Cyclic AMP and Cyclic GMP Concentrations in Serum- and Density-Restricted Fibroblast Cultures

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ABSTRACT Mouse fibroblasts transformed by simian virus 40 (SV3T3 cells) are characterized by cyclic AMP and cyclic GMP levels, respectively, about half and twice those found in growing untransformed 3T3 cells. Density-dependent inhibition of growth is correlated with reduced cyclic AMP concentrations in 3T3 and four different density-restricted revertant lines derived from SV3T3. The levels of cyclic AMP are not increased at confluence. Upon serum restriction, serum-dependent cell lines show a greater increase in intracellular cAMP than serum-insensitive lines. Cyclic AMP levels are greatly reduced, even in serum-insensitive density revertants, but not in SV3T3. Serum readdition to all serum-dependent lines is followed by a rapid decrease in cyclic AMP and increase in cyclic GMP concentrations. The magnitude of these responses is decreased in SV3T3 and density revertants.

The growth of nontransformed fibroblasts, such as the mouse 3T3 line, is regulated by serum factors and cell-to-cell contact (1–3). Transition from a growing state to a resting state occurs upon depletion or deprivation of serum and upon cell confluence. Simian virus 40 (SV40) virus-transformed 3T3 clones often lose sensitivity to these environmental factors (3). Using negative selection pressures on SV40-transformed 3T3 fibroblasts, one can isolate revertant cell lines; they have regained growth control by serum or cell density or by both signals (4–6).

Recent work from various laboratories has implicated 3':5'-cyclic AMP (cAMP) and 3':5'-cyclic GMP (cGMP) in the regulation of proliferation in fibroblast cultures.

On one hand, the induction of fibroblast proliferation, by a variety of mitogenic signals, is associated with early decreases in intracellular cAMP (7–15) and increases in the cGMP content (14, 15).

On the other hand, the addition of dibutyryl cyclic AMP to the culture medium partially prevents the stimulation of cell growth (12, 13), whereas exogenous addition of cyclic GMP counteracts the inhibitory effects of dibutyryl cyclic AMP upon a number of biochemical processes activated early in the mitogenic response (16).

Since cAMP and cGMP seem to exert antagonistic regulatory effects on cell proliferation (16, 17), we have investigated the influence of cell density and serum concentration on the intracellular levels of the cyclic nucleotides in 3T3, SV3T3 (transformed by SV40), and different classes of revertant cell lines derived from SV3T3 by Pollack and coworkers (4–6). Using the same cell lines, Oey et al. have reached the conclusion that the intracellular cAMP concentration responds specifically to growth regulation by serum (9), in agreement with earlier reports of increased cAMP levels in serum-restricted fibroblast cultures (13, 18). In contrast, the mediation by cAMP of density-dependent inhibition of growth remains controversial, since some (7, 19, 20), but not other (8, 9) investigators have observed a rise in cAMP when cells approach confluence.

We report here that density-dependent inhibition of growth is correlated with decreased cGMP concentrations in 3T3 and four different lines of density-dependent revertant cell lines. In contrast, the cGMP levels remain almost unaffected over the same range of cell densities, in the non “contact-inhibited” clone SV101 of SV40-transformed 3T3 fibroblasts.

MATERIALS AND METHODS

Cell Lines. The cell lines used in this study are Swiss 3T3, clone SV101 of SV40-transformed 3T3, and four revertant lines derived from SV101 by Pollack and coworkers (4–6). The density-dependent revertant clones FISV101 and BuSV2 were obtained by negative selection with fluorodeoxyuridine and bromodeoxyuridine, respectively (4, 5). The serum-dependent clones (LSV2 and AγSV5) were selected for their inability to grow at low serum concentrations (1%) and in gamma-depleted (= agamman) 10% calf serum, respectively (6). The density revertants have regained density-dependent inhibition of cell growth comparable to that of normal 3T3, whereas the serum revertants are in addition sensitive to serum restriction. All cell lines were checked periodically for and found free of contamination by mycoplasma.

Culture Conditions. The cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 50 μg/ml of gentamycin under 5% CO2–95% air atmosphere. Stock cultures were carried in Falcon 75 cm2 flasks. Experiments were performed in Greiner 75 cm2 tissue culture plates.

Cyclic Nucleotide Assays. The medium was aspirated and the plates were quickly washed with 5 ml of K-phosphate-buffered physiological saline at 0°C. This washing did not significantly alter the results. The cells were scraped with a rubber policeman in 2 ml of 50% ethanol (at −20°C) plus 0.2 ml of Zn acetate 1 M and the plates were rinsed once with 2 ml of 50% ethanol. [3H]CAMP and [3H]cGMP were added to monitor recovery of intracellular cyclic nucleotides after purification (usually 65–80%). Further purification of cAMP and cGMP was performed in most experiments, on QAE-Sephadex according to Schults et al. (21). Similar results were obtained when cells were collected in 5% trichloroacetic acid and the extracts were fractionated on Dowex-50, for cAMP, and on Dowex-1 (formate form), for cGMP. Intracellular cAMP concentrations were assayed by Gilman’s method (22) and cGMP by the radioimmunoassay of Steiner et al. (23). Results were corrected for the values found in phosphodiesterase.
Cyclic nucleotide concentrations in growing cultures

<table>
<thead>
<tr>
<th>Class</th>
<th>Cell line</th>
<th>cAMP</th>
<th>cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3T3</td>
<td>23</td>
<td>0.44</td>
</tr>
<tr>
<td>Transformed</td>
<td>SV3T3-101</td>
<td>10</td>
<td>0.82</td>
</tr>
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<td>Density-revertants</td>
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<td>0.58</td>
</tr>
<tr>
<td></td>
<td>BuSV2</td>
<td>20</td>
<td>0.71</td>
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<td>Serum-revertants</td>
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</tr>
<tr>
<td></td>
<td>AγSV5</td>
<td>10</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* All assays were performed on sparse cultures (about 1.5 × 10^6 cells per 75 cm² plate) growing in medium supplemented with 10% serum.

† Levels measured in separate experiments did not usually deviate from the reported mean values by more than ±3 pmol/mg of protein and ±0.05 pmol/mg of protein for cAMP and cGMP, respectively.

Table 2. Cyclic nucleotide concentrations in serum-restricted cultures

<table>
<thead>
<tr>
<th>Class</th>
<th>Cell line</th>
<th>cAMP</th>
<th>cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
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<td>0.06</td>
</tr>
<tr>
<td>Transformed</td>
<td>SV3T3-101</td>
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<td></td>
<td>BuSV2</td>
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<td>0.11</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>AγSV5</td>
<td>52</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* All assays were performed 1 day after replacing medium + 10% serum by medium + 0.5% serum in sparse cultures (about 1.5 × 10^6 cells per 75 cm² plate, at time of harvesting).

RESULTS

Cyclic nucleotide concentrations in growing cultures

In agreement with previous studies (7–9, 19, 24, 25), we find in SV3T3 a cAMP concentration (10 pmol/mg of protein) about half that observed in 3T3 (23 pmol/mg of protein) (Table 1). However, as reported by Oey et al. (9), the values found in the different classes of revertants indicate a lack of correlation between decreased cAMP levels and transformed phenotype. The revertant line AγSV5 retains the low cAMP concentration of SV3T3, whereas the other revertants contain the higher cAMP concentration characteristic of 3T3 (Table 1). Comparison of cGMP levels between normal and transformed fibroblasts shows a pattern which is the reciprocal of that observed for cAMP. Indeed, SV3T3 is characterized by a cGMP concentration (0.82 pmol/mg of protein) about twice that found in 3T3 (0.44 pmol/mg of protein). The serum-dependent revertants contain the low level characteristic of 3T3, whereas intermediate concentrations are found in the density revertants.

Cyclic nucleotide concentrations as a function of cell density

Cultures were grown to confluence, in 10% serum, with replacements of fresh medium every other day to prevent changes in cyclic nucleotide concentrations due to serum depletion. Under these conditions, the 3T3 and revertant cell lines exhibit density-dependent inhibition of growth, reaching saturation densities between 4 and 6 × 10^6 cells per 75 cm² dishes. SV101 fibroblasts, on the contrary, continue to grow exponentially up to cell densities higher than 20 to 30 × 10^6 cells, where the cultures begin to peel off (Fig. 1).

In agreement with Sheppard (8) and Oey et al. (9), we did not detect an increase in cAMP content in density-restricted cell lines, when monolayers are formed. However, an earlier increase in cellular cAMP occurring below a density of 10^4 cells per cm² (26) might have been missed.

At confluence, the cAMP concentrations tend rather to decrease, especially in the case of crowded SV3T3 cultures (Fig. 1) as previously reported (9, 19). On the contrary, cGMP concentrations are quite dramatically affected by density-dependent inhibition of growth in all density-restricted cell lines, i.e., 3T3 as well as revertants: cGMP levels decrease gradually in sparse cultures and abruptly when the growth rates begin to decrease; they finally reach values, in confluent cells, about one-fifth of those found at a density of 10^6 cells per 75 cm² plate (Fig. 1). In contrast, the cGMP concentration in SV3T3 fibroblasts decreases only slightly over the same range of cell population. At densities higher than 10^6 cells per plate, their cGMP content is still 75% of that measured in sparse cultures of SV3T3 and is higher than in sparse cultures of 3T3. It is only when the transformed line reaches its saturation density, that its cGMP concentration drops abruptly (Fig. 1).

Cyclic nucleotide concentrations in serum-restricted cultures

Increased intracellular cAMP concentrations are found in all cell lines cultured in medium containing 0.5% serum (Table 2). However, cAMP rises to levels higher than 40 pmol/mg of protein in 3T3 and the serum-dependent revertants LSV2 and AγSV5. These values are somewhat lower than those reported by Oey et al. for the same lines assayed 2 days after inoculation in a medium containing 1% serum. In agreement with their results, we observe a smaller rise of cAMP in SV3T3 and the two serum-insensitive density revertants. Unlike the cAMP levels, which are elevated, the cGMP concentrations are lower in serum-restricted cultures of all cell lines. However, cGMP concentration drops in 3T3 to less than 0.1 pmol/mg of protein, whereas, in SV3T3, it goes down to 0.32 pmol/mg; this is not greatly decreased in comparison with the value found in sparse cultures of 3T3 fibroblasts growing in 10% serum. Serum-dependent revertants as well as density-dependent revertants show, on the other hand, cGMP concentrations which are as greatly reduced as in 3T3 fibroblasts, when cultured in low serum.

Cyclic nucleotide concentrations in response to serum readdition

Serum readdition to sparse cultures of 3T3 and serum-dependent revertant lines maintained in medium containing 0.5% serum induces rapid changes in the concentrations of both cyclic nucleotides. The elevated cAMP levels in 0.5%
serum drop by a factor of approximately 3-fold within 20 min after serum readdition (Fig. 2). Thus, cAMP decreases temporarily to levels lower than those found in sparse cultures growing in 10% serum; it then comes back to the latter values after 1–2 hr. The cGMP response to serum readdition follows the same kinetics, but with a pattern reciprocal of that of cAMP (Fig. 2). In 3T3 cells, as well as in serum-dependent revertant lines, the cGMP concentrations rise from less than 0.1 pmol/mg of protein to 2 pmol/mg of protein, then return gradually to the values found for growth in 10% serum (Table 1), prior to the shift down to 0.5% serum for 24 hr. As could be foreseen from the moderate elevation of cAMP after serum restriction of SV3T3 and density revertants, the cAMP response to serum readdition to these lines is much less pronounced than in 3T3 and serum revertants. The magnitude of the cGMP changes is also less than in 3T3 and serum-
dependence revertants (Fig. 2). Indeed, the highest cGMP concentrations, observed 20 min after serum readdition to SV3T3 and density revertants, are not as strikingly elevated as in serum-dependent lines, especially when compared to the levels found in cultures growing in 10% serum (Table 1).

DISCUSSION

The mechanisms of growth regulation in normal cells and their alteration in malignant cells are still poorly understood. Recent work has suggested that a defect in cAMP metabolism might be involved in the expression of the malignant phenotype in cultured fibroblasts. Lower adenylate cyclase and/or lower cAMP concentrations have been found in virus-transformed fibroblasts (7-9, 27). Studies with temperature-sensitive mutants of oncogenic viruses have indicated that a viral function alters the activity of cellular adenylate cyclase and lowers the cAMP levels (24, 25). Exogenous addition of dibutyril cyclic AMP and theophylline to the culture medium of transformed fibroblasts slows down their proliferation and corrects many of their abnormal properties (morphology, adhesiveness, and agglutinability) (28-30).

Although the molecular mechanisms of these cAMP effects remain unknown, the cyclic nucleotide seems to control cell growth by regulating the rates of a set of biochemical processes called the pleiotropic response (13). These intracellular effects of cAMP are blocked in cAMP-resistant clones of lymphoma cells, characterized by decreased activity of cAMP-activated protein kinases (31). It has been further shown that addition of cGMP counteracts the inhibitory effects of cAMP on serum-induced stimulation of the pleiotropic parameters in normal 3T3 fibroblasts (16).
The data presented here lend some support to the physiological relevance of this antagonism between cAMP and cGMP. Intracellular cyclic GMP concentrations in 3T3 fibroblasts are sensitive to environmental factors, which regulate their proliferation. Greatly reduced cGMP levels are found at confluence or in sparse cultures maintained in medium containing 0.5% serum. A transient and important increase in intracellular cGMP concentration occurs after serum readdition, in agreement with the changes found in its level following growth induction in quiescent confluent monolayers by either serum (14) or a purified fibroblast growth factor (15). A similar striking increase in cellular cGMP was also reported in circulating lymphocytes within 30 min after stimulation by phytohemagglutinin (17); however, it is not accompanied by a concomitant decrease in cAMP, as found in fibroblast cultures activated by serum (refs. 7-15, and this report).

We have also compared cGMP concentrations in normal and SV40-transformed 3T3 fibroblasts under various conditions. Sparse cultures of 3T3, growing in 10% serum, contain about half the concentration of intracellular cGMP found in SV3T3. As cell density increases, cGMP levels in 3T3 decrease gradually; in confluent monolayers, they reach values less than one-fifth of those found during the early exponential growth phase. In contrast, intracellular cGMP concentrations decrease only slightly in SV3T3 over the same range of cell densities. The reduced sensitivity of transformed fibroblasts to growth restriction by low serum concentrations is also reflected by less dramatically decreased cGMP levels than in normal 3T3 fibroblasts under similar conditions.

Finally, the use of revertant lines, selected from SV3T3, has allowed us to investigate whether the responses of the cyclic nucleotide levels to serum concentration in the medium and to cell density are specific. We find decreased cGMP and increased cAMP concentrations in serum-restricted cultures. This condition exerts more pronounced effects on cGMP than on cAMP levels in serum-dependent lines; however, greatly reduced cGMP concentrations are found also in the serum-insensitive density revertants, albeit not in SV3T3. This discrepancy can be accounted for on the following basis. Density revertants grow as well as SV101 in 1% calf serum, but reach a lower saturation density of 1.5 x 10^6 cells per cm^2 (5, 6). Thus, the extremely low cGMP concentrations present under serum-restricted conditions in sparse cultures of all cell lines, except SV3T3, can be explained by the fact that cell density (1.5 x 10^8 cells per 75 cm^2 plate) already exceeds the saturation densities in low serum medium, except in the case of SV3T3 (6). On the other hand, we can confirm the observation by Oey et al. (9), that serum-dependent cell lines show a greater rise in cAMP than SV3T3 and density revertants, which are serum-insensitive. They concluded from their data that intracellular cAMP concentration responds specifically to growth regulation by serum. The response of cGMP concentrations to serum restriction or readdition might be secondary to the changes in cAMP levels, since addition of prostaglandin E2 elevates cellular cAMP and prevents the rise in cGMP, induced by serum readdition to confluent 3T3 cultures in serum-depleted medium (32).

Our results suggest that decreased cGMP concentrations might specifically mediate sensitivity to density-dependent inhibition of growth. Since the cAMP levels do not increase concomitantly, it is unlikely that these reduced cGMP levels reflect a lower ability of dense cultures to utilize components of the medium, especially serum (33, 34). Taken together with the possibility of dissociating serum restriction from density restriction in some revertant lines, the data suggest that these two modes of regulation of fibroblast proliferation are distinct.

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