Phosphorylation of Acid-soluble Proteins in Isolated Nucleoli of Novikoff Hepatoma Ascites Cells

EFFECTS OF DIVALENT CATIONS*

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SUMMARY

Isolated nucleoli from rat Novikoff hepatoma ascites cells incorporated $^{32}$P of [$\gamma$-32P]ATP into acid-soluble nucleolar proteins in vitro in systems containing 0.25 M sucrose, 5 mM MgCl$_2$, 12.5 mM NaCl, and 0.05 M Tris-HCl buffer (pH 7.3) at 37°C. Although the total uptake of $^{32}$P reached a maximum after 15 min of incubation, the uptake into some protein bands increased with longer incubation times. As shown by two-dimensional gel electrophoresis and autoradiography, all the proteins labeled in vitro, including the high molecular weight proteins designated as C23-24, B27, and B13 were shown previously to be labeled in vivo (OLSON, M. O. J., ORRIEK, L. R., JONES, C., AND BUSCH, H. J. Biol. Chem. 249, 2823-2827). Divalent cations were necessary for phosphorylation in vitro but different profiles of phosphorylation were observed when Mg$^{2+}$ was replaced with Co$^{2+}$, Zn$^{2+}$, or Mn$^{2+}$. The presence of Zn$^{2+}$ produced the highest $^{32}$P uptake associated with a marked increase in phosphorylation in proteins A17-19 and B23-25 compared to Mg$^{2+}$ which produced high labeling in spots B13, and C23-24. These data indicate that the nucleolus contains phosphoprotein kinase(s) which exhibit different specificities in the presence of different divalent cations.

Phosphorylation of nuclear proteins has been suggested to serve a role in the regulation of gene activities (1-5). Recently, selective phosphorylation of nuclear acid-soluble proteins of Novikoff hepatoma ascites cells was shown to occur in vivo (6). Analyses of the phosphorylation of these nuclear acid-soluble proteins by two-dimensional polyacrylamide gel electrophoresis showed that approximately 40 nucleolar proteins were phosphorylated (6).

Since isolated nucleoli are simple subcellular organelles which provide a convenient system for the study of biochemical events involved in the control of genetic activities (7), they have been used for a number of enzymatic studies. Several studies have reported the presence of specific DNA-dependent RNA polymerases (8, 9), methylases for transfer (10) and preribosomal RNA (10, 11), and systems for the aminoacylation of transfer RNA (12) and incorporation of amino acids into nucleolar proteins (13) in isolated nucleoli.

The present study was designed as an initial approach to the investigation of the significance and factors controlling the phosphorylation of nucleolar proteins in vivo. It was found that the proteins phosphorylated in vivo by properly supplemented isolated nucleoli were also labeled in vivo following exposure to [$\gamma$-32P]orthophosphate (6).

MATERIALS AND METHODS

Isolation of Nucleoli from Novikoff Hepatoma Ascites Cells—Nucleoli were isolated from Novikoff hepatoma ascites cells by the sucrose-calcium procedure employing sonic oscillation as described previously (6, 7, 14-17).

In Vitro Incubation of Nucleoli—Isolated nucleoli containing about 10 mg of protein (determined by the absorbance ratio of 280/290 nm in 0.5 ml of 0.6 M guanidine hydrochloride and 0.02 M Tris HCl buffer, pH 8.0) (13) were incubated in a medium containing 0.25 M sucrose, 5 mM MgCl$_2$, 12.5 mM NaCl, and 0.05 M Tris-HCl buffer (pH 7.3) and 0.125 mCi of [$\gamma$-32P]ATP (64 to 120 Ci per mmole, International Chemical and Nuclear Co., Irvine, California) in a total volume of 2.1 ml. The mixtures were placed in disposable test tubes which were gently shaken during incubation at 37°C in the thermostatically controlled water bath of a gyrotary shaker (New Brunswick Scientific Co., N. J.). At appropriate time intervals, the reaction was stopped by cooling the reaction tubes in ice-cold water and the subsequent addition of 0.4 ml of ice-cold 2 N H$_2$SO$_4$. The nucleolar acid-soluble proteins were then extracted at 4°C and precipitated with 4 volumes of absolute ethanol (6, 19).

Electrophoresis of Nucleolar Acid-soluble Proteins—Two-dimensional polyacrylamide gels were prepared and subjected to electrophoresis by the method of ORRIEK et al. (6, 20, 21). The radioactive protein spots were detected on DuPont Cronex x-ray film as described earlier (6).

Quantitative Determination of Phosphorylation—After precipitation from ethanol, 3 to 8 mg of acid-soluble proteins were washed twice with 3-ml portions of ethanol-ether (3:1, v/v) and once with 3 ml of 25% trichloroacetic acid containing 0.2 M sodium pyrophosphate (5, 22). To remove the nucleic acids, the proteins were suspended in 1 ml of 5% trichloroacetic acid at 96°C for 15 min. The proteins were then precipitated by the addition of 3 ml of 30% trichloroacetic acid. After spinning at 20,000 × g for 15 min, the supernatant fraction usually contained less than 8% of the total radioactivity. The protein pellet was again washed.
FIG. 1. The time course of the in vitro phosphorylation of nucleolar acid-soluble proteins during incubation of isolated nucleoli at 37°C. The incubation mixture of 2.1 ml contained 0.25 M sucrose, 5 mM MgCl₂, 12.5 mM NaCl, 0.125 mCi of [γ-32P]ATP (64 to 120 Ci per mm), 0.05 M Tris-HCl buffer (pH 7.3), and isolated nucleoli containing 10 mg of protein.

was rapid for the first 5 min and a plateau was reached by 15 min of incubation.

On one-dimensional 10% polyacrylamide disc gel electrophoresis, no significant differences were found in the patterns of the stained protein (Fig. 2, bottom) during different time intervals of incubation of nucleoli in vitro. However, when the 32P was determined in the protein bands after slicing the gels, significant differences were found in the radioactivity in the stained protein bands. In addition, at different times, there were changes in labeling of specific protein bands. As shown in Fig. 2, a radioactive peak in slices 55 to 57 corresponding to the area of spot B27 on the two-dimensional slab gels (Fig. 3) was maximally phosphorylated within 5 min. However, the uptake of radioactive phosphate was found to increase with longer incubation times into peaks at slices 50 and 68, which correspond to spots B13 and C23-24, respectively, on the slab gels (Fig. 3).

Two-Dimensional Gel Electrophoretic Analysis of Nucleolar Acid-soluble Phosphoproteins—Two-dimensional gel electrophoresis (6, 20) not only demonstrated the heterogeneity of the nucleolar proteins in the incubated isolated nucleoli (Fig. 3, a and b) but also showed that no major changes in the protein profile occurred for the duration of these experiments. The nomenclature for the spots in the regions A, B, and C (Fig. 3, a and b) is the same as that described earlier (20, 21). At 24 hours of exposure, three intense spots were observed in the autoradiograph (Fig. 3c, inset) which correspond to C23-24, B27, and B13 on the gel slab. Further exposure up to 10 days (Fig. 3c) showed incorporation of radioactive phosphate into a number of protein spots including C17-18, C8, C2, B35, B20, B9, A25, A20, A16, and A7. Since many protein spots contained little or no radioactivity (Fig. 3, a and c), there is clearly selective phosphorylation of the nucleolar acid-soluble proteins in vitro.

Quantitative Estimation of Phosphorylation of Protein Spots—The amounts of radioactivity in the protein spots differed in the

RESULTS

Kinetics of in Vitro Phosphorylation of Nucleolar Acid-soluble Proteins—Fig. 1 shows the time course of phosphorylation of nucleolar acid-soluble proteins in vitro. The uptake of isotope twice with 3 ml of 25% trichloroacetic acid and then dissolved in 1.0 ml of 1 N KOH. Aliquots of the solution were analyzed for protein concentration and for radioactivity after dilution with Aquasol (New England Nuclear, Boston, Mass.). The protein concentrations were determined by the method of Lowry et al. (23) with bovine serum albumin (Miles Laboratories, Kankakee, Ill.) as a standard.

Determination of Radioactivity on One- and Two-Dimensional Gels—After destaining, the one-dimensional disc polyacrylamide gels were frozen in acetone and Dry Ice and cut into 1-mm slices using a Bio-Rad (Richmond, Calif.) model 190 gel slicer. Each slice was dried overnight at room temperature in a scintillation vial. After addition of 8 ml of Aquasol, the radioactivity was measured in a Beckman (Palo Alto, Calif.) LS-230 liquid scintillation counter. With the two-dimensional gels, the spots found to be radioactive by autoradiography were excised with a cork borer. The radioactivity of each spot was determined as above.
FIG. 3. a, two-dimensional polyacrylamide gel electrophoresis of 250 μg of Novikoff hepatoma acid-soluble nucleolar proteins from nucleoli after 15 min of incubation in vitro as described in Fig. 1. Samples were first loaded on disc gels of 10% acrylamide-4.5 M urea and run in the first dimension for 5.5 hours at 120 volts constant voltage. For the second dimension, a 12% acrylamide-0.1% sodium dodecyl sulfate slab gel was run for 16 hours at 50 mA constant amperage and then stained with Coomassie brilliant blue R. b, diagrammatic representation of the protein pattern of a showing the numbering system for the protein spots. The spots are in the order of decreasing intensity: closed circles > open circles. Minor spots are shown as broken circles. c, visualization of nucleolar phosphoproteins from Novikoff hepatoma ascites cells after 15 min of in vitro incubation with [γ-32P]ATP. The system for in vitro incubation was the same as described in Fig. 1. The acid-soluble nucleolar proteins were subjected to electrophoresis as described in a. After electrophoresis, the slab gel was sandwiched between DuPont Cronex x-ray films in the dark. The film was exposed for 1 day (insert) or 10 days and then developed. The radioactive spots on the x-ray film were matched with the protein spots on b as indicated by the same numbering system. A8P indicates this radioactive spot does not coincide exactly with the stained spot on the slab gel.

TABLE I

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>Radioactivity</th>
<th>Relative radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without divalent cation</td>
<td>2,400</td>
<td>1.6</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1,000</td>
<td>0.6</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>47,000</td>
<td>30.3</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>47,800</td>
<td>30.8</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>100,200</td>
<td>70.4</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>155,100</td>
<td>100</td>
</tr>
</tbody>
</table>

*The values are expressed as percentages of counts per min relative to the incorporation observed in the presence of Zn²⁺.*

Effects of Divalent Cations on In Vitro Phosphorylation of Nucleolar Acid-soluble Proteins—The presence of divalent cations was necessary for in vitro phosphorylation; in the absence of any divalent cations, only 2400 cpm per mg were incorporated into the nucleolar acid-soluble proteins (Table I). Addition of Mg²⁺ (8), Mn²⁺ (8), and Zn²⁺ increased the labeling of the proteins by factors of 20, 45, and 65, respectively (Table I). In the presence of Co²⁺, the uptake of isotope was similar to that with Mg²⁺. However, Ca²⁺ had inhibitory effects on the phosphorylation since it reduced the phosphorylation to half the endogenous radioactivity.

In addition to these quantitative differences in labeling of whole nucleolar proteins, Fig. 5 shows marked differences in the incorporation of 32P into different protein bands on one-dimensional polyacrylamide disc gels. By comparison with labeling of three regions and varied considerably from spot to spot within each region (Fig. 4 a to c). For example, in the C region spots C23 and C24 were so highly phosphorylated that a separate ordinate was required (Fig. 4c); spots C4 and C6 contained less than 50 cpm. In the A region the protein spot which contained the most radioactivity was spot A20 with only 90 cpm. Differences in labeling were also found in the B region. These results provide further evidence for selective phosphorylation of nucleolar protein.
FIG. 4. Quantitative estimation of uptake of $^{32}$P into protein spots in the two-dimensional electrophoretogram of acid-soluble nucleolar proteins from Novikoff hepatoma ascites cells. The numbers on the abscissa correspond to the spot numbers on the map of Fig. 3b. a, A region of slab gel map; b, B region of slab gel map; c, C region of slab gel map as shown in Fig. 3b (a, b, and c).

in the presence of Mg$^{2+}$, there was a decrease in the isotope content of peaks in slices 48 to 50 and 65 to 68 in the presence of Zn$^{2+}$. On the other hand, Zn$^{2+}$ greatly increased the radioactivity in the peak in slice 55 to 57 which corresponds to protein spots B27 and B23-25 (Figs. 3, b and c, and 6c). An additional peak in slice numbers 31 to 33 corresponding to A17-19 (Figs. 3 and 6, a and c) was also observed when Mg$^{2+}$ was replaced by either Zn$^{2+}$ or Co$^{2+}$.

The presence of the various divalent cations produced qualitative and quantitative differences in the radioactivity in various spots (Figs. 3c and 6; Table II). For example, spots B23-25 were increasingly labeled in the presence of Co$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ and not in the presence of Mg$^{2+}$ (Fig. 3c). Spot B13 was labeled more in the presence of Mg$^{2+}$ than with the other cations. Spots A18-19 (lysine-rich histones) were moderately labeled only in the presence of Zn$^{2+}$. On the other hand, spot B27 was moderately labeled only in the presence of Mg$^{2+}$ and Zn$^{2+}$ (Table II). There was a high level of phosphorylation of protein spots C23-24 (Figs. 3c and 6, a to c) in the presence of all four divalent cations. The incorporation of $^{32}$P into nucleolar acid-soluble proteins in the absence of divalent cations or the presence of Ca$^{2+}$ was too low to be detected by autoradiography. By comparison with the divalent ions, cyclic AMP and papaverine produced little change in phosphorylation of nucleolar proteins (Fig. 7).

**DISCUSSION**

In addition to phosphorylation of other nuclear proteins (4, 22, 24-27), phosphorylation of nucleolar acid-soluble proteins in vivo has recently been demonstrated (6, 28). Since several nucleolar proteins were found to be highly phosphorylated (Figs. 3c and 4) in nucleoli in vitro, phosphoprotein kinases (28) may be added to the list of nucleolar enzymes which includes

$^2$ The abbreviation used is: cyclic AMP, adenosine 3':5'-monophosphate.
TABLE II

Major qualitative and quantitative differences in incorporation of 32P into nucleolar protein spots in presence of various divalent cations

With 10-day exposures of slab gels on x-ray films, different protein spots contained different amounts of 32P as detected by autoradiography (Figs. 3a and 6). Differences in the amounts of radioactivity in the protein spots showed different intensities of spots on x-ray films. These were expressed in the order of decreasing radioactivity as ++++ for greater than 20,000 cpm, +++ for 700 to 20,000 cpm, ++ for 100 to 700 cpm, + for 40 to 100 cpm, and - for less than 40 cpm or absence of radioactivity.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Relative radioactivity in spot</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1–4a</td>
<td>-</td>
</tr>
<tr>
<td>A7</td>
<td>-</td>
</tr>
<tr>
<td>A8P</td>
<td>++</td>
</tr>
<tr>
<td>A20</td>
<td>+</td>
</tr>
<tr>
<td>A25</td>
<td>-</td>
</tr>
<tr>
<td>A16</td>
<td>-</td>
</tr>
<tr>
<td>A17</td>
<td>-</td>
</tr>
<tr>
<td>A18–19</td>
<td>++</td>
</tr>
<tr>
<td>B5</td>
<td>+</td>
</tr>
<tr>
<td>B9</td>
<td>+</td>
</tr>
<tr>
<td>B13</td>
<td>++</td>
</tr>
<tr>
<td>B23–25</td>
<td>+++</td>
</tr>
<tr>
<td>B27</td>
<td>++</td>
</tr>
<tr>
<td>C2</td>
<td>+++</td>
</tr>
<tr>
<td>C23–24</td>
<td>+++</td>
</tr>
</tbody>
</table>

* This is a radioactive spot which migrates in the center of the region encompassed by stained spots A1, 2, and 4. It was also phosphorylated in vivo (6).

FIG. 6. Visualization of nucleolar phosphoproteins from Novikoff hepatoma ascites cells after a 15-min incubation in vitro of isolated nucleoli with [γ-32P]ATP in the presence of 5 mM of either (a) CoCl2, (b) MnCl2, or (c) ZnCl2. The procedures for the two-dimensional gel electrophoresis and the exposure of the x-ray film were the same as for Fig. 3.

RNA polymerase (8, 9, 15), an RNase (17), NAD pyrophosphorylase (17), ATPase A (17), and RNA methylase (10, 11). Although these results demonstrate the presence of phosphoprotein kinase in nucleoli, they do not establish a unique localization for this phosphoprotein kinase activity.

Comparison of the phosphorylation of nucleolar acid-soluble proteins shows that the same protein spots, including spots C23–24, B27, and B13 (6) were labeled both in vivo and in vitro. In the system containing Mg2+ (Fig. 3c), several protein spots that were phosphorylated in vivo were not phosphorylated in vitro, including spots A17–19, B15, B22, B23–25, C6, and C25 (6, 20). However, under proper conditions with replacement of Mg2+ by Co2+, Mn2+, or Zn2+ (Table II), the isolated

FIG. 7. Effects of increasing concentrations of cyclic AMP on the phosphorylation of nucleolar acid-soluble protein in isolated nucleoli from Novikoff hepatoma ascites cells. The in vitro incubation systems were the same as for Fig. 1 except for the presence of various concentrations of cyclic AMP as indicated.
nucleoli phosphorylated all the proteins phosphorylated in vivo (6).

Quantitative differences in phosphorylation were also observed between in vivo and in vitro systems. This variation could be due to different turnover rates or different divalent cation concentrations in vivo and in vitro.

Zn²⁺ produced the highest level of phosphorylation of the nucleolar proteins (Table 1). In histological studies, a high Zn²⁺ concentration has been found in nucleoli of prostatic cancer cells (29). Also Zn²⁺ and Mn²⁺ caused higher endogenous phosphorylation of proteins in synaptic membrane fractions from rat brain; Ca²⁺ was inhibitory (30). Divalent cations may act to change phosphoprotein kinase activities, phosphoprotein phosphatase activities (30), or the availability of serine hydroxyl groups for phosphorylation.

Possible relationships have been suggested between the phosphorylation of nuclear nonhistone proteins and gene regulation (4, 31, 32), cell division (26) or protein binding to deoxyribonucleic acid (33). Studies are currently underway to isolate and purify the nucleolar phosphoprotein kinases to identify which of the proteins phosphorylated are involved in maturation of the nucleolar preribosomal RNP particles and are related to phosphorylated polysomal proteins.

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