

Tyrosylprotein kinase and phosphatase activities in membrane vesicles from normal and Rous sarcoma virus-transformed rat cells

(*src* protein/phosphotyrosine dephosphorylation/zinc inhibition)

BYRON GALLIS*, PAUL BORNSTEIN*†, AND DAVID L. BRAUTIGAN*

Departments of *Biochemistry and †Medicine, University of Washington, Seattle, Washington 98195

Communicated by Edwin G. Krebs, July 20, 1981

ABSTRACT Membrane vesicles, isolated from normal and Rous sarcoma virus-transformed rat cells, have an associated cyclic-AMP independent kinase that phosphorylates a M_r 37,000 protein in vesicles from normal cells and proteins of M_r 37,000, 50,000, and 67,000 in vesicles from transformed cells. Proteins in vesicles from normal and transformed cells contain 9% and 77%, respectively, of their labeled phospho amino acids as phosphotyrosine. Thus, isolation of vesicles and subsequent incubation with [γ - 32 P]ATP enriches the proportion of labeled phosphotyrosine in proteins (relative to other phospho amino acids) by two orders of magnitude over that found in intact cells. The *in vitro* phosphorylation of each of these proteins is enhanced in the presence of $10 \mu\text{M Zn}^{2+}$, a phosphotyrosylprotein phosphatase inhibitor. From these studies it appears that membrane vesicles may be a valuable system for examination of transformation-specific phosphorylation of proteins.

The *src* gene of Rous sarcoma virus (RSV) codes for a M_r 60,000 phosphoprotein known as pp60^{src} (1, 2), which initiates transformation of cultured avian and mammalian cells (3). This gene product also induces the development of sarcomas in chickens (3). Immunoprecipitates of pp60^{src} contain a cyclic AMP-independent protein kinase that phosphorylates the heavy chain of IgG (4). The kinase activity associated with pp60^{src} phosphorylates proteins in tyrosine residues (5–7), so that transformation of avian and mammalian cells by RSV correlates with a 5- to 10-fold increase in the level of phosphotyrosine in cellular proteins (5, 8).

Several proteins containing phosphotyrosine have been identified in RSV-transformed chicken and rat cells. Sefton and Hunter (5) identified a M_r 50,000 phosphoprotein associated with pp60^{src} in immune complexes precipitated from chicken cells with anti-pp60^{src} serum. Another phosphoprotein of M_r 34,000–36,000, which contains both phosphoserine and phosphotyrosine, has been identified in RSV-transformed chicken and mammalian cells (9–11). In RSV-transformed cells pp60^{src} has been localized in the cytoplasm (12), adhesion plaques (13), and plasma membranes (14, 15).

In this study we isolated membrane vesicles from RSV-transformed cells and from normal cells. Kinases in vesicles from normal cells phosphorylated serine, threonine, and tyrosine residues in M_r 37,000 protein, whereas kinases in the transformed cell vesicles phosphorylated predominantly tyrosine residues in M_r 37,000, 50,000, and 67,000 proteins. The phosphorylation of membrane proteins in vesicles was not inhibited by anti-pp60^{src} serum and was enhanced in the presence of $10 \mu\text{M Zn}^{2+}$, which is known to inhibit phosphotyrosylprotein phosphatase activity (16).

METHODS AND MATERIALS

Materials. The anti-pp60^{src} serum was a generous gift from Larry Rohrschneider and its properties have been described (12, 17). Phosphotyrosine was a gift from Linda Pike, Department of Pharmacology and Howard Hughes Medical Institute, University of Washington, Seattle. Phosphothreonine and phosphoserine were obtained from Sigma. [γ - 32 P]ATP (3000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear.

Cells. The normal and RSV-transformed rat cell lines have been described in detail (18). The normal rat cells, denoted N2, are a diploid rat embryo fibroblast line; this line was infected with the Schmidt–Ruppin strain D of RSV to generate a transformed line (denoted as RS-1). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum.

Immunoprecipitation of pp60^{src}. Immunoprecipitation of pp60^{src} from vesicles was performed by addition of 1 ml of buffer containing 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1% NaDodSO₄, 0.5% Nonidet P-40, and 0.5% deoxycholate, followed by addition of 2 μl of anti-pp60^{src} serum. Immunoprecipitation of pp60^{src} from cell lysates and all subsequent steps, including incubation of antigen-anti-pp60^{src} complexes with [γ - 32 P]ATP, NaDodSO₄/polyacrylamide gel electrophoresis, and autoradiography were performed as described (19).

Preparation of Membrane Vesicles. Membrane vesicles were prepared by dilution of cells into hypotonic sodium borate (pH 10.2) as described by Carpenter *et al.* (20) and modified by Brautigam *et al.* (16).

Phosphorylation Reaction. Phosphorylation reaction mixtures (100 μl) contained membrane vesicles (10 μg of protein), bovine serum albumin at 0.1 mg/ml, 2 mM manganese acetate, and 0.1 μM [γ - 32 P]ATP in 20 mM Hepes (pH 7.4). Reactions were carried out for 1–5 min in an ice bath. Maximal ^{32}P incorporation occurred at 1 min. Precipitations of 10- μl aliquots of reaction mixtures were performed by addition of 10% trichloroacetic acid.

Hydrolysis of ^{32}P -Labeled Proteins and Two-Dimensional Electrophoresis of Phospho Amino Acids. Membrane vesicle reactions were carried out for 1 min at 0°C; zinc was immediately added to a concentration of 10 μM . Vesicles were dialyzed against ice-cold 50 mM NH₄HCO₃ containing 10 $\mu\text{M Zn}^{2+}$ for 30 min, and the phosphorylated membrane proteins were digested for 10 min at 30°C with 5 μg of trypsin treated with tosylamidophenylethyl chloromethyl ketone (TosPheCH₂Cl). Phosphorylated peptides were then acid hydrolyzed, and the

phospho amino acids were fractionated by high-voltage two-dimensional electrophoresis (5). Phosphorylated proteins were removed from gel slices as described by Beemon and Hunter (21).

Atomic Absorption Spectrometry. Normal and transformed rat cells were removed from plates with trypsin and washed twice by centrifugation from 50 vol of isotonic saline containing EDTA. The cells were counted in a Coulter Counter and then digested in nitric acid at 50°C for 1 hr. Residue was removed by centrifugation and the supernatant was analyzed for zinc by atomic absorption, using an Instrumentation Laboratory IL551 spectrometer. The instrument was used in standard flame mode (acetylene-air) and a certified zinc standard (Fisher) was used for calibration.

RESULTS

Kinase Activity of pp60^{src} in RSV-Transformed Rat Cell Lysates and Membrane Vesicles. We prepared membrane vesicles from RSV-transformed rat cells and from normal cells to determine whether pp60^{src} kinase activity was present in lysates and in vesicles. We found that pp60^{src} kinase activity is associated with vesicles from the transformed cells; immunoprecipitation of vesicles with anti-pp60^{src} serum followed by incubation with [γ -³²P]ATP (19) caused phosphorylation of the M_r 55,000 heavy chain of the immunoglobulin (Fig. 1, lane D). Antiserum against pp60^{src} also precipitated a kinase activity from whole RSV-transformed rat cells (Fig. 1, lane C) but not from lysates of normal cells (Fig. 1, lane A) or from normal cell vesicles (data not shown). No kinase activity was precipitated from RSV-transformed rat cells with nonimmune serum (Fig.

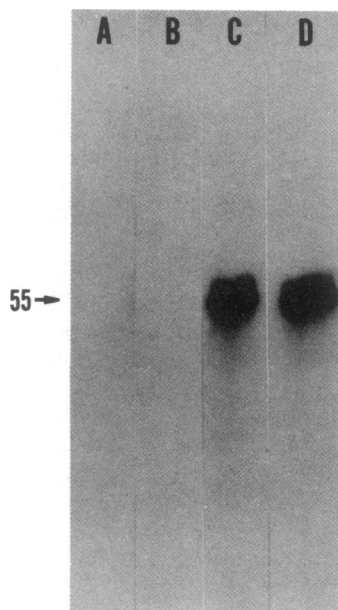


FIG. 1. Protein kinase activity in immunoprecipitates of normal and transformed cells and membrane vesicles. The autoradiogram of a NaDodSO₄/12.5% polyacrylamide slab gel shows immunoprecipitations from: lane A, a lysate of 3×10^6 normal cells with anti-pp60^{src} serum; lane B, a lysate of 3×10^6 transformed cells with nonimmune serum; lane C, a lysate of 3×10^6 transformed cells with anti-pp60^{src} serum; lane D, 20 μ g of transformed cell vesicle protein with anti-pp60^{src} serum. Cell lysis and immunoprecipitation with antiserum and *Staphylococcus aureus* cells were performed as in *Materials and Methods* and as described (19). Immunoprecipitates were incubated with 0.1 μ M [γ -³²P]ATP for 5 min on ice, washed, and boiled with NaDodSO₄ sample buffer and 5 mM dithiothreitol. The antigen-antibody complexes released from *S. aureus* were fractionated on the slab gel, stained, and subjected to autoradiography (19). $M_r \times 10^{-3}$ is given on the left.

1, lane B). These data show that pp60^{src} is present in the RSV-transformed rat cells and in membrane vesicles prepared from them. This antiserum does not precipitate the endogenous pp60^{src} from uninfected cells (15, 17).

Enhancement of Protein Phosphorylation in Vesicles from Normal and Transformed Rat Cells by Zn²⁺. We examined the phosphorylation of individual proteins in vesicles from normal and transformed rat cells after incubation in the presence of [γ -³²P]ATP and Mn²⁺. Vesicles from normal cells incorporate half as much [³²P]phosphate per mg of protein as do those from transformed cells, as determined by trichloroacetic acid precipitation of the reaction mixtures onto filter paper (data not shown). In membranes from normal cells, the most highly phosphorylated protein is a doublet corresponding to M_r 37,000 (Fig. 2, lane C). Vesicles from transformed cells have three major phosphorylated proteins, of M_r 37,000, 50,000, and 67,000 (Fig. 2, lane A), and two minor phosphoproteins of M_r 85,000 and 100,000. Of the proteins phosphorylated in these vesicles, only the M_r 100,000 can be seen as a stained band on polyacrylamide gels; the other phosphoproteins are present at concentrations too low to be seen by Coomassie blue staining. The phosphoprotein of M_r 67,000 is not the bovine serum albumin usually added to the reaction, because this protein is phosphorylated to the same extent when bovine serum albumin is omitted from the reaction (data not shown).

When the phosphorylation reactions were performed in the presence of 10 μ M Zn²⁺, the ³²P content of the M_r 37,000 pro-

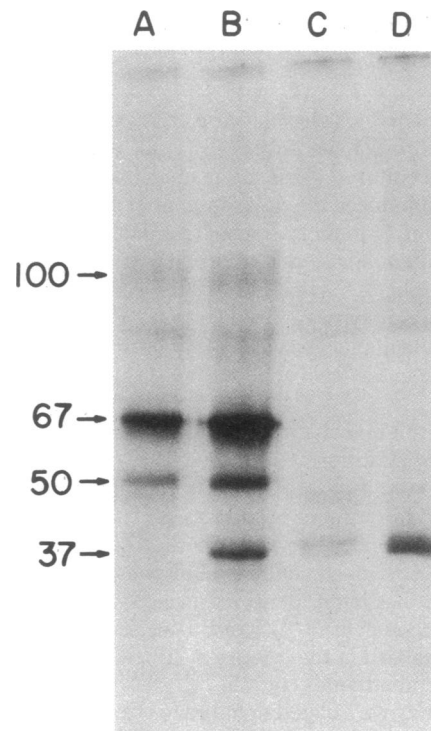


FIG. 2. Phosphoproteins labeled in vesicles from normal and transformed cells in the presence and absence of Zn²⁺. Normal or transformed cell vesicles (10 μ g of protein) were incubated with 0.1 μ M [γ -³²P]ATP in the presence or absence of Zn²⁺ for 5 min at 0°C. Aliquots of the reaction mixtures were removed and boiled in NaDodSO₄ buffer containing reducing agent before electrophoresis on a NaDodSO₄/10% polyacrylamide slab gel. The autoradiogram of the gel shows: [³²P]phosphoproteins of transformed cell vesicles incubated in the absence of Zn²⁺ (lane A) or with 10 μ M Zn²⁺ (lane B); [³²P]phosphoproteins of normal cell vesicles incubated in the absence of Zn²⁺ (lane C) or with 10 μ M Zn²⁺ (lane D). The relative molecular mass of the proteins was determined from standards: phosphorylase b, M_r 97,500; albumin, M_r 67,000; ovalbumin, M_r 43,000; and carbonic anhydrase, M_r 29,000. $M_r \times 10^{-3}$ is given on the left.

tein in normal cell vesicles (Fig. 2, lane D) and of the three lower molecular weight proteins in transformed cell vesicles was enhanced (Fig. 2, lane B). Membrane-associated kinase was dependent upon the presence of Mn^{2+} ; incubation of vesicles with [γ - ^{32}P]ATP in the presence of Zn^{2+} alone (between 10 μM and 1 mM) failed to support phosphorylation of proteins, hence Zn^{2+} was apparently not activating another kinase. In order to quantify the increased ^{32}P phosphorylation of proteins in the presence of Zn^{2+} , the autoradiograms in Fig. 2 representing proteins labeled in the presence and the absence of Zn^{2+} were scanned with a Quick Quant gel scanner (Helena Laboratories, Beaumont, TX). The phosphorylation of the M_r 37,000 protein from normal cell vesicles increased 3.1-fold in the presence compared to the absence of Zn^{2+} , whereas phosphorylation of the M_r 37,000, 50,000, and 67,000 proteins from transformed cell vesicles increased 4.3-fold, 1.8-fold, and 2-fold, respectively. The [^{32}P]phosphate content of the M_r 100,000 protein was not altered when reaction mixtures were supplemented with Zn^{2+} .

Even though pp60^{src} kinase is associated with RSV-transformed cell vesicles, we find that pp60^{src} is not responsible for phosphorylation of the proteins in vesicles isolated from transformed cells. Vesicles were preincubated for 1 hr at 30°C with an excess of antiserum to pp60^{src} under reaction conditions described in *Materials and Methods*, except [γ - ^{32}P]ATP was omitted. Reaction mixtures were cooled to 0°C and [γ - ^{32}P]ATP was then added. After 1 min, the reactions were stopped by boiling after addition of Laemmli sample buffer and dithiothreitol. Reaction products were fractionated on polyacrylamide gels. Autoradiography showed that no reduction in the level of phosphorylation of the three major phosphoproteins was caused by the preincubation with pp60^{src} serum (data not shown). However, the specific IgG in the antiserum was phosphorylated intensely, showing that it was bound to pp60^{src}. Preincubation with anti-pp60^{src} serum has been shown to inhibit phosphorylation of exogenously added substrates such as casein (7, 22). We interpret these results to indicate that a kinase or kinases, which is not pp60^{src} but another tyrosylprotein kinase, is responsible for these phosphorylations.

Phospho Amino Acid Content of Individual Proteins in Membrane Vesicles from Transformed Rat Cells. To identify the phospho amino acids present in the M_r 37,000, 50,000, and

67,000 phosphoproteins from transformed cell vesicles, membrane vesicles were incubated for 1 min at 0°C with [γ - ^{32}P]ATP. Reactions were stopped by addition of Laemmli sample buffer and boiling and fractionated on a NaDodSO₄/10% polyacrylamide slab gel. Autoradiography exposed the location of the [^{32}P]phosphoproteins (Fig. 2, lane B), and the sections of the gel containing the labeled proteins were excised and eluted from the gel by incubation with protease (2). The peptides were subjected to partial acid hydrolysis prior to separation of the phospho amino acids by two-dimensional electrophoresis on cellulose thin-layer plates (5). The predominant phospho amino acid in the M_r 37,000, 50,000, and 67,000 proteins was phosphotyrosine (Fig. 3).

Phospho Amino Acid Content of ^{32}P -Labeled Proteins in Membrane Vesicles from Normal and Transformed Cells. We identified the phospho amino acids in the proteins of membrane vesicles isolated from normal and transformed cells because the level of phosphotyrosine in proteins has been correlated with RSV transformation (5, 8). Membranes were incubated for 1 min with [γ - ^{32}P]ATP and Mn^{2+} in the absence of Zn^{2+} . After addition of Zn^{2+} to inhibit the phosphatase, the ^{32}P -labeled proteins were digested with TosPheCH₂Cl-trypsin and subjected to limited acid hydrolysis, and the phospho amino acids were separated by two-dimensional high-voltage electrophoresis (5). Autoradiograms of the cellulose thin-layer plates revealed that the [^{32}P]phosphate in proteins from normal cell vesicles was present predominantly as phosphoserine, with smaller amounts of phosphothreonine and phosphotyrosine (Fig. 4A), whereas phosphotyrosine was the predominant phospho amino acid in proteins from transformed cell vesicles (Fig. 4B). These data show that most of the kinase activity that we detect in transformed cell vesicles is a tyrosylprotein kinase that is immunologically distinct from pp60^{src} (see above).

We have estimated the relative amounts of labeled phosphoserine, phosphothreonine, and phosphotyrosine in the normal and transformed cell vesicle proteins (Table 1) by scraping the cellulose from the ninhydrin-positive areas of each plate that corresponded to the phospho amino acid standards (Fig. 4) and measuring the radioactivity. Of the total phospho amino acids in transformed cell vesicles, 77% is phosphotyrosine whereas only 18% is phosphoserine and 5% is phosphothreonine (Table 1). However, in normal cell vesicles, 65% of the labeled phos-

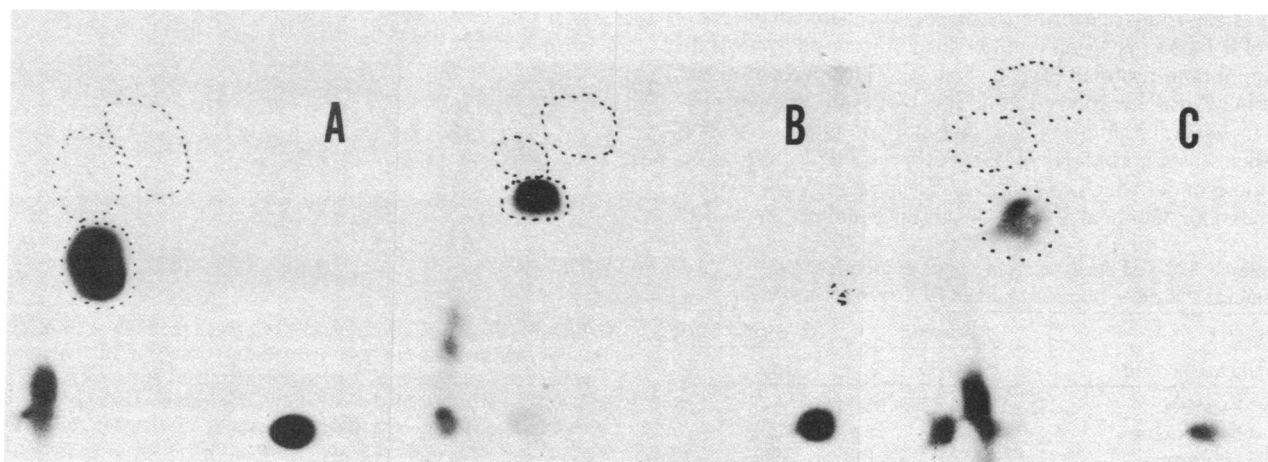


FIG. 3. Labeled phospho amino acids in particular proteins from vesicles of transformed cells. Proteins in transformed cell membrane vesicles were labeled in the presence of 10 μM Zn^{2+} and fractionated on gels as described in the legend to Fig. 2. Using the autoradiogram of the gel, slices corresponding to M_r 37,000, 50,000, and 67,000 proteins were excised, hydrated by addition of 0.05 M NH_4HCO_3 , and digested at 37°C for 18 hr with proteinase K at 40 $\mu g/ml$. The phosphopeptides were lyophilized, acid hydrolyzed, and subjected to two-dimensional electrophoresis. Phospho amino acids are from: the M_r 67,000 protein (A), the M_r 50,000 protein (B), and the M_r 37,000 protein (C). The origin is to the lower left in each case and the phospho amino acids are, in ascending order: phosphotyrosine, phosphothreonine, and phosphoserine. Other spots are presumably incompletely hydrolyzed phosphopeptides.

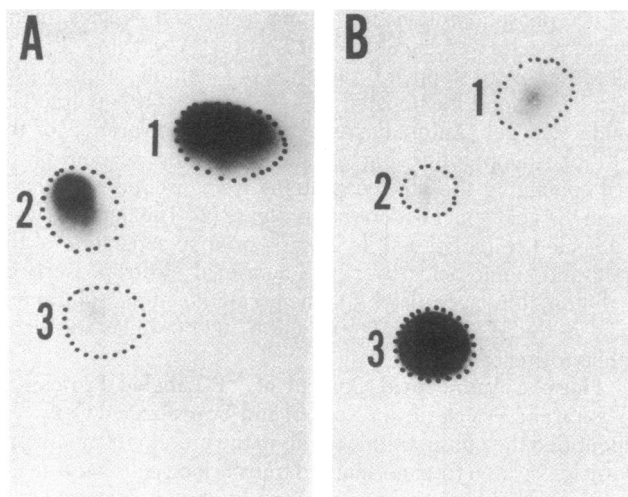


FIG. 4. Phospho amino acids in normal and transformed cell membrane vesicles. Vesicles from normal or transformed cells ($10 \mu\text{g}$ of protein) were labeled in the presence of $0.1 \mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]ATP for 1 min at 0°C . The mixtures were made $10 \mu\text{M}$ with Zn^{2+} , dialyzed, and digested with TosPheCH₂Cl-trypsin and subjected to partial acid hydrolysis (5). The hydrolysates, together with added phosphotyrosine, phosphoserine, and phosphothreonine, were subjected to electrophoresis at pH 1.9 in the first dimension (left to right) and at pH 3.5 in the second dimension (bottom to top). The plates were stained with ninhydrin to reveal the positions of the added standards, shown by dotted lines: 1, phosphoserine; 2, phosphothreonine; 3, phosphotyrosine. Autoradiograms of phospho amino acids labeled in normal vesicles (A) and in RSV-transformed vesicles (B) are shown.

pho amino acids is phosphoserine, whereas 25% is phosphothreonine and 9% is phosphotyrosine (Table 1). In contrast, normal and RSV-transformed cells labeled with [$\gamma\text{-}^{32}\text{P}$]phosphate contain approximately 0.04% and 0.48%, respectively, of their total phospho amino acids as phosphotyrosine and greater than 99% as phosphoserine and phosphothreonine (8). Thus, by isolating membrane vesicles we obtain a 150- to 200-fold enrichment of labeled phosphotyrosine, relative to the total phospho amino acids, compared to the intact RSV-transformed or normal cells from which the vesicles are prepared.

Effects of Zn^{2+} on the Dephosphorylation of ^{32}P -Labeled Proteins. The level of protein phosphorylation in the vesicles was enhanced by Zn^{2+} , which inhibits a phosphatase activity (16). Vesicles from transformed cells were incubated in the presence of $0.1 \mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]ATP on ice for 2 min to phosphorylate the membrane proteins (Fig. 5, lane A). Fifty percent of the [$\gamma\text{-}^{32}\text{P}$]ATP was hydrolyzed after incubation of reaction mixtures for several minutes on ice. Therefore, dephosphorylation of vesicles was monitored by incubation at 30°C for 30 min, conditions under which no [$\gamma\text{-}^{32}\text{P}$]ATP remained. Vesicles were incubated for 30 min at 30°C in the absence and presence of $10 \mu\text{M}$

Table 1. Labeled phospho amino acids in proteins from membrane vesicles from normal and RSV-transformed cells

Phospho amino acid	Normal		Transformed	
	cpm	%	cpm	%
Phosphoserine	11,213	65	717	18
Phosphothreonine	4,355	25	205	5
Phosphotyrosine	1,574	9	3905	77

Membranes containing ^{32}P -labeled protein were digested with TosPheCH₂Cl-trypsin and subjected to limited acid hydrolysis, and the phospho amino acids were separated by two-dimensional high-voltage electrophoresis (5). Relative amounts of phosphoserine, phosphothreonine, and phosphotyrosine were estimated by scintillation counting of the cellulose scraped from the ninhydrin-positive areas that corresponded to the position of the unlabeled standards.

μM Zn^{2+} . The proteins in each reaction were fractionated on a NaDodSO₄/10% polyacrylamide slab gel. An autoradiogram of the gel (Fig. 5) revealed that $10 \mu\text{M}$ Zn^{2+} inhibited the removal of ^{32}P from all proteins (lane C) compared to the marked dephosphorylation of the M_r 37,000 and 67,000 proteins in the absence of Zn^{2+} (lane B). A scan of the autoradiogram was used to quantify the dephosphorylation of the proteins. In the presence of Zn^{2+} , the [^{32}P]phosphate content of the M_r 37,000, 50,000 and 67,000 proteins was 4-fold, 1.5-fold, and 2-fold greater than in the absence of Zn^{2+} . To assess the specificity of the Zn^{2+} -inhibited phosphatase, we examined the distribution of phosphotyrosine, phosphoserine, and phosphothreonine in vesicles from transformed cells that were incubated in the presence and absence of $10 \mu\text{M}$ Zn^{2+} for 30 min at 30°C . Phosphoproteins were digested with TosPheCH₂Cl-trypsin and subjected to partial acid hydrolysis (5) prior to separation of the phospho amino acids by two-dimensional cellulose thin-layer electrophoresis. After ninhydrin staining and autoradiography, the phospho amino acids were scraped from the plate and their radioactivities were measured. Compared to reaction mixtures containing Zn^{2+} , mixtures incubated without Zn^{2+} lost 41% of their phosphotyrosine, but only 21% and 15% of their phosphoserine and phosphothreonine, respectively. Thus, Zn^{2+} preferentially inhibits hydrolysis of the phosphotyrosine residues in these proteins.

Zinc Content of Normal and Transformed Rat Cells. Because the phosphotyrosylprotein phosphatase present in both

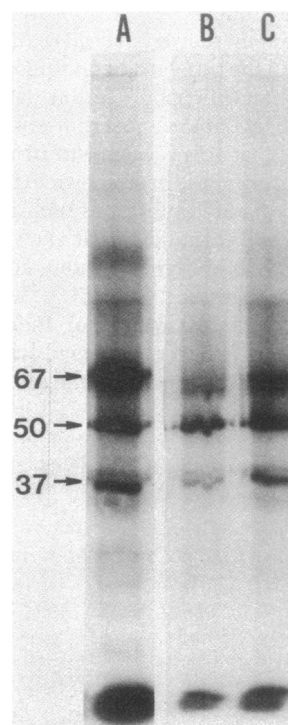


FIG. 5. Dephosphorylation of ^{32}P -labeled proteins in transformed cell vesicles incubated in the presence and absence of Zn^{2+} . Membrane vesicles ($10 \mu\text{g}$ of protein) were incubated in $0.1 \mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]ATP for 1 min at 0°C . After removal of an aliquot for fractionation of the protein labeled initially in the reaction, the remaining reaction mixture was divided into half and incubated either in the absence or presence of $10 \mu\text{M}$ Zn^{2+} for 30 min at 30°C . Dephosphorylation reactions were stopped by boiling with Laemmli sample buffer (23) containing 5 mM dithiothreitol. Equal volumes of the mixtures were subjected to electrophoresis on a 10% slab gel. The autoradiogram of the slab gel is shown: lane A, proteins labeled initially in the reaction; lane B, membrane vesicle phosphoproteins after incubation without Zn^{2+} ; lane C, membrane vesicle phosphoproteins after incubation with $10 \mu\text{M}$ Zn^{2+} . $M_r \times 10^{-3}$ is given on the left.

normal and transformed rat cells was inhibited by Zn^{2+} , we hypothesized that the phosphatase activity in transformed cells might be affected by an increased amount of zinc. The zinc content of normal and transformed cells and of the growth medium was measured by atomic absorption spectrometry. The level of zinc in transformed cells, 81 fg per cell, was nearly twice that in normal cells, 46 fg per cell. However, the protein content of transformed cells, as determined by Coomassie dye binding (24), paralleled the increase in zinc, 370 μ g of protein per 10^6 cells compared to 205 μ g of protein per 10^6 normal cells. Thus the zinc content would be 0.219 ng of Zn^{2+} per mg of protein in transformed cells and 0.224 ng of Zn^{2+} per mg of protein in normal cells. These determinations do not reveal a significant change in zinc content upon transformation, making it unlikely that changes in total cellular Zn^{2+} modulate the phosphatase activity under physiological conditions.

DISCUSSION

We have shown that tyrosylprotein kinases phosphorylate specific proteins associated with membrane vesicles prepared from normal and RSV-transformed rat cells. In normal cell vesicles the most highly phosphorylated protein appears as a doublet near M_r 37,000, whereas the major phosphorylated proteins in vesicles from transformed cells are of M_r 37,000, 50,000, and 67,000. Each of the proteins from transformed cells is phosphorylated predominantly on tyrosine residues. The phosphorylation of two additional proteins, of M_r 50,000 and 67,000, in RSV-transformed cells compared to normal cells could occur either by their *de novo* synthesis after transformation, or by the synthesis or the activation of the particular tyrosylprotein kinase responsible for this reaction. It is of interest that labeled phosphotyrosine is recovered in proteins of M_r 34,000–36,000 (9–11) and 50,000 (5) after whole RSV-transformed rat and chicken cells are incubated with [^{32}P]phosphate.

It is remarkable that substrates for the tyrosylprotein kinase and phosphatase are concentrated to such an extent in the membrane vesicles. The proportion of phosphotyrosine in proteins of membrane vesicles, relative to other phospho amino acids, is 150- to 200-fold higher than that attained with whole cells. Normal cell vesicles consistently had about two-thirds of the ^{32}P label in phosphoserine and 10–15% as phosphotyrosine, whereas RSV-transformed cell vesicles had three-quarters of the label in phosphotyrosine. This enrichment may result from the location of pp60^{src} on membranes (14, 15). The kinase activity in these vesicles is not inhibited by incubation with anti-pp60^{src} serum, suggesting that an enzyme other than pp60^{src} is present.

At a concentration of 10 μ M, Zn^{2+} inhibits phosphotyrosyl-protein phosphatase activity, causing increased labeling of the proteins in membrane vesicles of normal and transformed cells. Using human epidermoid carcinoma cell membranes, we have shown that the inhibition of phosphotyrosylprotein dephosphorylation by Zn^{2+} is specific (other divalent cations are ineffective) and reversible, because addition of EDTA restores the phosphatase activity (16). We believe that Zn^{2+} interacts directly with the phosphatase, rather than with the protein substrate, because it inhibits dephosphorylation of phosphotyrosylproteins of M_r 37,000 and 67,000 in vesicles from RSV-transformed rat cells as well as the M_r 150,000 protein in vesicles of the human epidermoid carcinoma cells. Inhibition involving different protein substrates in different cell types suggests a direct effect on the enzyme, although Zn^{2+} may bind specifi-

cally to the phosphotyrosine and surrounding residues. The effects of Zn^{2+} on the phosphate content of proteins of M_r 100,000 and 50,000 were much less pronounced, consistent with their relatively poor dephosphorylation after ^{32}P labeling (Fig. 5). It appears that these proteins may be less accessible to the phosphatase.

Physiological concentrations of Zn^{2+} in human plasma range between 11 and 18.5 μ M (25). Estimation of the free intracellular Zn^{2+} concentration is not practicable, but, because 10 μ M Zn^{2+} inhibits the phosphatase in these vesicles, it is possible that Zn^{2+} could inhibit the phosphatase activity intracellularly. This reduced phosphatase activity, together with the synthesis of pp60^{src} and the resulting increase in the phosphorylation of tyrosine residues, might serve to increase the levels of phosphotyrosine in proteins by a concerted mechanism

We thank Prof. Edmond H. Fischer for support throughout the course of this work and Clarice Martin for typing the manuscript. We thank Carol Quaife for performing atomic absorption spectrometry; these analyses were conducted under National Institute of Environmental Health Sciences Grant 00677. We also thank the Chemistry Division of the Department of Laboratory Medicine for zinc analyses. This investigation was supported by National Institutes of Health Grants DE 02600, AM 11248, and AM 07902, and by the Muscular Dystrophy Association. D.L.B. is a recipient of National Research Service Award F32 AM 06023.

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