MOLECULAR AND CELLULAR BIOLOGY, Feb. 1982, p. 199–206
0270-7306/82/020199-08$02.00/0

Vol. 2, No. 2

Tyrosine Phosphorylation of a 50K Cellular Polypeptide Associated with the Rous Sarcoma Virus Transforming Protein pp60^{src}

THOMAS D. GILMORE, KATHRYN RADKE, AND G. STEVEN MARTIN*

Department of Zoology, University of California, Berkeley, California 94720

Received 7 August 1981/Accepted 29 September 1981

We have examined the phosphorylation of a 50,000-dalton cellular polypeptide associated with the Rous sarcoma virus (RSV) transforming protein pp60^{src}. It has been shown that pp60^{src} forms a complex with two cellular polypeptides, an 89,000-dalton heat-shock protein (89K) and a 50,000-dalton phosphoprotein (50K). The pp60^{src}-associated protein kinase activity phosphorylates at tyrosine residues, and the 50K polypeptide present in the complex contains phosphotyrosine and phosphoserine. These observations suggest that the 50K polypeptide may be a substrate for the protein kinase activity of pp60^{src}. To examine this possibility, we isolated the 50K polypeptide by two-dimensional polyacrylamide gel electrophoresis from lysates of uninfected or virally infected cells. Tryptic phosphopeptide analysis indicated that the 50K polypeptide isolated by this method was the same polypeptide as that complexed to pp60^{src}. In uninfected cells or cells infected by a transformation-defective mutant, the 50K polypeptide contained phosphoserine but little or no phosphotyrosine. In cells infected by Schmidt-Ruppin or Prague RSV, there was a 40- to 50-fold increase in the quantity of phosphotyrosine in the 50K protein. Thus, the phosphorylation of the 50K polypeptide at tyrosine is dependent on the presence of pp60^{src}. However, the 50K polypeptide isolated from cells infected by temperature-sensitive mutants of RSV was found to be phosphorylated at tyrosine at both permissive and nonpermissive temperatures; this behavior is different from that of other substrates or putative substrates of the pp60^{src} kinase activity. It is possible that the 50K polypeptide is a high-affinity substrate of pp60^{src}.

Cellular transformation by Rous sarcoma virus (RSV) results from the expression of a single viral gene, the *src* gene. The product of this gene is a phosphoprotein (molecular weight, 60,000) designated pp60^{src} (2, 13, 20). This protein has, or is closely associated with, a protein kinase activity (4, 10, 13, 27) which phosphorylates at tyrosine residues (6, 12). In cells transformed by RSV, the total quantity of phosphotyrosine in protein rises approximately 10-fold (12). Several cellular polypeptides have been shown to become phosphorylated at tyrosine residues in transformed cells; the phosphorylation of these polypeptides at tyrosine residues is temperature dependent in cells infected by mutants of RSV which are temperature sensitive (*ts*) in their ability to transform (7, 9, 21–23, 26). These observations have led to the hypothesis that transformation by RSV results from the phosphorylation of cellular polypeptides at tyrosine residues by the pp60^{src} kinase activity.

When pp60^{src} is immunoprecipitated from RSV-infected cells by serum from rabbits bearing RSV-induced tumors (tumor-bearing rabbit serum), two cellular phosphoproteins with molecular weights of 89,000 (89K or pp89) and 50,000 (50K or pp50) are also precipitated (3, 12, 16, 17, 25). Sedimentation analysis indicated that a fraction of pp60^{src} is associated with these two cellular proteins to form a complex (3). The 89K phosphoprotein has been shown to be one of a set of proteins which are induced by heat-shock or anaerobiosis (17). The 50K protein in the complex contains phosphotyrosine and phosphoserine (3, 12, 16). This observation has suggested that it may be directly phosphorylated by pp60^{src}. However, when cells are infected by a *ts* mutant of RSV, the same quantity of phosphotyrosine is found in the complexed 50K at permissive and nonpermissive temperatures (16): this behavior is quite different from that of other substrates or putative substrates of the pp60^{src} kinase activity which become phosphorylated at tyrosine only at the permissive temperature (7, 22, 29).

To determine if phosphorylation of the 50K polypeptide at tyrosine occurs in uninfected cells or in cells infected by transformation-de-

fective mutants, it is necessary to use a method for its isolation which does not depend on complex formation with pp60*src* in vivo. We have therefore used two-dimensional polyacrylamide gel electrophoresis (PAGE) to isolate the 50K polypeptide from cell lysates. By tryptic phosphopeptide and phosphoamino acid analyses, we have examined the phosphorylation of this protein in uninfected cells and in cells infected by wild-type RSV or viral mutants. Our results indicate that phosphorylation of the 50K polypeptide at tyrosine is dependent on pp60*src* expression, but is not correlated with the transformed state of the cell.

MATERIALS AND METHODS

Virus infection and cell growth. Methods for the culture and infection of chick embryo fibroblasts have been previously described (31), as have the sources of the virus strains used in this work: Schmidt-Ruppin RSV subgroup A (SR-A), Prague RSV subgroup A (PR-A), the temperature-sensitive mutants *ts*NY68SR-A and *ts*LA29PR-A, and the transformation-defective *src* deletion mutant *td*NY106 (22). Virally infected cells and parallel cultures of uninfected cells were incubated at 35 or 41.5°C as indicated in the text and in the figure legends. On day 4 or 5 after infection, the cells were replated and labeled after 12 to 20 h; at this time, cells infected by transforming viruses were morphologically fully transformed. Nontransformed cells were plated at 10^5 cells per 16-mm well or 5×10^5 cells per 35-mm dish; transformed cells were plated at 2×10^5 cells per 16-mm well or 5×10^5 to 7.5×10^5 per 35-mm dish.

Radioactive labeling. For analytical purposes, cells growing in 16-mm wells were labeled for 4 h with 0.25 ml of phosphate-free medium containing 4% calf serum, 1% chick serum, and 1.5 to 2.0 mCi of $^{32}P_i$ (ICN) per ml. For preparative purposes, that is, for isolation of sufficient quantities of labeled material for tryptic phosphopeptide or phosphoamino acid analysis, cells growing in 35-mm dishes were labeled for 15 to 18 h with 1 ml of phosphate-free medium containing 2% tryptose broth, 4% calf serum, 1% chick serum, and 3 mCi of $^{32}P_i$ per ml.

Cell lysis. To prepare lysates for immunoprecipitation, cultures were chilled, washed twice with phosphate-buffered saline, then extracted with immunoprecipitation lysis buffer (0.2 ml per 16-mm well or 0.5 ml per 35-mm dish); this buffer contained 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-hydrochloride (pH 7.2), 1% (wt/vol) Nonidet P-40, 1 mg of bovine serum albumin per ml, and 1% (vol/vol) Aprotinin (Sigma Chemical Co.). Lysates were clarified by centrifugation for 5 min at $10,000 \times g$.

To prepare lysates for two-dimensional PAGE, cells were lysed and treated with nucleases as described previously (11, 22). The composition of the lysates was then adjusted to that of pH 4 to 6 lysis buffer: 9.5 M urea, 2% (wt/vol) Nonidet P-40, 5% (vol/vol) 2-mercaptoethanol, 1.6% carrier ampholytes, pH 4 to 6 (LKB Instruments), 0.4% carrier ampholytes, pH 3.5 to 10 (LKB Instruments). For analytical preparations, initial lysis was carried out at 1 mg of cellular protein per ml, and the final lysate contained approximately 200 μg of protein per ml; for preparative purposes, initial lysis was carried out at 2 mg of protein per ml, and the final lysate contained 500 μg of protein per ml.

Immunoprecipitation. All procedures were carried out at 0°C. Lysates were incubated for 30 min with tumor-bearing rabbit serum prepared as described by Brugge et al. (2); in general, 1 μl of antiserum per 2×10^5 cells was used to ensure complete precipitation. The immune complexes were adsorbed to fixed *Staphylococcus aureus* (20 volumes of a 10% suspension per volume of antiserum). The bacteria plus adsorbed complexes were then washed as described by Oppermann et al. (15), except that sodium dodecyl sulfate (SDS) was omitted from the second wash. For analysis by SDS-PAGE, the immunoprecipitated proteins were separated from the bacteria by incubation at room temperature with SDS gel sample buffer. For analysis by two-dimensional PAGE, proteins were separated from the bacteria by incubation with pH 4 to 6 lysis buffer. The bacteria were removed by centrifugation.

SDS-PAGE. Samples were boiled for 5 min, and proteins were separated by electrophoresis on 7.5% polyacrylamide gels as described (19). Analytical gels were fixed, stained, dried and exposed to Kodak XAR-5 film as previously described (22). Preparative gels were treated similarly, except that the gels were fixed for only 30 min in 40% methanol-5% acetic acid, then rinsed for 30 min in 5% acetic acid and dried; dried gels were marked with radioactive ink to allow alignment with autoradiograms and accurate excision of the bands.

Two-dimensional PAGE. Isoelectric focusing (pH 4 to 6) was done at 400 V for 12 h, 800 V for 1 h, and 1,000 V for 0.5 h (6,100 V/h) (20, 28). Preparative gels were loaded with between 2.5×10^6 and 8.0×10^6 cpm (^{32}P) in a volume of 50 to 60 μl (50 to 60 μg of protein) per gel; usually eight first-dimension gels were run per sample, i.e., a total load of 20×10^6 to 65×10^6 cpm.

Electrophoresis in the second dimension was as described previously (28). Separating gels contained 10% acrylamide (Bio-Rad Laboratories) and 0.13% methylenebisacrylamide (Bio-Rad Laboratories). Electrophoresis was carried out at 3.0 W per gel for approximately 8 h. Analytical and preparative gels were then processed as described above.

Tryptic peptide mapping. Spots or bands containing ^{32}P-labeled polypeptides (totaling 5×10^3 to 20×10^3 Cerenkov cpm in each preparation) were cut from dried gels, hydrated, and eluted without homogenization. The proteins were then precipitated, washed with ethanol, oxidized with performic acid, digested with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin, and lyophilized. These procedures have been previously described (1, 21). Between 10 and 60% of the radiolabel was recovered in the final digest.

Samples containing no more than 40 μg of carrier protein were separated in two dimensions on cellulose thin-layer plates (Whatman, Inc.): the first dimension was electrophoresis toward the anode at 1,000 V for 54 min in 1% ammonium carbonate (pH 8.9), and the second dimension was ascending chromatography in *n*-butanol–pyridine–glacial acetic acid–water (75:50:15:60) (12).

Phosphoamino acid separation. ^{32}P-labeled proteins were eluted from gels, precipitated, and washed with ethanol as described above. Samples were then suspended in 150 μl of 6 N HCl, hydrolyzed at 110°C in

vacuo for 80 min and separated in two dimensions (pH 1.9, then pH 3.5) by electrophoresis on thin-layer cellulose plates (12). Procedures for identification of phosphoamino acid markers (phosphoserine, phosphothreonine, and phosphotyrosine) by ninhydrin staining and for quantitation of ^{32}P-labeled phosphoamino acids have been previously described (19, 21).

To determine the phosphoamino acid content of an individual tryptic peptide, cellulose containing the peptide was scraped from the plate, the peptide was eluted with pH 1.9 buffer, and the sample was filtered and lyophilized. The sample was then subjected to partial acid hydrolysis and analyzed as described above.

RESULTS

Identification of the 50K phosphoprotein by two-dimensional PAGE and tryptic phosphopeptide analysis. The 50K phosphoprotein can be isolated by coprecipitation with pp60^{src} immunoprecipitated by tumor-bearing rabbit serum (3, 12, 16, 17). To determine the position of the 50K phosphoprotein after two-dimensional PAGE an immunoprecipitate prepared with tumor-bearing rabbit serum from ^{32}P-labeled, SR-A-infected cells was dissociated from the bacterial adsorbent with pH 4 to 6 lysis buffer. The dissociated proteins were analyzed by two-dimensional PAGE, using isoelectric focusing, pH 4 to 6, in the first dimension. Lysates of ^{32}P-labeled infected or uninfected cells were analyzed in parallel (Fig. 1). The 50K phosphoprotein in the immunoprecipitate migrated identically to a streak which appears at an isoelectric point of 5.18 in lysates of both uninfected and SR-A-infected cells. Mixing of the immunoprecipitated proteins with a cell lysate confirmed that the 50K phosphoprotein at an isoelectric point 5.18 comigrates with the 50K phosphoprotein immunoprecipitated with pp60^{src} (Fig. 1D). The identity of the 50K phosphoprotein isolated by the two different methods was confirmed by tryptic phosphopeptide mapping (see below). The 89K protein could also be identified in cell lysates by two-dimensional PAGE (small arrowhead, Fig. 1), but pp60^{src}, with an isoelectric point above pH 7 (23), does not enter the first-dimension pH 4 to 6 isoelectric focusing gel. The quantity of ^{32}P label in the 50K protein in RSV-transformed cells (Fig. 1B) does not appear to be significantly higher than in uninfected cells (Fig. 1A); however, as shown below, specific phosphorylation of the 50K does occur in RSV-infected cells. The streaking effect may be due to the presence of charge isomers with different levels of phosphorylation (see below) or to low solubility of the protein under isoelectric focusing conditions (14).

To confirm the identity of the 50K phosphoprotein isolated by the two different methods, we subjected ^{32}P-labeled samples isolated by two-dimensional PAGE or by coimmunoprecipitation with pp60^{src} to tryptic digestion. The tryptic phosphopeptides were then characterized by electrophoresis at pH 8.9 followed by chromatography in *n*-butanol–pyridine–acetic acid–water; this procedure was used so that the maps could be compared with those previously published (12) (Fig. 2).

The 50K polypeptide isolated from immunoprecipitates (Fig. 2A) contained two major phosphopeptides, numbers 1 and 4, which appear to be identical to those previously identified (12). In addition we detected three other phosphopeptides, numbers 2, 3, and 5 (5 is visible as a faint spot in reference 12, Fig. 4C); these may represent partial cleavage products or additional phosphorylation sites. The phosphopeptides of the 50K protein isolated by two-dimensional PAGE from RSV-infected cells (Fig. 2C) or uninfected cells (Fig. 2B) contained the same five phosphopeptides as the 50K isolated from immunoprecipitates, although with different intensities of labeling (see below). Mixing experiments confirmed that the corresponding peptides had identical mobilities (data not shown). These results indicate that the 50K protein isolated by two-dimensional PAGE is the same protein as that complexed to pp60^{src} and isolated from immunoprecipitates. However, peptides 4 and 5 accounted for 30% of the ^{32}P label in the 50K isolated by immunoprecipitation and for 23% of the label in the 50K isolated by two-dimensional PAGE from RSV-transformed cells, but in the 50K isolated by two-dimensional PAGE from uninfected cells, peptides 4 and 5 accounted for only 8% of the total ^{32}P label. These results suggested that there are differences in the level or sites, or both, of phosphorylation between the 50K protein recovered from uninfected cells, the 50K protein present in RSV-infected cells, and that fraction of the 50K protein in RSV-infected cells which is complexed to pp60^{src}.

To further characterize the differences in phosphorylation which result from RSV infection, we determined the phosphoamino acid composition of individual phosphopeptides by eluting each from the cellulose thin layer and subjecting it to partial acid hydrolysis. The phosphoamino acids were identified by two-dimensional electrophoresis. Peptides 1, 2, and 3 contained phosphoserine irrespective of the source of the 50K phosphoprotein. (Several minor phosphopeptides containing phosphoserine or phosphothreonine were found in some preparations; since the yield of these phosphopeptides was not reproducible, they are presumed to be derived from contaminants.) In contrast, the phosphoamino acid composition of phosphopeptides 4 and 5 varied according to the source of

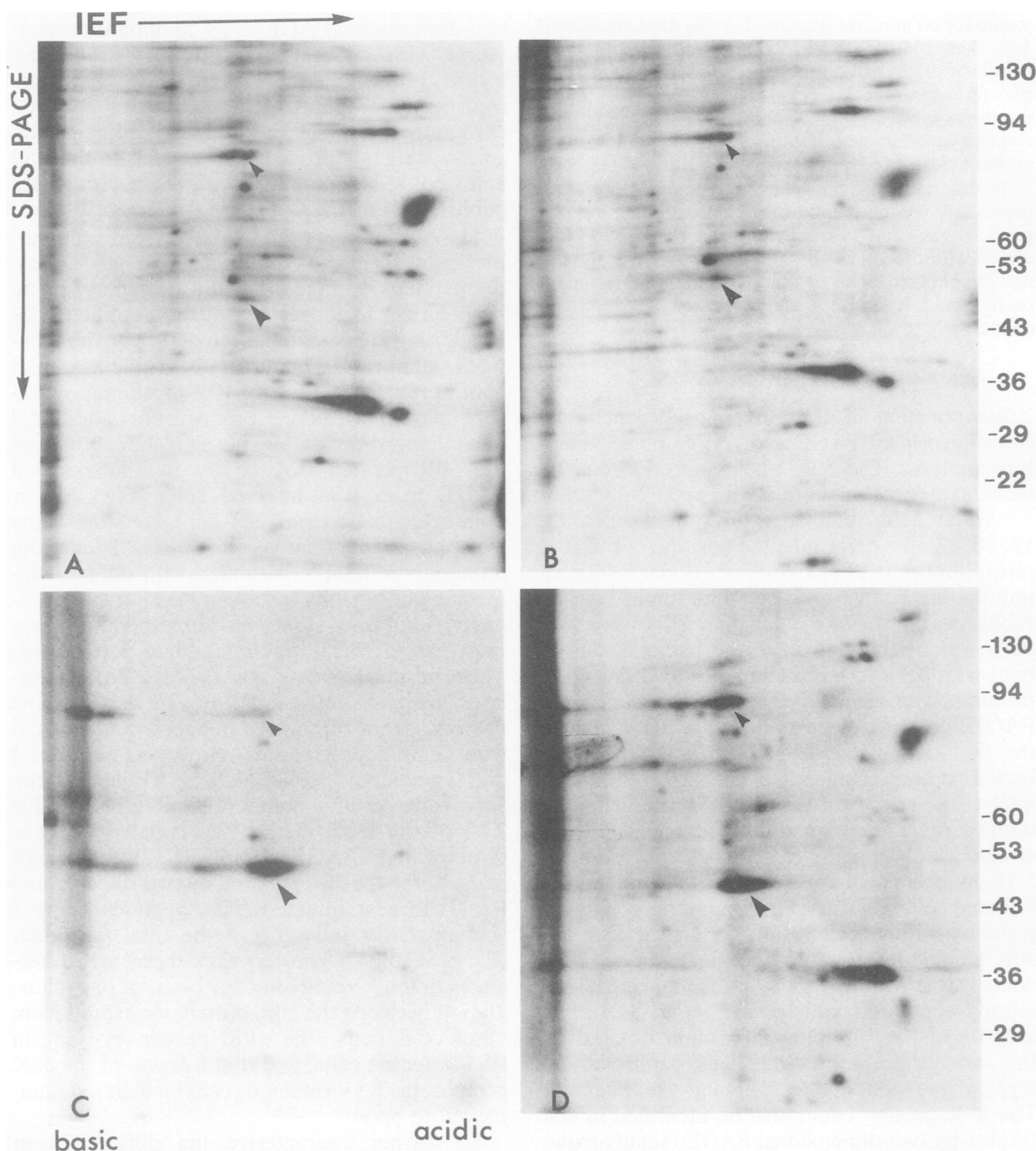

FIG. 1. Two-dimensional PAGE of the 50K and 89K phosphoproteins. Cells were labeled with $^{32}P_i$ for 4 h, and lysates or immunoprecipitates were prepared as described in Materials and Methods. As indicated in the figure, the proteins were separated in the first dimension by isoelectric focusing (pH 4 to 6) and in the second dimension by SDS-PAGE on a 10% slab gel. Proteins were visualized by autoradiography. Molecular weight markers ($\times 10^{-3}$) are indicated to the right. Large arrowheads point to the 50K phosphoprotein, and small arrowheads point to the 89K phosphoprotein. (A) Uninfected cell lysate, 3×10^5 cpm, exposed for 5 days; (B) SR-A-transformed cell lysate, 4×10^5 cpm, 3 days; (C) immunoprecipitate from SR-A-transformed cells, 1.2×10^4 cpm, 2 days with sensitized film and an intensifying screen; and (D) mixture of an immunoprecipitate from SR-A-transformed cells, 1.6×10^4 cpm, and a lysate of SR-A-transformed cells, 1.6×10^5 cpm, 1 day with sensitized film and an intensifying screen.

the 50K protein. An analysis of peptide 5 is shown in Fig. 3. In the 50K protein complexed to pp60^{src} and isolated by immunoprecipitation, peptides 4 and 5 contain only phosphotyrosine; this is in agreement with the results of Hunter and Sefton (12). In the total population of 50K molecules isolated by two-dimensional PAGE from RSV-infected cells, peptides 4 and 5 con-

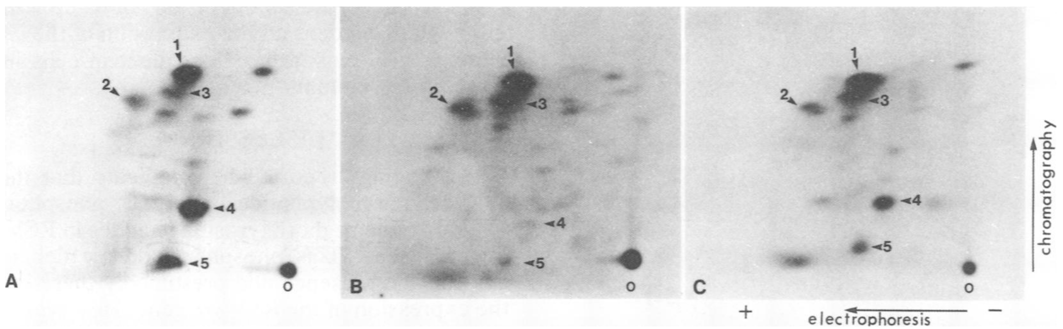

FIG. 2. Tryptic peptide mapping of the 50K phosphoprotein. ^{32}P-labeled spots or bands were cut from dried gels. The protein was eluted, precipitated, and digested with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin. The digests were separated on thin-layer cellulose plates in two dimensions: the first dimension was electrophoresis at pH 8.9, and the second dimension was ascending chromatography as described in Materials and Methods and as indicated in the figure. Phosphopeptides were visualized by autoradiography for 300,000 cpm × h with sensitized film and an intensifying screen. Numbered arrowheads indicate phosphopeptides referred to in the text. (A) 50K phosphoprotein from an immunoprecipitate of SR-A-transformed cells, isolated by one-dimensional SDS-PAGE; (B) 50K phosphoprotein from two-dimensional PAGE of an uninfected cell lysate; and (C) 50K phosphoprotein from two-dimensional PAGE of SR-A-transformed cell lysate.

tain both phosphoserine and phosphotyrosine. Since a doubly phosphorylated peptide would be expected to have different electrophoretic and chromatographic mobility, we conclude that in this case these peptides are mixtures of two populations, one phosphorylated at tyrosine and one phosphorylated at serine. In the 50K isolated from uninfected cells, peptides 4 and 5, which are only lightly labeled (see above), contain only phosphoserine. Thus, in uninfected cells, these peptides are either not phosphorylated or contain only phosphoserine. We conclude that transformation by RSV results in phosphorylation of peptides 4 and 5 at tyrosine.

Phosphoamino acid composition of the 50K protein isolated from mutant-infected cells. To determine if the tyrosine phosphorylation of the 50K protein observed in RSV-infected cells is dependent on expression of the RSV *src* gene, we examined the phosphoamino acid composition of the 50K protein isolated from cells infected by RSV mutants with conditional or nonconditional defects in transformation. The 50K protein was isolated by two-dimensional PAGE from ^{32}P-labeled infected cells and subjected to partial acid hydrolysis, and the phosphoamino acids were separated and quantitated. (Fig. 4 and Table 1).

The 50K polypeptide isolated from uninfected cells contains essentially no phosphotyrosine. The phosphotyrosine content, expressed as the counts recovered in phosphotyrosine relative to the total counts in phosphoserine, phosphothreonine, and phosphotyrosine, was only 0.14%. The 50K protein isolated from cells infected by Schmidt-Ruppin or Prague RSV contained 50-fold-elevated levels of phosphotyrosine (7.7%). The 50K protein isolated from immunoprecipitates of SR-A-transformed cells contained 15.6% phosphotyrosine. These results confirm the conclusions reached from analysis of tryptic phosphopeptides, namely, that phosphorylation of the 50K protein at tyrosine occurs in RSV-infected cells but not in uninfected cells and that the 50K protein associated with pp60^{src} is enriched for the phosphotyrosine-containing form. The 50K protein isolated by two-dimensional PAGE from cells infected by a transformation-defective *src* deletion mutant, *td*NY106, had a phosphotyrosine level (0.18%) similar to that of uninfected cells. This indicates that the presence (and presumably the expression) of the *src* gene is required for tyrosine phosphorylation of the 50K protein.

To determine whether the phosphorylation of the 50K protein was affected by mutations within *src* which result in temperature-dependent transformation, we examined the phosphoamino acid content of the 50K protein isolated from cells infected by various temperature-sensitive mutants. In agreement with the results of others (16), the 50K protein isolated from *ts*NY68-infected cells by immunoprecipitation with tumor-bearing rabbit serum contained essentially equal quantities of phosphotyrosine at 35°C (19.5% phosphotyrosine) and at 41.5°C (25.2% phosphotyrosine) (Table 1). However, the behavior of the 50K protein in the complex with pp60^{src} might not reflect the behavior of the total pool of the protein. We therefore isolated the 50K protein by two-dimensional PAGE from *ts*NY68-infected cells which were infected and maintained from the time of infection at either 35 or 41.5°C; the protein was found to contain 6.9%

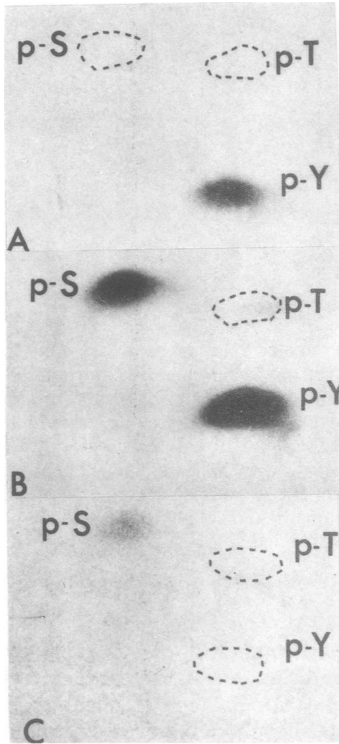

FIG. 3. Phosphoamino acid composition of peptide 5 of the 50K phosphoprotein. Tryptic digestions of the 50K phosphoprotein from different sources were performed as indicated in Fig. 2. Cellulose containing peptide 5 was aspirated from the thin layers; the peptide was eluted with pH 1.9 buffer, lyophilized and hydrolyzed in 6 N HCl at 110°C for 80 min. Phosphoamino acids were separated on thin layers by electrophoresis first at pH 1.9 (right to left) and then at pH 3.5 (bottom to top). Unlabeled phosphoamino acid markers were included in each run. p-S, Phosphoserine; p-T, phosphothreonine; and p-Y, phosphotyrosine. Labeled phosphoamino acids were visualized by autoradiography with sensitized film and an intensifying screen for times indicated below. (A) Peptide 5 of the 50K phosphoprotein from an immunoprecipitate of SR-A-transformed cells; 1-month exposure, (B) peptide 5 of the 50K phosphoprotein from an SR-A-transformed cell lysate isolated by two-dimensional PAGE, 2-week exposure; and (C) peptide 5 of the 50K phosphoprotein from an uninfected cell lysate isolated by two-dimensional PAGE, 1-month exposure.

phosphotyrosine at 35°C and 7.4% at 41.5°C. Since *ts*NY68 is somewhat "leaky," i.e., has residual function at the nonpermissive temperature (28), we also examined the phosphorylation of the 50K protein in cells infected by the nonleaky mutant *ts*LA29: again, phosphorylation of the 50K protein occurred at both 35°C (6.2% phosphotyrosine) and at 41.5°C (4.5% phosphotyrosine). These results indicate that tyrosine phosphorylation of the 50K protein, although dependent on the expression of the *src* gene, is not temperature-dependent in cells infected by *ts* mutants.

DISCUSSION

The findings reported here indicate that the 50K cellular polypeptide undergoes phosphorylation at one or more tyrosine residues in RSV-infected cells. This phosphorylation is dependent on the presence and presumably therefore the expression of the RSV *src* gene. However in *ts* mutant-infected cells grown at 41.5°C, which are phenotypically normal by most criteria, 50K is nevertheless phosphorylated at tyrosine. These observations raise two major questions: is the 50K protein directly phosphorylated by the $pp60^{src}$ kinase activity, i.e., is it a substrate of $pp60^{src}$?, and what is the functional significance of this phosphorylation in the process of transformation?

Several polypeptides phosphorylated at tyrosine residues in RSV-infected cells have now been identified. In all of the cases examined so far, the phosphorylation of tyrosine residues in

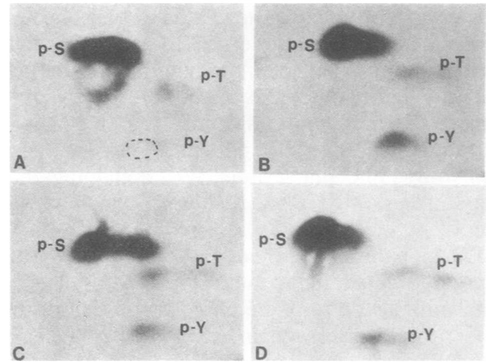

FIG. 4. Phosphoamino acid composition of the 50K phosphoprotein isolated by two-dimensional PAGE. ^{32}P-labeled 50K spots were excised from dried gels; the protein was eluted, precipitated with trichloroacetic acid, subjected to partial acid hydrolysis, and analyzed as described in Materials and Methods and in the legend to Fig. 3. Exposures were for approximately 3×10^5 cpm × h with sensitized film and an intensifying screen. Numbers in parentheses indicate the percentage of radioactivity found in a given phosphoamino acid out of the total radioactivity recovered in the three phosphoamino acids. p-S, Phosphoserine; p-T, phosphothreonine; and p-Y, phosphotyrosine. (A) 50K from uninfected cells (p-S, 95.3; p-T, 4.5; and p-Y, 0.14); (B) 50K from SR-A-transformed cells (p-S, 88.1; p-T, 4.2; and p-Y, 7.7); (C) 50K from *ts*NY68-infected cells grown and labeled at 35°C (p-S, 84.3; p-T, 8.8; and p-Y, 6.9); and (D) 50K protein from *ts*NY68-infected cells grown and labeled at 41.5°C (p-S, 85.3; p-T, 7.3; and p-Y, 7.4).

TABLE 1. Phosphotyrosine content of the 50K protein isolated from mutant-infected cells

Infecting virus	Growth temp (°C)	Isolation method[a]	Phosphotyrosine content (%)[b]
None	35	2D PAGE	0.14
SR-A	35	2D PAGE	7.7
	35	IPTN	15.6
*td*NY106	35	2D PAGE	0.18
*ts*NY68	35	2D PAGE	6.9
	41.5	2D PAGE	7.4
	35	IPTN	19.5
	41.5	IPTN	25.2
PR-A	35	2D PAGE	7.7
*ts*LA29	35	2D PAGE	6.2
	41.5	2D PAGE	4.5

[a] The 50K protein was isolated by either two-dimensional PAGE (2D PAGE) or immunoprecipitation (IPTN).

[b] Phosphotyrosine content is expressed as the counts recovered in phosphotyrosine as a percentage of the total counts in phosphoserine, phosphothreonine, and phosphotyrosine.

these polypeptides is temperature-sensitive in cells infected by RSV *ts* mutants. For example, the 36K cytoplasmic polypeptide (8, 9, 21–23) is phosphorylated at one or more tyrosine residues and also at a serine residue in *ts* mutant-infected cells grown at the permissive temperature, but is only weakly phosphorylated when the cells are grown at the nonpermissive temperature (22). Other polypeptides containing phosphotyrosine in RSV-infected cells have been identified by alkali treatment of gels containing fractionated ^{32}P-labeled phosphoproteins; again the tyrosine phosphorylation of these proteins is temperature-sensitive in cells infected by *ts* mutants (7). Thus the pattern of phosphorylation of 50K is anomalous. Some of these polypeptides may be directly phosphorylated by the pp60^{src} kinase activity. Others may be phosphorylated by other kinases activated directly or indirectly by pp60^{src}: this possibility is raised by the finding that uninfected cells contain a variety of tyrosine-specific protein kinases, including the kinase associated with the epidermal growth factor receptor (30) and, presumably, the cellular homologs of viral transforming protein kinases (5, 15, 24). Irrespective, however, of the mechanism by which these polypeptides become phosphorylated, it remains true that all with the exception of the 50K polypeptide described here show temperature-dependent phosphorylation in *ts* mutant-infected cells.

One possible explanation for this anomaly is that the 50K protein is a high-affinity substrate of pp60^{src}. This possibility is consistent with the resistance of the complex to 0.1% SDS (3). It is possible that in vivo at the nonpermissive temperature the pp60^{src}'s of *ts* mutants may not undergo an all-or-nothing inactivation, but may simply have a lowered affinity for their protein substrates. Thus, a high-affinity substrate might be phosphorylated under conditions where substrates with lower affinity would not. An alternative possibility is that the 50K protein is not phosphorylated by the pp60^{src} kinase activity but the tyrosine phosphorylation of 50K is affected in some way by its binding to pp60^{src}. For example, the binding of pp60^{src} or the 89K protein, or both, might protect the 50K phosphoprotein from the phosphatase which dephosphorylates phosphotyrosine residues (1a). It would clearly be of interest to determine if only that fraction of the 50K protein which is bound to pp60^{src} is phosphorylated at tyrosine.

Another question raised by these results is the functional significance of the RSV-induced phosphorylation of 50K. As we have shown, phosphorylation of the 50K protein occurs at the nonpermissive temperature in cells infected by *ts* mutants. Thus phosphorylation of the 50K protein is not sufficient to induce any of the more obvious phenotypic markers of transformation. However, in certain respects, cells infected by *ts* mutants do not behave like uninfected cells (18), and it is possible that the phosphorylation of 50K is responsible for changes such as these.

ACKNOWLEDGMENTS

We thank Dan Schleske and M. Namba for excellent technical assistance. While this manuscript was in preparation we learned that J. S. Brugge and D. Darrow have obtained similar results. We thank J. Brugge, H. Oppermann, and J. M. Bishop for communicating their results and for stimulating discussions.

TG is supported by Public Health Service training grant CA09041, and KR is supported by a senior fellowship from the American Cancer Society, California Division. This research was supported by Public Health Service grant CA17542.

LITERATURE CITED

1. **Beemon, K., and T. Hunter.** 1978. Characterization of Rous sarcoma virus *src* gene products synthesized in vitro. J. Virol. **28**:551–566.

1a. **Brautigan, D. L., P. Bornstein, and B. Gallis.** 1981. Phosphotyrosyl-protein phosphatase: specific inhibition by Zn^{2+}. J. Biol. Chem. **256**:6519–6522.

2. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–347.

3. **Brugge, J. S., E. Erikson, and R. L. Erikson.** 1981. The specific interaction of the Rous sarcoma virus transforming protein, pp60^{src}, with two cellular proteins. Cell **25**:363–372.

4. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2021–2024.
5. **Collett, M. S., E. Erikson, A. F. Purchio, J. S. Brugge, and R. L. Erikson.** 1979. A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product. Proc. Natl. Acad. Sci. U.S.A. **76**:3159–3163.
6. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus transforming protein, $pp60^{src}$, shows protein kinase activity specific for tyrosine. Nature (London) **285**:167–169.
7. **Cooper, J. A., and T. Hunter.** 1981. Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells. Mol. Cell. Biol. **1**:165–178.
8. **Erickson, E., R. Cook, G. J. Miller, and R. L. Erikson.** 1981. The same normal cell protein is phosphorylated after transformation by avian sarcoma viruses with unrelated transforming genes. Mol. Cell. Biol. **1**:43–50.
9. **Erikson, E., and R. L. Erikson.** 1980. Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus transforming gene product. Cell **21**:829–836.
10. **Erikson, R. L., M. S. Collett, E. Erikson, and A. F. Purchio.** 1979. Evidence that the avian sarcoma virus transforming gene product is a cyclic AMP-independent protein kinase. Proc. Natl. Acad. Sci. U.S.A. **76**:6260–6264.
11. **Garrels, J. I.** 1979. Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. J. Biol. Chem. **254**:7961–7977.
12. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. **77**:1311–1315.
13. **Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell **15**:561–572.
14. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
15. **Oppermann, H., A. D. Levinson, H. E. Varmus, L. Levintow, and J. M. Bishop.** 1979. Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (*src*). Proc. Natl. Acad. Sci. U.S.A. **76**:1804–1808.
16. **Oppermann, H., A. D. Levinson, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1981. Two cellular proteins that immunoprecipitate with the transforming protein of avian sarcoma virus. Virology **113**:736–751.
17. **Oppermann, H., W. Levinson, and J. M. Bishop.** 1981. A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein. Proc. Natl. Acad. Sci. U.S.A. **78**:1067–1071.
18. **Parry, G., J. C. Bartholomew, and M. J. Bissell.** 1980. Role of *src* gene in growth regulation of Rous sarcoma virus-infected chicken embryo fibroblasts. Nature (London) **288**:720–722.
19. **Pawson, T., J. Guyden, T.-H. Kung, K. Radke, T. Gilmore, and G. S. Martin.** 1980. A strain of Fujinami sarcoma virus which is temperature-sensitive in protein phosphorylation and cellular transformation. Cell **22**:767–775.
20. **Purchio, A. F., E. Erikson, J. S. Brugge, and R. L. Erikson.** 1978. Identification of a polypeptide encoded by the avian sarcoma virus *src* gene. Proc. Natl. Acad. Sci. U.S.A. **75**:1567–1571.
21. **Radke, K., T. Gilmore, and G. S. Martin.** 1980. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell **21**:821–828.
22. **Radke, K., and G. S. Martin.** 1979. Transformation by Rous sarcoma virus: effects of *src* gene expression on the synthesis and phosphorylation of cellular polypeptides. Proc. Natl. Acad. Sci. U.S.A. **76**:5212–5216.
23. **Radke, K., and G. S. Martin.** 1980. Transformation by Rous sarcoma virus: effects of *src* gene expression on the synthesis and phosphorylation of cellular polypeptides. Cold Spring Harbor Symp. Quant. Biol. **44**:975–982.
24. **Rohrschneider, L. R., R. N. Eisenman, and C. R. Leitch.** 1979. Identification of a Rous sarcoma virus transformation-related protein in normal avian and mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **76**:4479–4483.
25. **Sefton, B. M., K. Beemon, and T. Hunter.** 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vitro and in vivo. J. Virol. **28**:957–971.
26. **Sefton, B. M., T. Hunter, E. H. Ball, and S. J. Singer.** 1981. Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. Cell **24**:165–174.
27. **Sefton, B. M., T. Hunter, and K. Beemon.** 1979. Product of in vitro translation of the Rous sarcoma virus *src* gene has protein kinase activity. J. Virol. **30**:311–318.
28. **Sefton, B. M., T. Hunter, and K. Beemon.** 1980. Temperature-sensitive transformation by Rous sarcoma virus and temperature-sensitive protein kinase activity. J. Virol. **33**:220–229.
29. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20**:807–816.
30. **Ushiro, H., and S. Cohen.** 1980. Identification of phosphotyrosine as a product of epidermal growth-factor activated protein kinase in A431 cell membranes. J. Biol. Chem. **255**:8363–8365.
31. **Vogt, P. K.** 1969. Focus assay of Rous sarcoma virus, p. 198–211. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press, Inc. New York.