Multi-facet expressions of adenylate cyclase isoforms in B16-F10 melanoma cells differentiated by forskolin treatment

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Abbreviations: AC, adenylate cyclase; ACI, AC isoform I; cAMP, cyclic adenosine monophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; α-MSH, α-melanocyte stimulating hormone; PKA, cAMP dependent protein kinase; RT-PCR, reverse-transcription polymerase chain reaction

Abstract

The terminal differentiation of malignant melanoma cells is known to be induced by activating cAMP signaling pathway with α-MSH or cAMP analogues. However, sustained activation of cAMP signaling system that induces the differentiation of melanoma cells, also induces the desensitization of the pathway at the receptor level. Nevertheless, the adaptation of adenylate cyclase (AC) expression by sustained activation of cAMP signaling system has not been clearly understood. This study was performed to examine whether the sustained activation of cAMP system induce changes in the expression AC isoforms as an adaptation mechanism. Treatment of B16/F10 murine melanoma cells with 100 mM forskolin for 6 days resulted in differentiation, melanin accumulation and increased expression of tyrosine hydroxylase mRNA. In the forskolin-treated melanoma cells, change in expression of various AC isoform at the transcription level was detected by reverse-transcription polymerase chain reaction (RT-PCR). Expression of AC isoform mRNA: ACI, III, VI, VII, and IX increased to the level of 196-392% of the control whereas the level of ACII was decreased by 30%. The cAMP concentration was increased both in basal and α-MSH stimulated cells, but the AC activity was decreased in the forskolin treated cells. Thus, these results suggest that sustained activation of cAMP system induces differential expression of AC isoforms, which results in increase of cAMP accumulation.

Keywords: cAMP, adenylate cyclase, cyclic nucleotide phosphodiesterase, cAMP dependent protein kinase, G protein, RT-PCR

Introduction

Malignant melanoma originates from melanocytes in the basal layer of epidermis, and its incidence is increasing rapidly in the United States and Europe (Herlyn, 1993). Very high fatality could result from this cancer unless it is diagnosed and treated at an early stage. In rare cases, however, malignant melanoma regresses spontaneously by terminal differentiation of the cancer cells. Such spontaneous terminal differentiation can often be induced by activating cAMP signaling pathway with α-melanocyte stimulating hormone (α-MSH) (Sukhanov et al., 1993) or with dibutyryl cAMP (Giuffre et al., 1988). Understanding the mechanism of this spontaneous regression of melanoma can contribute to the development of a new therapy not only for melanoma but also for other cancers. Thus, defining the explicit molecular events underlying melanoma development and progression represents areas of intense investigation (Meier et al., 1998).

The differentiation-inducing α-MSH binds to its specific receptor located at the external surface of melanocytes and melanoma cells, and the resulting activated receptor then activates stimulatory GTP-binding proteins (Gs), which in turn stimulates adenylate cyclase to produce cAMP (Jeong et al., 1998 and Shah, 1999). The cAMP, as a second messenger, activates cAMP-dependent protein kinase (PKA), which in turn phosphorylates serine or threonine residue in many target proteins including those involved in diverse cellular functions such as metabolism, growth, differentiation, melanin synthesis, and cytokine production (Gilchrest et al., 1984; Rees and Healy, 1997). During the cAMP-induced melanoma cell differentiation, diverse signaling pathways are involved including phosphatidylinositol 3-kinase/p70(S6)-kinase (Busca et al., 1996), small G protein Rho (Busca et al., 1998), mitogen-activated protein kinase (Englaro et al., 1998), transcription factor microphthalmia (Bertolotto et al., 1998), proto-oncogene c-met (Rusciano et al., 1999), and cell cycle regulators (Haddad et al., 1999).
Sustained activation of cAMP signaling system that induces the differentiation of melanoma cells, on the other hand, also induces the desensitization of the pathway. Persistent stimulation of the cell surface receptors such as G protein coupled receptors (GPCR) dose not generally bring about a continuous response as the cell adapts to the presence of the stimulus and becomes desensitized. Receptor desensitization fulfills an important physiological role by acting as the feedback mechanism limiting both acute and chronic over-stimulation of the cascade. Furthermore, GPCR desensitization provides an effective mechanism by which information transduced by multiple GPCR inputs in a single cell can be filtered and integrated into a meaningful biological system (Ferguson and Caron, 1998). GPCR desensitization is known to involve the summation of several distinct events: uncoupling of receptors from their heterotrimeric G proteins, internalization of receptors to endosomes, and down-regulation. Sustained activation of cAMP system is achieved by increasing the concentration of cellular cAMP, the key regulator of the cAMP signaling pathway. Its formation from ATP is catalyzed by adenylate cyclase (AC) while its degradation is catalyzed by cyclic nucleotide phosphodiesterase (PDE). Therefore, it is potentially possible that the adaptation of cAMP system to the sustained activation can also take place downstream of the receptor such as AC, PDE, and cAMP dependent protein kinase (PKA) (Houslay, 1998). However, the adaptation of AC and PDE expression to sustained activation of cAMP signaling system has not yet been clearly recognized.

Mammalian AC is encoded by at least nine genes, and each isoform seems to have different kinetic and regulatory properties such as the response to calcium ions, beta gamma subunits of G proteins and protein kinase C (Sunahara et al., 1996). Such multiplicity of AC isoforms, together with the isoform multiplicity of PDE and PKA, is suggested to play an important role in the regulation of the cAMP signaling system in intracellular targeting, cross-talk between a wide variety of other signaling systems, and flux-controlled sensitivity (Houslay and Milligan, 1997).

This study was performed to examine whether the sustained activation of cAMP system induces changes in the expression of AC isoforms. Thus, using reverse-transcription polymerase chain reaction (RT-PCR), the expression of AC isoforms were analyzed in murine melanoma cells differentiated by forskolin treatment.

Materials and Methods

Materials

α-MSH, forskolin, bovine serum albumin, EGTA, HEPES, 3-isobutyl-1-methylxanthine (IBMX), and GTP were obtained from Sigma; agarose and reverse transcriptase [Superscript II RNase H(−)] Reverse transcriptase] were from GIBCO BRL; phosphocreatine, creatine phosphokinase were from Roche Diagnostics; Taq polymerase was from TaKaRa. All other reagents were of analytical grade.

Cell culture

B16-F10 mouse melanoma cells were purchased from American Type Culture Collection, and cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 100 IU/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere containing 5% CO2 in air at 37°C. The cells were treated with forskolin for 6 days by replacing fresh medium containing 100 µM forskolin in DMSO every other day.

Determination of melanin content

For melanin determination, cells from a confluent 6 cm diameter dish were harvested and homogenized in a 1.0 ml homogenization buffer containing 0.05 M Na2PO4, 200 nM PMSF, and 1X PIC. Part of the homogenate (800 µl) was solubilized in 200 µl of 1 M NaOH, and the samples were incubated at 60°C for 1 h and vortexed to solubilize the melanin. Absorbance of melanin was measured at 405 nm with a spectrophotometer, and melanin content was expressed as A405 per mg-protein. The protein content of the homogenate was determined by the Lowry method with bovine serum albumin as the reference standard (Lowry et al., 1951).

Quantification of tyrosine hydroxylase mRNA

The quantification of tyrosine hydroxylase mRNA was carried out by RT-PCR (Busca et al., 1996). In brief, total cellular RNA was prepared from control and forskolin-treated mouse melanoma B16 cells (Chomczynski and Sacchi, 1987), and the RNA (3 µg) was reverse transcribed using the reverse transcriptase. The cDNA obtained was subjected to 26 cycles of PCR (94°C, 1 min; 55°C, 1 min; 72°C, 2 min) using the following specific primers for the mouse tyrosine hydroxylase gene: 5'-CATTATTGAGTTGAGTCT and 5'-TGTGGTAGTGGTCTTGTGC, and an 1191-base pair PCR product was amplified. Specific primers for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were added as a control for the same reverse transcriptase product. The primers used were 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCTGTTGCTGA, and gave rise to an amplified PCR product of 495 base pair. Preliminary trials showed that, after 26 cycles of PCR, the reaction remained exponential. The PCR products were electrophoresed on 1% agarose gel containing ethidium bromide. The density of DNA band visualized by ultraviolet light was measured using a gel documentation system and the NIH Image J software.
RNA isolation and semi-quantitative RT-PCR of AC isoforms

The total RNA was isolated from cultured cells using the acid guanidinium thiocyanate phenol chloroform extraction method (Chomczynski and Sacchi, 1987). A series of four progressive dilutions was made by mixing varying amount of RNA from the forskolin-treated and the control sample to make a constant amount of total RNA in a constant volume to measure the relative amount of mRNA using the method of Nicoletti and Sassy-Prigent (1996). Three micrograms of the diluted RNA were reverse transcribed by reverse transcriptase and oligo-dT primers. The resulting cDNA was amplified using primers specific for each AC isoform. The primers used were as follows; for AC I 5'-AAG ATT AAC ACC ATT GGG AGC ACT TAC and 5’-CAC ATT GAC TGT GCT CCC CATGCG, for ACII (Sohma et al., 1999), for ACIII 5’-GCTGTCAAGCTCTATTACAT and 5’-CTGTGTCACTCTCTGTGTGACTC, for ACIV 5’-ATCAGAGCTCAGGTCCCCAG and 5’-AGGATGACCTGTGTCTCTTCTC, for AC V 5’-ACATGTGACGATGACGACGTG and 5’-AGGATGACCTGTGTCTCTTCTC, and 5’-AGGATGACCTGTGTCTCTTCTC and 5’-AGGATGACCTGTGTCTCTTCTC, for AC VI 5’-AGGATGACCTGTGTCTCTTCTC and 5’-AGGATGACCTGTGTCTCTTCTC, for AC VII 5’-AGGATGACCTGTGTCTCTTCTC and 5’-AGGATGACCTGTGTCTCTTCTC.

Measurement of cAMP Accumulation

From the B16-F10 melanoma cells in a 24-well plate, growth medium was removed and then cells were incubated in 500 µl serum-free Dulbecco modified Eagles medium containing 100 µM IBMX and in the presence or absence of 10 µM α-MSH for 30 min at 37°C. The treatment was terminated by aspiration of medium and addition of 2.5 M perchloric acid. Then, the acid extracts were neutralized with 4.2 M KOH, and the neutralized extract was frozen at -20°C until assay (Brown et al., 1971). The cAMP levels were determined by competitive binding with [3H]cAMP to a cAMP binding proteins, which was prepared by expressing the regulatory subunit Rlx of cAMP dependent protein kinase in E. coli. Production of cAMP was normalized to the amount of acid-insoluble protein.

Determination of AC activity

B16-F10 melanoma cells were harvested and homogenized in ice cold buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), and 10 mM MgCl2. Homogenates were centrifuged at 20,000 g at 4°C for 20 min. Pellets were rehomogenized and washed twice with the above buffers, recentrifuged, and resuspended in 10 mM Tris-HCl and 10 mM MgCl2, pH 7.5. Fresh membranes were used in all experiments. The adenylate cyclase assay was carried out as described by Ammer and Schulz (1997) with modification. Adenylate cyclase was determined in a reaction mixture (100 µl) containing 40 mM Tris-HCl, pH 7.4, 0.2 mM EGTA, 100 mM NaCl, 10 mM MgCl2, 0.5 mM ATP, 5 µg/ml phosphocreatine, 5 IU/ml creatine phosphokinase, 10 µM GTP, and 0.5 mM IBMX. Reactions were started by the addition of 5-100 µg of membrane protein, incubated for 10 min at 30°C, and stopped with 2.1 M perchloric acid followed by neutralization with 2.0 M KOH. The amount of cAMP generated was determined by cAMP binding assay (Brown et al., 1971), and adenylate cyclase activity was calculated as pmol/mg of protein min.

Statistical analysis

Results of mRNA expression were analyzed using the two tailed paired t-test, and other results were analyzed using Student t-test. Results were expressed as mean ± standard deviation (S.D.), and a probability level of 0.05 or smaller was used for statistical significance. All experiments were repeated at least three times in duplicate or triplicate.

Result

Differentiation of B16/F10 cells by forskolin treatment

To confirm whether B16/F10 melanoma cells were differentiated by forskolin treatment, cell morphology, melanin production, and expression of tyrosine hydroxylase were analyzed. The dendric morphology of the melanoma cells treated with 100 µM forskolin for 6 days became more pronounced as compared with the untreated cells (Figure 1A). The morphology of differentiated melanoma cells reverted to undifferentiated state when forskolin removed from the cultured medium (data not shown). The production of melanin, as one of the dif-
ferentiation marker of melanocytes and melanoma cells, was analyzed in B16-F10 melanoma cells following forskolin treatment. The melanin levels in these cells increased as the duration of the forskolin treatment increased, and reached the peak at day 6. When forskolin was removed after treatment for 6 days, the production of melanin decreased gradually and reached the basal level by 6th day (Figure 1D). In the forskolin treated B16-F10 melanoma cells, the expression of tyrosine hydroxylase, a key enzyme in the melanin synthesis pathways, increased sharply on the day 1 and reached peak on day 3. After removal of forskolin, the expression of tyrosine hydroxylase mRNA returned to the basal level on day 3 (Figure 1B and C).

Changes in the expression of AC isoforms in forskolin treated cells

The expression of mRNA of AC isoforms were analyzed by semi-quantitative RT-PCR in B16-F10 melanoma cells. The mRNA expression of ACI, III, VI, VII, and IX increased to 222%, 271%, 201%, 196%, and 392% of the control, respectively, by treatment with 100 µM forskolin for 6 days (Figure 2). In contrast, the expression of ACII decreased to 31.3% of the control. In addition, ACV was not amplified enough for detection, and ACIV and ACVIII were not amplified enough for a quantitative
Differential expression of AC isoforms in differentiated melanoma cells

Analysis by the RT-PCR method.

Changes in the AC activity in the forskolin-treated B16/F10 melanoma cells

AC activity was assayed to see the increment of mRNA expression of AC isoforms could result in a decrease of AC activity. Basal and forskolin stimulated AC activity were significantly decreased in the forskolin treated melanoma cells (Table 1).

CAMP response in the forskolin-treated B16/F10 melanoma cells

Table 1. Changes in AC activity in B16-F10 melanoma cells treated with forskolin. The AC activity was determined, in the presence or absence of 100 μM forskolin, in the fresh membranes prepared from the forskolin-treated and control cells. The formed cAMP was quantified by competitive binding with [3H]cAMP to a cAMP binding proteins after stimulation with buffer only (basal) or buffer containing 100 μM forskolin for 10 min.

| Condition          | AC activity (pmol/mg protein min) |
|--------------------|----------------------------------|
| Control            |                                   |
| Unstimulated       | 87.7 ± 13.1                       |
| Forskolin stimulated| 478 ± 105                         |
| Forskolin treated  |                                   |
| Unstimulated       | 10.9 ± 2.4                        |
| Forskolin stimulated| 345 ± 29                          |

Table 2. Changes in accumulation of cAMP in B16-F10 melanoma cells treated with forskolin. After B16/F10 melanoma cells were treated with 100 μM forskolin for 6 days, cAMP levels were analyzed by competitive binding with [3H]cAMP to a cAMP binding proteins after stimulation with buffer only (basal) or buffer containing 10 μM α-MSH for 30 min.

| Condition          | cAMP (pmol/mg protein) |
|--------------------|------------------------|
| Control            |                        |
| Basal              | 5.3 ± 3.2              |
| α-MSH-stimulated   | 2266 ± 489             |
| Forskolin treated  |                        |
| Basal              | 1109 ± 736             |
| α-MSH-stimulated   | 5083 ± 1102            |

Analysis of the cAMP levels in B16/F10 melanoma cells following forskolin treatment showed, the basal level of cAMP was 1109 pmol/mg protein and about 4.1-fold higher than the control (5.3 pmol/mg protein). Stimulation with α-MSH increased cAMP level sharply to 2266 pmol/mg protein in the control, and 5083 pmol/mg protein in the forskolin-treated B16/F10 melanoma cells. Thus 2.2-fold more cAMP was accumulated in the forskolin-treated cells when they were stimulated by α-MSH (Table 2).

Discussion

The present study was carried out to examine whether the sustained activation of cAMP system induces changes in the expression AC isoforms. We found that sustained activation of adenylate cyclase with forskolin induces differential expression of AC isoforms at the mRNA level, which results in accumulation of cAMP and decrease of AC activity in the B16-F10 melanoma cells. Such result demonstrates that the AC activity can be modulated depending the activation of cAMP system, therefore, the change in the AC expression may be considered as an adaptive response of the system. It may be an example of post-receptor adaptation of cAMP signaling system.

The up regulation of all the AC isoforms except ACII indicates that there is some positive feedback mecha-
such positive feedback loop of AC expression might play an important role in forskolin-induced differentiation of B16-F10 melanoma cells. However, it is not clear whether such positive feedback loops could apply to other mechanisms that activate cAMP systems, induced by β-adrenergic agonists or cAMP analogues, and expression of a constitutively activated mutant of stimulatory G protein. It should make it clear whether there exists any cell type specificity in the positive feed back loop of AC expression induced by forskolin treatment, with regard to the specific role of activated cAMP system in the cellular proliferation, differentiation, and apoptosis.

The extent of increase in AC isoform mRNAs in this study was significantly different among each AC isoforms; some increased markedly and the others slightly. Such differential expression imply that the same stimuli, the forskolin treatment, resulted in different effects on the expression of each AC isoform, reflecting the fact that mammalian AC's are encoded by at least 9 separate genes which are not clustered, and localized on different chromosomes in the human genomes (Hanoune et al., 1997). The promoter regions of all the AC genes are not known, but the expression of individual AC isoform, based our results, would likely be regulated by different mechanisms. The expression of AC isoforms changed in a similar pattern to that of tyrosine hydroxylase as the duration of forskolin treatment increased. The mRNA expression reached the peak on the day of 3, and then decreased sharply or maintained higher level depending on the isoforms. The expression of ACII mRNA reached the peak on day 3 but decreased sharply after the peak so that it reached a decreased level compared to the control on the day 6 (data not shown). In addition, the expression of ACII is suggested to be regulated under different mechanisms from other isoforms.

AC isoforms have different kinetic and regulatory properties, such as regulation by inhibitory G proteins, beta-gamma subunits of G proteins, calcium, calmodulin, and protein kinases (Hanoune et al., 1997). Thus the observed differential increase in the expression of AC isoforms may result in both the increase of the enzyme activity and the changes in the regulatory properties in the melanoma cells. The changes in the regulatory properties of cAMP system can change the cellular response of signaling systems coupled to the cAMP system including hormones and neurotransmitters. Such changes might contribute to the differentiation process of B16-F10 melanoma cells by changing the expression patterns of many target genes of cAMP dependent protein kinase.

Though treatment with forskolin increased the expression of AC isoform mRNA, AC activity decreased significantly in this study. Such decrease can be resulted from decrease in protein concentration by either enhanced degradation or reduced synthesis. Thus, the analysis of protein expression of each isoform is needed to prove this possibility. The observed decrease in AC activity could result from the inhibition by protein kinases. This mechanism is supported by the reports that AC activity is reduced by cAMP dependent phosphorylation (Premont et al., 1992) and that the underlying mechanism may be the inhibitory phosphorylation of ACVI by PKA on Ser 674 (Chen et al., 1997). Protein kinase C also inhibits ACVI activity during desensitization of the A2a-adenosine receptor-mediated cAMP response (Lai et al., 1997). The observed decrease in the AC activity is suggested as a feedback inhibition of sustained activation of cAMP system to prevent excessive stimulation of the system.

The level of cAMP was significantly increased in the basal and the α-MSH stimulated B16/F10 melanoma cells treated with 100 µM forskolin for 6 days. The increase in cAMP levels can result from increased synthesis, decreased degradation, or both. Because both the basal and the forskolin-stimulated AC activity were decreased in forskolin treated cells, the increase in cAMP accumulation is more likely from reduced degradation of cAMP. Thus the analysis of the activity change of the cAMP-degrading enzyme, cyclic nucleotide phosphodiesterase, would be helpful for explaining the mechanism of increased cAMP response in the forskolin-treated SH-SY5Y neuroblastoma cells (Cho et al., 2000). Another possibility is the activation of AC by α-MSH is not inhibited in contrast to the activation by forskolin, which must be analyzed to understand the mechanism of the increased cAMP accumulation. The resulting increase in cellular cAMP level increases the activity of PKA, which regulates cellular metabolism and gene expression by phosphorylating various target genes including those involved in the differentiation of B16-F10 melanoma cells (Daniel et al., 1998).

Treatment with forskolin induces differentiation of B16/F10 melanoma cells as recognized by the morphological changes, increase in melanin synthesis, and increased expression of tyrosine hydroxylase in this study. Sustained activation of the cAMP pathway is known to result in accumulation of large quantities of melanin and terminal differentiation of melanocytes (Medrano et al., 1994). However, the changes in the melanin synthesis and tyrosine hydroxylase expression were reverted to its basal level when forskolin was removed from the medium after 6-days of treatment, suggesting that the observed changes are reversible in nature. It seems to be important to find what makes differentiation of melanoma cells irreversible, and studies like differentiation induction subtraction hybridization (DISH) may be useful for this purpose (Huang et al., 1999).

Because numerous genes and signaling systems are involved, including the small GTP-binding protein Rho, phosphatidylinositol 3-kinase/p70(S6)-kinase, extracellular signal-regulated kinase (ERK), NF-κB (Englaro et al., 1997), signal-regulated kinase (ERK), NF-κB (Englaro et al., 1997).
Differential expression of AC isoforms in differentiated melanoma cells

Differential expression of AC isoforms in differentiated melanoma cells (Aberdam et al., 1998), microphthalmia-associated transcription factor (Aberdam et al., 1999), microphthalmia-associated transcription factor (Aberdam et al., 1998), in the differentiation of B16-F10 melanoma cells, the contribution of differential expression of AC isoforms observed in this study to the differentiation of the forskolin-treated melanoma cells is not clear at present. Thus it is not certain whether such change in the AC isoform expression is a simple adaptation mechanism for sustained activation of cAMP system, or it is involved directly in the differentiation procedures. However, the differential expression of AC in concert with multi-potent AC signaling system play an important role in the differentiation process of the melanoma cells. Such implication is supported by some studies to show that cyclic AMP is a key messenger in the regulation of skin pigmentation (Busca and Ballotti, 2000), and that there exists a melanocyte-specific Ras exchange factor directly regulated by cAMP (Busca et al., 2000).

This study suggests that sustained activation of cAMP signaling system induces differential expression of AC isoforms, resulting in increase cAMP accumulation. This differential expression of AC isoforms may further indicate that there is a positive feedback loop in AC expression in B16 melanoma cells, and that it may be involved in the process of differentiation of B16-F10 melanoma cells by changing phosphorylation patterns of target genes.

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