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B16-G4F mouse melanoma cells: an MSH receptor-deficient cell clone

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The two mouse melanoma cell lines B16-F1 and B16-G4F retain their melanogenic capacity when cultured in vitro. Melanotropic peptides such as α-melanocyte-stimulating hormone (α-MSH) induce formation and release of melanin pigment in B16-F1 cells. In contrast, B16-G4F cells do not respond to α-MSH. Using receptor-binding analysis and photoaffinity crosslinking we demonstrate that the lack of response of B16-G4F cells to α-MSH is due to the absence of functional MSH receptors from the cell surface. Northern blot analysis of receptor mRNA revealed that MSH receptor mRNA is not expressed in B16-G4F cells. These cells represent a new tool for the study of signal pathways related to the control of melanogenesis in melanoma cells.

B16-F1; B16-G4F mouse melanoma cell; Melanocyte stimulating hormone; Receptor expression

1. INTRODUCTION

Melanin pigments are produced in subcellular organelles, the melanosomes, which are distributed throughout the cytoplasm of melanocytes, and serve important physiological functions [1,2]. Intracellular melanin levels undergo alterations in response to various physiological stimuli [3]. However, the precise molecular mechanisms involved in skin pigmentation are still poorly understood. α-MSH is a well known stimulator of melanogenesis in mammalian melanocytes and melanoma cells and acts via the cAMP pathway on tyrosinase (EC 1.14.18.1) [4]. A specific membrane receptor for α-MSH has been characterized by structure–activity and binding studies [2] and by use of photoaffinity labelling techniques [5,6,7]. Recently the cDNAs encoding the human and mouse MSH receptor genes have been cloned and sequenced [8, 9]. This report presents evidence for the identification of a B16 murine melanoma subclone lacking MSH receptors.

2. MATERIALS AND METHODS

2.1. *Chemicals*

α-MSH was a gift of Ciba-Geigy AG (Basel, Switzerland). The preparation of the radioligands ([^{125}I]Tyr^2,Nle^4,D-Phe^7)α-MSH and of photoreactive ([^{125}I]Tyr^2,Nle^4,D-Phe^7,Trp(Naps)^9)α-MSH ([^{125}I]Naps-MSH) was performed by the chloramine-T method as previously described [6]. All chemicals and solvents were of the highest purity available.

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2.2. *Tissue culture*

The B16-F1 and B16-G4F cell lines were maintained at 37°C in a humidified 95% air/5% CO_2 atmosphere using modified Eagle's medium (MEM) with Earle's salts (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Amimed, Basel, Switzerland), 2 mM L-glutamine (Gibco), 1% MEM non-essential amino acid solution (Gibco), 1% MEM vitamine solution (Gibco), 50 U/ml of penicillin and 50 μg/ml of streptomycin.

2.3. *Bioassays*

The melanogenic response was determined with the in situ melanin assay as described previously [10].

2.4. *Photoaffinity labeling*

500 μl of cell suspension (10^7 cells/ml) were incubated at 25°C together with 100 μl of a 2 nM [^{125}I]Naps-MSH solution in the presence or absence of a 3000-fold excess of α-MSH. After 90 min, the samples were UV-irradiated on ice for 5 min, using the whole 310–550 nm spectrum of a 1 kW Oriel mercury–xenon UV-irradiation apparatus (irradiation intensity: 180 mW/cm^2). Irradiated cells were extensively washed with cold 0.2% EDTA in PBS, pH 7.4. They were then lysed by resuspension in 2 mM Tris-HCl, pH 7.4, containing 0.3 mM 1,10-phenanthroline (15 min on ice) followed by homogenization. The pellets were resuspended in SDS-PAGE sample buffer and analyzed on gels as described previously [6].

2.5. *Competition binding assays*

The cell suspension was prepared at a density of 10^7 cells/ml in modified Eagle's medium with Earle's salts (Gibco) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.2% bovine serum albumine and 1 mM 1,10-phenanthroline (Merck, Darmstadt, Germany). Binding was initiated by adding 50 μl of ([^{125}I]Tyr^2,Nle^4,D-Phe^7]α-MSH to Eppendorf tubes containing 50 μl of [Nle^4,D-Phe^7]α-MSH peptide dilutions and 500 μl of cell suspension. For equilibrium binding the samples were incubated at 15°C for 3 h. Unbound radioactivity was removed by centrifugation through silicon oil. The binding parameters were determined by Ligand, an iterative non-linear regression analysis program [11].

2.6. *Plasma membranes from B16 cells*

Subconfluent monolayers were harvested with EDTA-PBS and

washed twice with PBS. Lysis of the cells was performed in 5 mM Tris-HCl, pH 7.4, containing 1 mM 1,10-phenanthroline. The homogenate was centrifuged at 10,000 × *g* for 30 min. The resulting supernatant was adjusted to 10 mM $MgCl_2$ and further centrifuged at 100,000 × *g* for 1 h at 4°C. The membranes were resuspended in 25 mM Tris-HCl, pH 7.4, and the protein concentration was determined according to Lowry [12].

2.7. *Adenylate cyclase activity assay*

Adenylate cyclase activity in B16-F1 and B16-G4F plasma membranes was determined using [α-^{32}P]ATP as substrate. The reaction mixture (100 μl final volume) contained 25 mM Tris-HCl, pH 7.5, 1 mM cAMP, 10 mM creatine phosphate, 0.25 μg/μl creatine kinase, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 mM [α-^{32}P]ATP (1–2 × 10^6 cpm/assay), 50 μM ATP, 50 μM GTP, 5 mM IBMX together with one of the adenylate cyclase agonists. Assays were initiated with the addition of 60 μg plasma membranes and were incubated at 37°C for 15 min. Termination of the assay was achieved by the addition of 200 μl of 0.5 N HCl. The samples were immersed in boiling water for 7 min, neutralized with 200 μl of 1.5 M imidazole and cAMP was quantified by the method of White [13].

2.8. *Polymerase chain reaction*

The oligonucleotide primers were designed according to the published mouse MSH receptor cDNA sequence [9].The primer sequence at the 5′ end was 5′-ACAAGACTATGTCCACTC-3′ and at the 3′ end 5′-CAGGAAGGGATGAGTACC-3′. PCR was performed according to Saiki et al. [14]. Briefly, 1 μg of mouse genomic DNA was subjected to amplification in a 50 μl reaction mixture containing 10 mM Tris-HCl, pH 9, 50 mM KCl, 0.01% gelatin and 1.5 mM $MgCl_2$. Sense and antisense primers were added at a concentration of 1 μM, followed by 200 μM of each dATP, dCTP, dTTP and dGTP (Pharmacia) with 0.25 U of Taq Polymerase. The PCR profile used was 94°C for 60 s, 45°C for 40 s and 72°C for 60 s, using a thermal cycler (Perkin Elmer Cetus, USA). The PCR product was gel purified and subcloned in pBluescript SK− (Stratagene). The resulting plasmid was sequenced using the chain termination method [15] in order to confirm the DNA sequence.

2.9. *Northern analysis of mRNA*

Total mRNA was isolated using the guanidium thiocyanate method [16]. 10 μg of total RNA was subjected to electrophoresis through a 1% formaldehyde agarose gel. The RNA was blotted on Hybond (Amersham). The hybridization was done for 16 h at 65°C in 0.5 M NaH_2PO_4, pH 7.2, containing 7% SDS, 1% BSA, 10 mM EDTA and the ^{32}P-labelled MSH receptor probe. The membrane was then washed for 30 min in 2 × SSC and 0.1% SDS followed by 30 min in 0.1 × SSC and 0.1% SDS. The membrane was exposed to a autoradiographic film for 20 h at −70°C.

3. RESULTS AND DISCUSSION

Terminal differentiation of B16-F1 cells in culture can be monitored by the production of melanin pigments [2,10]. While melanogenesis in the B16-F1 clone was stimulated by the addition of various concentrations of α-MSH, the results displayed in Fig. 1 show clearly that the hormone did not induce melanin production and release in the B16-G4F clone. This finding is in accordance with our previous results [17,18] which showed that α-MSH and ACTH did not stimulate tyrosinase activity during the logarithmic growth phase of B16-G4F cells, indicating a possible impairment in the signal transduction pathway of these peptides. We suggested that the defect is located before the activation of protein kinase A since 2 mM theophylline could elicit

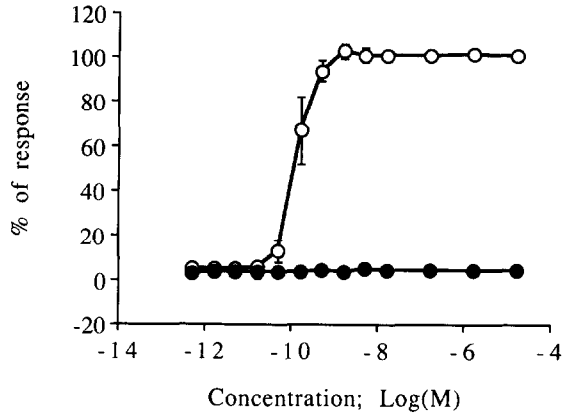

Fig. 1. Dose–response curves of B16 melanoma cell clones to α-MSH. The total melanin content of B16-F1 (○) and B16-G4F (●) cells was determined after 72 h of stimulation with serial dilutions of α-MSH in three independent experiments.

a rise in tyrosinase activity. In order to identify the putative defect(s) in the MSH signal transduction machinery we investigated cAMP production in B16-G4F cells as compared to B16-F1 cells. Adenylate cyclase activity in plasma membrane fractions from B16-F1 was highly stimulated by 10^{-6} M α-MSH, 10^{-4} M forskolin and 10^{-2} M sodium fluoride. The B16-G4F plasma membranes could also be stimulated by forskolin and NaF to produce cAMP, but not by α-MSH (Fig. 2). The lack of adenylate cyclase response to MSH is therefore clearly due to the absence of functional MSH receptors, since a functional α_s subunit of the heterotrimeric GTP-binding protein and adenylate cyclase must be present. This finding is further supported by competition binding analysis using ([^{125}I])Tyr^2,Nle^4,D-Phe^7)α-MSH as tracer and its non-

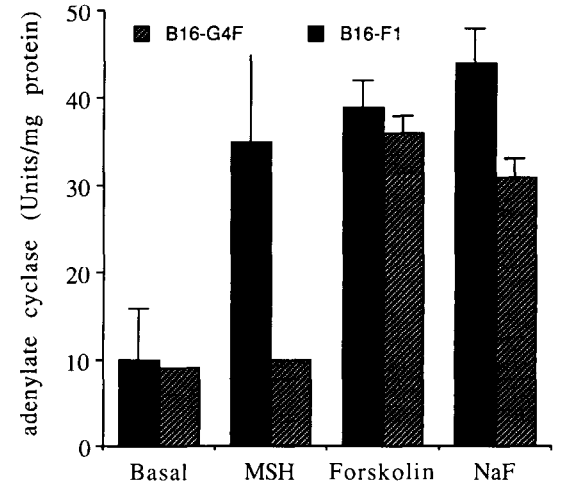

Fig. 2. Adenylate cyclase activity of B16 melanoma membranes after stimulation with various agonists. One unit of enzyme was defined as the amount catalyzing the formation of 1 pmole of cAMP/min. Results are expressed as the mean ± standard deviation of quadruplicate determinations.

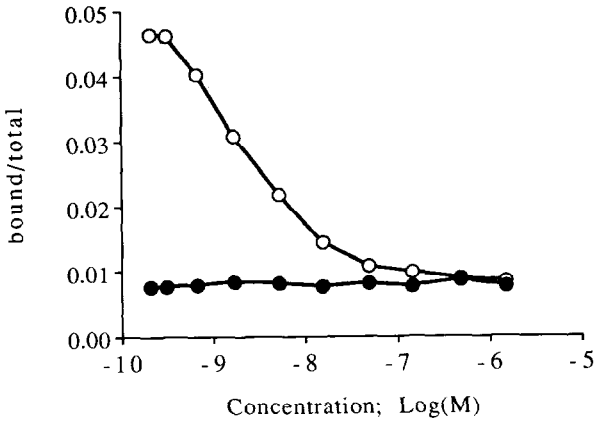

Fig. 3. Competition binding assays using constant amounts of ([^{125}I]Tyr^2,Nle^4,D-Phe^7]α-MSH and varying concentrations of [Nle^4,D-Phe^7]α-MSH as displacer. B16-F1 (○) and B16-G4F (●) cells were incubated for 3 h at 15°C in the presence of both peptides and unbound radioactivity was determined as described in section 2.

iodinated form as displacer (Fig. 3). We have also performed photoaffinity labeling experiments using [^{125}I]Naps-MSH as photoreactive probe [6] for the identification of MSH receptors on the cell surface of B16-G4F cells. As shown in Fig. 4 our results corroborate the previous finding since no molecule could be identified which had incorporated [^{125}I]Naps-MSH after photoactivation. Furthermore we have analysed the MSH receptor mRNA of both cell lines by Northern blot analysis. A mouse MSH receptor probe was generated by PCR from the published cDNA sequence [9]. Northern blot analysis revealed that B16-F1 cells contain a

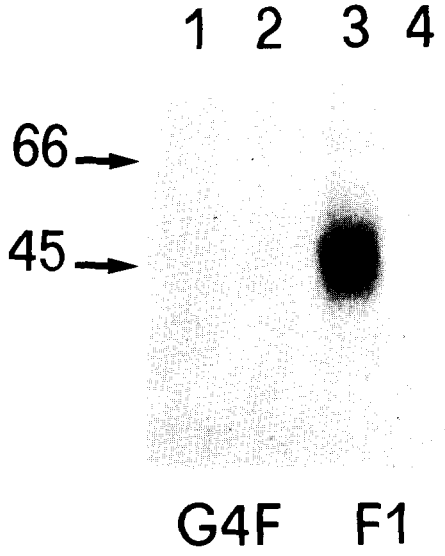

Fig. 4. Autoradiogram of an SDS-PAGE analysis of affinity-labelled B16 melanoma cells. Covalent labelling was achieved by incubating B16-G4F (1,2) and B16-F1 (3,4) cells with 0.2 pmoles of [^{125}I]Naps-MSH, in the presence (2,4) or in the absence (1,3) of a 3000-fold excess of α-MSH.

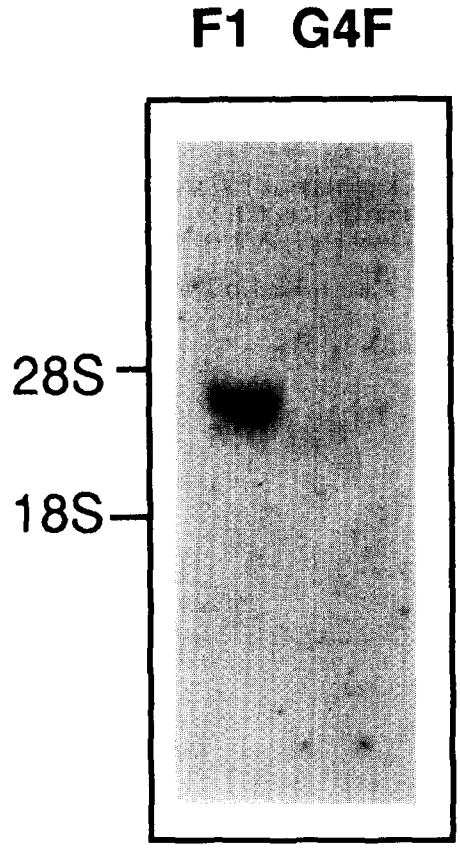

Fig. 5. Northern blot analysis of MSH receptor transcripts in B16-F1 and B16-G4F cells. 10 μg of total mRNA was separated through a 1% formaldehyde agarose gel, blotted on Hybond (Amersham) and hybridized with the ^{32}P-labelled mouse MSH receptor probe. Locations of 28 S and 18 S ribosomal RNA are indicated.

transcript of ~4kb. In contrast no MSH receptor mRNA was observed in B16-G4F cells (Fig. 5).

Taken together these results show clearly that the lack of functional MSH receptors in B16-G4F is not caused by a mutated receptor protein but by the absence of expression of MSH receptor mRNA. Thus the B16-G4F melanoma cell clone will become a very useful tool for the study of signal pathways controlling melanogenesis.

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