

Transforming gene product of Rous sarcoma virus phosphorylates tyrosine

(phosphotyrosine/protein kinase/*src* gene/phosphoproteins)

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ABSTRACT The protein kinase activity associated with pp60^{src}, the transforming protein of Rous sarcoma virus, was found to phosphorylate tyrosine when assayed in an immunoprecipitate. Despite the fact that a protein kinase with this activity has not been described before, several observations suggest that pp60^{src} also phosphorylates tyrosine *in vivo*. First, chicken cells transformed by Rous sarcoma virus contain as much as 8-fold more phosphotyrosine than do uninfected cells. Second, phosphotyrosine is present in pp60^{src} itself, at one of the two sites of phosphorylation. Third, phosphotyrosine is present in the 50,000-dalton phosphoprotein that coprecipitates with pp60^{src} extracted from transformed chicken cells. We infer from these observations that pp60^{src} is a novel protein kinase and that the modification of proteins via the phosphorylation of tyrosine is essential to the malignant transformation of cells by Rous sarcoma virus. pp60^{src}, the closely related cellular homologue of viral pp60^{src}, is present in all vertebrate cells. This normal cellular protein, obtained from both chicken and human cells, also phosphorylated tyrosine when assayed in an immunoprecipitate. This is additional evidence of the functional similarity of these structurally related proteins and demonstrates that all uninfected vertebrate cells contain at least one protein kinase that phosphorylates tyrosine.

The product of the *src* gene of Rous sarcoma virus (RSV) is a 60,000-dalton phosphoprotein, pp60^{src} (1-4), which is necessary for the transformation of cells in culture and for the formation of sarcomas in birds (5). Immunoprecipitates containing pp60^{src} invariably have a protein kinase activity that is capable of phosphorylating the immunoglobulin heavy chain (2, 4, 6, 7). It has been proposed that the protein kinase activity associated with pp60^{src} is critical in its transforming function (6). Substantial support for this idea comes from the observation that temperature-sensitive mutations that render the virus unable to transform also decrease the protein kinase activity induced at the nonpermissive temperature and cause this activity to be extremely labile after lysis of infected cells (2, 4, 6-9).

All normal vertebrate cells possess one or a few genes (*sarc*) that are homologous to the viral *src* gene (10, 11). It has been shown that these sequences are expressed, at a low level, as a 60,000-dalton phosphoprotein, pp60^{sarc} (12-16). Like viral pp60^{src}, this protein possesses a protein kinase activity capable of phosphorylating the immunoglobulin heavy chain (13-16). Hanafusa and colleagues (17, 18) and Vogt and colleagues (19) have shown that viruses with a partial deletion in the *src* gene can recover a fully functional *src* gene during passage through a chicken. Partial analysis of the sequence of the *src* gene of the recovered viruses suggests that much of the gene is derived, by some unknown mechanism, from the cellular *sarc* gene (18). This in turn suggests that RSV transforms by producing abnormally high levels of a protein very similar to a normal cellular protein kinase.

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Immunoprecipitates prepared from polyoma virus-infected cells and antipolyoma tumor antiserum contain a novel activity that is capable of phosphorylating a tyrosine in the 60,000-dalton large tumor antigen of polyoma virus present in the precipitate (20). This observation stimulated us to examine whether pp60^{src} also had such an activity. We have found that viral pp60^{src} and the endogenous pp60^{sarc} of birds and of mammals catalyze the phosphorylation of a tyrosine in the immunoglobulin heavy chain when their protein kinase activity is assayed in an immunoprecipitate. It appears that viral pp60^{src} and cellular pp60^{sarc} are protein kinases with unique specificities.

MATERIALS AND METHODS

Cells and Viruses. The preparation and growth of normal and infected cultures of chicken cells were as described (3, 21). The origin and the cultivation of the normal human mammary cell line HBL-100 is described elsewhere (21).

Radioactive Labeling, Cell Lysis, and Immunoprecipitation. Chicken cells were labeled with [³²P]orthophosphate (carrier-free, 0.3-1.0 mCi/ml; 1 Ci = 3.7 × 10¹⁰ becquerels; ICN) for 15-18 hr (3). Cell lysis was as before (3) except that the modified RIPA buffer was supplemented with 2 mM EDTA. Immunoprecipitation was as described (3, 8); the antitumor antisera were prepared by the protocol of Brugge and Erikson (1).

Protein Kinase Assay and Gel Electrophoresis. Incubation of immunoprecipitates with [³²P]ATP (2000-3000 Ci/mmol; New England Nuclear) and isolation of the phosphorylated heavy chain were as described (7).

Extraction of Gel-Purified Proteins for Acid Hydrolysis and Tryptic Peptide Mapping. Proteins were recovered from gels exactly as described (22) up to and including the organic solvent washes of the trichloroacetic acid precipitates. For acid hydrolysis the precipitates were dissolved directly in 6 M HCl by heating at 100°C for 1 min and then were incubated for 2 hr at 110°C under N₂. The HCl was removed under reduced pressure and the hydrolysates were dissolved in a marker mixture containing phosphoserine, phosphothreonine, and O⁴-phosphotyrosine [Tyr(P)], each at 1 mg/ml. For peptide mapping the protein precipitates were treated with performic acid and digested with trypsin (22).

Electrophoresis and Chromatography. Acid hydrolysates were analyzed on cellulose thin-layer plates (100 μm) by electrophoresis at pH 1.9 for 60 min at 1.5 kV in glacial acetic acid/formic acid (88% by vol)/H₂O, 78:25:897 (vol/vol), and at pH 3.5 for 45 min at 1 kV in glacial acetic acid/pyridine/H₂O, 50:5:945 (vol/vol). Ascending chromatography in the second dimension was performed with isobutyric acid/0.5 M NH₄OH, 5:3 (vol/vol). The markers were detected by staining with ninhydrin.

Abbreviations; RSV, Rous sarcoma virus; Tyr(P), O⁴-phosphotyrosine.

Tryptic digests were resolved by electrophoresis at pH 8.9 for 27 min at 1 kV in buffer containing 1% ammonium carbonate. Ascending chromatography in the second dimension was performed with *n*-butanol/pyridine/glacial acetic acid/H₂O, 75:50:15:60 (vol/vol).

Extraction of ³²P-Labeled Proteins from Cell Lysates. ³²P-Labeled cells were lysed in RIPA buffer, and insoluble nuclear and cytoskeletal elements were removed by centrifugation. Phosphoproteins were then extracted by treatment with an equal volume of phenol saturated with 0.1 M NaCl/0.05 M Tris-HCl, pH 7.5/5 mM EDTA. The first aqueous phase was reextracted with an equal volume of phenol. The combined phenol phases including the interfaces in both cases were extracted three times with 2 vol of buffer, the aqueous phase being discarded in each case. The final phenol phase with the interface was diluted 1:40 with water and the proteins were precipitated by addition of trichloroacetic acid to a concentration of 15%. The precipitate was recovered by centrifugation and extracted twice with a large volume of chloroform/methanol, 2:1 (vol/vol). The resulting proteins were dissolved in 6 M HCl and hydrolyzed as outlined above.

Materials. Phosphoserine and phosphothreonine were obtained from Sigma. Tyr(P) was synthesized in two different ways: by reaction of tyrosine with POCl₃ as reported elsewhere (20), and by condensation of P₂O₅ with tyrosine (23). Both preparations of Tyr(P) had identical mobilities on four different chromatographic and electrophoretic separation systems. Treatment of the preparations of Tyr(P) with alkaline phosphatase led to release of orthophosphate and generation of free tyrosine.

RESULTS

Amino Acid Substrate Specificity of Viral pp60^{src}. The protein kinase activity associated with pp60^{src} phosphorylates the heavy chain of immunoglobulin when immunoprecipitates containing pp60^{src} are incubated with Mg²⁺ and ATP (2, 4, 6, 7). The linkage of the phosphate incorporated into the heavy chain was completely stable to treatment with 1 M HCl for 2 hr at 55°C. This ruled out the possibility that the phosphate was attached to the protein via histidine or as an acyl phosphate (24). The phosphate linkage was much more stable to alkali (60% resistance to 1 M KOH for 2 hr at 55°C) than expected for either phosphoserine or phosphothreonine (24). This suggested that the phosphate acceptor in the heavy chain was not threonine as reported (6) but rather some other amino acid. Hydrolysis of the isolated heavy chain with 6 M HCl for 2 hr at 110°C and two-dimensional separation of the products revealed that about 25% of the radioactivity was released as Tyr(P) which, in turn, comprised >95% of the radioactivity in phosphoamino acids (Fig. 1). Identification of the phosphorylated amino acid as Tyr(P) was corroborated by the comigration of the phosphate label with authentic Tyr(P) during electrophoresis at pH 3.5 (see Fig. 2) and also on chromatography in saturated ammonium sulfate/1 M sodium acetate/isopropanol, 40:9:1 (23) (data not shown). It seems unlikely that the occurrence of Tyr(P) in the heavy chain is an artifact of acid hydrolysis because Tyr(P) was released from the phosphorylated heavy chain by exhaustive digestion with Pronase (data not shown).

Strong support for the idea that the protein kinase activity associated with pp60^{src} isolated from transformed cells is due to pp60^{src} itself and not to a contaminating protein kinase is the observation that pp60^{src} isolated from a reticulocyte lysate after synthesis by *in vitro* translation has protein kinase activity (7, 25). We therefore examined which amino acid became phosphorylated when the protein kinase activity associated with pp60^{src} produced by *in vitro* translation of RSV virion RNA was assayed in the immunoprecipitate. Tyr(P) was again the

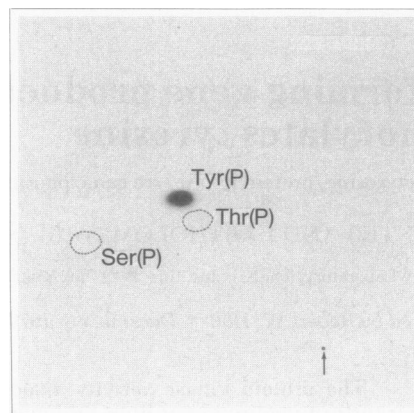


FIG. 1. Identification of phosphoamino acids in phosphorylated immunoglobulin heavy chain. Immunoglobulin heavy chain phosphorylated in an immunoprecipitate containing Schmidt-Ruppin RSV-D pp60^{src} was isolated and subjected to partial acid hydrolysis as described in *Materials and Methods*. A sample (2500 cpm) of the products was subjected to electrophoresis toward the anode at pH 1.9 and chromatography in isobutyric acid/ammonia. The origin is indicated with an arrow. Here and in subsequent figures, the positions of the stained marker phosphoamino acids are indicated by broken lines. The orthophosphate was run off the plate during electrophoresis. The minor spots are probably incompletely hydrolyzed phosphopeptides. Autoradiography was performed with a fluorescent screen for 12 hr. Thr(P), phosphothreonine; Ser(P), phosphoserine.

principal phosphorylated amino acid (Fig. 2).

Amino Acid Substrate Specificity of Endogenous pp60^{src}. Tryptic peptide analysis has shown that although

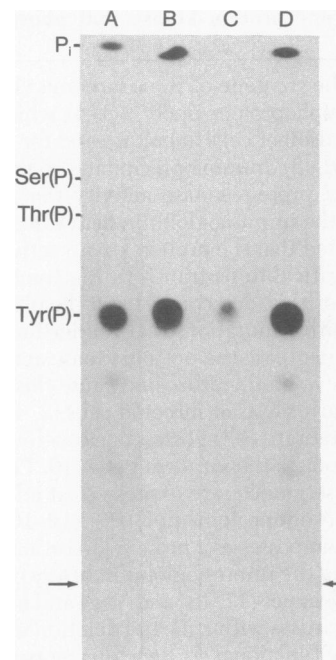


FIG. 2. Identification of amino acids phosphorylated by viral pp60^{src} and endogenous pp60^{src}. Immunoglobulin heavy chains phosphorylated by pp60^{src} from SR-RSV-D infected cells, pp60^{src} of SR-RSV-D synthesized *in vitro* (7), and pp60^{src} from chicken and human cells were isolated and subjected to partial acid hydrolysis. The hydrolysates were analyzed on a single plate by electrophoresis toward the anode at pH 3.5. The origin is indicated by arrows. Lanes, the exposure times (with a fluorescent screen), and the protein that phosphorylated the heavy chain were: A, pp60^{src} from infected cells, 25,000 cpm applied, 1 hr; B, pp60^{src} synthesized *in vitro*, 1400 cpm; 2 days; C, human pp60^{src}, 800 cpm, 10 hr; D, chicken pp60^{src}, 4000 cpm, 10 hr.

the pp60^{src} and pp60^{src} polypeptides are closely related they are not identical (21). We examined whether the protein kinase activity associated with chicken and human pp60^{src}s shared with that associated with viral pp60^{src} the ability to phosphorylate tyrosine when assayed in the immunoprecipitate. In both cases the heavy chain of the phosphorylated immunoglobulin was found to contain predominantly Tyr(P) (Fig. 2).

Phosphoamino Acids in pp60^{src}. pp60^{src} contains two sites of phosphorylation: one in the NH₂-terminal half of the molecule (which has been shown clearly to be a phosphoserine), and one in the COOH-terminal half (26). The phosphoamino acid in the COOH-terminal half was identified as phosphothreonine on the basis of its electrophoretic mobility at pH 1.9 (26). Stimulated by indications that pp60^{src} could undergo auto-phosphorylation and knowing that Tyr(P) and phosphothreonine are difficult to resolve by electrophoresis at pH 1.9, we decided to reexamine the question of what phosphorylated amino acids were contained in pp60^{src}.

In addition to pp60^{src}, two other polypeptides, of 80,000 and 50,000 daltons, are found in immunoprecipitates made from ³²P-labeled transformed chicken cells with antitumor antiserum (Fig. 3) (3). Neither of these is related to pp60^{src} in amino acid sequence (ref. 3; see below). Both appear to be cellular proteins that coprecipitate with pp60^{src} because they are in physical association with some fraction of the pp60^{src} molecules in a transformed chicken cell (our unpublished observations; J. Brugge, personal communication). We analyzed the phosphotryptic peptides and the phosphorylated amino acids present in all three phosphoproteins.

pp60^{src} contained two phosphorylated tryptic peptides (Fig. 4), designated here as α and β (9), as was first observed by Collett *et al.* (26). The 50,000-dalton protein contained two major and one minor phosphotryptic peptides; the 80,000-

dalton protein contained an indeterminate number of phosphotryptic peptides that were poorly resolved. The phosphotryptic peptides of the three polypeptides were obviously unrelated. Hydrolysis of each protein and two-dimensional separation of the phosphorylated amino acids revealed that both pp60^{src} and the 50,000-dalton protein contained both Tyr(P) and phosphoserine and that the 80,000-dalton protein contained only phosphoserine (Fig. 4).

We had previously determined (9) that pp60^{src} phosphopeptide α is derived from the 34,000-dalton NH₂-terminal fragment generated from pp60^{src} by partial proteolysis with *Staphylococcus aureus* V8 protease, whereas peptide β is derived from the COOH-terminal 24,000-dalton fragment (9). Partial acid hydrolysis of the purified peptides revealed that peptide α , as expected, contained phosphoserine and β contained Tyr(P). In the 50,000-dalton protein, peptide I contained Tyr(P) and peptide II contained phosphoserine (data not shown).

Tyr(P) in Normal and Transformed Cells. Because Tyr(P) had not been detected in normal cells (28, 29) and because we now knew that at least two proteins that contained Tyr(P) were present in RSV-transformed cells, a comparison of the amounts of Tyr(P) in uninfected and RSV-transformed chicken cells seemed warranted. We isolated proteins from cells that had been labeled for 18 hr with [³²P]orthophosphate and carried out partial acid hydrolysis. RSV-transformed chicken cells contained 7- to 8-fold more Tyr(P) than did uninfected cells or cells infected with RSV carrying a deletion in the *src* gene (Fig. 5; Table 1).

The hydrolysis conditions chosen here provide a compromise between the efficient release of phosphoamino acids and the competing hydrolysis of the phosphomonoester linkage. Under our conditions, approximately 50% of the ³²P was released as orthophosphate and 20–25% as phosphoamino acids. Because of this, we have expressed the radioactivity in the individual phosphoamino acids as a percentage of the sum of the radioactivity recovered as phosphoamino acids. These ratios will deviate from those in whole cells if the three phosphoamino acids are released or destroyed by acid at different rates. Although free Tyr(P) has been reported to be somewhat more acid labile than phosphoserine or phosphothreonine (27), this may not be true for Tyr(P) in a polypeptide. We have not found a major difference between the recovery of phosphoserine and Tyr(P) from pp60^{src} and the 50,000-dalton protein. Therefore, we think that we are not underestimating seriously the relative abundance of Tyr(P). Both the rate of release of any particular amino acid residue and the stability of the phosphomonoester bond will be affected by its polypeptide environment. However, there is no reason to assume *a priori* that the rate of release of Tyr(P) from proteins in transformed cells is significantly greater than the rate of release from proteins in normal cells. We believe therefore that we detect significantly more Tyr(P) in RSV-transformed cells because there is increased phosphorylation of tyrosine in these cells and not because the yield of Tyr(P) is greater from these cells.

DISCUSSION

The protein kinase activity associated with pp60^{src}, the transforming protein of RSV, phosphorylates tyrosine when assayed in an immunoprecipitate. This observation is surprising because protein modification by way of phosphorylation of tyrosine is unprecedented (28, 29). It is nonetheless real. We have found that chicken cells (Table 1) and mouse, rat, and hamster cells (data not shown) all contain readily detectable amounts of Tyr(P). This modified amino acid appears to have escaped detection before because it is rare (phosphoserine and phos-

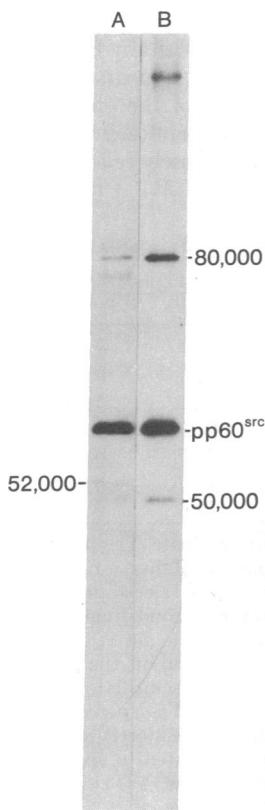


FIG. 3. Analysis of immunoprecipitates from cells infected with RSV and labeled with [³⁵S]cysteine (lane A) and [³²P]orthophosphate (lane B). Infected cells were labeled for 15 hr at 41°C with [³⁵S]cysteine at 40 μ Ci/ml (New England Nuclear; 665 Ci/mmol) or [³²P]orthophosphate at 1 mCi/ml and immunoprecipitates were prepared by using antitumor antiserum preabsorbed with disrupted RSV virions. Electrophoretic separation was on a 15% NaDodSO₄/polyacrylamide gel. ³⁵S was detected by fluorography for 2 days; ³²P was detected by autoradiography with a fluorescent screen for 10 hr. Numbers are size in daltons. The 52,000-dalton polypeptide in lane A is related to pp60^{src} (3).

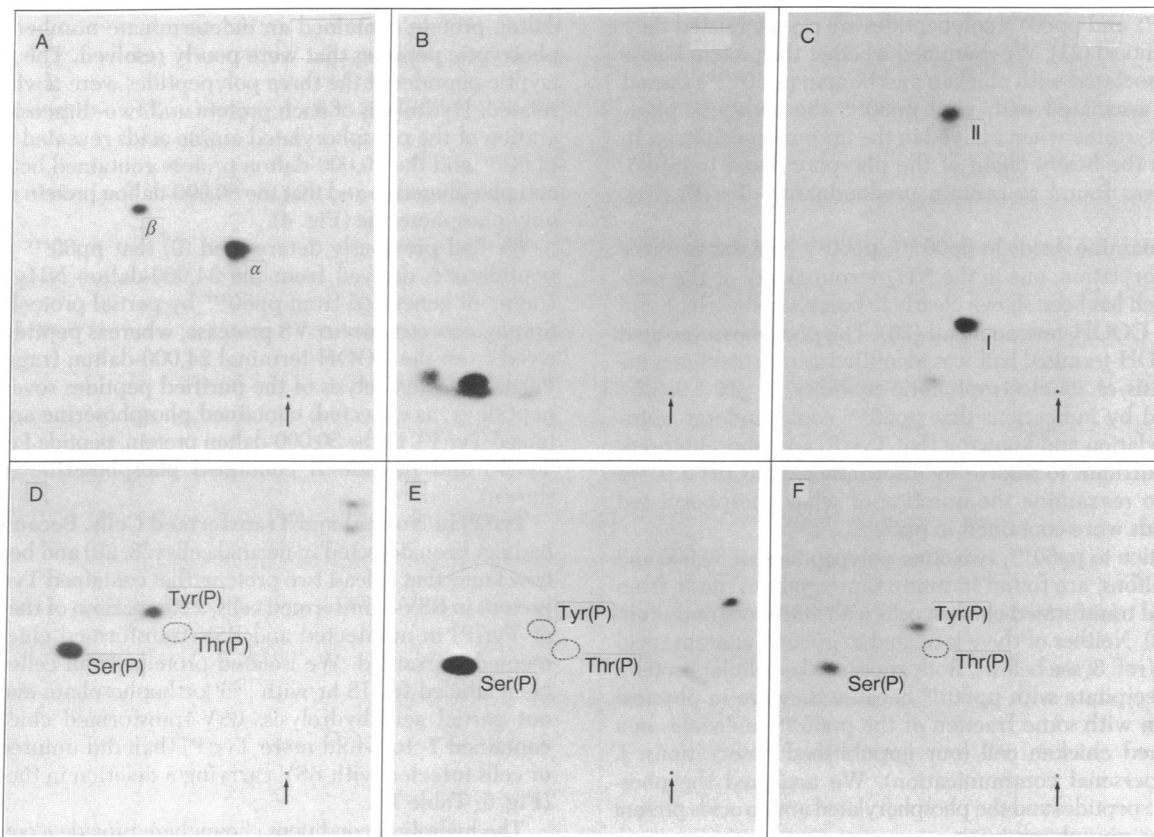


FIG. 4. Analysis of ^{32}P -labeled pp60^{src} and the 80,000- and 50,000-dalton proteins. ^{32}P -Labeled proteins were isolated from the preparation in Fig. 3 and part of each sample was subjected to tryptic digestion and analyzed. The remainder was subjected to partial acid hydrolysis and analyzed as in Fig. 1. The origins were all on the right and are indicated with arrows. All exposures were with a fluorescent screen. (A) pp60^{src}; tryptic digest; 6400 cpm applied; 5 hr exposure. (B) 80,000 protein; tryptic digest; 2000 cpm; 20 hr. (C) 50,000 protein; tryptic digest; 1500 cpm; 20 hr. (D) pp60^{src}; acid hydrolyzate; 1300 cpm; 2 days. (E) 80,000 protein; acid hydrolyzate; 900 cpm; 4 days. (F) 50,000 protein; acid hydrolyzate; 500 cpm; 4 days.

phothreonine together being about 3000 times more abundant) and because it and phosphothreonine are difficult to separate by traditional electrophoretic procedures. Because there is a 7-fold increase in the abundance of Tyr(P) in proteins in cells transformed by RSV and because pp60^{src} itself contains Tyr(P), it seems likely that pp60^{src} phosphorylates tyrosine *in vivo* as well as *in vitro*. We suggest that pp60^{src} is a protein kinase and

that the modification of proteins by phosphorylation of tyrosine is essential to the malignant transformation of cells by RSV.

The viral *src* gene, which encodes pp60^{src}, appears to be descended from the homologous *sarc* gene of the chicken (10–13). The peptide composition of the polypeptide product of the *sarc* gene of the chicken, pp60^{sarc}, is closely related to that of all viral pp60^{src}s but identical to none (21). A funda-

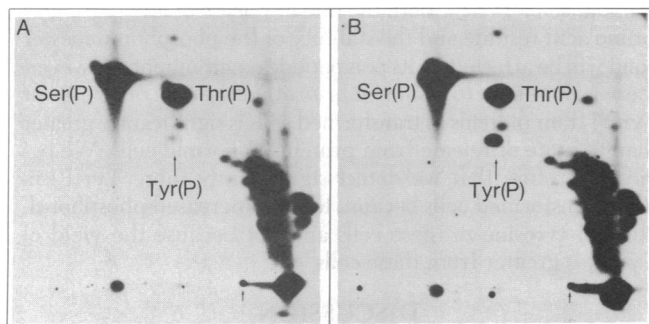


FIG. 5. Analysis of phosphoamino acids of whole cells. ^{32}P -Labeled proteins were extracted and subjected to partial acid hydrolysis; 1.5×10^6 cpm of each hydrolyzate was resolved in two dimensions by electrophoresis toward the anode at pH 1.9 followed by electrophoresis toward the anode at pH 3.5. Autoradiography with a fluorescent screen was for 10 hr. The radioactive spots corresponding to the markers are indicated. The origins are designated by arrows. The material migrating toward the cathode at pH 1.9 corresponds to incompletely hydrolyzed phosphopeptides. (A) Uninfected chicken cells. (B) Chicken cells transformed with Schmidt-Ruppin RSV-A.

Table 1. Abundance of phosphoamino acids in cells

Cells*	Temp., °C	% of total cpm		
		Ser(P)	Thr(P)	Tyr(P)
Uninfected	36	92.19	7.77	0.03
Uninfected	41	91.42	8.53	0.04
SR-RSV-A	36	92.48	7.24	0.28
SR-RSV-A	41	92.73	6.96	0.31
Uninfected	38	92.40	7.55	0.04
td SR-RSV-D	38	92.22	7.75	0.04
SR-RSV-D	38	92.02	7.66	0.32

Chicken cells were labeled with [^{32}P]orthophosphate. The protein was extracted, and hydrolyzed with acid. Approximately 2×10^6 cpm of each hydrolyzate was analyzed by two-dimensional electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension. The thin-layer plate was autoradiographed with the aid of a fluorescent screen for 10 hr, and the spots were scraped off the plate, eluted, and assayed for radioactivity in an aqueous scintillator. The radioactivity of each phosphoamino acid is expressed as a percentage of the total radioactivity recovered in phosphoserine [Ser(P)], phosphothreonine [Thr(P)], and Tyr(P), which amounted to approximately 20% of the applied radioactivity in every case.

* SR, Schmidt-Ruppin; td, transformation defective.

mental question is whether RSV transforms a cell because of the overproduction of a polypeptide functionally equivalent to the normal cellular pp60^{src} or through the production of an altered form of the cellular protein. It is not yet possible to answer this question definitively, but it does appear that the two proteins are functionally similar: both are protein kinases with the unusual property of phosphorylating tyrosine. Because both chicken and human pp60^{src}s have the ability to phosphorylate tyrosine it is possible that the modification of proteins through the phosphorylation of tyrosine is an indispensable cellular function that has been conserved during evolution.

It has been shown recently that a tyrosine in both the 60,000-dalton tumor antigen of polyoma virus (20) and p120 of Abelson virus (30) can undergo phosphorylation in an immunoprecipitate. The significance of these reactions is not yet clear because neither protein contained Tyr(P) when isolated from infected cells. It has been suggested that these phosphorylated tyrosines are observed only *in vitro* because they are normally short-lived reaction intermediates that are trapped *in vitro* due to the absence of the appropriate acceptor (20, 30).

We favor the idea that the phosphorylation of tyrosine carried out by pp60^{src} is an end-state protein modification that is analogous to the phosphorylation of serine or threonine. Several observations suggest this. One is that Tyr(P) is detectable, albeit at a low level, in a wide variety of cells that have been labeled for 18 hr with [³²P]orthophosphate. Another is that the reaction that occurs in precipitates that contain pp60^{src} is clearly a transphosphorylation reaction which appears to involve the pp60^{src}-catalyzed transfer of phosphate from ATP to the heavy chain of immunoglobulin. The third is that pp60^{src} and the 50,000-dalton phosphoprotein that coprecipitates with pp60^{src} both contain a Tyr(P) when isolated from chicken cells. Although we cannot quantify precisely the amount of Tyr(P) in pp60^{src} or the 50,000-dalton phosphoprotein, it appears to be at least half the amount of phosphoserine in the two molecules. This is much more than we would expect were the phosphorylated tyrosine only a reaction intermediate.

Phosphorylated tyrosines are rare in uninfected cells and significantly more abundant in cells transformed by RSV. It is conceivable that all of this increase is due to the presence in these cells of pp60^{src} and its substrates. We suspect that it may be possible to identify cellular substrates of pp60^{src} simply on the basis of their containing a Tyr(P). We have already found one such protein, the 50,000-dalton phosphoprotein that coprecipitates with pp60^{src}. It is possible that this protein is one of the normal substrates of pp60^{src} and that it coprecipitates because of an association with pp60^{src}.

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