CYCLIC-3',5'-AMP-DEPENDENT AND INDEPENDENT PROTEIN KINASE LEVELS IN NORMAL AND FELINE SARCOMA VIRUS TRANSFORMED CELLS

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SUMMARY

The results of these studies have revealed no differences in the level of the cyclic-3',5'-AMP (cAMP)-dependent or independent protein kinases, using calf thymus histone as substrate, in normal and feline sarcoma virus transformed cells. Similarly, the degree of responsiveness of the basal protein kinase activity to cAMP was also identical in the two cell types. These experiments have been carried out in normal, bovine-derived (thymic) fibroblasts and confirmed in feline-derived, embryonic mixed cell cultures. Thus, these results are consistent with the conclusion that one of the major amplification mechanisms for cAMP is not altered following viral transformation.

Associated with the loss in the density-dependent inhibition of cell replication following viral transformation of many mammalian cells in culture is a decrease in the level of cAMP (1,2) and alterations in the structure of several macromolecular components of the cell periphery (3-9). When normal cells in culture collide, a signal may be transmitted from the cell membrane to the nucleus which may be involved in regulating cell division (10). cAMP has been well established as an important "mediator-messenger" for a number of hormones and other cellular processes (11). The signal for the inhibition of growth in normal cells may also be mediated by cAMP which is synthesized by the membrane-associated adenyl cyclase. When cAMP or its dibutyryl analogues are added to transformed cells, there is a transitory restoration of growth control as well as morphological changes which resemble the normal cell (12,13). The change is reversible, since removal of the nucleotide returns the cells to their original transformed character.

Steady state levels of cAMP in some transformed cell lines are approximately one-half the levels in the corresponding normal cells (1,2). Evidence from several laboratories indicates that cAMP levels in different cells are related to their growth rate (1,2,14). Further, there is a rapid fall in the level of this nucleotide just prior to cell division (15). Thus, these observations suggest that the intracellular level

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of cAMP may regulate mitotic activity and that transformed cells may be considered to have a "permanent" mitotic character.

The exact mechanism for the inhibitory action of cAMP on cell growth is presently unknown. cAMP is a known effector of protein kinases (16) which may also be involved in the regulation of nuclear activity (17,18). It was of interest to look at the comparative level of these enzymes in normal and C-type virus transformed cells since it is possible that the level of coenzymes and effectors could also have an influence on the level of the enzyme upon which they act.

EXPERIMENTAL PROCEDURE

Cell Culture - Normal bovine monolayer cell cultures (BNT) were initiated from a thymic biopsy of a newborn calf. All cultures were propagated in 250 ml plastic flasks (Falcon) containing 30 ml Leibovitz medium supplemented with 10-15% (v/v) heat inactivated fetal calf serum (complete medium). The cultures were maintained at 37° and split 1: 2 - 1: 6 as the cultures became confluent.

Feline Sarcoma Virus (FSV) Infected Cells - Normal bovine cell cultures were altered morphologically by infection with known feline sarcoma virus (19). The sarcoma virus was purified initially from feline fibrosarcoma by density gradient and used to infect feline tissue culture cells free of known agents.

Growth Characteristics - The growth characteristic of the normal and virus infected cell cultures were determined by counting the number of cells per cm^2 in 30 ml plastic flasks (Falcon). Three independent counts were made twice per day and the average growth rate was used to plot the growth curve.

Preparation of Protein Kinases from Normal and Transformed Cells - After four days of growth the cells were washed once in cold Dulbecco's phosphate buffered saline (PBS) and harvested by scraping with PBS containing 0.5% EDTA. Cell viability was determined by exclusion of Trypan blue. The cell pellets were resuspended in either sucrose-Tris buffer, pH 7.2, (De Robertis et al. (20)) or the same buffer containing 0.1% Triton X-100 and disrupted by 15 strokes in a Potter homogenizer. The homogenates were centrifuged at 48,000 g for 30 min and aliquots from the supernatant fractions (Su-1) were analyzed directly for protein

kinase activity. As a precautionary measure, transformed cells were disrupted in the presence of 0.1% Triton X-100 since this non-ionic detergent disrupts the FSV particles present in these cells. In order to determine the effect of Triton X-100 on the activity of protein kinase, normal cells were treated under identical conditions and compared to nontransformed cells disrupted in the absence of Triton X-100.

Protein Kinase Assay - Protein kinase activity was determined at pH 6.9 using a modification of procedure B as described by Corbin et al. (21). The mixed fraction from calf thymus histone (Sigma, Type IIA) was used as the phosphoryl acceptor. Each incubation mixture contained the following components in a final volume of 80 µl: 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.9, 2 µ moles; magnesium acetate, 0.3 µ moles; EGTA, 0.02 µ moles; [γ-^{32}P]ATP, 0.01-0.04 µ moles (3.5×10^4 - 1.2×10^6 dpm/nmole); cAMP (when added), 0.1 nmoles; histone, 0.6 mg; and enzyme protein, 3-15 µ g. The reaction was initiated by the addition of [γ-^{32}P]ATP. cAMP (when present) was added to the incubation mixture just prior to initiation. Protein kinase activity determined in the absence of cAMP is defined as cAMP-independent activity. All incubations were carried out at 37° for 10 minutes or less, conditions under which the rate of phosphorylation was proportional to enzyme concentration and time. Reactions were terminated by transferring 50 µl samples onto filter paper discs and washing in cold 10% trichloroacetic acid, ethanol and ether as described by Corbin et al. (22). The amount of ^{32}P-labeled histone which remained on the filter paper disc was determined by counting in a vial which contained a toluene-based scintillation fluid as previously described (23). Results are expressed as the nmoles of ^{32}P incorporated per mg protein per 10 min. Protein was determined by the method of Lowry et al. (24).

RESULTS

A comparative growth curve characteristic of normal and FSV infected cultures is shown in Fig. 1. The normal and virus infected cultures were seeded initially with equal number of cells and showed nearly identical growth rates with generation times of approximately 24 hours. The nontransformed cell cultures de-

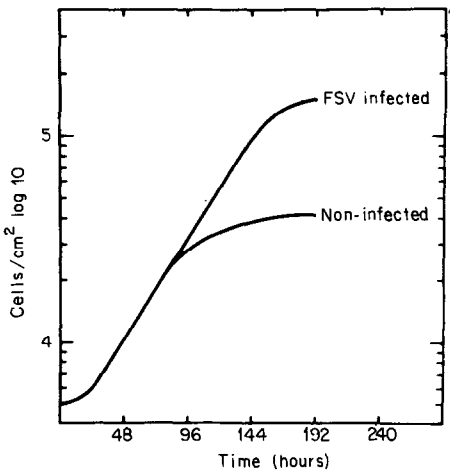

Figure 1. Growth characteristics of normal and FSV transformed bovine fibroblasts. Cells were grown as described under "Experimental Procedures".

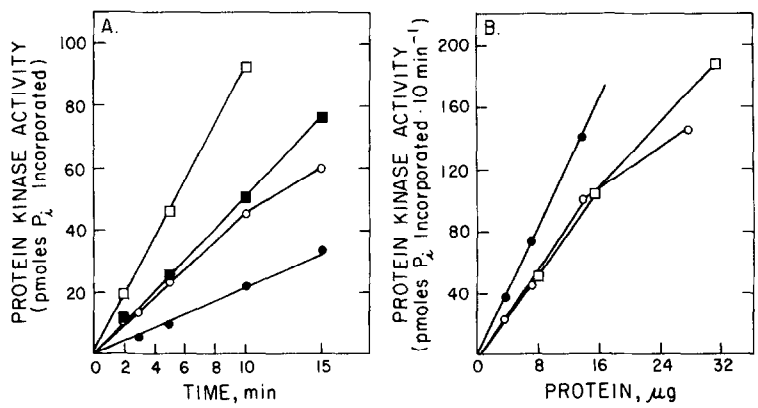

Figure 2. Properties of the cAMP-dependent protein kinases in normal and FSV transformed fibroblasts. The effect of the incubation time (A), and protein concentration (B) on histone phosphorylation catalyzed by protein kinases isolated from the two cell types as described under "Experimental Procedures". In A, 3.43 μg of protein (●——●) and 6.9 μg of protein (○——○) from normal BNT cells and 7.8 μg (■——■) and 15.6 μg of protein (□——□) from FSV transformed cells. In B, (●——●), normal BNT cells disrupted in the absence of Triton X-100; (○——○), normal BNT cells disrupted in the presence of 0.1% Triton X-100; and (□——□), FSV transformed cells disrupted in the presence of 0.1% Triton X-100. Incubation time, 10 min.

creased in growth rate as the cultures reached confluency while virus infected cultures continued to increase in number until the total number of cells was 4 to 5 fold greater than the normal cultures. Thus, the virus infected cultures exhibited a loss in the property of density-dependent inhibition of cell replication which is a characteristic of the transformed state. In addition, these cells also showed mor-

Table I. cAMP-dependent and independent protein kinase activity in normal and feline sarcoma virus transformed thymic fibroblasts. The protein kinase assay was carried out as described under "Experimental Procedures". The results reported in each column represent the average values calculated from assays carried out at three different protein concentrations under conditions where the rate was constant with time and proportional to enzyme concentration. The viability of all cell types used was > 95%. For Experiment 1, 2.7×10^7 normal and 6.96×10^7 transformed cells were disrupted. The normal cells were at passage level 15. In Experiment 2, 3.72×10^7 normal cells (passage level 15; 97% viable) and 7.75×10^7 transformed cells (passage level 16; 98% viable) were disrupted.

Cell Type	Treatment*	Protein Kinase Specific Activity (nmoles ^{32}P mg protein^{-1} 10 min^{-1})		Stimulation By cAMP
Experiment 1		minus cAMP‡	plus cAMP	
Normal BNT	None	5.52	10.41	1.88
Normal BNT	TX-100	3.87	6.62	1.72
FSV Transformed	TX-100	3.40	6.19	1.82
Experiment 2				
Normal BNT	None	11.21	22.63	1.98
Normal BNT	TX-100	7.66	14.75	1.92
FSV Transformed	TX-100	7.47	14.78	1.98

* Refers to disruption of the cells in sucrose-Tris buffer containing 0.1% Triton X-100 as described in the text. The final concentration of detergent in incubation mixtures from cells disrupted in the presence of Triton X-100 was only 0.0012-0.012% since the volume of Su-1 required for assay was only 1.0 to 10 µl.

‡The maximal concentration of cAMP in the incubation mixtures in Experiment 1 was 0.142-1.63 nM and in Experiment 2, 0.186-4.57 nM. These minute amounts were contributed by the enzyme preparation (Su-1 fraction) itself.

phological alterations (hyper-refractive, rounded cells) and produced infectious viral particles in vitro.

As shown in Fig. 2, the rate of the protein kinase catalyzed incorporation of ^{32}P from [γ-^{32}P]ATP into histone was constant with time of incubation (A) and proportional to enzyme concentration (B) in extracts prepared from both transformed and nontransformed cells. Deviation from linearity with respect to protein concentration was generally observed in standard incubation mixtures at enzyme protein concentrations above ca. 15 µg.

Table I summarizes the results from experiments designed to assess the comparative levels of the cAMP-dependent and independent protein kinases in normal and viral transformed cells. These results clearly show no differences between the two cell types in the activity of either of these enzymes or in the level of responsiveness (approximately 2-fold) of the basal protein kinase activity to stimulation by exogenous cAMP at 1.25 μM. Thus, these results support the contention that neither the cAMP-dependent protein kinase, which is one of the major amplification mechanisms for cAMP, nor the cAMP independent protein kinase, are altered following viral transformation. Also of interest from Table I is the consistent observation that the activity of both the cAMP-dependent and independent protein kinases are decreased approximately 30-35% when cells are disrupted in buffer containing 0.1% Triton X-100.

Confirmation of these results and therefore support for the above conclusions was obtained using a feline embryonic mixed cell population which was also infected with FSV. The results of these experiments are described in Table II. Two types of viral transformed cells, kindly provided by Dr. Charles Aldrich of the Department of Pathology, were used. The first, designated "virus producing", exhibited morphological characteristics of transformation and produced viruses in vitro while the second, designated "non-virus producing", appeared morphologically transformed yet did not, at the time these experiments were run, produce detectable viruses in culture. Subsequently, however, these cells did begin to elaborate detectable FSV particles[1]. The precise mechanism for this "pseudo" or apparent latency in viral production is not known but it appears evident from the data in Table II that it is a phenomenon which is also not related to the level of cAMP-dependent or independent protein kinases.

DISCUSSION

The results reported here reveal no differences in the activity of the cAMP-dependent or independent protein kinases from normal and FSV transformed cells

[1]Personal communication from Dr. Charles Aldrich.

Table II. cAMP-dependent and independent protein kinase activity in non-infected feline embryo and FSV transformed feline embryo cells. All three cell types (passage level 15) were grown to confluency (4 days) as described for the BNT cells in 75-cm^2 T-flasks. Fresh growth medium was added and the cells were harvested after an additional 24 hours of growth as described under "Experimental Procedure". The density of all three cultures at the time of isolation was 1-2 x 10^7 cells per flask. The protein kinase was assayed exactly as described in the text and the results represent average determinations as described in the legend to Table I.

Cell Type	Protein Kinase Specific Activity (nmoles ^{32}P mg protein^{-1} 10 min^{-1})		Stimulation By cAMP
	minus cAMP‡	plus cAMP	
Non-infected Feline Embryo	11.10	21.38	1.93
FSV Transformed			
-non-virus producing	11.63	22.23	1.93
-virus producing	10.16	21.50	2.11

‡The maximal concentration of cAMP in these incubation mixtures was 0.32-3.76 nM (see legend, Table I).

when calf thymus histone is used as substrate. Furthermore, the level of responsiveness of the basal protein kinase activity to cAMP was also identical in the two cell types. Thus, these results suggest that the major amplification mechanism for cAMP is not altered following viral transformation. At present, nearly all the protein kinase activity directly related to histone phosphorylation is thought to be due to the free catalytic subunit (C) which arises by dissociation from the inactive regulatory-catalytic (RC) complex in the presence of cAMP (25). Thus, under these experimental conditions, these data are in accord with the conclusion that the concentration of C, measured both as the free catalytic subunit and as the RC complex, is identical in normal and transformed cells. It also follows from this conclusion that neither transcriptional nor translational control of the C subunit is altered following viral transformation. Furthermore, protein kinase synthesis does not appear to be regulated by intracellular levels of cAMP, although it is well established that the activity of protein kinase is modulated by this nucleotide. A

definitive answer as to whether the regulatory subunits are identical in normal and viral transformed cells awaits the provision of additional experimentation. Similarly, extension and verification of these results in other normal and transformed cell lines should provide critical insight into at least one functional parameter associated with cellular transformation.

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