

For competition binding assay, cell suspensions of transfected and non-transfected B16-G4F cells were plated at a density of 107 cells/ml in modified Eagle’s medium with Earle’s salts (Gibco) containing 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 0.2% bovine serum albumin and 1 mM 1,10-phenanthroline (Merck, Darmstadt, FRG). Radioligand binding was initiated by adding 50 μl (approx. 200,000 cpm) of [125I]Ty2, Nle5, D-Phe6]-αMSH to Eppendorf tubes containing 50 μl of [Nle5, D-Phe6]-αMSH at serial dilutions and 500 μl of cell suspension. The cells were incubated at 37°C for 2 hours as described for human melanoma cells (Siegrist et al., 1989). Bound ligand was separated from free radioligand by centrifugation through a silicon oil layer as described by Siegrist et al. (1988). The radioactivity was counted in a Packard γ-counter. The competition binding data were analyzed with a recent Macintosh version of the Ligand program (Munson and Rodbard, 1980) for receptor numbers and affinities. It has previously been demonstrated by Siegrist et al. (1989) that MC1 receptor numbers on human melanoma cells and dissociation constants calculated from competition binding data correspond very well with those obtained from saturation binding experiments.

For cAMP assay, the cells were grown in 24-well Costar tissue culture plates at a density of 400,000 cells per well. After 24 hours, the medium was replaced by MEM containing 25 mM Hepes, 0.2% BSA, and either 100 nM αMSH or 10 μM forskolin. After 30 minutes of incubation at 37°C (in the absence of phosphodiesterase inhibitors), the cells were detached with 0.3 ml trypsin (0.2% trypsin in Ca2+- and Mg2+-free 50 mM phosphate-buffered saline, pH 7.2; PBS), counted again and frozen at −70°C. Extraction of cAMP was performed at 4°C: the cells were thawed, incubated with 0.2 ml of cold 5% HClO4 for 30 minutes and centrifuged. To 500 μl of supernatant, 62 μl 4.2 M KOH were added and the KClO4 precipitate was removed by centrifugation. The supernatant was acetylated and cAMP determined by radioimmunoassay using the kit from IBL GmbH (Hamburg, FRG).

For melanin assay with transfected and non-transfected B16-G4F cells, 2,500 cells per well were seeded in 96-well tissue culture plates at a density of 400,000 cells per well. After 24 hours, the medium was replaced by MEM containing 25 mM Hepes, 0.2% BSA, and either 100 nM αMSH or 10 μM forskolin. After 30 minutes of incubation at 37°C (in the absence of phosphodiesterase inhibitors), the cells were detached with 0.3 ml trypsin (0.2% trypsin in Ca2+- and Mg2+-free 50 mM phosphate-buffered saline, pH 7.2; PBS), counted again and frozen at −70°C. Extraction of cAMP was performed at 4°C: the cells were thawed, incubated with 0.2 ml of cold 5% HClO4 for 30 minutes and centrifuged. To 500 μl of supernatant, 62 μl 4.2 M KOH were added and the KClO4 precipitate was removed by centrifugation. The supernatant was acetylated and cAMP determined by radioimmunoassay using the kit from IBL GmbH (Hamburg, FRG).

For melanin assay with transfected and non-transfected B16-G4F cells, 2,500 cells per well were seeded in 96-well tissue culture plates in the presence of 0.3 mM L-tyrosine, as described for B16-F1 cells by Siegrist and Eberle (1986). The cells were incubated with medium alone or with αMSH, cholera toxin, forskolin, or combinations of αMSH and mouse or human agouti protein or αMSH and αMSH antibodies. Since the onset of melanogenesis in B16-G4F cells and its transfectants was slower than in B16-F1 cells, the assay period had to be extended to 8 days (in some experiments, melanogenesis was followed for up to 10 days). In general, absorbance at 405 nm was determined in a microplate reader after 4, 7 and 8 days of incubation. It was noted that readdition of hormone or other stimulants after 3 or 4 days did not significantly affect the final result. All experiments were
performed at least twice or three times, each yielding sextuplicate values. The data represent the means of all values of the different experiments, except for the studies of the time-course where one representative experiment is shown because of small variations between individual experiments during the initial phase of onset of melanogenesis.

**Plasma membrane preparation and immunoblotting**

Plasma membranes from B16-F1 and the different B16-G4F clones were prepared according to the procedure developed by Solca et al. (1989). Briefly, the cells were washed in PBS and 10 mM Tris-HCl, pH 7.4, before incubating them for 15 minutes in 5 mM Tris-HCl, pH 7.4, containing 1 mM 1,10-phenanthroline, followed by gentle disruption in a dounce homogenizer. The membranes were isolated by three consecutive centrifugation steps at 4°C, first at 8000 g for 25 minutes, then at 10,000 g for 30 minutes (Sorvall SS-34 rotor), and finally at 100,000 g for 60 minutes (Beckman L7, SW 50.1 rotor). The resulting plasma membrane pellet was suspended in 50 mM Tris-HCl, pH 7.4, containing 1.4 mM CaCl₂, 9.8 mM MgCl₂, 109 mM NaCl, 5.4 mM KCl and 0.3 mM 1,10-phenanthroline. The protein concentration was determined according to the method of Lowry et al. (1951).

For sodium dodecyl sulphate gel electrophoresis (SDS-PAGE), 20 μg of crude membranes were loaded onto a 10% SDS gel and run in parallel with prestained molecular mass markers (Sigma). The protein bands were then transferred to nitrocellulose (Bio-Rad) in a Bio-Rad Mini Trans Blot apparatus at 70 V for 3 hours using 190 mM glycine, 25 mM Tris-HCl and 20% methanol as transfer buffer. Blocking of the non-specific binding sites, probing with rabbit anti-Gs antibody (RM/1; NEN, Boston, MA), sheep-anti-rabbit antibody and detection was performed by the Western-Light™ Chemiluminescent Detection System (Tropix Inc., Bedford, MA). The blot was subsequently exposed to Kodak T-MAT G film (Kodak, Rochester, NY).

**RESULTS**

Transfection of B16-G4F cells with the expression vector pRc/CMV containing the MC1-DNA insert, followed by subcloning and screening of the mRNA of the individual clones by northern hybridization with the human MC1 receptor probe produced 17 stable cell clones all of which expressed the human MC1 receptor (Chluba-de Tapia et al., 1995). Microscopic inspection showed that most of these clones produced melanin. We have now determined the morphology as well as the binding and signalling characteristics of those four clones yielding the strongest hybridization signal, namely G4F-12, 14, and 15 (melanotic cell clones) and G4F-7 (an amelanotic clone). Clone G4F-6, a stable clone transfected with pRc/CMV vector without MC1 insert and hence only expressing the neomycin resistance gene, served as negative control.

**Cell morphology and melanin production**

The stable transfectants G4F-7, 12, 14 and 15 differed from the parental cell, B16-G4F, as well as from the control clone, G4F-6, with respect to their morphology and their capacity to produce melanin (Fig. 1). Confluent cultures of both B16-G4F and G4F-6 cells appeared as an homogeneous population of mostly bipolar cells which did not produce or secrete melanin. G4F-7 cells had a similar shape but slightly increased size as compared to B16-G4F cells although they did not synthesize melanin either. Confluent cultures of G4F-12 and 14 cells were heterologous populations with large melanin-containing cells and small cells presenting a shape similar to B16-G4F cells. In these two cell lines, melanin production was clearly accompanied by cell hypertrophy; the melanin was stored in the cells and not released into the medium. By contrast, G4F-15 cells were not hypertrophic after confluency but formed cell aggregates and released massive amounts of melanin into the medium which is typical for B16-F1 cells stimulated by αMSH for three to four days (see Eberle, 1988c). Therefore, G4F-15 cells could be used for the microplate-based melanin assay previously developed for B16-F1 cells (Siegrist and Eberle, 1986), which allows easy recording of time-response curves. It should be noted, however, that the onset of melanin formation in G4F-12, 14 and 15 cells as well as the onset of melanin secretion by G4F-15 cells was delayed by 2-3 days as compared to B16-F1 cells, necessitating a prolonged assay period.

In order to determine whether melanin synthesis in the transfectants is influenced by αMSH or other known stimulators of melanogenesis, we compared the melanin production of G4F-15 cells with that of B16-G4F cells in the absence and presence of 1 μM αMSH, 1 nM CT, or 1 μM forskolin. The time-response curves shown in Fig. 2 demonstrate that in G4F-15 cells αMSH and CT led to an increased melanin production as compared to non-stimulated controls. At day 7 when the cell viability in all cultures was still virtually 100%, the melanin content in αMSH- and CT-stimulated cells was 24% and, respectively, 46% above

![Fig. 1. Light microscopy of a confluent culture of control cells B16-G4F (A), and G4F-6 (B) and of stable transfectants G4F7 (C), G4F-12 (D), G4F-14 (E) and G4F-15 (F) expressing the human MC1 receptor. The cells were kept in normal medium without addition of hormone for 5 days under usual culture conditions. The cells were stained with Shandon. Each of the six photographs depicts an area of 0.41 mm ×0.33 mm of the culture plate.](image-url)
controls. Forskolin slightly delayed the onset of melanogenesis due to a reduced cell growth and by day 7, the melanin content of the culture medium did not differ from that of controls; however, by day 10, it was significantly increased. Higher concentrations of forskolin (3 and 10 mM) completely blocked cell proliferation and melanin synthesis (not shown), paralleling the finding with αMSH-stimulated B16-F1 cells where 3 mM forskolin antagonizes melanogenesis (Siegrist et al., 1996b). B16-G4F cells could not be stimulated with αMSH; forskolin induced a clear but delayed melanin production and CT had only a marginal effect (Fig. 2). G4F-6 and G4F-7 cells did not produce melanin in the presence of any of these stimulators (not shown).

### MC1 receptor binding

The binding characteristics of G4F-7, 12, 14 and 15 cells in a cellular competition binding assay using [(125)I]Tyr2, Nle4, D-Phe7-αMSH as radioligand and [Nle4, D-Phe7]-αMSH as displacer are shown in Fig. 3. All four curves were parallel and the dissociation constants (K_D) varied from 0.19 nM for G4F-7 to 0.71 nM for G4F-12 (Table 1). These data, except for clone G4F-12, correspond to the dissociation constants previously reported for the human MC1 receptor on different human melanoma cells (Siegrist et al., 1989). The number of receptors found on the transfectant cell lines, which ranged between 7,000 and 27,000 per cell (Table 1), was generally higher than that determined in most human melanoma cells, except for HBL cells (Siegrist et al., 1994). Preliminary binding experiments done at different temperatures showed that the binding kinetics for transfected G4F cells are optimal at 37°C (unpublished results) and thus correspond with those for human melanoma cells (Siegrist et al., 1989), but differ from the findings with mouse B16-F1 cells (Siegrist et al., 1988).

### cAMP

In order to study whether human MC1 receptors expressed in B16-G4F cells are functionally coupled to adenylate cyclase, we compared the basal, αMSH-stimulated and forskolin-stimulated intracellular cAMP contents of G4F-7, 14 and 15 cells; B16-G4F and G4F-6 cells served as controls. Table 2 shows that the basal cAMP content of the clones expressing the MC1 receptor ranged between 11.4 and 17.2 pmol/10⁶ cells whereas for control cells the values ranged from 3.9 to 4.5 pmol/10⁶ cells. Whereas G4F-6 cells could not be stimulated by αMSH, the cAMP content of G4F-7, 14 and 15 cells rose to 27.9, 29.6 and 17.3 pmol/10⁶ cells after stimulation by hormone, corresponding to a 1.5- to 1.7-fold increase in cAMP. Forskolin-stimulated production of cAMP, which served as control for maximal activity of the adenylate cyclase in controls and in transfected cells, reached values of 2,593 to 5,377 pmol/10⁶ cells and was comparable to that observed with B16-F1 cells, indicating that the adenylate cyclase was fully functional. It should be noted that all these experiments were done in the absence of phosphodiesterase inhibitors as the hydrolysis of cAMP apparently is slower in melanoma cells as compared to other cell types (unpublished observation).

### G_s subunit

Since MC1 receptors are coupled to adenylate cyclase via G_s protein, we next analyzed the presence of the G_s subunit in G4F-7 and 15, as compared to B16-G4F cells; B16-F1 cells

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### Table 1. Dissociation constants (K_D) and number of MC1 receptors on transfected G4F cells clones

<table>
<thead>
<tr>
<th>Cell clone</th>
<th>K_D (nM/l)</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4F-7</td>
<td>0.19±0.05*</td>
<td>7,428±800*</td>
</tr>
<tr>
<td>G4F-12</td>
<td>0.71±0.01</td>
<td>27,506±1,481</td>
</tr>
<tr>
<td>G4F-14</td>
<td>0.29±0.02</td>
<td>7,256±1,043</td>
</tr>
<tr>
<td>G4F-15</td>
<td>0.22±0.01</td>
<td>20,298±348</td>
</tr>
</tbody>
</table>

*The values are the means of three independent experiments ± s.e.m.

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**Fig. 2.** Time-response curves of melanin production in G4F-15 cells (filled symbols) and B16-G4F cells (open symbols) exposed to 10⁻⁶ M αMSH (●, ○), 10⁻⁹ M CT (■, □), or 10⁻⁵ M forskolin (●, ○) or without stimulants (▲, △). The data represent the means of sextuplicate values from one representative experiment; s.e.m. values ranged from ±5 to ±8%. This experiment was repeated twice with very similar results.

**Fig. 3.** Competition binding assays with G4F-7 (▲), G4F-12 (●), G4F-14 (△) and G4F-15 (○) cells using [(125)I]Tyr2, Nle4, D-Phe7]-αMSH as radioligand. The cells were incubated at 37°C for 2 hours in the presence of a fixed amount of radioligand and of increasing concentrations of non-radioactive [Nle4, D-Phe7]-αMSH as competitor, as described in Materials and Methods. The data represent the mean of 3× triplicate values from three independent experiments; s.e.m. values ranged from ±5 to ±10%.
were included as additional control. The content of Gα was identified by quantitative western blot analysis of SDS-PAGE-separated membrane proteins using one of the most specific anti-Gα antibodies currently available. All four cell lines expressed Gα which yielded a distinct band at 45 kDa (Fig. 4). In addition, the four cell lines expressed a fainter band at 43 kDa which most likely corresponds to Golf (Robishaw et al., 1986; Yagami, 1995) because of cross-reaction of the antiserum with the short and long form of Gα (Mumby et al., 1986). It seems therefore that B16-G4F, G4F-7 and G4F-12 cells only express the short form of Gα, with no difference in the amount.

Effect of agouti and αMSH antibodies

We have previously demonstrated that agouti protein blocks basal and αMSH-induced melanogenesis in B16-F1 cells (Siegrist et al., 1996a). Similarly, αMSH in the incubation medium can be neutralized by αMSH antibodies (Eberle, 1988b) which also leads to suppression of melanogenesis. Therefore, it was of interest to test whether mouse or human agouti protein inhibits the constitutive stimulation of MC1 receptor signalling. As shown in Fig. 5A, mouse agouti protein did not have any effect on the basal melanogenesis of G4F-15 cells. However, it blocked the melanogenic effect of added αMSH partially but not completely. Similarly, human agouti protein did not alter basal melanin production and exhibited about the same partial blockage of melanogenesis as mouse agouti protein (not shown). The observation that agouti protein did not completely block αMSH-induced melanogenesis may have been caused by partial breakdown of agouti during the prolonged incubation conditions required for the melanin assay with G4F-15 cells. The conclusion that the constitutive activation of G4F-15 cells was not caused by endogenous αMSH present in the incubation medium could be confirmed by the addition of αMSH antiserum 118/36 which inhibited the

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**Table 2. Intracellular cAMP content in transfected and control cells before and after stimulation**

<table>
<thead>
<tr>
<th>Cell clone</th>
<th>No stimulation</th>
<th>αMSH stimulation</th>
<th>Forskolin stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16-G4F</td>
<td>4.54±0.59</td>
<td>4.64±1.09</td>
<td>2929±342</td>
</tr>
<tr>
<td>G4F-6</td>
<td>3.87±0.58</td>
<td>4.88±0.60</td>
<td>3132±42</td>
</tr>
<tr>
<td>G4F-7</td>
<td>16.07±3.37*</td>
<td>27.91±7.31†</td>
<td>5377±963</td>
</tr>
<tr>
<td>G4F-14</td>
<td>17.24±3.04‡</td>
<td>29.61±5.30§</td>
<td>4307±943</td>
</tr>
<tr>
<td>G4F-15</td>
<td>11.35±0.09¶</td>
<td>17.34±4.86</td>
<td></td>
</tr>
</tbody>
</table>

Cells were grown in 24-well Costar plates at a density of 400,000 cells/well. After stimulation with either αMSH (5×10^{-5} M) or forskolin (5×10^{-5} M) for 30 minutes at 37°C, the cells were detached, counted and intracellular cAMP was extracted and measured by radioimmunoassay. The results are the means of triplicate determinations ± s.e.m. of three experiments. *P=0.023; †P=0.011; ¶P=0.009; §P=0.031; ||P=0.008; |||P=0.036.
DISCUSSION

Our results show that the human MC1 receptor, expressed in MC1 receptor-deficient B16-G4F mouse melanoma cells, displays binding characteristics for [Nle⁴, D-Phe⁷]- αMSH which are very similar to those previously reported for different human melanoma cell lines (Siegrist et al., 1989). These include optimal binding kinetics at 37°C and dissociation constants in the subnanomolar range. In contrast, optimal binding to the mouse MC1 receptor in B16-F1 cells was found at 15°C and the binding affinities were generally lower than in human cells (Eberle et al., 1993). The number of MC1 receptors found in G4F-7 and 14 cells was approximately the same as that found in human HBL melanoma cells (Siegrist et al., 1994), one of the few αMSH-responsive human melanoma cell lines producing melanin in vitro (Ghanem et al., 1988). G4F-15 cells and also G4F-12 cells expressed >20,000 receptors per cell, i.e. 3-fold more than HBL cells and about 10- to 20-fold more than most cultured human melanoma cells studied to date (Siegrist et al., 1989). However, the higher number of MC1 receptors in G4F-15 cells did not result in a higher basal cAMP content when compared with G4F-7 and 14 cells. The same is true for human melanoma cells where no correlation between receptor number and basal cAMP content could be observed (Siegrist et al., 1994). A comparison of the binding data determined for MC1 receptors in cultured human melanocytes with those obtained in this study shows that both receptor numbers and dissociation constants are considerably lower in human melanocytes than in transfected mouse cells (Donatien et al., 1992; De Luca et al., 1993). Since it is likely that human melanocytes embedded in their natural environment of the skin express a higher number of MC1 receptors than when kept in culture, transfected human MC1 receptors may represent a useful model to analyze molecular details of melanogenesis.

All three transfectant cell lines expressing human MC1 receptor had a 3- to 4-fold higher basal level of cAMP content than the controls without receptor. This indicates that the presence of 7,000 or more human MC1 receptors per cell are sufficient to induce a marked activation of adenylate cyclase in the absence of ligand, leading to an almost maximal response in melanogenesis. In twelve different human melanoma cell lines studied previously (Siegrist et al., 1994), the basal level of intracellular cAMP ranged between 0.76 and 4.43 pmol/10⁶ cells, the latter corresponding to the values found for B16-G4F and G4F-6 control cells in this study. The addition of αMSH peptide to G4F-7, 14 and 15 cells raised the intracellular cAMP content by a factor of 1.5- to 1.7-fold above basal levels. A similar relative increase in cAMP of αMSH-stimulated cells was also found in most human melanoma cells (1.0- to 1.5-fold), except for HBL cells which showed a 5.5-fold increase after αMSH stimulation (Siegrist et al., 1994).

Considering the 3- to 4-fold higher basal cAMP levels of the G4F transfectant cell lines, their final response to stimulation with the ligand reached about the same plateau as that of αMSH-stimulated HBL cells. This indicates that the ability of human MC1 receptors to induce cAMP production in the mouse G4F cell line resembles the situation in human melanoma cells, except that in mouse cells non-ligated human receptors induce cAMP formation per se.

The appearance under the microscope of the melanotic G4F-12, 14 and 15 cells, whether stimulated by [Nle⁴, D-Phe⁷]- αMSH or not stimulated, was about the same as that of B16-F1 cells fully stimulated by [Nle⁴, D-Phe⁷]-αMSH or αMSH, and there was no difference between B16-F1 and G4F-15 cells in the extent of melanogenesis in the melanin assay. Also the amelanotic G4F-7 cells, whose defect to melanize is localized after the cAMP response, show the same increase in cell size. On the other hand, there are marked differences between B16-F1 cells and the transfectant clones: firstly, the cAMP content of B16-F1 cells rises much higher above basal levels after maximal stimulation by αMSH (Siegrist and Eberle, 1993) and secondly, the western blot of the Gαα subunit of the G4F clones only displayed the smaller of the two Gαα bands, indicating that G4F-derived cells do not express the long form of Gαα. This assumption, however, must be verified at the molecular level, e.g. by northern analysis for Gαα-L and Gαα-S using specific probes. A detailed analysis of the different G protein subunits (for review see Birnbaumer and Birnbaumer, 1995) and/or their putative regulation by other membrane proteins (Sato et al., 1995) as well as a study of phosphodiesterase activities in the different cell lines may also help to explain the differential responses of B16-G4F and G4F-15 cells to forskolin and CT: while forskolin induced about the same maximal cAMP production in both cell lines, its melanogenic response was maximal in G4F-15 cells but markedly reduced in B16-G4F cells. CT induced only marginal melanogenesis in B16-G4F cells but a full response in G4F-15 cells. It could be argued that spontaneous mutations, e.g. in G protein subunits, may have occurred, explaining some of the effects observed. However, upon prolonged cultivation of the transfectant cell lines over many passages, spontaneous loss of expression of the MC1 receptor did occasionally occur, resulting in characteristics of the cells that were indistinguishable from B16-G4F cells.

The finding that specific αMSH antibodies block the αMSH-induced melanogenesis in G4F-15 cells but not the constitutive part, proves that the basal activation of MC1-transfected G4F cells did not originate from endogenous αMSH acting in an autocrine manner. This is an important control as it has been reported that melanoma cells produce and secrete αMSH-like peptides which may induce autostimulation of the cells (Lunec et al., 1990; Ghanem et al., 1992). Similarly, mouse or human agouti protein did not affect the constitutive activation of the transfectant cell clones but antagonized the αMSH-induced melanogenesis. It has been shown by Hunt and Thody (1995) and Siegrist et al. (1996a) that in B16-F1 melanoma cells agouti protein not only inhibits the binding of αMSH to MC1 receptors but also reduces the basal MC1 receptor activity. Therefore, the finding that the interaction of the human MC1 receptor with the agonist (αMSH) or antagonist (agouti) is not altered by expression in a mouse cell, is of particular interest because it demonstrates that the ligand binding site of the MC1 receptor is not involved in evoking the constitutive activation. This is in contrast to the previously reported constitutive activation of the mouse MC1 receptor associated with the E¹⁰⁻³¹ sombre allele which causes black coat colour but is not responsive to αMSH (Robbins et al., 1993). This MC1 receptor
variant contains a mutation in the second transmembrane domain (Glu92 to Lys) disrupting the salt bridge between Glu92 and Lys182/His183 which is thought to keep the MC1 receptor in an inactive conformation, accessible to ligand binding (Robbins et al., 1993).

Constitutive activity of G protein-coupled receptors is a well established phenomenon in cells, isolated membranes and reconstituted systems (Costa et al., 1992) and has also been associated with various pathological conditions of man (for review see Birnbaumer, 1995). Studies with mutated forms of α1B, α2A and β2 adrenergic receptors containing mutations in the C terminus of the third intracellular loop (Lefkowitz et al., 1993), known to interact with Gq subunits (see Birnbaumer, 1995), have demonstrated that modifications within this part of a G protein-coupled receptor may markedly alter the coupling to Gα. The finding that the sequence of the mouse and human MC1 receptor differs considerably in this area (Cone et al., 1993) may explain the altered coupling between human MC1 and mouse Gα. Although the assumption that the constitutive activity of the transfected B16-G4F clones is caused by tight MC1-Gα coupling must be confirmed at the molecular level, our findings may initiate pathophysiological studies of MC1 receptor-G protein coupling, e.g. in those cases of hyperpigmentary disorders where melanocortin hormone levels have been shown to be normal and no other responsible endocrine factor for increased pigmentation could be identified (A. N. Eberle, unpublished). Conversely, diminished or missing coupling between MC1 and Gα may well be the reason for certain forms of hypopigmentary disorders and also for the lack of response to αMSH of cultured melanoma cells.

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