ACTIVATION OF MOUSE MELANOMA CELL CYCLIC AMP-DEPENDENT PROTEIN KINASE BY MELANOCYTE STIMULATING HORMONE

by

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STATEMENT BY AUTHOR

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ABSTRACT

The purpose of this study was to determine the activity of cyclic AMP-dependent protein kinase in Cloudman S91 melanoma cell cultures treated with MSH (melanocyte stimulating hormone), and to relate any changes in activity to other changes in these cells in response to the hormone. To carry out the study the assay for cyclic AMP-dependent protein kinase was adapted to the Cloudman cells. The parameters of cell concentration, incubation time, and sodium fluoride and sodium chloride concentrations were defined. With this assay it was shown that the activity of the kinase is higher in cells treated with MSH. Both the MSH dose response and the time course of stimulation of the kinase were observed to closely parallel the previously observed MSH induced changes in cyclic AMP levels. This evidence gives support to the theory that the effect of MSH on mouse melanoma tyrosinase activity is mediated by cyclic AMP and cyclic AMP-dependent protein kinase.
CULTURED CLOUDMAN MOUSE MELANOMA CELLS RESPOND TO MELANOCYTE
STIMULATING HORMONE (MSH) WITH AN INCREASE IN TYROSINASE ACTIVITY (23).
MSH HAS BEEN SHOWN TO EXERT ITS EFFECT ON THESE CELLS THROUGH THE "SECOND MESSAGER" CYCLIC AMP (23). A SERIES OF EXPERIMENTS HAVE BEEN INITIATED IN OUR LAB TO INVESTIGATE THE CHAIN OF BIOCHEMICAL EVENTS WHICH LEADS TO THE HORMONALLY INDUCED INCREASE IN THE ACTIVITY OF TYROSINASE, THE RATE LIMITING ENZYME IN THE SYNTHESIS OF MELANIN.

The proposed mechanism by which cyclic AMP mediates hormonal action involves a "cascade" of events which begins with the binding of the hormone to a membrane receptor. A membrane-bound adenylate cyclase system is then activated resulting in an increase in cytosolic cyclic AMP. The increase in cyclic AMP results in the activation of cyclic AMP-dependent protein kinase. The kinase has been shown to be able to phosphorylate many cellular proteins including enzymes, ribosomal proteins, membrane proteins, and various nuclear proteins (11,19). Phosphorylation of any (or any combination) of these proteins could play a role in mediating the cells ultimate response to the hormone.

Cyclic AMP-dependent protein kinase is thought to be universally present in animal cells. The enzyme has been isolated and characterized in many tissues and has been shown to vary little in structure or function (18,19). The kinase has two subunits and seems to exist as a tetramer (1). A catalytic subunit contains the phosphotransferase activity. It catalyzes the transfer of the \( \gamma \)-phosphate of ATP to serine and threonine residues in the substrate protein. A regulatory subunit has the ability to either bind and inhibit the catalytic subunit or bind cyclic AMP. The stoichiometry of the activation of the enzyme by cyclic AMP has been determined to be as follows:

\[
2\text{cAMP} + R_2\text{C}_2 \rightleftharpoons R_2\text{cAMP}_2 + 2\text{C}
\]

where \( R \) is the regulatory subunit, \( C \) is the catalytic subunit and \( R_2\text{C}_2 \) is called the holoenzyme (1).

Two major forms of the holoenzyme can be isolated in most tissues. The forms have been designated type I and type II in respect to
the order of their elution from DEAE cellulose with a salt gradient. The two types differ in their regulatory subunit while apparently sharing identical catalytic subunits (19). Many differences between the holoenzymes have been demonstrated. Other than the difference in their affinity for anion exchange medium the enzymes show differences in their size and molecular weight, autophosphorylation, cyclic AMP affinity, and their disassociation and reassociation characteristics in respect to substrate protein, cyclic AMP, MgATP, and salt concentration (19,4). The ratio of type I to type II holoenzyme varies from tissue to tissue and in the same tissue in different species (18,19).

Cloudman mouse melanoma has been shown to exhibit the classic response to a peptide hormone. Studies in our lab and others have shown that MSH treatment of Cloudman cell cultures elicits an increase in cyclic AMP levels in the cells (7,23) and that specific cytosolic binding of cyclic AMP occurs (6). It has also been shown that the plasma membrane of these cells contains adenylate cyclase activity which can be stimulated by MSH (16). Finally, another lab has reported cyclic AMP-dependent protein kinase activity in Cloudman melanoma and linked the kinase activity to stimulation of tyrosinase (14,22). Because of these findings we have carried out studies to determine if MSH stimulates the kinase and if the stimulation coordinates in a temporal and dose response fashion with the other components of the cascade system.
CHAPTER 2
MATERIALS AND METHODS

Compounds Studied
The α-MSH used in these studies was obtained from Dr. Victor Hruby of the Department of Chemistry, University of Arizona. Fetal calf serum and horse serum were obtained from Flow Labs and the penicillin/streptomycin from Microbiological Associates. Adenosine 5'-triphosphate, adenosine 3',5'-cyclic monophosphoric acid, and calf thymus histone type II-AS were purchased from Sigma Chemical Company. 1-methyl-3-isobutylxanthine was obtained from Aldrich Chemical Company. ATP-γ-32P, with a specific activity of 10-40 Ci/mmol, was purchased from New England Nuclear.

Cell Culture
Cloudman S91 NCTC 3960 (CCL 53.1) melanoma cells were obtained from the American Type Culture Collection Cell Repository. Cells were grown as a monolayer in 150 cm² Corning flasks in Ham's F-10 medium fortified with 2% fetal calf serum and 10% horse serum. Penicillin/streptomycin (100 units/ml, 100 ug/ml) was also present in the medium.

Preparation of Cell Fractions
For experiments cells grown to confluency were removed from their flasks as described in the figure legends. The cells were homogenized in 5.0 mM sodium phosphate buffer (pH 7.4) containing 1.0 mM...
EDTA, 0.5 mM 1-methyl-3-isobutylxanthine (MIX), and 5.0% glycerol. The homogenization was carried out in a Sorval Omni-mixer at setting six using two 30-second bursts with a one-minute cooling period between bursts. The homogenate was centrifuged at 30,000Xg for 30 minutes at 4°C. The supernatant was assayed or stored in liquid nitrogen. Cell counts were done with a hemocytometer.

**Assay for Cyclic AMP-dependent Protein Kinase Activity**

The method used was an adaptation of the method described by Corbin and Reimann (5). 20 ul samples of enzyme preparation were incubated with 50 ul of 20 mM potassium phosphate buffer (pH 6.5) containing 6.0 mM magnesium acetate, 0.5 mM MIX, 5.0 mg/ml histone (type II-A S), 0.2 mM AT^32P, and 5.0 x 10^{-4} M cyclic AMP. One uCi of ^32P activity was used per 50 ul of reaction buffer for a final specific activity, in the reaction buffer, of 0.1 uCi/mmol ATP. Reactions were carried out for the indicated times at 30°C and terminated by pipetting a 50 ul aliquot onto a Whatman 3MM filter disc. The filters were washed once in cold 10% TCA for 20 minutes, twice in 5% TCA for 10 minutes, and finally in methanol for 5 minutes. The filters were dried and counted in a Beckman LS-8000 scintillation counter.
CHAPTER 3

RESULTS

Kinase Assay Parameters

The assay for cAMP-dependent protein kinase has many variables which have not been well defined for melanoma. Thus, it was necessary to modify the assay procedure, as described by Corbin and Reimann (5), in order to optimize the assay conditions for melanoma.

The kinase assay takes advantage of the enzyme's high affinity for histone protein. In the assay the enzyme preparation is incubated with ATP-$\gamma^3^2$P and histone. The phosphorylated histone is then precipitated onto filter papers and the filter papers are counted for $3^2$P activity. The incorporation of $3^2$P into histone is taken to represent kinase activity. The cyclic AMP-dependence of the kinase activity is determined by assaying each enzyme preparation twice, adding an excess of cyclic AMP to one of the assays. The state of activation of the kinase in the enzyme preparation can then be expressed as a ratio of the sample assayed without added cyclic AMP (in situ activity) to the sample assayed with excess cyclic AMP (100% activity). Cyclic AMP-dependent protein kinase activity is thus described in terms of an activity ratio (-cAMP/+cAMP). Using this system an enzyme preparation in which the kinase is 100% activated will have an activity ratio of 1.0, since addition of exogenous cyclic AMP to the reaction mixture will have no added effect. It is important to realize that other histone kinase
activity might be present in the cells. For this reason the assay described can only be considered as a qualitative indication of cyclic AMP-dependent protein kinase.

The amount of protein kinase varies among different cells. Therefore, it is necessary to determine the optimum number of cells and incubation time to be used in the assay. Figures 1a and 1b represent data from the same experiment in which the effects of cell concentration in the reaction mixture were examined. Figure 1a demonstrates that at concentrations below 0.05 million cells/reaction the $^{32}$P incorporation is so low that the accuracy of the assay is impaired. Figure 1b, which expresses the data in terms of $^{32}$P incorporation/10$^6$ cells, shows that with concentrations of up to 0.40 million cells/reaction the incorporation per cell decreases but the ratio of -cAMP to +cAMP remains constant. The constancy of the activity ratio is an important consideration. At high concentrations of cells it would be expected that the reaction containing the highest activity, the +cAMP reaction, would be more affected by substrate availability. If this were the case, then the activity ratio would be artificially raised.

In figures 2a and 2b the effects of incubation time are demonstrated. Once again the same experiment is presented in two fashions. As the incubation time increases better $^{32}$P incorporation per reaction is seen. From figure 2a the incorporation in both reactions seems to be linear with time after the first few minutes. However, figure 2b shows that the values for the +cAMP reaction and the -cAMP reactions begin to converge with longer incubation. Based on the above data the subsequent
Figure 1. Effects of cell concentration on the kinase assay.

a, data expressed in $^{32}\text{P}$ incorporation/number of cells; b, data expressed in protein kinase activity/number of cells. Cells were removed from their flasks by treatment with Tyrode's buffer containing EDTA. The cells were washed twice by centrifugation and suspension in Ham's medium. Cell pellets were resuspended in the homogenization buffer to give the cell concentrations/reaction indicated. The suspensions were homogenized and assayed as described in the Materials and Methods section, using an incubation time of 10 minutes. Each point represents an assay done in quadruplicate, ± s. e. Assays were done with (o) and without (o) cyclic AMP. One unit of protein kinase activity represents the incorporation into histone of one pmol of $^{32}\text{P}$/minute.
Figure 1. Continued
Figure 2. Effects of incubation time on the kinase assay.

a, data expressed in $^{32}$P incorporation/number of cells; b, data expressed in protein kinase activity/number of cells. Cells were prepared as described in Figure 1, to give a concentration of approximately $0.10 \times 10^6$ cells/reaction. The kinase assay was carried out as described in the Materials and Methods section using the incubation times indicated. Each point represents an assay done in quadruplicate either with (○) or without (ο) cyclic AMP, ± s. e. One unit of protein kinase activity represents the incorporation into histone of one pmol of $^{32}$P/minute.
Figure 2. Continued

Protein Kinase Activity
(Units/10^6 cells)

32P Incorporated (cpm x 10^-3)
experiments were carried out using cell concentrations of 0.05 to 0.20 million cells/reaction and an incubation time of 10 minutes.

To assess the effects of MSH on kinase activity growing cells were treated with the hormone, removed from their flask, and assayed for cAMP-dependent kinase activity. For these studies it was important that nothing in the homogenization or incubation mixtures artificially altered the state of activation of the enzyme. For example, it has been shown that salt concentration can have an effect on the disassociation and reassociation of the subunits of the kinase (4). Type I kinase is susceptible to dissociation by high concentrations of salt while type II kinase tends to reassociate in low salt concentrations. Thus, when tissues containing primarily type I kinase, such as rat heart, are assayed, low ionic strength buffers are used. For tissues, like adipose, which contain predominantly type II enzyme, ionic strengths of up to 0.50 M are used. As shown in Table 1, high salt concentration in the homogenization buffer slightly increases the activity of melanoma kinase under the assay conditions employed. Since it has been reported that melanoma has primarily type I kinase, no salt was used when assaying for MSH stimulation.

Because ATPase can compete with kinase for substrate, kinase assays usually include sodium fluoride to inhibit ATPase activity. However, sodium fluoride can also inhibit kinase activity. In our cells the use of sodium fluoride gives no improvement over controls and slightly inhibits the kinase activity at higher concentrations (see figure 3). Therefore, sodium fluoride was not used in the assays.
Table 1. Effects of salt concentration on kinase activity. Cells were prepared as described in the captions of Figures 1a and 1b to give a concentration of approximately $0.15 \times 10^6$ cells/reaction. The homogenization buffer used contained the indicated concentrations of sodium chloride. The incubation time was 10 minutes. Each preparation was assayed in quadruplicate with cyclic AMP (+cAMP) or without (-cAMP). For a discussion of activity ratio see the Results section.

<table>
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<th>NaCl (M)</th>
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<tr>
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<td>0.42 ± 0.03</td>
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<tr>
<td>0.08</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>0.15</td>
<td>0.44 ± 0.05</td>
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<tr>
<td>0.30</td>
<td>0.64 ± 0.06</td>
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Figure 3. Effects of sodium fluoride on kinase activity.

Cells were prepared as described in Figure 1 using a cell concentration of approximately 0.10 x 10^6 cells/reaction, and using an incubation time of 10 minutes. Sodium fluoride was present in the reaction buffer at the concentrations indicated. Each point represents an assay done in quadruplicate ± s. e., either with (o) or without (o) cyclic AMP.
Effects of MSH on Kinase Activity in Melanoma

With a sensitive assay for cyclic AMP-dependent protein kinase activity in Cloudman melanoma it was possible to demonstrate stimulation of the kinase by incubation of the cells with MSH. The peak of kinase activity occurs after the cells are incubated with MSH for 30 minutes (figure 4). Activity declines after 30 minutes but remains slightly elevated above controls for as long as 90 minutes. An increase in activity after a 30 minute incubation can be detected in cells treated with as little as $1 \times 10^{-8}$ M$\alpha$-MSH (figure 5). A separate experiment which more closely examined the effects of $10^{-9}$ M$\alpha$-MSH showed that there is no stimulation over controls when cells are treated with that concentration of hormone.
Figure 4. Effects of MSH on kinase activity over time.

Cells were treated with MSH (10^{-7} M) for the times indicated. The cells were then washed, in the flask, with cold PBS. Homogenization buffer was added to the flasks and the cells were removed by scraping. The cells were homogenized and assayed as described in the Materials and Methods section. The cell concentrations used were approximately 0.10 x 10^6 cells/reaction and the incubation time was 10 minutes. Each point represents an assay done in quadruplicate, ± s. e. For a discussion of activity ratios see the Results section.
Figure 5. Dose-response for the effects of MSH on kinase activity.

Cells were treated with the indicated concentrations of MSH for 30 minutes. They were then removed from the flasks, homogenized, and assayed as described in Figure 4. Each point represents an assay done in quadruplicate, ± s. e.
Treatment of cultured Cloudman melanoma cells with melanocyte stimulating hormone (MSH) results in an increase in tyrosinase activity after a lag period of 6-9 hours (7,21). Because either dibutyryl cyclic AMP or theophylline will mimic the effects of MSH it has been suggested that MSH exerts its effects through cyclic AMP (21). Pawelek et al. have described an increase in cellular cyclic AMP levels in mouse melanoma in response to MSH stimulation (23). Fuller and Viskochil have shown that when the cells are exposed to MSH the concentration of cyclic AMP rises to a peak approximately 2 times control by 60 minutes (7). The cyclic AMP level remains at 1.5 to 2 times control level for at least 4 hours. Dose-response studies have shown that treatment of cells with concentrations of MSH as low as $10^{-8}$ M will produce significant increases in cAMP (6).

The evidence presented in this study shows that the activation of a cyclic AMP-dependent protein kinase in melanoma in response to MSH stimulation closely parallels the MSH-induced change in cyclic AMP concentrations. Korner and Pawelek have demonstrated that addition of partially purified cyclic AMP-dependent protein kinase preparations to the 30,000 Xg supernatant of melanoma cells will increase the tyrosine hydroxylase activity of tyrosinase (increased melanin formation was not seen) (14). Addition of protein kinase inhibitor blocked this
activation. The above data suggest a role for cyclic-AMP-dependent protein kinase in the regulation of tyrosinase activity.

Two theories have been proposed as to how cyclic AMP might exert control over tyrosinase activity. Both theories could involve cyclic AMP-dependent protein kinase in roles similar to those which have been attributed to the enzyme in other systems.

Körner and Pawelek have suggested that activation of tyrosinase results from the kinase-dependent phosphorylation and resultant deactivation of a tyrosinase inhibitor (14). They have found tyrosinase inhibitor activity but have not linked the activity to a phosphorylation event. They suggest that the lag period which occurs between hormone stimulation and tyrosinase activation is due to "an antagonism between kinase-mediated phosphorylation and phosphatase-mediated dephosphorylation."

On the other hand, Fuller and Viskochil suggest that the increase in tyrosinase activity is the result of de novo synthesis of the enzyme (7). They have shown that stimulation of tyrosinase activity by MSH, dibutyryl cyclic AMP, or theophylline can be blocked by cyclohexamide or actinomycin D. With double labeling experiments they have presented evidence that tyrosinase is synthesized in response to MSH stimulation. They suggest that the lag period for induction is due to the time needed for synthesis of the enzyme and its incorporation into melanosomes.

It has been clearly demonstrated that the activities of several enzymes are regulated by direct phosphorylation. A classic example is
the role of phosphorylation in the control of glycogen metabolism. Cyclic AMP-dependent protein kinase has been shown to inactivate glycogen synthetase and activate phosphorylase kinase and thus promote glycogen breakdown (26,19). Regulation by phosphorylation has also been demonstrated for hormone sensitive lipase (27), pyruvate kinase (28), tyrosine hydroxylase (30), and glycerol phosphate acyltransferase (20). A role for cyclic AMP-dependent protein kinase has been shown in each of these cases.

A strong correlation has been shown between the activation of cyclic AMP-dependent protein kinase and the induction of several enzymes including tyrosine amino transferase (29), ornithine decarboxylase (2), and tyrosine hydroxylase (9). The ability of several effectors to induce these enzymes corresponds closely to their ability to activate the kinase. The induction of these enzymes could occur at the transcriptional or the translational levels. However, studies on cyclic AMP-mediated phosphorylation of ribosomes has shown that phosphorylation can occur but does not result in increased protein synthesis (8). Thus, a transcriptional site has been suggested for the control of enzyme production.

Many studies have addressed the possible role of cyclic AMP-dependent protein kinase in the regulation of gene expression (for a review see Jungman and Russel) (13). A cyclic AMP-mediated translocation of kinase activity from the cytosol to the nucleus has been observed in several systems and cyclic AMP-mediated specific binding of cyclic AMP-binding protein and catalytic subunit to chromatin acceptor
sites has been shown in ovarian tissue (12). Based on this evidence a model has emerged in which the cytosolic kinase migrates to the nucleus and, through phosphorylation of nuclear proteins, regulates the synthesis of specific RNA species.

It is clear that neither of the theories on the regulation of tyrosinase activity is unprecedented. However, the phosphorylation of a tyrosinase modulator of non-histone chromosomal proteins is not the only means by which the kinase might exert its effects. For instance, cyclic AMP-dependent phosphorylation of membrane proteins has been indicated in the control of anion transport (24). Since tyrosinase resides primarily in melanosomes, membrane phosphorylation could certainly play a role in the enzyme's activity. The number of possible models for regulation by phosphorylation seem to be unlimited.

Krebs has described a set of criteria for the mediation of an effect of cyclic AMP by phosphorylation of a protein (15). The first criterion has been met for melanoma: the presence of a cyclic AMP-dependent protein kinase. The remainder, which involve the identification of a substrate and its activation and deactivation by phosphorylation and dephosphorylation, have yet to be studied. Phosphorylation of tyrosinase or of melanosomes has not been investigated. And though other protein substrates have been suggested none have been isolated.

Perhaps the most interesting finding in the study of melanoma is that of the apparent stimulation of de novo synthesis of tyrosinase by MSH through cyclic AMP. Though the data are not conclusive, if induction is involved then cultured melanoma presents an ideal system in
which to study one of the more interesting roles suggested for the action of cyclic AMP-dependent protein kinase.
REFERENCES CITED


