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$ACTH_{4–12}$ Is the Minimal Message Sequence Required to Induce the Differentiation of Mouse Epidermal Melanocytes in Serum-Free Primary Culture

TOMOHISA HIROBE[1,2*] AND HIROYUKI ABE[3]
[1]*Division of Biology and Oncology, National Institute of Radiological Sciences, Anagawa, Chiba 263-8555, Japan*
[3]*Graduate School of Science and Technology, Chiba University, Yayoi-cho, Chiba 263-8522, Japan*
[3]*Research Institute of the Functional Peptides, Shimojo, Yamagata 990-0823, Japan*

ABSTRACT It is well known that α-melanocyte stimulating hormone (MSH) induces the differentiation of mouse epidermal melanocytes in vivo and in vitro. Although adrenocorticotropic hormone (ACTH) possesses the same amino acid sequence as MSH does, it is not clear whether the peptide and its fragments induce the differentiation of mouse epidermal melanocytes. In this study, the differentiation-inducing potencies of human ACTH and its fragments were investigated by adding them into a culture medium (0.001–1,000 nM) from the initiation of primary culture of epidermal cell suspensions. Their potencies were compared with the potency of α-MSH. After 2–4 days of primary cultures with $ACTH_{1–13}$, $ACTH_{1–17}$, $ACTH_{1–24}$, $ACTH_{1–39}$, $ACTH_{4–12}$, $ACTH_{4–13}$, and α-MSH, pigment granules appeared in the cytoplasms and dendrites of melanoblasts that were in contact with the adjacent keratinocyte colonies. By 14 days, cultures contained mostly pigmented melanocytes. The order of potencies of ACTH fragments and α-MSH shown by the ED_{50} value was as follows: α-MSH = $ACTH_{1–13}$ = $ACTH_{1–17}$ = $ACTH_{4–12}$ = $ACTH_{4–13}$ > $ACTH_{1–24}$ > $ACTH_{1–39}$. The length of their peptide chains was inversely proportional to the potency. On the contrary, $ACTH_{1–4}$, $ACTH_{11–24}$, and $ACTH_{18–39}$ failed to induce the differentiation of melanocytes. In contrast, $ACTH_{1–10}$, $ACTH_{4–10}$, $ACTH_{4–11}$, and $ACTH_{5–12}$ possessed a weak potency at high doses only (100 and 1,000 nM). These results suggest that $ACTH_{4–12}$ is the minimal message sequence required to induce the differentiation of mouse epidermal melanocytes in culture completely. The amino acids of Met^4 and Pro^{12} are suggested to be important for its potency. *J. Exp. Zool. 286:632–640, 2000.*
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Melanocortins are produced from a common precursor glycoprotein, the proopiomelanocortin (POMC), by post-translational processing (Smith and Funder, ’88). These peptides include adrenocorticotropic hormone (ACTH) and α-, β-, and γ-melanocyte stimulating hormones (MSH). The POMC gene is expressed mainly in the pars distalis and intermedia of the pituitary and in the arcuate nucleus of the hypothalamus, but at a lower level also in a wide variety of peripheral tissues including the skin (Eberle, ’88; Thody and Graham, ’98).

It has been shown that α-MSH plays an important role in the regulation of melanogenesis in mammals and lower vertebrates (Hadley, ’96; Thody and Graham, ’98). In mice, α-MSH is one of the important hormones that regulate the function of melanocytes in the epidermis (Hirobe, ’92b) and hair follicles (Hirobe, ’95; Ermak and Slominsky, ’97). Especially, the differentiation of mouse epidermal melanocytes at neonatal stage is thought to be mainly regulated by α-MSH (Hirobe, ’92b, ’95; Hirobe and Abe, ’99). In the epidermis of newborn mouse skin, MSH induces the differentiation of melanocytes in vivo (Hirobe and Takeuchi, ’77a, ’78). In serum-free organ culture system of mouse skin, MSH also induces the differentiation of melanocytes (Hirobe and Takeuchi, ’77b). Moreover, in serum-free primary culture of mouse epidermal cells, α-MSH induces the differentiation of

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*Correspondence to: Tomohisa Hirobe, Division of Biology and Oncology, National Institute of Radiological Sciences, Anagawa, Inage-ku, Chiba 263-8555, Japan. E-mail: thirobe@nirs.go.jp
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mouse epidermal melanocytes in the presence of keratinocytes (Hirobe, '92c).

ACTH is also known to stimulate the melanogenesis of mammalian skin. ACTH possesses the same amino acid sequence within its sequence as α-MSH does. Namely, a tridecapeptide with a sequence of the first 13 amino acid ($ACTH_{1–13}$) is identical to α-MSH. Alpha-MSH is identical to N-acetyl (Ac)-$ACTH_{1–13}$-NH_2. It is well known that in poikilothermal animals ACTH stimulates melanophore differentiation in vivo and in vitro during development of fish (Chen et al., '74) and frog (Wahn et al., '76). In mammals, the first evidence came from a study of Lerner and McGuire ('64), in which increased skin color was observed in humans injected with high doses of ACTH. However, it has not been well established that ACTH is involved in regulating the melanogenesis of human epidermal melanocytes owing to a lack of suitable culture system of human epidermal melanocytes. Recently, Hunt et al. ('94) reported that ACTH increased melanin content and tyrosinase activity of human epidermal melanocytes in culture. Their results suggest that in human skin ACTH plays an important role in the regulation of melanogenesis. However, it is not known whether the differentiation of mammalian melanocytes can be induced by ACTH. Moreover, it is also remained unsolved what amino acid sequence of ACTH is primarily important for its potency. Recently, a serum-free primary culture system for mouse epidermal melanoblasts has been developed by one of us (Hirobe, '92c). This culture system enables us to provide a good system for testing the potency of peptides to induce the differentiation by adding them into a serum-free culture medium. The object of this study is to investigate the role of ACTH in the regulation of melanocyte differentiation in the mouse epidermis as well as to make clear its active site by using the serum-free primary culture system.

MATERIALS AND METHODS

Mice

All animals used in this study belonged to strain C57BL/10JHir of the house mouse, *Mus musculus*. They were given water and a commercial diet, OA-2 (Clea Japan, Tokyo, Japan) ad libitum. They were maintained at 24 ± 1°C with 40–60% relative humidity: 12 hr of fluorescent light was provided daily.

Primary culture of melanoblasts

The sources of tissue for the culture of melanoblasts were dorsal skin from 0.5-day-old mice. Unless stated otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The method for obtaining epidermal cell suspensions was reported previously (Hirobe, '94, '96, Hirobe et al., '98). Briefly, the skin samples were floated onto a 0.25% trypsin (Gibco, Grand Island, NY) solution in phosphate buffered saline (PBS, pH 7.2) at 2°C for 16–18 hr. Epidermal sheets were separated from the dermis and floated onto a 0.02% ethylenediaminetetraacetate (EDTA) solution in calcium- and magnesium-free PBS (CMF-PBS, pH 7.4), and then gently shaken repeatedly to produce an epidermal cell suspension. Disaggregated epidermal cell suspensions were pelleted by centrifugation and suspended in Ham's F-10 medium (Gibco). The cell pellet after centrifugation was resuspended in a melanoblast-defined medium (MDM; F-10 plus 10 μg/ml insulin [bovine], 0.5 mg/ml bovine serum albumin [Fraction V], 1 μM ethanolamine, 1 μM phosphoethanolamine, 10 nM sodium selenite, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 50 μg/ml gentamycin sulfate, and 0.25 μg/ml amphotericin B). The same lots of these supplements were used in this study. The cells in the epidermal cell suspension were counted in a hemocytometer chamber and plated onto type I collagen-coated dishes (Falcon, Lincoln Park, NJ) at an initial density of 1×10^6 cells/35 mm dish (1.04×10^5 cells/cm^2). Cultures were incubated at 37°C in a humidified atmosphere composed of 5% CO_2 and 95% air (pH 7.2). Medium was replaced by fresh medium four times a week. After 14 days, almost pure cultures of melanoblasts were obtained. Various doses (0–1,000 nM) of human ACTH fragments; $ACTH_{1–4}$, $ACTH_{1–10}$, $ACTH_{1–13}$, $ACTH_{1–17}$, $ACTH_{1–24}$, $ACTH_{1–39}$, $ACTH_{4–10}$, $ACTH_{4–11}$ (Bachem, Bubendorf, Switzerland), $ACTH_{4–12}$ (Funakoshi, Tokyo, Japan), $ACTH_{4–13}$ (Funakoshi), $ACTH_{5–12}$ (Funakoshi), $ACTH_{11–24}$, and $ACTH_{18–39}$. Alpha-MSH (0–1,000 nM) was also supplemented to MDM similarly.

Assay of melanocyte differentiation

The numbers of melanoblasts and melanocytes were determined per dish by phase contrast and bright field microscopy, and the calculations were based on the average number of cells from ten randomly chosen microscopic fields covering an area of 0.581 mm^2. Bipolar, tripolar, dendritic, polygonal, or epithelioid cells, as seen by phase contrast, which contained brown or black pigment granules, as observed by bright field microscopy, were scored melanocytes. These cells were confirmed as melanocytes by dopa cytochemistry

(Hirobe, '92a). In contrast, bipolar, tripolar, dendritic, or polygonal cells, as seen by phase contrast, which contained no pigments, as observed by bright field microscopy, were scored melanoblasts. Some of these cells were stained by combined dopa-premelanin reaction (combined dopa-ammoniacal silver nitrate staining; Mishima, '60; Hirobe, '82a, '92a). This preferential staining reveals undifferentiated, nonpigmented melanoblasts that contain stage I and II melanosomes in addition to tyrosinase-containing differentiated melanocytes (Mishima, '64; Hirobe, '82b).

The statistical significance of the differences in the number of melanoblasts and melanocytes or in the percentage of melanocytes in the melanoblast-melanocyte population was determined by Student's *t*-test for comparisons of groups of equal size.

Melanin assay

Pure primary melanoblasts or melanocytes cultured for 14 days were treated with a solution of 0.05% trypsin and 0.02% EDTA in CMF-PBS at 37°C for 10 min. After trypsinization was inhibited by the addition of 2,000 U/ml of soybean trypsin inhibitor, the cell suspensions were centrifuged at 2,000 rpm for 5 min. The cell pellet was resuspended in CMF-PBS, and the cells in the suspension were counted in a hemocytometer chamber. After centrifugation at 2,000 rpm for 5 min, the cell pellet was frozen at –20°C and stored. After thawing at room temperature, cells were solubilized in 100 μl of 1 N NaOH and diluted with 400 μl of redistilled water. The OD_{475} was measured and converted to melanin content via a standard curve using synthetic melanin.

Electron microscopy

Pure primary melanoblasts or melanocytes cultured for 14 days were similarly collected and fixed in chilled (2°C) 2.5% glutaraldehyde (Wako, Osaka, Japan) solution in 0.1 M phosphate buffer, pH 7.4. After washing with chilled 0.1 M phosphate buffer, cells were postfixed in chilled 1% osmium teroxide (Taab Laboratories Equipment Ltd., Berkshire, UK) in 0.1 M phosphate buffer. After washing with chilled 0.1 M phosphate buffer, the cells were dehydrated in a series of graded ethanols and embedded in epoxy resin (Taab Laboratories Equipment Ltd.). Ultra thin sections were cut with a diamond knife on an ultramicrotome (Reichert Ultracuts, Leica, Heerbrugg, Switzerland), stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (IEM-1210, Jeol, Tokyo, Japan).

RESULTS

Within 1 day after initiation of culture with MDM (0 nM $ACTH_{1–4}$), small keratinocyte colonies could be seen in the dishes. Small bipolar, tripolar, or dendritic cells were scattered between the keratinocyte colonies. No pigments were found in these cells when examined under the bright field microscope. After 2 days, these presumed melanoblasts without pigments were in contact with the adjacent keratinocyte colonies via a dendrite. The "melanoblast" is defined here as a nonpigmented cell that possesses no tyrosinase activity. After 3–7 days, the keratinocyte colonies increased in size and number, and the melanoblasts also increased in number. After 8–13 days, the keratinocyte colonies were smaller and refractile in appearance with progressive detachment of cells, whereas the melanoblasts were more numerous than before. By 14 days, the cultures contained mostly melanoblasts (Fig. 1A). Electron microscopic observations showed that these melanoblasts were devoid of stage I and II melanosomes, though they possessed well-developed Golgi apparatus (Fig. 2A). When the epidermal cell suspensions were cultured with various doses of $ACTH_{1–4}$ from 0.001 to 1,000 nM, a similar tendency of keratinocyte proliferation as well as melanoblast proliferation was observed in all cultures. However, no induction of melanocyte differentiation was observed (Fig. 3). $ACTH_{11–24}$ and $ACTH_{18–39}$ also failed to induce the differentiation of melanocytes at all doses tested (Table 1).

The results of $ACTH_{4–10}$ (Table 1) at doses from 0.001 to 10 nM were similar to those of $ACTH_{1–4}$. However, higher doses (100 and 1,000 nM) of the peptide induced the differentiation of melanocytes (Fig. 1B). Pigment granules were observed in the cytoplasm and dendrites after 2–4 days of primary culture. After 4–9 days, differentiated melanocytes increased in number. They were more pigmented than before and extended dendrites into the surrounding keratinocytes. By 14 days, cultures contained a lot of fully pigmented melanocytes that were polygonal or epithelioid in morphology (Fig. 1B). Twenty to thirty percent of the cells were differentiated melanocytes (Table 1). Higher doses of $ACTH_{4–10}$ are thought to be capable of inducing the differentiation of melanocytes, though not all. Similar tendencies were observed in the cultures with $ACTH_{1–10}$, $ACTH_{4–11}$, and $ACTH_{5–12}$ (Fig. 3). In contrast, $ACTH_{4–12}$ induced the differentiation of melanocytes even at the lowest dose (0.001 nM; Table 1). The percentage of melanocytes in the

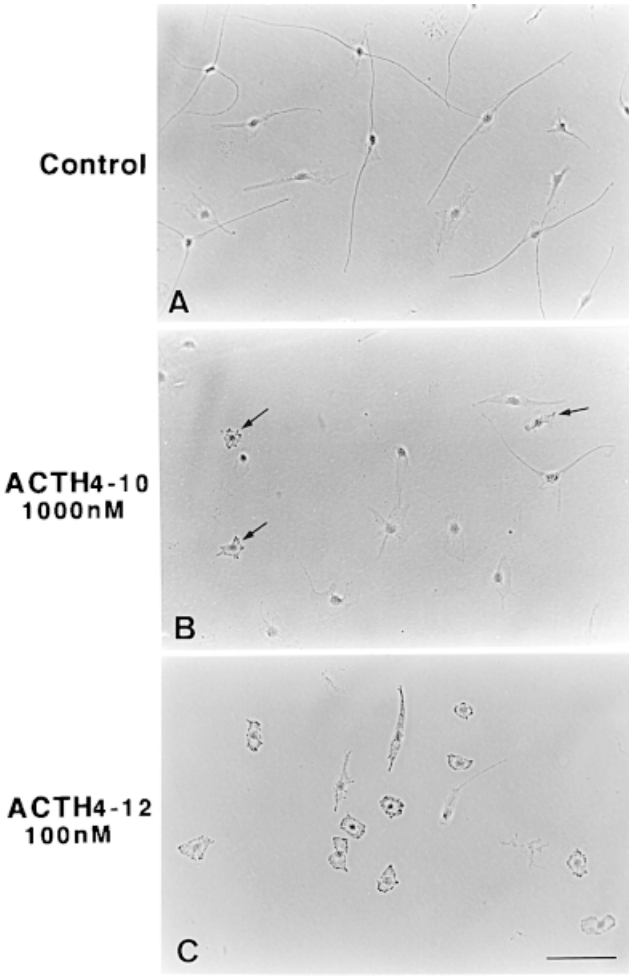

Fig. 1. Melanoblasts and/or melanocytes in pure culture of epidermal cell suspensions derived from mouse skin in three different media: (**A**) Control; (**B**) 1,000 nM $ACTH_{4–10}$; and (**C**) 100 nM $ACTH_{4–12}$. After 14 days in culture. Pure cultures of melanoblasts (A); melanoblasts plus melanocytes (arrows, B); or melanocytes (C) which are bipolar, tripolar, dendritic, polygonal, or epithelioid in morphology. Phase-contrast microscopy. Bar = 100 μm.

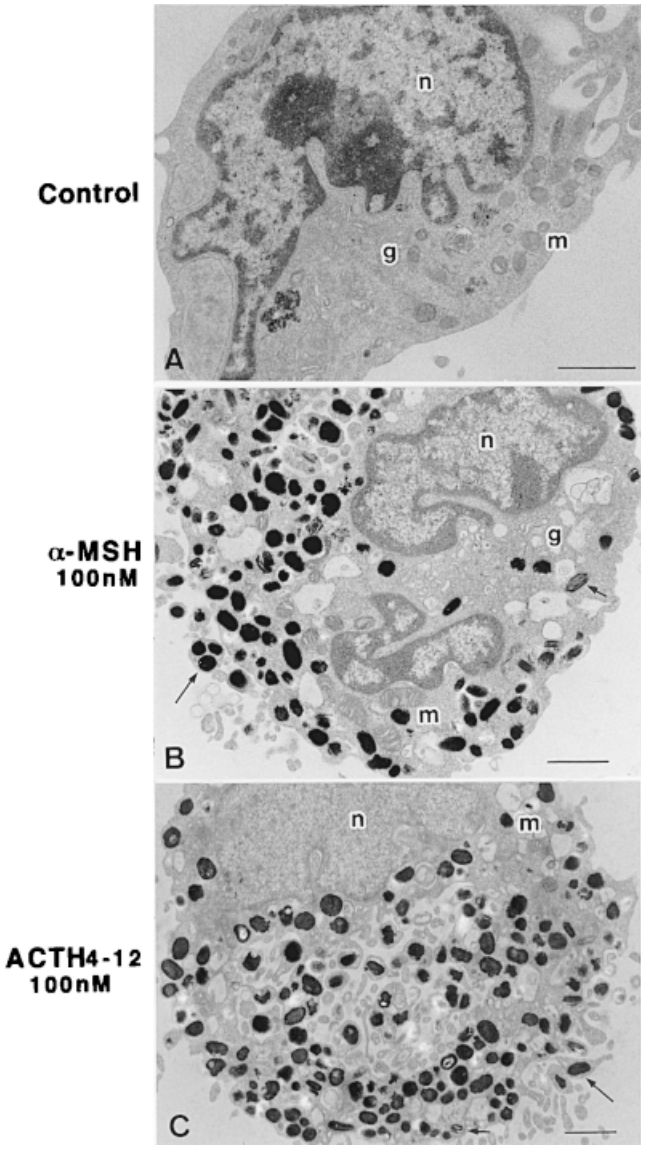

Fig. 2. Electron micrograph of melanoblast and melanocytes in pure culture of epidermal cell suspensions derived from mouse skin in three different media: (**A**) Control; (**B**) 100 nM α-MSH; and (**C**) 100 nM $ACTH_{4–12}$. After 14 days in culture. In the melanoblast, no stage I and II melanosomes are observed. In melanocytes, numerous stage III (short arrows) and IV (long arrows) melanosomes are observed. n, nucleus; m, mitochondria; g, Golgi apparatus. Bar = 1 μm.

melanoblast-melanocyte population increased in a dose-dependent manner (Fig. 3). The differences in the percentage of melanocytes in the melanoblast-melanocyte population between control and $ACTH_{4–12}$ were statistically significant ($P < 0.05$) at all doses from 0.001 to 1,000 nM. At the doses more than 10 nM almost all cells were differentiated melanocytes which were polygonal or epithelioid in morphology (Fig. 1C) at 14 days of primary culture. At the dose of 10 nM, $ACTH_{4–10}$ possessed no potency, but $ACTH_{4–12}$ reached a maximal potency. The result of $ACTH_{4–12}$ is similar to that of α-MSH which was reported previously (Hirobe, '92c). Electron microscopic observation revealed that numerous stage III and IV melanosomes were evenly distributed within the cytoplasm and dendrites of the melanocytes cultured with α-MSH (Fig. 2B) or $ACTH_{4–12}$ (Fig. 2C). The ultrastructural features of the cultured melanocytes with α-MSH or $ACTH_{4–12}$ are similar to those of in vivo melanocytes injected with α-MSH which were reported previously (Hirobe and Takeuchi, '77a, '78; Hirobe, '78), suggesting that our culture conditions are suitable for maintaining melanocytes in vitro.

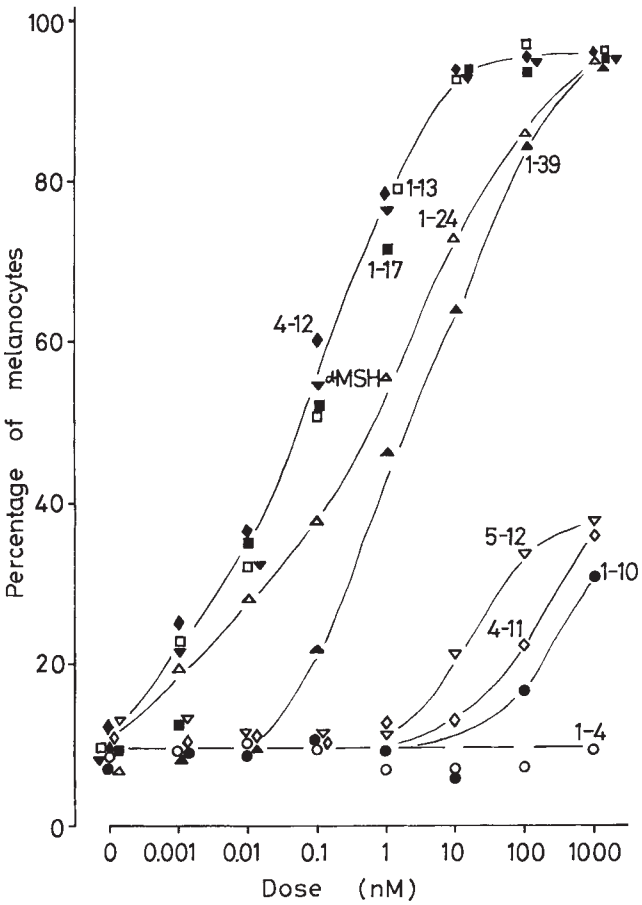

Fig. 3. Dose-response curves for the differentiation of mouse epidermal melanocytes cultured for 14 days in media that consisted of melanoblast-defined media (MDM) supplemented with $ACTH_{1-4}$ (○); $ACTH_{1-10}$ (●); $ACTH_{1-13}$ (□); $ACTH_{1-17}$ (■); $ACTH_{1-24}$ ( ); $ACTH_{1-39}$ ( ); $ACTH_{4-11}$ ( ); $ACTH_{4-12}$ ( ); $ACTH_{5-12}$ ( ); α-MSH ( ) at various doses (0–1,000 nM). The numbers of melanoblasts and melanocytes were counted by phase-contrast and bright-field microscopy. The percentages of melanocytes in the melanoblast-melanocyte population were scored. The epidermal cell suspensions of the ten different groups (0–1,000 nM) of each peptide were derived from the same litter of mice. The data are the averages of results from triplicate experiments. Each experiment was performed with different litters of mice. Standard errors of the mean are not shown in the figure.

Chemical analysis showed that melanin contents in the cells cultured with α-MSH or $ACTH_{4-12}$ are similar level (ca. 100 pg/cell), though those of control cultures are about 7 pg/cell (Table 2). The level of 100 pg/cell of differentiated melanocytes is comparable to that of melan-a cells reported by Sviderskaya et al. ('98). The results of $ACTH_{1-13}$, $ACTH_{1-17}$, $ACTH_{1-24}$, $ACTH_{1-39}$, and $ACTH_{4-13}$ are similar to the result of $ACTH_{4-12}$ (Table 1, Fig. 3). However, the potency gradually decreased according to the increase in the length of their peptides (Fig. 3). To measure the potency of ACTH fragments, doses of the peptides required to induce 50% of maximal response (ED_{50} value) were estimated (Table 3). ED_{50} values for $ACTH_{1-13}$, $ACTH_{1-17}$, $ACTH_{1-24}$, and $ACTH_{1-39}$ were 0.098, 0.098, 0.90, and 2.50, respectively (Table 3). These results suggest that the length of the peptide chains of ACTH was inversely proportional to the potency. Maximal activity was observed in $ACTH_{1-13}$, $ACTH_{1-17}$, $ACTH_{4-12}$, $ACTH_{4-13}$, and α-MSH (Table 3).

In all cultures treated with human ACTH fragments or α-MSH, the number of melanoblasts and melanocytes at 14 days of primary culture did not differ significantly from that of control (Table 1), suggesting that ACTH fragments and α-MSH do not induce a proliferation of melanoblasts or melanocytes in serum-free primary culture system.

DISCUSSION

In the present study, $ACTH_{1-39}$ was demonstrated to be effective for stimulating the differentiation of melanocytes in serum-free primary culture of mouse epidermal cell suspensions. However, the differentiation-inducing potency of $ACTH_{1-39}$ (ED_{50} = 2.50 nM) was much less than that of α-MSH (ED_{50} = 0.090) or $ACTH_{1-13}$ (ED_{50} = 0.098). Since $ACTH_{1-39}$ possesses the same amino acid sequence ($ACTH_{1-13}$) as α-MSH does, the amino acid sequence of $ACTH_{14-39}$ seems to decrease its potency. The order of potency was α-MSH = $ACTH_{1-13}$ = $ACTH_{1-17}$ > $ACTH_{1-24}$ > $ACTH_{1-39}$. The length of their peptide chains was inversely proportional to the potency. Recently, Hunt et al. ('94) reported that human $ACTH_{1-39}$ increased melanin content and tyrosinase activity of human epidermal melanocytes in culture. $ACTH_{1-39}$ exhibited a biphasic dose-response curve, whereas α-MSH exhibited a sigmoidal one. ED_{50} value of $ACTH_{1-39}$ was 0.0014 nM and 6.3 nM for melanin content, and 0.0014 nM and >20 nM for tyrosinase activity, respectively, while that of α-MSH was 0.008 nM for melanin content and 0.01 nM for tyrosinase activity, respectively. However, the present study showed that dose-response curve for $ACTH_{1-39}$ was sigmoidal, and ED_{50} value was 2.50 nM. Although the differences between their results and our present findings cannot be fully explained at present, they might be attributed to the differences in the species tested (human versus mouse) and the assay system (stimulation of melanogenesis in cultured melanocytes versus induction of melanocyte differentiation in primary culture). Part of the failure to observe greater sensitivity to $ACTH_{1-39}$ might lie in the fact that a heterologous bioassay was used

TABLE 1. The number of melanoblasts and melanocytes and percentage of melanocytes in the melanoblast-melanocyte population of C57BL/10JHir mice at 14 days in serum-free primary culture[1]

	Dose (nM)							
Peptide	0	0.001	0.01	0.1	1	10	100	1000
A								
1–4	2.56 ± 0.24	2.51 ± 0.19	2.46 ± 0.16	2.45 ± 0.06	1.97 ± 0.12	1.91 ± 0.11	1.85 ± 0.13	1.80 ± 0.14
1–10	2.71 ± 0.43	2.70 ± 0.45	2.73 ± 0.45	2.86 ± 0.49	2.57 ± 0.46	2.40 ± 0.32	2.31 ± 0.26	2.59 ± 0.32
1–13	2.23 ± 0.07	2.19 ± 0.24	2.14 ± 0.17	2.18 ± 0.12	2.09 ± 0.22	2.05 ± 0.13	2.02 ± 0.18	1.93 ± 0.23
1–17	2.19 ± 0.11	1.89 ± 0.09	2.12 ± 0.06	1.80 ± 0.13	2.23 ± 0.36	1.95 ± 0.23	1.96 ± 0.28	1.96 ± 0.16
1–24	2.20 ± 0.49	2.21 ± 0.37	2.38 ± 0.47	2.25 ± 0.34	2.25 ± 0.38	2.49 ± 0.41	2.08 ± 0.53	2.08 ± 0.68
1–39	2.69 ± 0.36	2.83 ± 0.22	2.46 ± 0.31	2.53 ± 0.25	2.50 ± 0.21	2.65 ± 0.41	2.75 ± 0.41	2.77 ± 0.34
4–10	2.25 ± 0.21	2.33 ± 0.46	2.32 ± 0.55	2.10 ± 0.40	2.02 ± 0.26	2.12 ± 0.29	1.94 ± 0.22	2.07 ± 0.35
4–11	2.81 ± 0.08	2.64 ± 0.04	2.65 ± 0.16	2.51 ± 0.08	2.45 ± 0.18	2.24 ± 0.23	2.26 ± 0.21	2.54 ± 0.35
4–12	2.59 ± 0.15	2.53 ± 0.20	2.62 ± 0.22	2.38 ± 0.23	2.15 ± 0.15	2.13 ± 0.23	2.38 ± 0.28	2.44 ± 0.09
4–13	2.23 ± 0.12	2.21 ± 0.12	1.85 ± 0.11	1.76 ± 0.09	1.84 ± 0.07	2.07 ± 0.14	2.02 ± 0.35	2.20 ± 0.24
5–12	2.43 ± 0.14	2.60 ± 0.10	2.49 ± 0.12	2.35 ± 0.22	2.15 ± 0.17	1.94 ± 0.15	2.30 ± 0.06	2.39 ± 0.29
11–24	2.60 ± 0.43	2.32 ± 0.48	2.65 ± 0.48	2.61 ± 0.43	2.47 ± 0.52	2.37 ± 0.44	2.31 ± 0.40	2.12 ± 0.33
18–39	2.40 ± 0.15	2.31 ± 0.16	2.47 ± 0.25	2.39 ± 0.18	2.24 ± 0.20	1.88 ± 0.20	2.03 ± 0.24	1.83 ± 0.26
α-MSH	2.33 ± 0.25	2.60 ± 0.20	2.37 ± 0.29	2.56 ± 0.29	2.30 ± 0.25	2.46 ± 0.24	2.55 ± 0.35	2.46 ± 0.24
B								
1–4	8.59 ± 2.03	9.17 ± 0.31	10.07 ± 1.18	9.44 ± 1.65	6.95 ± 1.91	7.65 ± 1.52	7.02 ± 1.46	9.30 ± 1.47
1–10	6.63 ± 1.45	9.33 ± 1.83	8.79 ± 1.75	10.43 ± 3.89	9.44 ± 1.65	5.78 ± 1.52	16.89 ± 2.68	30.90 ± 6.54
1–13	8.80 ± 0.61	22.77 ± 1.35	31.92 ± 2.33	50.72 ± 4.02	79.71 ± 2.44	92.77 ± 1.08	96.89 ± 0.42	95.88 ± 1.18
1–17	9.89 ± 0.82	12.55 ± 1.86	35.24 ± 3.47	51.75 ± 5.79	75.63 ± 2.07	93.93 ± 0.96	93.44 ± 2.78	95.47 ± 0.63
1–24	6.76 ± 3.40	19.41 ± 6.61	27.77 ± 6.56	37.51 ± 9.16	55.46 ± 10.71	72.69 ± 10.07	85.84 ± 5.45	95.28 ± 5.88
1–39	9.08 ± 0.38	8.64 ± 0.99	8.88 ± 1.74	21.94 ± 1.85	46.32 ± 4.88	64.03 ± 4.55	84.31 ± 2.93	94.52 ± 0.45
4–10	7.92 ± 0.31	8.71 ± 0.13	7.17 ± 0.21	8.35 ± 0.62	6.53 ± 0.83	7.36 ± 0.59	18.75 ± 3.80	31.76 ± 5.45
4–11	10.84 ± 1.20	9.83 ± 1.26	11.19 ± 0.71	10.37 ± 0.41	12.23 ± 0.45	12.80 ± 1.68	22.40 ± 4.53	35.83 ± 2.89
4–12	12.00 ± 1.86	25.01 ± 3.50	36.11 ± 3.57	60.35 ± 2.90	78.16 ± 1.38	93.84 ± 0.90	95.18 ± 0.74	95.95 ± 0.62
4–13	8.07 ± 1.15	23.34 ± 3.34	33.71 ± 2.87	48.88 ± 4.48	77.24 ± 2.51	94.91 ± 0.38	93.69 ± 1.20	93.81 ± 1.19
5–12	13.01 ± 0.70	13.26 ± 2.02	10.49 ± 0.88	11.52 ± 3.16	11.79 ± 0.29	21.03 ± 3.16	33.55 ± 5.35	37.72 ± 7.39
11–24	9.15 ± 2.30	7.14 ± 0.58	10.37 ± 1.92	8.46 ± 2.22	8.42 ± 1.44	9.95 ± 3.38	10.80 ± 2.42	10.71 ± 2.52
18–39	7.59 ± 1.21	7.02 ± 0.74	6.76 ± 1.16	4.86 ± 0.58	6.28 ± 0.28	6.57 ± 1.51	8.85 ± 1.82	5.60 ± 1.05
α-MSH	8.12 ± 2.26	21.36 ± 3.75	32.26 ± 3.31	54.40 ± 4.62	76.27 ± 1.96	93.00 ± 2.51	94.93 ± 0.94	95.11 ± 0.62

[1]Epidermal cell suspensions derived from 0.5-day-old C57BL/10JHir mice were cultured with a melanoblast-defined medium (MDM). ACTH fragments and α-MSH were supplemented to MDM from the initiation of culture. Pure cultures of melanoblasts and/or melanocytes were obtained after 14 days. The number of melanoblasts and melanocytes was counted by phase contrast and bright field microscopy and are shown in A. The percentage of melanocytes in the melanoblast-melanocyte population was also scored and is shown in B. The data of the percentage of melanocytes in the melanoblast-melanocyte population are underlined for values above background. The data are the averages of results from triplicate experiments. Each experiment was performed with different litters of mice. Average ± standard error of the mean.

in the present study, with human $ACTH_{1–39}$ and mouse melanoblasts.

The present study also showed that $ACTH_{4–12}$ was equipotent to α-MSH, though $ACTH_{4–11}$ and $ACTH_{5–12}$ possessed a weak potency only at high doses. These results suggest that $ACTH_{4–12}$ is the minimal message sequence required to induce completely the differentiation of mouse epidermal melanocytes in culture, and amino acids of Met^4 and Pro^{12} are important for its potency. Since α-MSH, β-MSH, and ACTH contain an identical central sequence ($ACTH_{4–10}$), it has been thought that this sequence is the minimal message sequence (Sawyer et al., '90). However, in our present study $ACTH_{4–10}$ was shown to possess a limited potency. In poikilothermal animals, the sequence of ACTH required for hormone activity (melanosome dispersion) has been determined in the skin bioassay (Hruby et al., '87, Castrucci et al., '89). The minimal sequence possessing nearly equipotently to α-MSH is Ac-$ACTH_{4–12}$-NH_2 in the frog skin bioassay (Hruby et al., '87) or Ac-$ACTH_{4–11}$-NH_2 in the lizard skin bioassay (Castrucci et al., '89). The

TABLE 2. Melanin content in melanocytes cultured with ACTH and α-MSH at 14 days in serum-free culture[1]

Peptide	Melanin content (pg/cell)
α-MSH	93.5 (82.9, 96.4, 101.1)
$ACTH_{4–12}$	97.6 (105.8, 88.2, 98.9)
Control	6.6 (3.8, 7.6, 8.3)

[1]Homogenates contain cells treated with a solution of trypsin and EDTA. Samples were derived from 10-20 dishes of cells cultured for 14 days in a serum-free medium. They were examined for melanin content. Values in parentheses are individual data.

TABLE 3. Relative potency of ACTH fragments and α-MSH to induce the differentiation of mouse epidermal melanocytes in serum-free primary culture

Peptide	Approximate differentiation-stimulative potenticy (%)	ED_{50} (nM)	Amino acid sequence
$ACTH_{1–4}$	0	—	SerTyrSerMet
$ACTH_{1–10}$	30	—	SerTyrSerMetGluHisPheArgTrpGly
$ACTH_{1–13}$	100	0.098	SerTrySerMetGluHisPheArgTrpGlyLysProVal
$ACTH_{1–17}$	100	0.098	SerTyrSerMetGluHisPheArgTrpGlyLysProValGlyLysLysArg
$ACTH_{1–24}$	100	0.90	SerTyrSerMetGluHisPheArgTrpGlyLysProValGlyLysLysArgArgProValLysVal TyrPro
$ACTH_{1–39}$	100	2.50	SerTyrSerMetGluHisPheArgTrpGlyLysProValGlyLysLysArgArgProValLysVal TyrProAsnGlyAlaGluAspGluSerAlaGluAlaPheProLeuGluPhe
$ACTH_{4–10}$	30	—	MetGluHisPheArgTrpGly
$ACTH_{4–11}$	30	—	MetGluHisPheArgTrpGlyLys
$ACTH_{4–12}$	100	0.087	MetGluHisPheArgTrpGlyLysPro
$ACTH_{4–13}$	100	0.098	MetGluHisPheArgTrpGlyLysProVal
$ACTH_{5–12}$	30	—	GluHisPheArgTrpGlyLysPro
$ACTH_{11–24}$	0	—	LysProValGlyLysLys-----ArgPro
$ACTH_{18–39}$	0	—	ArgPro-----Phe
α-MSH	100	0.090	N-Ac-SerTyrSerMetGluHisPheArgTrpGlyLysProVal-NH_2

nonacetylated and nonamidated peptides and shorter fragments were reported not to be equipotent to α-MSH (Hruby et al., '87). Our present study showed that nonacetylated and nonamidated peptides ($ACTH_{1–13}$, $ACTH_{4–12}$, $ACTH_{4–13}$, $ACTH_{1–17}$) were equipotent to α-MSH. The differences in the minimal message sequence required to stimulate melanocyte function between their results and our present findings might be due to the differences in the species tested (frog or lizard versus mouse) and the assay system (stimulation of melanosome dispersion versus induction of melanocyte differentiation in primary culture).

Recently, five receptor genes for MSH and ACTH have been cloned that encode a family of G-protein coupled receptors showing high homology in their amino acid sequences. These receptors were termed melanocortin receptors 1–5 (MC1R–MC5R according to their order of cloning (Cone et al., '96). In the mouse MC1R, MSH and ACTH increased the levels of adenosine 3´:5´-cyclic monophosphate (cAMP) with the following order of potency: α-MSH = β-MSH > $ACTH_{1–39}$ > γ-MSH (Mountjoy et al., '92), while in the human MC1R with the following order of potency: α-MSH = $ACTH_{1–39}$ > β-MSH > γ-MSH > $ACTH_{4–10}$ (Chhajlani and Wirkberg, '92). The expression of MC1R mRNA is detected only in melanoma cells (Mountjoy et al., '92). The effects of MSH and ACTH on melanocytes are thought to be mediated specifically by binding to and activation of MC1R. Schiöth et al. ('98) reported that in ligand binding assays with cells transiently expressing the human MC1R, N-terminal segment (Ser^1-Tyr^2-Ser^3) of [Nle^4, D-Phe^7] α-MSH (NDP) was not important for binding to MC1R, but C-terminal segment (Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}) of NDP was important for binding to MC1R. Their results suggest that C-terminal segment plays an important role in the binding between MC1R and α-MSH. The present findings well agree with their results in that the C-terminal region is important for the potency of α-MSH. The present study also showed that intact amino acid residues of Met^4 and Pro^{12} were required for the potency of MSH. Schiöth et al. ('97) reported the binding affinities of the MSH analogue, MSH-B on a cell line transiently expressing the human MC1R. They found that MSH-B possessed a potency similar to that of α-MSH. The sequence of MSH-B differs from that of α-MSH in the following amino acids, Gly^1, Arg^3, Gln^5, Glu^{11}, Leu^{13}. Their results suggest the important role of Met^4 and Pro^{12} in the interaction between MC1R and α-MSH. However, it remains to be investigated in a future study what sort of three-dimensional structure of $ACTH_{4–12}$ is important for the binding to MC1R, and why the $ACTH_{4–11}$ or $ACTH_{5–12}$ loses almost all activity. Furthermore, the present study demonstrated that α-MSH and $ACTH_{1–13}$ were equipotent. One possibility is that the N-acetylation and C-amidation of the α-MSH molecule play a role in the maintenance of its stability in vivo. However, it remains to be investigated in a future study.

Wakamatsu et al. ('97) demonstrated that ACTH activated the MC1R in cultured human epidermal melanocytes and in human embryonic kidney HEK 293 cells transfected with the human MC1R. $ACTH_{1–17}$ were as active as α-MSH in stimulating melanocyte dendricity and melanogenesis. ACTH peptides also increased the levels of cAMP with the following order of potency: $ACTH_{1–17}$ > α-MSH > $ACTH_{1–39}$ > $ACTH_{1–10}$. Their results suggest that ACTH and its fragments are capable of activating the human MC1R. On the other hand, Suzuki et al. ('96) reported different results. They determined potency of MSH and $ACTH_{1–39}$ to stimulate MC1R expression, cAMP formation, and tyrosinase activity of human epidermal melanocytes in culture. The order of potency was α-MSH = $ACTH_{1–39}$ > β-MSH > γ-MSH. The binding affinities of MSH and $ACTH_{1–39}$ for MC1R were well correlated with their abilities to stimulate melanogenesis. Our present findings are consistent with the results of Wakamatsu et al. ('97) in that the potency of $ACTH_{1–39}$ was less than that of α-MSH or $ACTH_{1–17}$. It remains to be investigated in a future study how the MC1R expression is regulated by α-MSH and ACTH during the differentiation of mouse epidermal melanocytes.

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