The Effect of Dibutyryl Cyclic Adenosine Monophosphate on Synthesis of Sulfated Acid Mucopolysaccharides by Transformed Fibroblasts

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SUMMARY

The effect of dibutyryl-cyclic adenosine 3',5'-monophosphate and theophylline on the incorporation of [35S]sulfate into acid mucopolysaccharides by 3T3 and SV3T3 fibroblasts has been studied. SV40 transformation of 3T3 fibroblasts produced a marked decrease in the rate of synthesis of acid mucopolysaccharides, in agreement with the data of others. When the transformed cells were grown in medium supplemented with dibutyryl-cyclic AMP and theophylline for 3 days, they secreted, on the basis of cell protein, approximately 250% more labeled acid mucopolysaccharides during a 24-hour labeling period than did the nontreated controls. The secretion of a variety of sulfated acid mucopolysaccharides was increased including chondroitin-4- and -6-sulfate and dermatan sulfate. Comparison of the rate of sulfate incorporation into acid mucopolysaccharides and the rate of loss of previously labeled acid mucopolysaccharides from the cells indicated that the effect was due to a greater rate of synthesis of these compounds at the time of labeling rather than due to a decrease in the rate of their degradation.

Viral transformation alters many of the characteristics of fibroblasts grown in culture. Marked biochemical as well as morphological changes have been demonstrated in transformed cells compared with their parent cell lines. One of these, the synthesis and secretion of acid mucopolysaccharides, has been reported to be markedly decreased by SV40 transformation of mouse 3T3 cells (1, 2). The levels of cyclic adenosine 3',5'-monophosphate are also frequently decreased in transformed fibroblasts (3). This observation suggested to us that cyclic AMP might regulate the synthesis of acid mucopolysaccharides; therefore, we have investigated the effects of dibutyryl-cyclic AMP on the synthesis and secretion of sulfated acid mucopolysaccharides in normal 3T3 cells and in a line of SV40-transformed 3T3 cells which has very low levels of cyclic AMP.

MATERIALS AND METHODS

H235SO4 (carrier-free) and [1-14C]glucosamine (9 mCi per mm) were obtained from New England Nuclear Corporation. Dulbecco-Vogt modified Eagle’s medium was prepared in the National Institutes of Health media kitchen. Calf serum was from Flow Laboratories and theophylline from Mann. N,N'-dibutyryl 3',5' cyclic AMP was obtained from Schwarz-Mann and further purified as previously described (4). Hyaluronic acid (Grade I), chondroitin sulfate (Grade III, mixed isomers), and testicular hyaluronidase (Type IV, ~750 N.F. units per mg) were obtained from Sigma Chemical Company. Chondroitinase ABC was from Miles Laboratories and DEAE-cellulose (Whatman DE52) from H. Reeve Angel and Company. Preparations of standard chondroitin-4-sulfate and dermatan sulfate were kindly supplied by Dr. J. A. Cifonelli, Department of Pediatrics, The University of Chicago, Chicago, Illinois. All other chemicals were reagent grade.

Cell Culture—Swiss 3T3-4 fibroblasts were a gift of Dr. H. Greene; 3T3SV40 clone X were purchased from Flow Laboratories which had received them from Dr. G. Todaro. The cells were cultured in Dulbecco-Vogt modified Eagle’s medium containing 10% calf serum, 50 units per ml of penicillin, and 50 μg per ml of streptomycin. The cells were cultured in 75-cm² Falcon flasks in a moist 5% CO₂ atmosphere. Treated cells were grown and labeled in medium supplemented with 1.2 mM (but)cAMP and 1.0 mM theophylline. The 3T3 control cells were planted at a concentration of approximately 4 x 10⁶ cells per flask, and those planted with (but)cAMP and theophylline at 6 x 10⁶ cells per flask. Unless otherwise indicated, the control SV3T3 were planted at 3 x 10⁵ cells per flask and the treated at 9 x 10⁵ cells per flask. The cells were cultured for 3 days with one change of medium 24 hours prior to labeling. At these concentrations, the cells had not reached confluence at the time of labeling.

Analysis of Sulfate Incorporation into Acid Mucopolysaccharides—The cells were incubated in fresh medium containing 5 μCi per ml of H235SO4 for varying periods of time. Inorganic sulfate in the medium was approximately 1.2 mm. The

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† The abbreviations used are: cyclic AMP, cyclic adenosine 3',5'-monophosphate; (but)cAMP, N,N'-dibutyryl 3',5' cyclic adenosine monophosphate.
corporation of the labeled sulfate into sulfated acid mucopolysaccharides was assayed by the method of Fratantoni et al. (5).

Chromatography—Aliquots of the media were dialyzed exhaustively against 0.05 M Tris-HCl, pH 7.2, and chromatographed at room temperature on a DEAE-cellulose column (1.8 by 7 cm) equilibrated with the same buffer. The samples were eluted with 0.05 M Tris-HCl, pH 7.2, upon which was superimposed a linear gradient of 0 to 1.0 M LiCl over a volume of 250 ml. Three-milliliter fractions were collected and radioactivity was measured on aliquots using standard liquid scintillation counting procedures. Standard nonradioactive preparations of hyaluronic acid, chondroitin-4-sulfate, and dermatan sulfate were also chromatographed under similar conditions and fractions were analyzed for uronic acid by the method of Gregory (6).

Electrophoresis—The labeled acid mucopolysaccharides were also subject to cellulose acetate electrophoresis. Aliquots of the medium were dialyzed against 0.1 M sodium acetate buffer, pH 5.5, and then cysteine and EDTA were added to a final concentration of 5 mM, and the samples were digested with 0.3 mg per ml of papain for 24 hours at 60°. The residual protein was precipitated with 10% trichloroacetic acid and the supernatants dialyzed exhaustively against water, lyophilized, and taken up in a minimal volume of water. Aliquots were applied to cellulose acetate strips (1 by 6 inches) and were subjected to electrophoresis in 0.15 M NaSO₄ for 3 hours at a constant current of 1.5 ma per strip (7). Samples of standard preparations of chondroitin-4-sulfate and dermatan sulfate were included in each run. Strips with the standards were stained with 0.1% alcian blue in 20% ethanol containing 5% acetic acid and decolorized in 5% acetic acid. The strips with the labeled acid mucopolysaccharides from the fibroblasts were cut into segments which were counted directly in a liquid scintillation spectrometer.

Estimation of Proportions of Groups of Polysaccharides—To estimate the proportions of the different groups of sulfated acid mucopolysaccharides that were secreted by the cells, we used a modification of the method of Toole and Gross (8) which depends on the substrate selectivity of various polysaccharidases. Chondroitin-4- and -6-sulfates are markedly degraded by testicular hyaluronidase, whereas dermatan sulfate is resistant to chondroitinase ABC. Keratan sulfate, heparan sulfate, and heparin are not degraded by either enzyme (9). The media were deproteinized with papain digestion and trichloroacetic acid precipitation followed by dialysis against water. Aliquots were then digested with 50 µg per ml of testicular hyaluronidase (0.1 M sodium acetate, pH 5.1, containing 0.15 M NaCl) or 0.2 units per ml of chondroitinase ABC (0.05 M Tris-HCl, pH 8, containing 0.1 M NaCl and 0.01% bovine serum albumin). Controls consisted of samples incubated without enzymes. The tubes were incubated for 3 hours at 37°. Following in


duction, 0.25 mg per ml of carrier chondroitin sulfate, mixed isomers, was added to each tube and the polysaccharides were isolated by cetylpyridinium chloride and alcohol precipitation (8). Under these conditions 0.2 unit of chondroitinase ABC completely degraded 0.25 mg chondroitin sulfate within 1 hour. Recovery of the polysaccharides from the cetylpyridinium chloride complex ranged between 85 and 105% as judged by the carbazole reaction.

To calculate the proportions of the different groups of sulfated acid mucopolysaccharides in each sample, the difference between the recovery of the labeled acid mucopolysaccharides in the control and the hyaluronidase digested material was taken as a measure of the content of chondroitin-4- and -6-sulfates. The difference between the material recovered after chondroitinase ABC digestion and that recovered after hyaluronidase digestion was taken as a measure of the dermatan sulfate; and finally, the material recovered after chondroitinase ABC digestion was taken as a measure of the nondegradable sulfated acid mucopolysaccharides.

All determinations were conducted in duplicate and the data presented represent the means. Protein was determined with the Miller modification of the Lowry method (10).

RESULTS

Sulfate and Glucosamine Incorporation—Both the normal and transformed fibroblasts incorporated [³⁵S]sulfate into acid mucopolysaccharides. Table I shows the results of a typical experiment. There was a marked decrease in the amount of sulfated acid mucopolysaccharides secreted by the transformed fibroblasts when compared to the normals after a 24-hour labeling period. On the basis of cell protein, the SV3T3 cells secreted only one-fourth of the amount of sulfated acid mucopolysaccharides as did the 3T3 cells. There was little difference in the accumulation of labeled intracellular material by the transformed fibroblasts in the same period of time.

Treatment of the normal and transformed fibroblasts with (but)cAMP and theophylline produced little change in the accumulation of labeled intracellular material. There was, however, an increase in the amount of labeled acid mucopolysaccharides secreted by both cell types compared to their non-treated controls. The greatest percentage of increase by

<table>
<thead>
<tr>
<th>Fibroblasts</th>
<th>Sulfate Incorporation</th>
<th>Ratio of extracellular to control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra.</td>
<td>Extrap.</td>
</tr>
<tr>
<td>3T3</td>
<td>Control</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>21</td>
</tr>
<tr>
<td>SV3T3</td>
<td>Control</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>19</td>
</tr>
</tbody>
</table>

Effect of (but)cAMP and theophylline on incorporation of [³⁵S]sulfate into acid mucopolysaccharides by 3T3 and SV3T3 fibroblasts

Cells were plated at densities described in the text and grown for 3 days with one change of medium during that period. The cells were then incubated in 10 ml of fresh medium containing approximately 5 µCi per ml of [³⁵S]sulfate for 24 hours. The treated cells were grown and labeled in medium containing 1.2 mM (but)cAMP and 1.0 mM theophylline. Sulfate incorporation into acid mucopolysaccharides was determined by the method of Fratantoni et al. (5).
Effect of (but)cAMP and theophylline on incorporation of [14C]glucosamine into extracellular sulfated acid mucopolysaccharides by SV3T3 fibroblasts

The treated and control cells were planted and grown as described in the text. After 3 days growth the cells were incubated in medium containing approximately 2.5 μCi per ml of [14C]glucosamine for 24 hours. Extracellular sulfated acid mucopolysaccharides were isolated by DEAE-cellulose chromatography as described in the text.

<table>
<thead>
<tr>
<th>Fibroblasts</th>
<th>Glucosamine incorporation</th>
<th>Ratio of treated to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>633</td>
<td>3.72</td>
</tr>
</tbody>
</table>

Treatment with (but)cAMP and theophylline was seen with the transformed fibroblasts which showed an increase of approximately 250% in the amount of labeled acid mucopolysaccharides secreted. On the basis of cell protein, the treated SV40-transformed cells secreted an amount comparable to that of the 3T3 control cells. When treated with (but)cAMP and theophylline, the 3T3 fibroblasts showed an increase in secretion of sulfated acid mucopolysaccharides of about 50%. The marked increase in secretion of sulfated acid mucopolysaccharides by the treated SV3T3 cells was confirmed using [14C]glucosamine as a precursor (Table II).

Kinetics—Since the marked increase in the amount of labeled extracellular acid mucopolysaccharides found with treatment of the SV3T3 cells could result from either an increase in the rate of synthesis or a decrease in the rate of breakdown, we followed the kinetics of secretion as well as loss of previously labeled material from the cells. After the cells had been grown for 3 days with and without (but)cAMP and theophylline, the increased rate of secretion of labeled acid mucopolysaccharides by the treated cells was evident from the earliest time period of labeling (Fig. 1). In contrast, the loss of previously labeled material from the treated and control cells proceeded at a similar rate (Fig. 2). After 8 hours in unlabeled medium, 60% of the previously labeled material from the control cells and 50% from the treated cells was dialyzable. Since the rate of loss of previously labeled acid mucopolysaccharides from fibroblasts serves as a measure of their rate of breakdown (5), it appears that the observed effect of treatment is that of a greater rate of synthesis of these compounds by the treated cells at the time of labeling, as opposed to a decreased rate of breakdown.

To study how rapidly (but)cAMP and theophylline acted, the transformed cells were planted in control medium and the cultures were allowed to establish for 24 hours. The cells were then treated with (but)cAMP and theophylline, and the rate of sulfated acid mucopolysaccharide synthesis was determined with a 2-hour pulse of the cells with [35S]sulfate at various times after the initiation of the treatment (Table III). There was little difference in the rate of total synthesis between the treated and control cells during the first 26 hours after the initiation of treatment.

When the incorporation was expressed as a function of the total amount of cell protein present in the flask at the time of labeling, the incorporation in the control cultures fell. This was therefore added to the medium when calculating total extracellular acid mucopolysaccharides.

Fig. 1. Rate of secretion of labeled acid mucopolysaccharides by cells incubated in medium containing approximately 5 μCi per ml of [35S]sulfate. SV3T3 cells had been grown for 3 days prior to labeling as described in the text. Aliquots of medium were removed at various times after the initiation of labeling and analyzed for sulfate incorporation. The cell protein content of the cultures at the end of the experiment was used for calculations. ○—○, cells grown and labeled in control medium; ●—●, cells grown and labeled in the presence of 1.2 mM (but)cAMP and 1.0 mM theophylline.

Fig. 2. Percentage of previously labeled acid mucopolysaccharides remaining in cells after chase in unlabeled medium. SV3T3 cells had been grown for 3 days in either control medium or in medium containing 1.2 mM (but)cAMP and 1.0 mM theophylline prior to labeling for 24 hours with approximately 5 μCi per ml of [35S]sulfate and chasing in unlabeled medium. ○—○, cells grown, labeled, and chased in control medium; ●—●, cells grown, labeled, and chased in medium containing (but)cAMP and theophylline.
may indicate that as the cells become heavier they lose their ability to make sulfated mucopolysaccharides. In contrast the incorporation in the cells treated with (but)cAMP and theophylline remained relatively constant when expressed in this manner.

To correct for the previously described decreased growth rate of the (but)cAMP-treated cells (11), the amount of cell protein present in control and treated cells at various times of the experiment was plotted as a line graph. From the line best fitting the points, the increase in cellular protein during each labeling period was determined and the differential rate of synthesis was calculated: that is the amount of [35S]sulfate incorporated into polysaccharides in a 2-hour labeling period divided by the increase in cell protein over that same period. When the data are expressed in this way it is evident that the degree of sulfation of the acid mucopolysaccharides in the treated cells was comparable to that of the controls. Upon cellulose acetate electrophoresis of the material from the medium of the treated SV3T3 cells, two migrating bands were detected (Fig. 4). The slower migrating band had a mobility corresponding to that of authentic dermatan sulfate, and the more rapidly migrating band had a mobility corresponding to that of authentic chondroitin-4-sulfate. The material remaining at the origin presumably consisted of nondegraded protein-polysaccharides. Under these conditions chondroitin-4-sulfate is not separated from chondroitin-6-sulfate and heparan sulfate has a mobility similar to that of dermatan sulfate.

We also investigated which particular groups of sulfated acid mucopolysaccharides were affected by treatment of the cells with (but)cAMP and theophylline. On the basis of their susceptibility to degradation by different polysaccharidases the effect did not appear to be highly specific since there was greater synthesis of all fractions by the treated cells (Table IV). This was most marked in the chondroitin-4- and -6-sulfate and dermatan sulfate fractions of the transformed fibroblasts; however, there was also greater synthesis of the nondegradable fraction by these cells as well as all fractions of the normal cells.

**DISCUSSION**

Treatment of transformed fibroblasts with (but)cAMP produces a variety of changes in the behavior of the cells. These include a more normal morphology (4, 11), a decreased growth rate (11), decreased motility (12), decreased agglutinability by plant agglutinins (19), an increased adhesive-

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**TABLE III**

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Total Incorporation</th>
<th>Protein</th>
<th>Incorporation</th>
<th>Protein</th>
<th>Differential Incorporation</th>
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<td></td>
<td>cpm X 10^-3/dish</td>
<td>mg/dish</td>
<td>cpm x 10^-3/mg protein</td>
<td>mg/dish</td>
<td>cpm X 10^-3/mg protein</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-4</td>
<td>7.2</td>
<td>0.08</td>
<td>90.0</td>
<td>0.036</td>
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<td>0.11</td>
<td>70.0</td>
<td>0.018</td>
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<tr>
<td>12-14</td>
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<td>0.18</td>
<td>54.4</td>
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<tr>
<td>24-26</td>
<td>14.2</td>
<td>0.34</td>
<td>41.8</td>
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<tr>
<td>48-50</td>
<td>32.6</td>
<td>0.82</td>
<td>30.8</td>
<td>0.069</td>
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<tr>
<td>Treated</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2-4</td>
<td>7.5</td>
<td>0.08</td>
<td>93.8</td>
<td>0.010</td>
<td>750</td>
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<tr>
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<td>1130</td>
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<tr>
<td>24-26</td>
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<td>71.2</td>
<td>0.011</td>
<td>1100</td>
</tr>
<tr>
<td>48-50</td>
<td>23.3</td>
<td>0.33</td>
<td>70.6</td>
<td>0.016</td>
<td>1450</td>
</tr>
</tbody>
</table>
SV3T3 cells, this was studied in more detail. The data indicated that the rate of synthesis of mucopolysaccharides was increased, whereas the degradation rate was unaffected.

Mucopolysaccharide synthesis may be controlled either by the rate of synthesis of the carbohydrate precursors or at the level of synthesis of the protein moiety (16). There are a number of proteins already known whose synthesis is induced by cyclic AMP (17), but stimulation of polysaccharide synthesis has not been described. Indeed, the reaction first described for cyclic AMP was stimulation of glycogen dissimilation (17). Later inhibition of glycogen synthesis was described (17).

One key enzyme in the synthesis of glycoproteins and proteoglycans is UDP-galactose 4-epimerase. The activity of this enzyme is known to be markedly inhibited in rapidly growing fibroblasts (18). NADH is an inhibitor of the enzyme and therefore the ratio of NADH to NAD is important in regulating its activity. Since glycolysis is rapid in growing cells, the ratio of NADH:NAD may be increased enough to inhibit this epimerase. The activity of the epimerase, which catalyzes the synthesis of UDP-L-iduronic acid (19) and UDP-N-acetylgalactosamine (20) is also inhibited by NADH. Inhibition of growth by cyclic AMP might decrease the NADH:NAD ratio and thereby increase epimerase activity.

The synthesis of some proteins is limited to specific parts of the cell cycle. The decrease in the growth rate of cells treated with (but)cAMP and theophylline does not appear to expand all parts of the cell cycle proportionately. For example, treatment of 3T3 cells with (but)cAMP causes them to be arrested in early G1 or in G2, depending on where in the cell cycle the cells were at the time treatment was initiated.3 If the synthesis of sulfated mucopolysaccharides were limited to early G1 or G2, their synthesis could thus be increased. The isolation of cell lines in which (but)cAMP fails to inhibit growth may help clarify this point.

Cyclic AMP regulates many differentiated functions of cells growing in tissue culture. (But)cAMP induces axon formation in neuroblastoma cells (21, 22) and pigment production in melanoma cells (23). Hsie et al. (24) reported that (but)cAMP induces collagen synthesis in Chinese hamster ovary cells. The results of this study indicate that cyclic AMP regulates the expression of another differentiated function of fibroblasts, the synthesis and secretion of sulfated acid mucopolysaccharides.

Acknowledgments—We wish to acknowledge the technical assistance of Lloyd C. Bihora, Elizabeth Lovelace, Pat Middleton, and C. James Wheeler.

References


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