DIBUTYRYL CYCLIC ADENOSINE 3′ 5′ MONOPHOSPHATE, SUGAR TRANSPORT, AND REGULATORY CONTROL OF CELL DIVISION IN NORMAL AND TRANSFORMED CELLS

WILLIAM J. GRIMES and JUDITH L. SCHROEDER

From the Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85721

ABSTRACT

Swiss 3T3 cells exhibit contact-regulated cell growth and have a lower ability to transport 2-deoxyglucose than polyoma (Py)-transformed 3T3 cells. Py3T3 cells treated with dibutyryl cyclic adenosine 3′5′ monophosphate (dBcAMP) and theophylline have reduced cell growth and transport 2-deoxyglucose at the same rate as normal 3T3 cells. Evidence that the cessation of cell growth and reduced transport abilities in Py3T3 cells does not represent a return to contact-regulated growth comes from the following observations. First, treating high density Py3T3 cells with dBcAMP allows more than two doublings of cell number, even though ability to transport 2-deoxyglucose is returned to levels equal to those of normal 3T3 cells. Second, dBcAMP prevents serum-stimulated increases in 2-deoxyglucose transport in Py3T3 but not in 3T3 cells.

Growth of normal cells is characterized by density-dependent regulatory control of cell division. Spontaneous or virally-transformed cells lack growth control and continue to divide even when in contact with other cells. These phenomena have led to the current dogma that cell surface alterations are primary in neoplastic transformation. Two biological parameters, agglutination by plant lectins and transport, have been used to study cell surface alterations in transformed cells.

Studies using plant lectins have shown that RNA and DNA virus-transformed cells are agglutinated by much lower concentrations of wheat germ agglutinin (WGA) or concanavalin A than their normal counterparts (1–4). Since dilute protease treatment renders normal cells agglutinable at low lectin concentrations, it is believed that the differences are primarily architectural (2, 5). The involvement of these changes in growth control is evidenced by observations that dilute protease treatment can stimulate contact-inhibited cells to divide (6, 7). Reversion of transformed cells to contact-regulated cell growth by addition of trypsinized concanavalin A further implicates these cell surface sites in contact inhibition (8). Further support for this notion comes from the recent report that concentrations of lectins required for agglutinating chick cells transformed by a temperature-sensitive mutant of Rous sarcoma virus (RSV) were low at the permissive temperature for transformation and high at the nonpermissive temperature for transformation (9).

The transport of amino acid analogues, uridine, and sugars, including 2-deoxyglucose, has been reported to be altered in transformed cells (10–14). In every case, the transformed cells are capable of taking up nutrients at much greater rates than their normal counterparts This phenomenon is related to growth control since the growth response seen when fresh serum is added to contact-inhibited normal cells is accompanied by rapid increases in the rates of transport of uridine and 2-deoxyglucose (11, 15, 16). In addition, treating normal cells with dilute trypsin causes a rapid

stimulation in 2-deoxyglucose uptake (16). As was observed in studies using lectins, chick cells transformed by temperature-sensitive mutants of RSV are temperature sensitive in 2-deoxyglucose transport (17).

In the past year, cyclic adenosine 3′5′ monophosphate (cAMP) has been implicated in the regulation of cell growth. These conclusions resulted from the observation that adding dibutyryl cyclic adenosine 3′5′ monophosphate (dBcAMP) altered the morphology of transformed cells grown in tissue culture (18, 19). The most clear-cut demonstration of the involvement of dBcAMP in growth control came from studies of 3T3 cells transformed by polyoma virus (Py3T3) (21). Adding dBcAMP and theophylline to Py3T3 cells caused a cessation of cell division at cell densities where 3T3 cells exhibit contact-regulated growth. That this phenomenon was related to cell surface alterations was shown by the fact that both 3T3- and dBcAMP-treated Py3T3 cells require much higher concentrations of WGA for agglutination than do Py3T3 cells (20). Evidence has now been presented that transformed cells have reduced levels of cAMP as compared with normal cells, and that treatments with fresh serum and proteases could cause a rapid drop in the cAMP levels of normal cells (21, 22).

In this communication, effects of dBcAMP on growth and 2-deoxyglucose transport have been determined. We have attempted to ask whether alterations caused in transformed cells by dBcAMP leads to a membrane which exhibits properties similar to that of normal cells.

METHODS

3T3 and Py3T3 cells were obtained from Dr. Howard Green, Massachusetts Institute of Technology and from Dr. Jack R. Sheppard, University of Colorado Medical School. Cells were grown in media composed of Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) with four times the usual concentration of vitamins and amino acids (4 × MEM), 10% fetal bovine serum and penicillin and streptomycin at concentrations of 76 U and 50 μg per ml, respectively. Cell lines were routinely tested and found to be negative for mycoplasma contamination. Cells were grown in 35-mm tissue culture dishes containing 2 ml of medium. For measurements of cell saturation densities in the presence and absence of dBcAMP, control medium or medium containing 2 mm of theophylline and 0.2 mM dBcAMP was changed each day during the course of the experiment. Uptake of 2-deoxy[^{3}H]-glucose was measured by methods similar to those described previously (16). Cells in a monolayer were washed with warmed PBS (0.8% NaCl, 0.05% KCl, 0.001 M KPO_4, pH 7.4, and containing 100 mg/liter $CaCl_2$ and $MgCl_2$). 1 ml of PBS containing 0.25 μCi of 2-deoxy[^{3}H]glucose (New England Nuclear Corp., Boston, Mass.; sp act 7.2 Ci/mmole) was then added. After 10 min at 37°C, cells were washed four times with 2 ml of ice-cold PBS. Washed cells were dissolved in 1 ml of Lowry C solution. One aliquot was neutralized by adding 100% trichloroacetic acid (TCA) and placed in a scintillation vial containing 10 ml of Patterson-Greene solution (23). Radioactivity was determined in a Beckman LS250 scintillation counter. A second aliquot was used for protein determination by the method of Lowry et al. (24).

dBcAMP Effects on Growth and Transport

Py3T3 cells are not sensitive to density-dependent growth, and form multilayered colonies on tissue culture dishes. Adding dBcAMP and theophylline to Py3T3 cells results in an inhibition of cell division at cell densities similar to the limiting growth densities of normal 3T3 cells (Fig. 1). This confirms results reported previously (20). Measurements of uptake show that transport rates correlate with ability of cells to maintain uncontrolled growth. Py3T3 cells grown in the presence of dBcAMP and theophylline are indistinguishable from 3T3 cells in ability to transport 2-deoxyglucose. It has been reported that rates of amino acid uptake measured in cells can be influenced by the intracellular pools (14). Therefore, uptake rates of 2-deoxyglucose were measured after cells had been washed and incubated for up to 45 min in the presence of PBS. Preincubation had no effect on rates of 2-deoxyglucose uptake in any of the cells. Time courses of uptake were linear for up to 1 h. It has previously been reported that levels of hexokinase did not differ between normal and transformed cells (13). The uptake rates measured in these experiments should therefore indicate ability of cells to transport 2-deoxyglucose.

Effects of Cell Density

Measurements of uptake rates were independent of cell density and the length of time cells had been growing on plates (Fig. 2). Even transformed cells that had reached high densities transported 2-deoxyglucose at the same rate as low density transformed cells. Adding dBcAMP and theophylline to Py3T3 cells had markedly different results, depending on the density of the cells (Fig. 3). Cells at very low density were killed by addition of dBcAMP and theophylline. Cells at intermediate density responded to dBcAMP by cessation of cell division after reaching densities

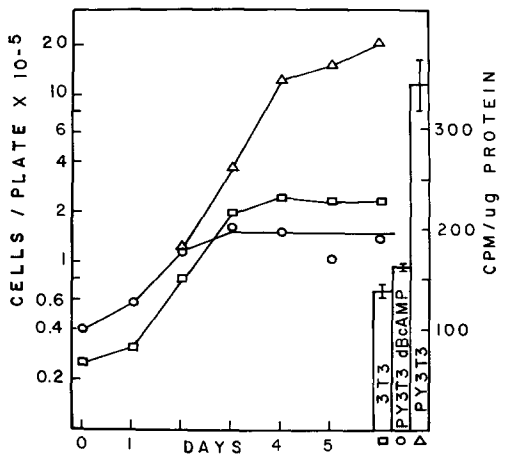

FIGURE 1 Effect of dBcAMP on cell growth and 2-deoxy[^{3}H]glucose uptake. 3T3 and Py3T3 cells were plated on 35-mm tissue culture plates on day 0. On day 1, one half of the Py3T3 cells were changed to medium containing 2 mM theophylline and 0.2 mM dBcAMP. Fresh medium with or without dBcAMP and theophylline as indicated was changed each day on all plates. One plate was used for cell counts and a second was used for 2-deoxy[^{3}H]glucose-uptake measurements on each day of the experiment. The bar graph indicates the average of the uptake measurements for Py3T3 dBcAMP, control Py3T3, and 3T3 cells. □, 3T3 cells. ○, Py3T3 cells treated with dBcAMP. △, control Py3T3 cells.

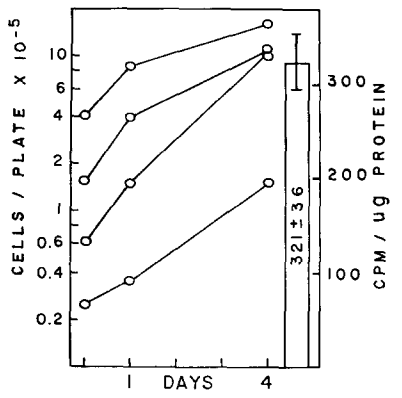

FIGURE 2 Effect of cell density on growth and 2-deoxy[^{3}H]glucose uptake in Py3T3 cells. On day 0, Py3T3 cells were plated on 35-mm tissue culture dishes at the indicated densities. Medium was changed on each day of the experiment. On days 1 and 4, duplicate plates were used for cell counts and uptake measurements. The bar graph shows the average of the uptake rates for all points in the experiment.

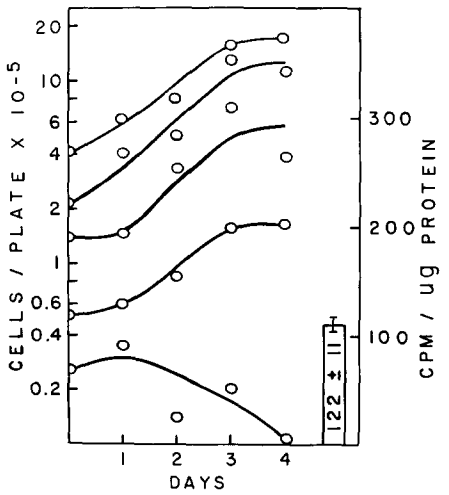

FIGURE 3 Effect of cell density on the growth and 2-deoxy[^{3}H]glucose uptake in Py3T3 cells grown in the presence of dBcAMP. Py3T3 cells were plated at densities of 0.2, 0.4, 1, 2, and 4 × 10^5 cells per plate. On day 0, medium containing 2 mM theophylline and 0.2 mM dBcAMP was added to each plate. Medium containing theophylline and dBcAMP was changed each day of the experiment. On each day, one plate from cells at each density was used for cell counts and a second was used for uptake measurements. The bar graph is the average of uptake measurements from each day of the experiment.

where 3T3 cells are contact inhibited. Cells at high density continued to grow even in the presence of cell contacts. However, the rate of 2-deoxy-glucose transport was reduced to the level of that observed in 3T3 cells, regardless of whether cells responded with death, cessation of cell division, or noncontact-inhibited growth. When theophylline- and dBcAMP-treated Py3T3 cells stop dividing at cell densities similar to 3T3 cells, the morphology of the cell monolayer is quite different from that of confluent 3T3 cells. The cells do not appear to be contact inhibiting, but instead are simply inhibited from cell division, even though there are places where there are no cell-to-cell contacts. Rather than suggesting that these cells are contact inhibited, we favor the explanation that addition of dBcAMP to cells above a critical density results in cell growth for a limited number of generations (less than three), regardless of the initial densities. If the Py3T3 cells are at the appropriate density, then addition of dBcAMP leads to a monolayer that does not divide when the cells approach the density of confluent 3T3 cells. However, treating high density cell populations results in cell divisions even in the presence of cell contacts. Other transformed cell lines SV40 3T3, RSV-transformed

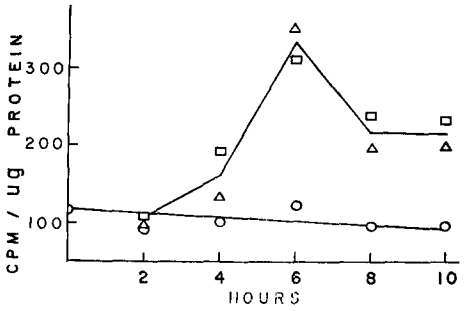

FIGURE 4 Effect of fresh serum and dBcAMP on 2-deoxy[^{3}H]glucose transport in confluent 3T3 cells. 3T3 cells were grown to confluency on 35-mm tissue culture dishes. At time 0, one third of the plates received fresh medium, one third received fresh medium containing 0.2 mM dBcAMP and 2 mM theophylline and one third were controls. The uptake of 2-deoxy[^{3}H]glucose was measured every 2 h. ○, control. △, fresh medium. □, fresh medium plus dBcAMP and theophylline.

chick cells, and Hamster sarcoma virus-transformed Nil cells (Grimes, unpublished observations) all show the same growth phenomena in the presence of dBcAMP. Thus, cells responded to dBcAMP with a limited growth which is independent of cell contacts. Removal of dBcAMP in all but low density cell cultures resulted in rapid recovery of the ability of cells to divide and an increase in 2-deoxyglucose transport. These effects were measurable within 24 h.

Fresh Serum Response

Further study of the membrane change which is caused by treating Py3T3 cells with dBcAMP is shown in Figs. 4 and 5. Confirming previous results, addition of fresh serum to confluent 3T3 cells results in a stimulation of 2-deoxyglucose uptake. Addition of theophylline and dBcAMP did not prevent the response to fresh serum. In other experiments, 2 mM theophylline and 0.2 mM dBcAMP were added 24 and 48 h before the addition of fresh serum, and still could not prevent the stimulation of 2-deoxyglucose uptake. Since dBcAMP did not prevent the serum response, and since treated Py3T3 cells had 2-deoxyglucose uptake rates smimilar to those of 3T3 cells, we asked whether adding fresh serum to dBcAMP-treated Py3T3 cells would result in stimulation of transport. In other words, could the treated Py3T3 cells show a normal cell response to fresh serum? The results presented in Fig. 5 show that treated Py3T3 cells are not similar to normal cells by the criteria of serum-stimulated 2-deoxyglucose transport.

The transport of 2-deoxyglucose in the cell lines grown under various conditions is shown in Table I. Little difference was observed between growing and confluent 3T3 cells, and dBcAMP had little effect on transport in 3T3 cells. Fresh serum caused an alteration in the membrane of 3T3 cells such that their transport ability was indistinguishable from that of Py3T3 cells. Addition of theophylline and dBcAMP to Py3T3 cells caused their uptake rates to be similar to normal. The kinetics of the change in transport has not been studied in detail, but upon changing

TABLE I

Effect of dBcAMP and Theophylline on 2-deoxy-[^{3}H]glucose Uptake by 3T3 and Py3T3 Cells

Cell	Uptake (cpm/μg protein)	Number of experiments
3T3 growing	138 ± 9	5
3T3 confluent	113 ± 17	5
3T3 (dBcAMP)	112 ± 10	5
Py3T3	333 ± 36	14
Py3T3 (dBcAMP)	140 ± 18	8
3T3 (fresh serum)	337 ± 27	2

The rate of 2-deoxy[^{3}H]glucose uptake was measured as described in Methods. The uptake rate for Py3T3 (dBcAMP) and 3T3 (dBcAMP) cells was determined 24 h after adding theophylline and dBcAMP. The uptake rate of 3T3 cells (fresh serum) was measured 6 h after adding medium containing fresh serum.

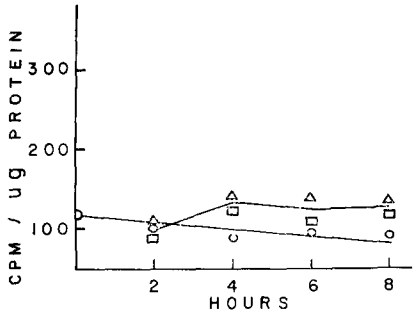

FIGURE 5 Effect of fresh medium and dBcAMP on 2-deoxy[^{3}H]glucose uptake in Py3T3 cells grown in the presence of dBcAMP and theophylline. Cells were plated at 0.4 × 10^5 cells per 35-mm tissue culture dish. The next day, cells were changed to medium containing 2 mM theophylline and 0.2 mM dBcAMP. On each day, fresh medium containing theophylline and dBcAMP was added to the plates. After the cells had stopped growing and were at a density similar to that of contact-inhibited 3T3 cells (Fig. 1), one third of the cells were changed to fresh medium, one third were changed to fresh medium containing dBcAMP and theophylline, and one third were controls. The uptake of 2-deoxy[^{3}H]-glucose was measured every 2 h. ○, control. △, fresh medium. □, fresh medium plus dBcAMP and theophylline.

treated Py3T3 cells to normal medium the transport of 2-deoxyglucose requires at least 24 h before returning to normal levels.

DISCUSSION

Changes in agglutination and 2-deoxyglucose uptake have been correlated with lack of contact inhibition (12–14, 16, 17, 25). Inhibiting cell growth by dBcAMP reverses the agglutinability change occurring upon cell transformation (20). Results reported here show that 2-deoxyglucose transport is also returned to levels found in normal cells by dBcAMP. It may be that both agglutination and 2-deoxyglucose transport are measuring related cell surface alterations. Ability of fresh serum to both stimulate cell division and to change 2-deoxyglucose transport from levels of normal cells to that of transformed cells further correlates transport with cell surface alterations leading to cell growth. Adding dBcAMP to Py3T3 cells at various cell densities allows cell divisions for only a few generations. Ability to transport 2-deoxyglucose is reduced to levels of 3T3 cells within 24 h after adding dBcAMP even though cells are continuing to divide. High density cells are thus dividing in the presence of cell contacts, and yet have normal cellular levels of ability to transport 2-deoxyglucose. These observations coupled with the fact that Py3T3 cells treated with dBcAMP are incapable of responding to fresh serum by increased 2-deoxyglucose uptake indicate that dBcAMP does not cause the transformed cell membrane to revert to normal. Transformed cells treated with dBcAMP at low densities stop cell division at densities where 3T3 cells contact inhibit. Under these conditions, the cells are not agglutinated by low concentrations of lectin (20). It will be of interest to determine if transformed cells at high cell densities respond to dBcAMP with decreased agglutinability even though the cells continue to divide in the presence of cell contacts. These studies are in progress. Whether cAMP plays any role in growth regulation of normal and transformed cells is unknown. These studies certainly do not rule out such an involvement. However, it appears that addition of dBcAMP to transformed cells does not return ability to contact inhibit, even though several parameters of transformation do revert to normal. Further, reduced 2-deoxyglucose transport may be necessary but is not sufficient for a return to contact-regulated growth.

This work was supported by Grant Number CA-12753-01 from the National Institute of Cancer.

Received for publication 20 July 1972, and in revised form 18 September 1972.

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