Role of $p60^{src}$ Kinase Activity in the Induction of Neuroretinal Cell Proliferation by Rous Sarcoma Virus

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Expression of the *src* gene of Rous sarcoma virus (RSV) in chicken embryo neuroretinal (NR) cells results in morphological transformation and sustained proliferation of a normally resting cell population. We have previously reported the isolation of mutants of RSV which retain full growth-promoting activity while displaying reduced transforming properties. Two such mutants, PA101 and PA104, were used to investigate whether the $p60^{src}$ -associated kinase activity is required for the mitogenic function of *src*. A comparison of the patterns of phosphorylation of wild-type and mutant $p60^{src}$ revealed that the phosphorylation of tyrosine residues of $p60^{src}$ of PA104 was markedly reduced, whereas the relative amount of phosphotyrosine in $p60^{src}$ of PA101 was comparable to that of the wild-type protein. In vitro kinase activity of $p60^{src}$ immunoprecipitated from NR cells infected with PA101 or PA104 as measured by phosphorylation of the heavy chains of specific immunoglobulin G molecules was 1/10 that of the wild-type molecule. Moreover, when NR cells infected with mutants temperature sensitive for mitogenic capacity were maintained at a temperature either permissive or restrictive for cell growth, quantitation of kinase activity indicated that proliferation of NR cells could not be linked to the absolute level of in vitro kinase activity of $p60^{src}$. Transformation of NR cells by wild-type RSV resulted in a 10-fold increase in total cellular phosphotyrosine and in the phosphorylation of tyrosine residues of a 34K protein, a possible in vivo substrate for $p60^{src}$. In contrast, phosphorylation of tyrosine residues of cellular targets was markedly reduced in NR cells infected with PA101 or PA104. These results indicate that the mitogenic capacity of RSV in NR cells does not require elevated levels of $p60^{src}$ kinase activity.

Changes in growth regulation which take place during malignant transformation by Rous sarcoma virus (RSV) can be conveniently studied by using differentiating cells from the chicken embryo neuroretina (NR) as a host system. The characteristics of this system are as follows: (i) when these cells are cultured in vitro their capacity to synthesize DNA and to multiply declines rapidly, and (ii) infection of NR cells with RSV leads to morphological transformation and induction of sustained proliferation (21). Both morphological changes and cell multiplication appear to require the expression of a functional *src* gene since they are not seen in cells infected with avian leukosis virus or with transformation-defective mutants of RSV lacking the *src* gene (3).

We have previously reported the isolation of RSV mutants which induce and maintain NR cell multiplication in the absence of morphological transformation. In addition, the mitogenic property of these viruses is temperature sensitive (5, 6).

Preliminary characterization of two such mutants, PA101 and PA104, has shown that their genome contains most, if not all, of the *src* sequence (Pluquet and Stehelin, personal communication). The mutated genes encode a 60K protein similar in size to the standard $p60^{src}$ which has been identified as the product of the *src* gene (1, 8). Therefore, this type of mutant represents a useful tool for studying the molecular mechanisms of the mitogenic function of $p60^{src}$.

In the present work, we characterize the *src* gene product of PA101 and PA104 and investigate the relationship of the mitogenic property of $p60^{src}$ to its associated protein kinase activity (9, 18). Our results show that the mitogenic effect of

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$p60^{src}$ does not directly correlate with the levels of its phosphorylating activity in vitro and in vivo.

MATERIALS AND METHODS

Cell cultures and viruses. NR cultures were prepared from 7-day-old Spafas chicken embryos and infected as previously described (21). The cells were maintained and subcultured in basal medium of Eagle supplemented with 5% fetal calf serum. Schmidt-Ruppin RSV subgroup A (SR RSV-A) was used as the wild-type (wt) virus. PA101 and PA104 are two mutants derived from SR RSV-D and SR RSV-A, respectively. Their isolation and properties have been reported elsewhere (5, 6).

tsNY72-4 is a temperature-sensitive mutant of SR RSV-A (Hanafusa, unpublished data). Avian erythroblastosis virus (RAV 50 pseudotype) was obtained from S. Anderson.

Measurement of NR cell growth. Growth of infected NR cells was measured by counting the number of cells at various intervals after extensive dissociation with trypsin and EDTA.

Isotopic labeling of cells. Infected NR cells were subcultured three to four times to select for growing cells and subsequently seeded in 35-mm tissue culture plates. All experiments were performed on confluent cultures.

Cells were incubated for 2 h in 2 ml of methionine-free minimum essential medium (GIBCO Laboratories) or phosphate-free medium 199 (GIBCO), each supplemented with 1% dialyzed calf serum. The medium was replaced with 0.7 ml of the same medium containing, respectively, 150 μ Ci of L-[35 S]methionine (specific activity, 900 to 1,200 Ci/mmol, Amersham Corp.) per ml or 1.5 mCi of carrier-free 32 P_i orthophosphate (Amersham) per ml. The cells were harvested 8 h later.

Preparation of cell extracts. Culture plates were placed on ice, and all subsequent operations were carried out at 0 to 4°C with precooled glassware and solutions. The cells were washed twice with isotonic buffer (25 mM Tris [pH 7.4]–135 mM NaCl–5 mM KCl–0.4 mM Na₂HPO₄–5.5 mM glucose) and lysed in 200 μ l of either buffer C (20 mM Tris maleate [pH 8]–10 mM KCl–10 mM dithiothreitol–1 mM EDTA–10% glycerol–1% Triton X-100–1 mM phenylmethylsulfonyl fluoride–100 kIU of Trasylol per ml) prepared by the method of Krueger et al. (15) or RIPA buffer (50 mM Tris-hydrochloride [pH 7.4]–0.15 M NaCl–1% Triton X-100–1% sodium deoxycholate–0.1% sodium dodecyl sulfate [SDS]–20 mM EDTA–100 kIU of Trasylol per ml).

The cells were scraped off the plates with a rubber policeman. The lysates were mixed three times for 10 s in a Vortex mixer and set back on ice for 1 min in between. Lysates were then clarified at 10,000 $\times$ g for 5 min at 4°C.

Preparation of antisera. Antisera directed against $p60^{src}$ were prepared as first described by Brugge and Erikson (1). Sarcomas were induced in newborn rabbits by injecting them subcutaneously with about 10⁶ focus-forming units of SR RSV-D. The same lot of serum obtained from a tumor-bearing rabbit (TBR) 8 weeks after inoculation was used throughout these experiments.

Immunoprecipitation. Samples of cell extracts were incubated with saturating amounts of TBR serum for 60 min on ice. Four serum volumes of protein A-Sepharose CL4B (a 50% [wt/vol] slurry in RIPA buffer; Sigma Chemical Co.) was added and mixed for 30 min at 4°C to absorb the immune complex as described by Kessler (14). The Sepharose pellet was washed five times with RIPA buffer and twice with water. It was then suspended in 50 μ l of electrophoresis sample buffer (1) or assayed for protein kinase activity.

Protein kinase assay. A 10- μ l sample of reaction mixture containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.0)–0.15 M KCl–10 mM magnesium acetate–5 mM dithiothreitol–0.1 μ M [γ - 32 P]ATP (3,000 Ci/mmol, Amersham) was added to the pellet of immune complex protein A-Sepharose. The tube was gently mixed in a Vortex mixer and incubated at 37°C for 8 min. The reaction was stopped by the addition of 50 μ l of cold electrophoresis sample buffer.

Under these conditions, the amount of transferred 32 P radioactivity reflects the total extent of the reaction and is, therefore, presumably proportional to the number of enzymatically active molecules in the precipitates.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 8.5% slab gels as described by Laemmli (16). Immunoprecipitates or kinase assay samples were heated in boiling water for 3 min and loaded into the gel slots. Gels were run at 7 to 15 mA constant current until the tracking dye reached the bottom of the gel. Gels were not fixed or stained, except to identify marker proteins. Gels were dried onto filter paper before exposure to film.

Quantitation of $p60^{src}$. Determinations of $p60^{src}$ were done essentially as described by Collett et al. (7), except for the following: (i) the cells were labeled as described above, and (ii) the gel slices containing $p60$ were solubilized in 5 ml of a solution containing 15% NCS tissue solubilizer (Amersham)–0.006% 1,4-bis-(5-phenyloxazolyl) benzene–0.5% 2,5-diphenyloxazole–82% toluene–2.5% water for 6 h at 55°C before measurement of radioactivity. The protein content of cell extracts was assayed by the method of Lowry et al. (19).

Quantitation of phosphate transfer. Gel slices containing the heavy chain of immunoglobulin G (IgG) were cut out and solubilized as described above before counting. Kinase activity was expressed as femtomoles of 32 P_i transferred to the heavy chain of IgG per milligram of cell extract protein.

Partial hydrolysis of proteins with protease V8 of *Staphylococcus aureus*. Protein samples in gel slices were partially digested with *S. aureus* V8 protease (Miles Laboratories), and peptide maps were generated as described by Levinson and Levine (17). Gel slices containing $p60^{src}$ were cut out, incubated five times for 15 min in 10% methanol at room temperature, lyophilized, and applied on a 12% SDS-polyacrylamide gel. Slots were filled up with V8 solution (2 μ g of protease per ml in 0.625 M Tris [pH 6.8]–10% sucrose–0.15% SDS). Gels were run at 7 mA constant current.

Measurement of the amount of phosphotyrosine in total cellular proteins. NR cells were labeled for 14 to 18 h in 1 ml of phosphate-free medium 199 containing

FIG. 1. Autoradiogram of immunoprecipitates of extracts prepared from uninfected and infected NR cells. Uninfected NR cells (lanes 1 and 2) or NR cells infected with SR RSV-A (lanes 3 and 4), PA101 (lanes 5 and 6), or PA104 (lanes 7 and 8) were labeled with either [^{35}S]methionine (A) or $^{32}\text{P}_i$ (B). Cell extracts were prepared by using buffer C, immunoprecipitated with nonimmune serum (lanes 1, 3, 5, and 7) or TBR serum (lanes 2, 4, 6, and 8) and washed with RIPA buffer. The products of the reaction were run on an 8.5% polyacrylamide gel. Catalase (60K) was included as a molecular weight marker.

1.5 mCi of carrier-free $^{32}\text{P}_i$ per ml. Cell extracts were prepared in RIPA buffer and subsequently treated as described by Hunter and Sefton (13). Proteins were extracted with phenol, dissolved in 6 M HCl, and hydrolyzed at 110°C for 2 h under nitrogen atmosphere. The hydrolysates were lyophilized, washed once with water, lyophilized again, and dissolved in a marker mixture containing phosphoserine, phosphothreonine, and phosphotyrosine, each at 1 mg/ml. The hydrolysates were resolved in two dimensions on thin-layer cellulose-coated glass plates (Merck & Co.) by electrophoresis toward the anode at pH 1.9 (750 V for 75 min) followed by electrophoresis toward the anode at pH 3.5 (1,000 V for 60 min).

Immunoprecipitation of radiolabeled 34K protein from infected NR cells. Cells were radiolabeled in culture with either [^{35}S]methionine or $^{32}\text{P}_i$ for 2 h. The cells were washed, scraped from the dishes, and lysed. Samples of the clarified lysate were immunoprecipitated with preimmune or anti-34K serum under conditions of antigen excess. The immunoprecipitated proteins were resolved by PAGE and visualized by fluorography or autoradiography.

RESULTS

Identification of p60^{src} in NR cells infected with PA101 and PA104. Lysates of [^{35}S]methionine-labeled uninfected NR cells and NR cells infected with SR RSV-A, PA101, and PA104 were immunoprecipitated with either nonimmune serum or with serum raised in rabbits bearing regressing tumors induced by SR RSV-D (TBR serum). Immune complexes were collected on *S.*

aureus protein A-Sepharose and analyzed by SDS-PAGE.

A protein of about 60,000 daltons was specifically precipitated by TBR serum from all virus-infected cells (Fig. 1A, lanes 4, 6, and 8). We confirmed the identification of these proteins as p60^{src} by analysis of their proteolytic digests generated by V8 protease of *S. aureus* (see below). Small amounts of cellular endogenous p60^{src} were detected in extracts of uninfected NR cells (Fig. 1A, lane 2). Immunoprecipitation of lysates from $^{32}\text{PO}_4$ -labeled cultures showed that these p60^{src} proteins were phosphorylated (Fig. 1B). Therefore, proteins analogous to the *src* gene product of RSV are encoded by PA101 and PA104. Although p60^{src} proteins of SR RSV-A and PA104 were efficiently immunoprecipitated by all TBR sera, relatively few sera recognized the *src* protein of PA101, suggesting that the structure of p60^{src} of the two mutants is not identical.

Phosphorylation patterns of p60^{src} of PA101 and PA104. p60^{src} is phosphorylated at two sites: a phosphoserine site in the N-terminal portion of the molecule (8) and a phosphotyrosine site in its carboxy portion (8, 13). Partial hydrolysis of p60^{src} with the V8 protease of *S. aureus* yields two primary fragments, V_1 and V_2 , containing the phosphoserine and phosphotyrosine residues, respectively (8, 13). Further digestion of V_1 generates fragment V_3 and subsequently V_4 which both contain the phosphoserine residue. Analysis of the V8 digestion products thus permits a determination of the ratio of phosphate groups at the serine and tyrosine sites.

Duplicate cultures of NR cells infected with SR RSV-A virus, PA101, or PA104 and maintained at 36°C were labeled with either [^{35}S]methionine or $^{32}\text{P}_i$, and cell lysates were immunoprecipitated with TBR serum. The total extent of phosphorylation of each p60^{src} was determined by calculating the ratio of ^{32}P to ^{35}S radioactivity associated with the various p60^{src} . The phosphorylation pattern of the *src* gene products was then analyzed by partial hydrolysis with V8 protease of ^{32}P -labeled p60^{src} .

As shown in Fig. 2, the digestion pattern of p60^{src} of the two mutants was similar to that of wt virus, although the smaller primary cleavage fragment (V_2) of PA101 and PA104 p60^{src} displayed a slower electrophoretic mobility relative to the corresponding peptide of wt virus. These differences suggest that the mutations in PA101 and PA104 may be located in the region of *src* coding for the C-terminal portion of p60^{src} .

The total extent of phosphorylation of p60^{src} of wt and mutants and the relative incorporation of phosphate into the serine and tyrosine sites of the molecule are presented in Table 1. These data show that the p60^{src} of both PA101 and

FIG. 2. *S. aureus* V8 protease partial digest patterns of ³²P_i-labeled p60^{src}. Infected NR cells were labeled with ³²P_i for 8 h. Extracts were submitted to immune precipitation (Fig. 1), and gel bands containing p60^{src} from wt SR RSV-A (lane 1), PA101 (lane 2), and PA104 (lane 3) were loaded on a 12% acrylamide gel. Fifty microliters of a 2-μg/ml solution of *S. aureus* V8 protease was added in each slot. The gel was run at 7 mA constant current.

PA104 were underphosphorylated as compared with wt p60^{src}. The level of phosphorylation of serine residues in p60^{src} of the two mutants was about half that found in p60^{src} of wt virus. The relative amount of phosphotyrosine in p60^{src} of PA101 represented 86% of the wt, but it amounted to only 21% of wt levels in p60^{src} of PA104. In addition, when infected NR cells were shifted to nonpermissive temperature, the pattern of phosphorylation of the mutant proteins was not modified (data not shown).

p60^{src} of PA101 and PA104 have low protein kinase activity. Lysates of uninfected NR cells

and of proliferating cultures infected at 36°C with SR RSV-A, PA101, and PA104 were prepared by lysing the cells with buffer C, immunoprecipitated with TBR serum, and incubated with [γ-³²P]ATP. The reaction products were analyzed by SDS-PAGE (Fig. 3). Immunoprecipitates from PA101- and PA104-infected cells had much lower kinase activity than the immunoprecipitate from wt-infected cells. To further quantitate the levels of kinase activity, we calculated the ratio of radioactivity incorporated into the heavy chain of IgG per nanogram of ³⁵S-labeled p60^{src} immunoprecipitated under the same conditions. This defines a specific activity for the enzyme. The specific activity of p60^{src} of PA101 was about 1/10 that of SR RSV-A (Table 2). Nevertheless, this residual kinase activity was three- to fourfold higher than that of immunoprecipitates from normal cells.

Levels of in vitro kinase activity do not correlate with the induction of NR cell proliferation by p60^{src}. We previously reported that the mitogenic property of both PA101 and PA104 was temperature sensitive (5). Growth of NR cells infected with either mutant is strictly temperature dependent and can be controlled by reciprocal shifts from permissive temperature (35 to 37°C) to nonpermissive temperature (40 to 42°C). To examine the relationship between the mitogenic function of p60^{src} and its levels of in vitro kinase activity, we measured the levels of p60^{src}-associated kinase activity in PA101- and PA104-infected NR cells as well as in cells infected with the mutant tsNY72-4, which is temperature sensi-

TABLE 1. Pattern of phosphorylation of different p60^{src} ^a

Virus	Total extent of phosphorylation (%) ^b	Relative amount of phosphoserine (%) ^c	Relative amount of phosphotyrosine (%) ^d
SR RSV-A	0.65 (100)	0.51 (100)	0.14 (100)
PA101	0.36 (56)	0.24 (47)	0.12 (86)
PA104	0.29 (45)	0.26 (51)	0.03 (21)

^a p60^{src} were immunoprecipitated from extracts of [³⁵S]methionine- or ³²P_i-labeled infected cells as shown in Fig. 1. The p60^{src} regions of the gels were excised, and the radioactivity was quantitated. The ³²P-labeled p60^{src} were then submitted to partial hydrolysis with V8 protease of *S. aureus* as shown in Fig. 2, and the relative amounts of phosphoserine and phosphotyrosine were determined by measuring the radioactivity respectively associated with V₁, V₃, and V₄ fragments and with V₂ fragment (Fig. 2).

^b ³²P counts per minute (cpm) in p60^{src}/³⁵S cpm in p60^{src}.

^c ³²P cpm in V₁ + V₃ + V₄ fragments/³⁵S cpm in p60^{src}.

^d ³²P cpm in V₂ fragment/³⁵S cpm in p60^{src}.

FIG. 3. Reaction products of protein kinase assay performed on immunoprecipitates of uninfected and infected NR cells after electrophoresis in an 8.5% acrylamide gel. Extracts from uninfected NR cells (lane 1) and from NR cells infected with SR RSV-A (lane 2), PA101 (lane 3), and PA104 (lane 4) were immunoprecipitated with TBR serum and assayed for kinase activity as described in the text.

tive for transformation. At 36°C, NR cells infected with this mutant are transformed and induced to proliferate, whereas both transforming and mitogenic properties are suppressed at the non-permissive temperature (Fig. 4).

NR cells infected with wt virus and with the various mutants were subcultured at 36°C, and duplicate cultures were shifted to the non-permissive temperature. Cell extracts from infected

cultures were then assayed for immunoprecipitable kinase activity (Table 2). Comparable levels of kinase activity were immunoprecipitated from NR cells transformed by wt virus and by *ts*NY72-4 at permissive temperature. From PA101- and PA104-infected cells, however, the *src*-associated kinase activity was considerably lower. Extracts from NR cells infected with *ts*NY72-4 contained more precipitable kinase activity under conditions restrictive for cell growth than those from NR cells infected with PA101 or PA104 at permissive temperature.

Sensitivity of p60^{src} to ionic detergents and thermal inactivation. The stability of p60^{src} of PA101 and PA104 was further investigated by testing the effects of ionic detergents and of thermal inactivation of the kinase activity in vitro.

Proliferating NR cells infected with wt virus, PA101, or PA104 were lysed in either buffer C, which does not contain ionic detergents, or in RIPA buffer, and the amounts of precipitable kinase were measured in the various cell extracts. As shown in Table 2, the lability of kinase activity to ionic detergents depended on the origin of p60^{src}. The kinase activity of p60^{src} from SR RSV-infected cells was not sensitive to ionic detergents, and the use of RIPA buffer actually improved the yield in kinase activity. There was no difference in kinase activity of extracts from PA101-infected NR cells prepared either in RIPA buffer or in buffer C. In contrast, the kinase levels of p60^{src} from PA104-infected cells were seven to eight times lower in RIPA buffer than in buffer C. The low recovery of kinase activity from these cells by RIPA buffer probably reflects the denaturing effect of this buffer rather than a more efficient extraction of p60^{src} by buffer C since in each case comparable amounts of ³⁵S-labeled p60^{src} were immunoprecipitated. In addition, the enzyme activity was stable once bound to the antibody since the

TABLE 2. Quantitation of cellular and viral p60^{src} and associated protein kinase activities in uninfected and infected NR cells

Virus	Growth temp (°C)	Mitogenic capacity	ng of p60/mg of cell protein	fmol of PO ₄ transferred to IgG/mg of cell protein		Sp act (fmol of PO ₄ /ng of p60)	
				Buffer C	RIPA	Buffer C	RIPA
None	36	—	30	0.2	ND ^a	0.007	ND
SR RSV-A	36	+	830	240	1,046	0.290	1.26
SR RSV-A	42	+	780	203	ND	0.260	ND
PA101	36	+	460	9.2	10.4	0.020	0.023
PA101	42	—	391	4.7	ND	0.012	ND
PA104	36	+	2,000	58	8.55	0.029	0.004
PA104	42	—	1,478	30	ND	0.020	ND
<i>ts</i> NY72-4	36	+	1,210	293	ND	0.242	ND
<i>ts</i> NY72-4	42	—	1,170	80	ND	0.068	ND

^a ND, Not determined.

FIG. 4. Growth kinetics of NR cells infected with *tsNY72-4*. NR cells were infected and subcultured twice at 37°C. They were subsequently seeded at low density (2×10^5 cells per 60-mm dish) and maintained at 36°C for 24 h. The following day, the medium was renewed, and half of the cultures were transferred to 42°C. The medium was changed daily, and cells were counted at intervals as indicated.

phosphorylating capacity of PA104 $p60^{src}$ extracted in buffer C remained intact whether the immunoprecipitates were washed in buffer C or in RIPA buffer (data not shown).

The in vitro protein kinase activity of $p60^{src}$ of certain transformation defective *ts* mutants of RSV has been shown to be more sensitive to heat inactivation than that of wt viruses (20, 24, 25). We have compared the rate of heat inactivation of $p60^{src}$ kinase activity of wt virus and of three mutants temperature sensitive for the induction of NR cell proliferation. Cell extracts were prepared from NR cells infected with SR RSV-A or with the mutants PA101, PA104, or *tsNY72-4* and maintained at 35°C. The extracts were heated at 40°C for various lengths of time before immunoprecipitation and assayed for kinase activity (Fig. 5). Under these conditions, heating for up to 3 min did not result in any significant reduction in the amounts of precipitable ^{35}S -labeled $p60^{src}$ (data not shown), in agreement with other reports (2, 18, 24). The kinase activities of both wt and PA101 $p60^{src}$ decreased at about the same rate, that is, 50% of the molecules were inactive within 2 min. In contrast, $p60^{src}$ of PA104 and *tsNY72-4* appeared to be more thermolabile, since 50% of their kinase activities were lost in 0.75 min.

Taken together these results indicate that the $p60^{src}$ of PA101 is as stable as that of wt SR RSV, whereas the $p60^{src}$ of PA104 is more labile to both ionic detergents and heat inactivation. This argues that, at least in the case of PA101, the low level of *src*-associated in vitro kinase activity is an intrinsic property of the protein

rather than the result of denaturation during extraction.

Levels of phosphotyrosine in total cell phosphoproteins. Hunter and Sefton (13) have reported that transformation of chicken embryo fibroblasts by RSV results in a dramatic increase in the abundance of phosphotyrosine in total cellular phosphoproteins. Analysis of cells infected with transformation-defective, temperature-sensitive mutants has suggested that the phosphorylation of tyrosine is essential for transformation by RSV (26).

To investigate whether *src*-induced proliferation caused similar biochemical changes in NR cells, we measured the relative abundance of phosphotyrosine in phosphoamino acids of NR cells infected with wt virus, PA101, or PA104 and maintained at 37°C (Fig. 6). Control cultures included uninfected cells and cells infected with avian erythroblastosis virus, an acute leukemia virus whose oncogene is unrelated to *src* (23, 27), but which induces sustained growth of NR cells (4, 5). Transformation of NR cells by RSV

FIG. 5. Heat inactivation of kinase activity. Lysates of infected NR cells maintained at 36°C were prepared by using buffer C. Samples were incubated for 1, 2, and 3 min at 40°C. Heat inactivation was stopped by the addition of 50 μ l of ice-cold buffer C, and the samples were subjected to immune precipitation and kinase assay. The amount of ^{32}P radioactivity transferred to the heavy chain of the IgG was determined. Results are expressed as percentage of transferred radioactivity in the corresponding unheated sample.

FIG. 6. Relative abundance of acid-stable phosphoamino acids in uninfected and infected NR cells. NR cells maintained at 37°C were labeled with $^{32}\text{P}_i$ for 16 h. Phosphoproteins were then extracted, hydrolyzed, and resolved in two dimensions as described in the text. Only the central portion of the thin-layer plates is shown here. The upper panel indicates the relative position of the phosphoserine (P. Ser), phosphothreonine (P. Thr), and phosphotyrosine (P. Tyr) markers. The arrows indicate phosphotyrosine. The radioactivity associated with each phosphoamino acid is expressed as a percentage of the total radioactivity recovered in phosphoserine, phosphothreonine, and phosphotyrosine together.

resulted in a 10-fold increase in total cellular phosphotyrosine. In contrast, levels of phosphotyrosine in NR cells induced to grow by avian erythroblastosis virus and by PA101 or PA104 were only slightly above those of uninfected cells.

Phosphorylation of 34K protein. A protein of 34K, a major constituent of normal cells (0.5% of total cellular proteins), is phosphorylated on both serine and tyrosine residues upon transformation by RSV. In addition, partially purified 34K protein can be phosphorylated on its tyrosine site(s) by p60^{src} in vitro. Therefore, this protein is considered to be a candidate target for p60^{src} phosphotransferase activity in vivo (11, 22).

To compare the pattern of phosphorylation of the 34K protein from NR cells infected with SR RSV, PA101, or PA104, [^{35}S]methionine- and $^{32}\text{P}_i$ -labeled cell extracts were immunoprecipitated with anti-34K protein serum (Fig. 7). Equal amounts of 34K protein can be detected in NR

cells infected with wt virus, PA101, or PA104 and maintained at 35 or 41°C.

The extent of phosphorylation of 34K protein extracted from either PA101- or PA104-infected NR cells is comparable, but at 35°C and at 41°C, it is respectively 1/4 and 1/10 as phosphorylated as 34K protein extracted from transformed NR cells (Fig. 7 and Table 3). Moreover, these differences in the extent of phosphorylation reflect the differences in the relative content in phosphotyrosine of 34K protein (Table 3).

DISCUSSION

We have investigated the relationship between the mitogenic function of the *src* gene of RSV and the protein kinase activity associated with p60^{src} . In the present work, the term mitogenic describes the ability of the virus to induce and maintain the proliferation of chicken embryo NR cells, a cell population whose growth capacity is physiologically restricted in vivo and

FIG. 7. Immunoprecipitation of radiolabeled 34K protein. Extracts from [^{35}S]methionine-labeled (top panel) or [^{32}P]labeled (bottom panel) NR cells infected with SR RSV-A (1), PA101 (2), or PA104 (3) maintained at 35 or 41°C were immunoprecipitated with nonimmune serum (a) or anti-p34 serum (b). Only the 34K region of each gel is displayed.

in vitro. To study this characteristic property of RSV, we have used two viruses, PA101 and PA104, which, as a consequence of small mutations in the *src* gene, are capable of inducing NR cell proliferation in the absence of morphological transformation. That the mutant-induced cell growth is temperature sensitive represents an additional advantage in analyzing the mitogenic capacity of *src*. Our results show that the p60^{src} proteins encoded by the *src* gene of these mutants are largely defective in their phosphorylating ability and lead us to conclude that the mitogenic function of the *src* gene is compatible with reduced levels of protein kinase activity of p60^{src} as measured in vivo and in vitro.

PA101 and PA104 induced similar phenotypic changes in NR cells. However, a study of the phosphorylation patterns of their p60^{src} revealed differences between the mutants. Although both proteins were significantly underphosphorylated in comparison to wt p60^{src} , the relative distribution of phosphate groups into serine and tyrosine residues was different in PA101 and PA104 *src* proteins. It has been reported that the extent of phosphorylation of tyrosine residues in p60^{src} of transformation-defective *ts* mutants is reduced at nonpermissive temperature and parallels the decrease in kinase activity (8). Such coincidence between a low kinase activity and underphosphorylation at tyrosine sites of p60^{src} was verified in the case of PA104, but not in PA101. Moreover, shifting NR cells infected with PA101 to the temperature restrictive for cell growth did not result in a significant decrease in phosphorylation of tyrosine residues in the mutant p60^{src} (data not shown). Therefore, we conclude that the expression of the mitogenic function of the

src gene probably does not depend on the extent of phosphorylation at tyrosine sites in p60^{src} .

p60^{src} -associated kinase activity is measured in vitro by its ability to transfer phosphate from ATP to the heavy chains of specific IgG molecules. The specific activity of p60^{src} extracted from proliferating NR cells infected with PA101 or PA104 was about 1/10 of wt levels, indicating

TABLE 3. Effects of growth temperature on the phosphorylation of 34K protein in infected NR cells^a

Virus	Growth temp (°C)	Relative phosphate content	Relative phosphotyrosine content
SR RSV-A	35	11.1	10.0
	41	11.8	8.0
PA101	35	2.8	1.9
	41	0.8	0.2
PA104	35	3.3	2.2
	41	1.0	0.3

^a 34K protein was immunoprecipitated from extracts of [^{35}S]methionine- or [^{32}P]labeled NR cells as shown in Fig. 7. The 34K regions of the gels were excised. Relative phosphate content is expressed as ^{32}P cpm/100 cpm of ^{35}S recovered in the 34K region of the gel. The radioactivity recovered in the corresponding nonimmune serum tracks was subtracted. Relative phosphotyrosine content was determined after elution of ^{32}P -labeled 34K protein from the gel and hydrolysis at 100°C for 3 to 5 h in 6 N HCl. Phosphoaminoacids were resolved by two-dimensional electrophoresis at pH 1.9 and 3.5 with authentic phosphoaminoacids included in each sample. The values presented are phosphotyrosine cpm/phosphoserine cpm. Radioactivity was quantified by liquid scintillation spectrometry. The data presented are the average of two separate experiments.

that the growth of NR cells is compatible with significantly reduced levels of kinase activity. In addition, quantitation of this function in NR cells infected with either mutant or with tsNY72-4 under conditions permissive and restrictive for cell growth has shown that the absolute level of in vitro kinase activity did not correlate with the expression of the mitogenic function of *src*.

Coinciding with a low protein kinase activity in vitro, the capacity of the mutant p60^{src} to phosphorylate tyrosine residues of cellular targets was also reduced. Thus, the levels of total phosphotyrosine in proliferating NR cells infected with PA101 or PA104 were much lower than those in NR cells transformed by wt virus. Similarly the tyrosine residue(s) of a 34K protein suspected of being an in vivo substrate of p60^{src} kinase (11, 22) was four to five times less phosphorylated in NR cells infected with PA101 or PA104 at the temperature permissive for cell growth than in NR cells transformed by wt virus.

Our results show significant reduction in the phosphorylating capacities of p60^{src} of PA101 and PA104 as compared with wt protein. Interpretation of the dissociation between the mitogenic and transforming properties of the *src* gene must take into account such quantitative differences. Therefore, one is confronted with two possibilities. It may be that the mitogenic effect of p60^{src} is mediated through another—yet unknown—activity distinct from its phosphotransferase property. The residual levels of phosphorylation of specific targets by the mutant p60^{src} would then be insufficient to lead to transformation, but the activity responsible for mitogenesis would remain intact. Alternatively, it is conceivable that both transformation and cell proliferation result from the same enzymatic activity of p60^{src}, the phosphorylation of different primary targets within the cell. Indeed, several proteins have been shown to be phosphorylated at their tyrosine sites in RSV-transformed cells (10, 12). Phosphorylation of one—or a few—of these targets, even at reduced levels, could be sufficient to induce NR cell proliferation. That the 34K protein may serve as a candidate for this function is suggested by the fact that the residual phosphorylation of this protein is abolished when NR cells infected with either mutant are shifted to the nonpermissive temperature.

It is difficult, for the time being, to decide which alternative is likely to be correct. Isolation and characterization of more mutants with similar phenotypic properties are required. Meanwhile, it should prove useful to study the phosphorylation of other putative targets of p60^{src} in NR cells infected with PA101 and PA104 in relation to their possible role in initiating DNA synthesis and cell multiplication.

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