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α-MSH-induced changes in protein phosphorylation of Cloudman S91 mouse melanoma cells

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Summary

The role of protein phosphorylation in MSH-induced melanogenesis was investigated with an in vivo phosphorylation assay using intact cultured Cloudman S91 mouse melanoma cells preincubated with [^{32}P]orthophosphate. Exposure of the cells to α-MSH increased the extent of labelling of two protein bands on SDS gel electrophoresis with estimated molecular weights of 43 and 34 kDa, respectively. The ^{32}P incorporation was concentration-dependent and reached a maximal value at 10^{-8} M α-MSH for the 43 kDa band (156% of controls) and at 10^{-5} M α-MSH for the 34 kDa band (250% of controls). The corresponding ED_{50}s were 5×10^{-10} M (43 kDa) and 3×10^{-8} M (34 kDa). The ^{32}P incorporation into the 34 kDa band reached a maximum after a 5 min exposure to α-MSH whereas 43 kDa phosphorylation was maximal after a 30–60 min incubation with hormone. The effect was completely reversible after removal of the hormone and specific for melanotropic peptides. Dibutyryl cAMP (10^{-3} M) and forskolin (10^{-4} M) together with isobutylmethylxanthine (10^{-4} M) mimicked the effect of α-MSH, pointing to an involvement of adenylate cyclase activation in the phosphorylation of both the 34 kDa and the 43 kDa protein. Preliminary observations showed that the 34 kDa protein is membrane-bound whereas the 43 kDa protein is of mitochondrial or melanosomal origin.

Introduction

α-Melanotropin (α-MSH) is well known to stimulate pigment dispersion in dermal melanophores of lower vertebrates (Bagnara and Hadley, 1973) and pigment formation in epidermal melanocytes (Snell, 1967) and melanoma cells (Wong and Pawelek, 1973). Murine melanoma cells have been shown to contain specific MSH receptors (cf. Eberle, 1981) which are linked to an adenylate cyclase system (Bitensky and Demopoulos, 1970). Elevation of intracellular levels of cAMP leads to the activation of tyrosinase, the key enzyme for melanogenesis, and possibly of other regulators of melanin biosynthesis (Pawelek et al., 1973, 1980). The mechanism of tyrosinase stimulation is, however, poorly understood. Fuller and Viskochil (1979) have shown that the activation of tyrosinase by MSH is dependent on de

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novo RNA and protein synthesis which are transiently raised after a preceding sharp increase in cAMP levels.

As in many other systems in which cAMP has been shown to mediate cellular responses through cAMP-dependent protein kinases (Greengard, 1978), such kinase activity was also found in Cloudman S91 cells (Niles and Logue, 1979) and was increased after stimulation with α-MSH (Burnett et al., 1981). Furthermore, treatment of intact melanoma cells with α-MSH was shown to affect the phosphorylation of a number of protein bands in cell lysates assayed for post hoc in vitro phosphorylation with (γ-^{32}P]ATP (Pawelek et al., 1985). In a similar post hoc in vitro phosphorylation assay, α-MSH induced an increase in ^{32}P incorporation into a 53 kDa protein in *Xenopus* melanophores (de Graan, 1984, 1985a, b). The phosphorylation of this 53 kDa melanophore protein was also stimulated with low concentrations of forskolin (de Graan, 1984), a specific activator of adenylate cyclase. Recently, the role of protein phosphorylation in goldfish erythrophores was studied with an in vivo phosphorylation assay using [^{32}P]orthophosphate to label the endogenous ATP pool: a group of → 57 kDa proteins was found whose ^{32}P incorporation was markedly increased by ACTH or cAMP (Lynch et al., 1986). These examples demonstrate that protein phosphorylation may play a role in the mechanism of α-MSH action on pigment cells.

In this paper we report the first direct evidence for a role of protein phosphorylation in the mechanism of action of α-MSH on Cloudman S91 mouse melanoma cells. We have developed an in vivo phosphorylation assay to monitor ^{32}P incorporation into phosphoproteins in intact melanoma cells. Using this assay, we show that treatment with α-MSH results in a time- and concentration-dependent increase in the degree of phosphorylation of at least two phosphoproteins.

Materials and methods

Reagents

α-MSH was prepared in our laboratory by a classical solution approach (Eberle and Hübscher, 1979). $ACTH_{1-24}$, $ACTH_{1-10}$ and $ACTH_{11-24}$ were a gift from Ciba-Geigy, Basel. β-Endorphin, dibutyryl cAMP (db-cAMP) and isobutylmethylxanthine (IBMX) were purchased from Sigma (St. Louis, MO, U.S.A.). Vasoactive intestinal peptide (VIP) was obtained from Calbiochem-Behring (San Diego, CA, U.S.A.). Culture media and sera were from Gibco (Glasgow, U.K.) and ^{32}P-labelled orthophosphate ($^{32}P_i$, carrier-free) from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of the highest grade commercially available.

Melanoma cells

The Cloudman S91 mouse melanoma cell line (clone P3) was a gift from Prof. B.B. Fuller (University of Oklahoma, Oklahoma City, OK, U.S.A.). Cells were grown in Ham's F-10 medium supplemented with 10% horse serum, 100 units/ml of penicillin, 100 μg of streptomycin and 1% L-glutamine (w/v). The doubling time was approximately 24 h. The cells were detached from the culture flasks in Ca^{2+}- and Mg^{2+}-free EDTA buffer consisting of 117 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 11 mM sucrose, 1.5 mM EDTA, pH 7.4, gassed with 95% O_2/5% CO_2. Cells were collected by centrifugation and resuspended in phosphate-free Krebs-Ringer buffer at 5×10^6 cells/ml and kept on ice until the phosphorylation experiment was started.

In vivo phosphorylation assay

The cell suspension (5×10^6 cells/ml) was preincubated with $^{32}P_i$ (250 μl/ml) in phosphate-free Krebs-Ringer buffer at 36°C for 60 min under gentle shaking and continuous gassing with 95% O_2/5% CO_2. 100 μl aliquots of the cell suspension were distributed in Eppendorf tubes and further incubated with different concentrations of peptide for 30 min under light shaking in a humidified 95% O_2/5% CO_2 atmosphere. The vials were then put on ice in order to stop the ^{32}P incorporation and diluted with 1 ml of ice-cold Krebs-Ringer buffer containing 1.2 mM K-phosphate. The cells were centrifuged in a MicroCentauer centrifuge (MSE) for 30 s and the pellets dissolved in 100 μl solubilization mixture (62.5 mM Tris/HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue and 5% 2-mercaptoethanol). The samples were frozen in liquid nitrogen and then boiled for 15 min in order to facilitate solubilization.

Proteins were separated by SDS-polyacrylamide gel electrophoresis using 7 → 15% linear gradient gels. The samples were diluted 1 : 1 with fresh solubilization mixture before application onto the gel. Routinely, 10 μg were applied per slot. Electrophoresis was performed at 120 V/30 mA for 6–7 h. Proteins were stained with 0.1% Fast Green according to Zwiers et al. (1976). Gels were dried under vacuum at 50 °C and subjected to autoradiography. ^{32}P incorporation into protein bands was quantified by densitometric scanning of the autoradiograms with a linear gel scanner (slit width 0.03 mm) and expressed as peak height above background (PAB; Ueda et al., 1973). The linearity between radio-activity and grain density per band was checked by cutting the bands from the gel and counting them in a liquid scintillation counter. Comparisons were made only between samples on one gel. For a comparison of different experiments, the PAB values were normalized by setting the controls as 100%. Statistical analysis of the data was performed with an analysis of variance (completely randomized design), followed by a supplemental *t*-test (Bruning and Kintz, 1977).

Results

Incubation of Cloudman S91 mouse melanoma cells with $^{32}P_i$ resulted in ^{32}P incorporation into > 30 protein bands separated by one-dimensional SDS-gel electrophoresis (Fig. 1). Variations in the time of preincubation with $^{32}P_i$ showed that a 60 min period was superior to a 30 min or a 120 min period with respect to MSH-stimulated phosphorylation changes. The binding of ^{32}P to proteins was not affected by acid treatment but was susceptible to treatment with 1 N NaOH for 1 h at 40 °C (not shown). This indicates that the ^{32}P is covalently bound to proteins by ester linkage on seryl or threonyl residues.

Incubation of ^{32}P-preloaded cells with 10^{-6} M α-MSH did not affect the protein pattern but led to an increase in ^{32}P incorporation into two protein bands with apparent molecular weights of 34 kDa and 43 kDa, respectively (Fig. 1). Quantification of these effects by densitometry of autoradiograms showed that α-MSH-induced changes in protein phosphorylation were dependent on the hormone concentration (Fig. 2) and confined to

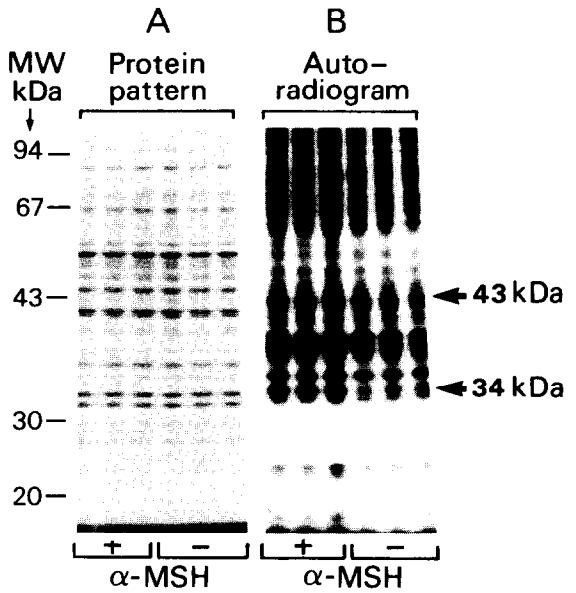

Fig. 1. Protein pattern and autoradiogram after gel electrophoresis of solubilized Cloudman S91 cells which had been preincubated with $^{32}P_i$ and then incubated with or without 10^{-6} M α-MSH, as described in the text.

the two bands. (This does not, however, rule out the possibility that ^{32}P incorporation into additional proteins, not detectable by one-dimen-

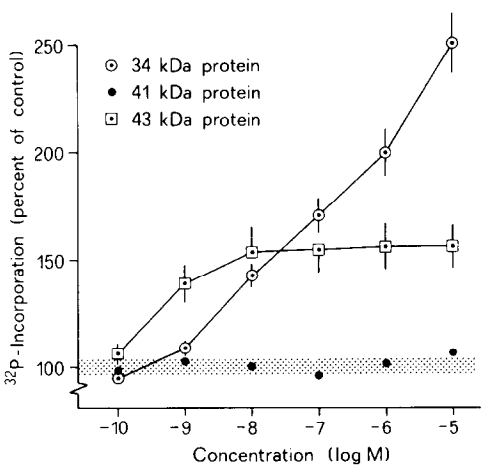

Fig. 2. Dependence on α-MSH concentration of the ^{32}P incorporation into the 34 kDa, 41 kDa (control) and 43 kDa protein bands. Each gel track was scanned and peak height above background (PAB) determined. PAB values of MSH-stimulated cells were then normalized against those of non-stimulated cells (→ 100%) of the same experiment (gel). Each value is the mean of $n = 6$ determinations, except for 10^{-9} M ($n = 5$) and 10^{-5} M α-MSH ($n = 4$) and for the 41 kDa control band ($n = 4$). Bars indicate SEM values.

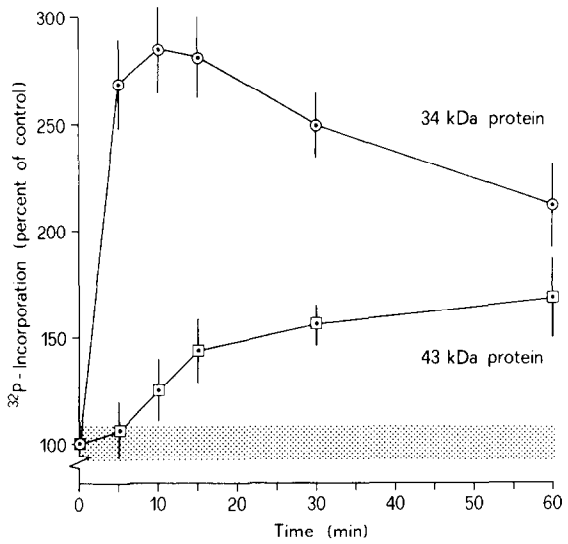

Fig. 3. Time course of the ^{32}P incorporation into the 34 kDa and 43 kDa protein band induced by 10^{-6} M α-MSH. PAB values were calculated as described in Fig. 2. Each value is the mean of $n = 4$ determinations from two experiments. Bars indicate SEM values.

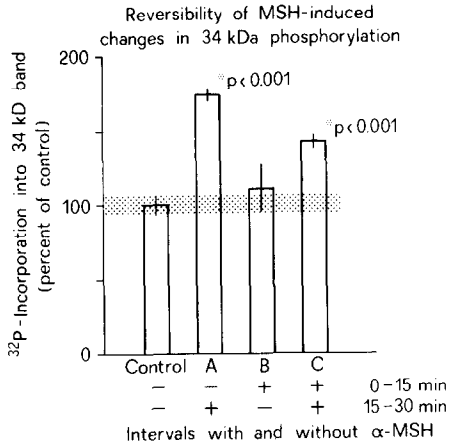

Fig. 4. Reversibility of ^{32}P incorporation into the 34 kDa protein band induced by 10^{-6} M α-MSH. The incubation was performed in two periods of 15 min each with a washing procedure in between. A: first period without, second period with α-MSH; B: first period with, second period without α-MSH; C: both periods with α-MSH. Each value is the mean of three determinations. Bars indicate SEM values; $^{*}P < 0.001$.

sional separation, is affected by α-MSH.) ^{32}P incorporation into the 43 kDa band was detectable at 10^{-10} M α-MSH and reached a plateau at 10^{-8} M α-MSH with a value of 156% of controls not exposed to hormone. ^{32}P incorporation into the 34 kDa band was first visible at 10^{-9} M α-MSH but did not reach a plateau and was highest at 10^{-5} M α-MSH with a value of 250% of controls (Fig. 2). The corresponding EC_{50} values were 5×10^{-10} M (43 kDa) and 3×10^{-8} M (34 kDa). Phosphorylation of a control band (41 kDa) did not change in the MSH concentration range tested (10^{-10} to 10^{-5} M).

After a 5 min incubation with α-MSH, the 34 kDa phosphorylation was almost maximal whereas 43 kDa phosphorylation did not significantly differ from non-stimulated controls (Fig. 3). After a 30–60 min incubation with hormone, there was a decline in the 34 kDa phosphorylation whereas the 43 kDa phosphorylation was still increasing. This shows that the onset of the 34 kDa phosphorylation is much more rapid than that of the 43 kDa phosphorylation.

The ^{32}P incorporation into both bands was reversible as illustrated in Fig. 4 for the 34 kDa band. After a 15 min incubation in 10^{-6} M α-MSH, cells were centrifuged, washed with KRB, resuspended in 100 μl KRB containing 25 μCi $^{32}P_i$ and incubated for another 15 min. This procedure completely reversed the α-MSH-induced effect on 34 kDa phosphorylation. Similar results were obtained for the 43 kDa band (not shown). This indicates that phosphate turnover in both the 34

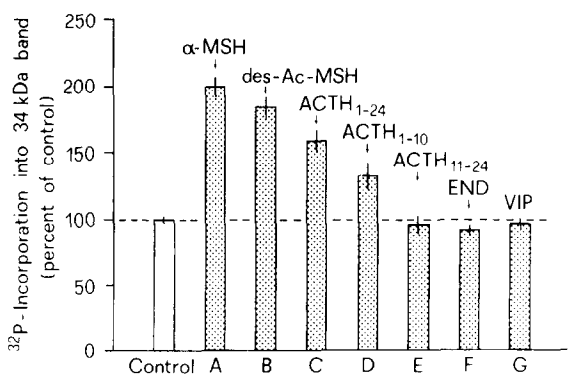

Fig. 5. Effect of 10^{-6} M α-MSH (A), des-acetyl-α-MSH (B), $ACTH_{1-24}$ (C), $ACTH_{1-10}$ (D), $ACTH_{11-24}$ (E), β-endorphin (F) and vasointestinal peptide (G) on ^{32}P incorporation into the 34 kDa protein band. Each value is the mean of $n = 12$ (A), $n = 6$ (C) or $n = 4$ (all others). Bars indicate SEM values.

kDa and the 43 kDa protein band is rapid. Since we focussed our main interest on the 34 kDa protein, the results of the subsequent experiments will be restricted to this band.

The specificity of the MSH-induced increase in 34 kDa phosphorylation was tested with α-MSH and ACTH analogues (Fig. 5). α-MSH was the most potent of the peptides tested. At a 10^{-6} M concentration, stimulation of ^{32}P incorporation by des-acetyl-α-MSH was 85% and by $ACTH_{1-24}$ 59% as compared to α-MSH. $ACTH_{1-10}$ still induced a significant increase in 34 kDa phosphorylation (33%) whereas $ACTH_{11-24}$, β-endorphin and VIP were inactive. Thus, only peptides stimulating tyrosinase and melanin production also induced an increase in 34 kDa phosphorylation. These data indicate that the effects on 34 kDa phosphorylation are specific for melanotropic peptides. ^{32}P incorporation into the 43 kDa band (not shown) was also restricted to the melanotropic peptides tested although the effects were less conspicuous.

A possible role for cAMP in mediating the effects of α-MSH on 34 kDa phosphorylation was investigated with substances known to elevate intracellular levels of cAMP and known for their ability to mimick the effects of α-MSH. As shown in Table 1, dibutyryl cAMP (10^{-3} M) in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 10^{-4} M) increased 34 kDa phosphorylation by 228% of controls and forskolin (10^{-4} M) plus IBMX by 195%. IBMX alone also stimulated 34 kDa phosphorylation but to a lesser degree. This shows that substances known to elevate intracellular levels of cAMP also induce an increase in 34 kDa phosphorylation.

TABLE 1

EFFECT OF DIBUTYRYL-cAMP, FORSKOLIN AND ISOBUTYLMETHYLXANTHINE ON ^{32}P INCORPORATION INTO THE 34 kDa BAND (30 min ASSAYS)

Treatment	PAB [a]	Percent of control
Control	6.7 ± 0.3 ($n = 9$)	100 ± 5
α-MSH (10^{-6} M)	13.3 ± 0.5 ($n = 4$)	199 ± 8 *
db-cAMP (10^{-3} M) + IBMX (10^{-4} M)	15.2 ± 1.4 ($n = 4$)	228 ± 20 *
Forskolin (10^{-4} M) + IBMX (10^{-4} M)	13.0 ± 0.6 ($n = 7$)	195 ± 9 *
IBMX (10^{-4} M)	9.2 ± 0.5 ($n = 5$)	138 ± 8 *

[a] Peak height above background.

* Significantly different from control ($P < 0.001$).

Discussion

Incubation of Cloudman S91 mouse melanoma cells in a medium containing $^{32}P_i$ results in the labelling of numerous phosphoproteins. The labelling appears to be the result of a specific kinase-mediated reaction since the chemical linkage of the phosphate to the proteins was stable in acid but labile in alkali. Incubation of ^{32}P-prelabelled cells with α-MSH induced a specific increase in ^{32}P incorporation into two protein bands with estimated molecular weights of 34 and 43 kDa. The effect of the hormone is not due to effects on the specific activity of the endogenous γ-labelled ATP pool since (i) the specific activity of the ATP pool was unchanged during MSH-treatment (not shown), (ii) α-MSH did not affect overall ^{32}P incorporation into the proteins, and (iii) reversal of the α-MSH effect on 34 kDa and 43 kDa phosphorylation was not accompanied by changes in ^{32}P incorporation into other proteins.

It appears that α-MSH-induced changes in ^{32}P incorporation, at least into the 34 kDa protein band, are the result of MSH receptor stimulation since the effect is specific for MSH/ACTH peptides which have previously been shown to induce tyrosinase stimulation and melanin formation (Eberle et al., 1985). Furthermore, the effect is likely to be mediated through a cAMP-dependent protein kinase because forskolin, db-cAMP and IBMX stimulate ^{32}P incorporation into the 34 kDa protein band. Although cAMP-dependent protein kinase activity in Cloudman S91 cells was found to be increased by MSH (Burnett et al., 1981), we cannot rule out the possibility that the 34 kDa phosphorylation may also be dependent on MSH-induced changes of phosphatase activity or on other kinases. Such direct effects have been reported, e.g., for $ACTH_{1-24}$ which affects the activity of protein kinase C in rat brain membranes (Zwiers et al., 1976, 1980; Aloyo et al., 1982).

^{32}P incorporation into both the 34 kDa and the 43 kDa protein band was dose-dependent but

α-MSH concentrations required for inducing threshold or maximal values were not identical. In addition, the onset of 34 kDa phosphorylation was very short and comparable to that of cAMP production (see Fuller and Viskochil, 1979) whereas the induction of 43 kDa phosphorylation lasted longer. These findings are not surprising in view of preliminary experiments (Eberle et al., unpublished) which indicated that the two proteins are located in different cellular compartments (34 kDa: enriched in membrane fractions; 43 kDa: predominantly in the mitochondrial/melanosomal fraction) and that therefore the process of their stimulation would involve different steps.

The 34 kDa phosphorylation appeared to be the more interesting for the study of MSH receptor activation and signal transduction through the membrane. The fact that ^{32}P incorporation did not reach a plateau at α-MSH concentrations inducing maximal tyrosinase stimulation (EC_{50}: 2.5×10^{-9} M, and EC_{100}: 10^{-7} M; Eberle et al., 1985) or melanin production (EC_{50}: 2.5×10^{-8} M, and EC_{100}: 3×10^{-7} M; unpublished), may either point to an effect not directly involved in tyrosinase stimulation or to the possibility that only a small change in the degree of 34 kDA phosphorylation is required for eliciting the effect. This is reminiscent of the concept of intracellular regulation by cAMP where in many biological systems a fractional change was thought to be sufficient for maximal stimulation. It should also be noted that Bitensky et al. (1972) found maximal stimulation of adenylate cyclase in Cloudman S91 cell membranes at 10^{-5} M α-MSH and an EC_{50} at concentrations similar to that required for inducing changes in 34 kDa phosphorylation.

The effect of α-MSH on melanoma cells involves a multistep mechanism at different levels of cell regulation, such as changes in cAMP production, protein phosphorylation, RNA synthesis, protein synthesis, activation of tyrosinase and possibly other regulators of melanin biosynthesis and cell proliferation. Although the 34 kDa phosphorylation seems to be an 'early' event in this regulatory network, it is not possible to assign a specific role to this protein before it is further characterized, e.g. with two-dimensional separation, and its kinase identified. This may also help to detect other phosphoproteins involved in α-MSH action.

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