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Highly Purified pp60^{src} Induces the Actin Transformation in Microinjected Cells and Phosphorylates Selected Cytoskeletal Proteins In Vitro

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The *src* gene product of Rous sarcoma virus (pp60^{src}) was highly purified from a rat tumor cell line and shown to have physiological actin transformation activity in a cellular microinjection assay that measures the dissolution of actin microfilament bundles in vivo. The purified pp60^{src} fraction consisted of two major proteins, seen on silver-stained sodium dodecyl sulfate-polyacrylamide gels: a 60,000-dalton (60K) protein, identified as pp60^{src} by immunoprecipitation with tumor-bearing rabbit immunoglobulin G (IgG) and peptide mapping, and an unrelated 65K protein. There was no evidence for proteolytic cleavage of pp60^{src}. A 7,000-fold purification of the tyrosine-specific protein kinase activity of pp60^{src} was achieved by this procedure. Purified pp60^{src} phosphorylated tumor-bearing rabbit IgG heavy chains, casein, histones H1 and H2B, tubulin, and microtubule-associated proteins when assayed in vitro. When incubated with [γ-^{32}P]ATP in the absence of exogenous phosphoacceptor substrates, purified pp60^{src} became labeled with ^{32}P at the tyrosine residues exclusively. Phosphatase and cyclic AMP-dependent protein kinase activities were undetectable in the purified fraction. Microinjection of highly purified pp60^{src} into the cytoplasm of normal Swiss 3T3 mouse fibroblasts caused rapid and reversible dissolution of actin stress fibers, as visualized by indirect immunofluorescence with actin antibodies. The actin-disrupting activity was thermolabile and sensitive to inhibition by coinjection of tumor-bearing rabbit IgG, and purified to about the same extent (8,000-fold) as did the IgG kinase activity of pp60^{src}, thus implicating pp60^{src} as the active agent. Examination of actin-associated proteins as substrates for the pp60^{src} kinase in vitro showed that vinculin was phosphorylated directly by pp60^{src}, although to a small extent. Actin, myosin, and tropomyosin were not phosphorylated. Thus, pp60^{src} purified by this procedure retains native functional properties and provides a useful probe for analyzing transformation-dependent changes in actin cytoarchitecture.

Transformation of fibroblasts by Rous sarcoma virus (RSV) causes a dramatic dissolution of actin microfilament bundles (reviewed in reference 21). This actin transformation is due to the action of the RSV *src* gene product, pp60^{src}, as studies with mutant viruses have shown (12, 34). The mechanism by which pp60^{src} causes the actin transformation is not known but may involve phosphorylation of cellular proteins, as pp60^{src} is known to function as a tyrosine-specific protein kinase (6, 8, 18, 20). In vivo labeling studies with ^{32}P have revealed a number of proteins with increased phosphotyrosine content in cells transformed by RSV that may be direct targets of pp60^{src} action (9, 14, 24, 28, 30). One of these proteins, vinculin, is an attractive candidate for cytoskeletal alterations, as it is located within the cellular adhesion plaques at which actin microfilament bundles insert (4, 16). Studies such as these have been important in identifying potential pp60^{src} substrates; however, elucidation of the actin transformation mechanism will require demonstrating that the direct phosphorylation of one or more of these proteins by pp60^{src} leads to expected changes in cytoskeletal organization. This will depend on the availability of purified pp60^{src} and on suitable assays for the actin transformation.

To address this task, we developed a microinjection assay for measuring the actin-disrupting activity of purified pp60^{src}. We previously showed that microinjection of extracts of cells transformed by RSV into the cytoplasm of normal cells induces actin changes phenotypically identical to those seen in RSV-infected cells (23, 25). Furthermore, actin-disrupting activity was

temperature sensitive in extracts of cells infected with temperature-sensitive *src* mutants, suggesting that the activity was due to pp60^{src} and not to a component induced secondarily by the action of pp60^{src}. Direct demonstration that the activity in extracts is due to pp60^{src} has awaited its purification, which we describe here.

We first reported a protocol for partially purifying the pp60^{src} kinase from the rat tumor cell line RR1022 (22, 23). Other procedures for purifying pp60^{src} from small amounts of RSV-transformed avian (15) and mammalian (20, 27) cells and from RSV virions (11) and tumors (1) have also been reported. These studies provide strong evidence that the kinase activity is an intrinsic property of pp60^{src} and elucidated some interesting properties of the enzyme, including its cyclic AMP (cAMP)-independent activity (15, 20), physical association with specific cellular proteins (3), and evidence for autophosphorylation (27). Further work has been limited by the small quantities of pp60^{src} obtained and by the marked tendency of pp60^{src} to undergo proteolytic cleavage from a 60,000-dalton (60K) native form to a 52K fragment (1, 20). Isolation of uncleaved pp60^{src} from RSV-transformed cells with good yields of activity in amounts convenient for extensive enzymatic analysis has not been described.

Here we report a method for purifying uncleaved pp60^{src} 7,000-fold from RSV-transformed mammalian cells and show that highly purified pp60^{src} rapidly disrupts actin microfilament bundles in microinjected cells. In addition, selected cytoskeletal proteins, including vinculin, can serve as primary substrates for the pp60^{src} protein kinase when assayed in vitro. These results show that pp60^{src} purified by our method retains native physiological properties and therefore should prove to be useful for dissecting the mechanism of transformation-related changes in the cytoskeleton.

MATERIALS AND METHODS

Cells and culture methods. The RR1022 cell line, derived from a tumor induced in an Amsterdam rat after injection of the Schmidt-Ruppin strain of RSV, was obtained from the American Type Culture Collection and grown in Dulbecco modified eagle medium containing 5% calf serum in plastic roller bottles. Cells were harvested when the cultures were 80% confluent and stored at −70°C.

Purification of pp60^{src}. Thawed RR1022 cells (30 ml) were diluted with 90 ml of buffer A (27 mM potassium phosphate [KP] [pH 7.1], 1.3 mM EDTA, 20 mM β-mercaptoethanol [β-ME], 0.66% [wt/vol] sodium deoxycholate, 1.3% [vol/vol] Nonidet P-40 [NP-40], 13 mM NaF, 100 Kallikrein inhibitor units [KIU] of aprotinin per ml) containing 100 μl of diisopropyl fluorophosphate and lysed by Dounce homogenization. Phenylmethylsulfonyl fluoride (2 mM) was added during homogenization.

The homogenate was centrifuged at 100,000 × *g* for 1.5 h. The supernatant extract was brought to 10% glycerol (vol/vol) and applied to a column of ω-aminohexyl agarose (1.3 by 13 cm) which had been preequilibrated with buffer B (20 mM KP [pH 7.1], 1 mM EDTA, 9 mM β-ME, 10 mM NaF, 100 KIU of aprotinin per ml, 10% glycerol). The column was washed with 40 ml of buffer C (buffer B with 0.05% NP-40), and pp60^{src} kinase activity was eluted in a 180-ml linear NaCl gradient (0 to 1 M NaCl) in buffer C.

Fractions containing pp60^{src} kinase activity were pooled, dialyzed overnight against buffer C, and applied to a phosphocellulose column (2.5 by 15 cm) preequilibrated with buffer C. The column was washed with 50 ml of buffer C, and pp60^{src} kinase activity was eluted in a 350-ml linear KP gradient (0.02 to 0.4 M) in buffer C. pp60^{src} activity eluted at about 0.15 M KP. The active fractions were pooled and dialyzed against buffer D [20 mM KP (pH 7.1), 1 mM EDTA, 9 mM β-ME, 10 mM NaF, 100 KIU of aprotinin per ml, 0.34 M sucrose, 0.4 M $(NH_4)_2SO_4$].

The sample was applied to a phenyl-Sepharose column (1.2 by 12 cm) preequilibrated with buffer D. The column was washed with 30 ml of buffer D, and pp60^{src} was eluted in a 200-ml gradient of starting buffer D and final buffer B with 50% (vol/vol) ethylene glycol and 0.5% NP-40. pp60^{src} eluted at about 45% ethylene glycol and 60 mM $(NH_4)_2SO_4$. The active fractions were pooled and stirred with Cibacron blue-agarose (20 ml, preequilibrated in buffer E [buffer B with 0.5% NP-40]).

The Cibacron blue-agarose mixture was dialyzed overnight against buffer E and then poured into a glass column (1.2-cm diameter). The column was washed with 20 ml of buffer E, and pp60^{src} kinase activity was eluted with buffer E supplemented with 10 mM ATP and 5 mM $MgCl_2$. The active fractions were pooled and stirred with casein-Sepharose (3 ml) preequilibrated with buffer F (25 mM Tris-hydrochloride [pH 7.1], 9 mM β-ME, 0.05% NP-40, 10 mM NaF, 100 KIU of aprotinin per ml, 0.34 M sucrose).

The casein-Sepharose mixture was dialyzed overnight against buffer F and then poured into a small column (1-cm diameter). The column was washed with buffer F, and pp60^{src} was eluted in a 30-ml linear NaCl gradient (0 to 3 M) in buffer F. Active fractions were pooled and brought to 0.5% NP-40 and 50% (vol/vol) glycerol to stabilize pp60^{src} activity. This pool was stored at −20°C. Activity was stable for more than 2 months.

Microinjection. Microinjection of cells was performed at high magnification (500×) by the method of Diacumakos (10), using fine-tipped glass micropipettes with an outer diameter of about 0.5 μm. For microinjections we used as recipient cells early-passage Swiss mouse 3T3 fibroblasts. Cells were grown on glass cover slips in Dulbecco modified Eagle medium containing 10% calf serum. A small square was etched on the back of the cover slip with a diamond pen to indicate which cells were to be injected. Injection was made directly into the cytoplasm of the cells within one square with pp60^{src}-containing samples in a physiological buffered saline solution containing 0.34 M sucrose, 114 mM KCl, 20 mM NaCl, 3 mM NaH_2PO_4, and 3 mM $MgCl_2$ (pH 7.4). Cells on another cover slip were injected with the buffered saline solution alone. From 50 to 100 cells were injected on each cover slip.

During this time cells remained in warm medium at pH 7.4. Cells were injected with approximately 10 fl of solution. This approximate injection volume is routinely delivered with the Diacumakos microinjection method (10) and was experimentally determined elsewhere (33). Cells within the squares on the cover slips were identified as having been successfully injected by visually monitoring the change in refractive index of the cell as the injected material entered. Any cells damaged by overinjection were identifiable by marked granulation of the cytoplasm minutes after injection and were dislodged from the square by micromanipulation with the micropipette. After being injected, the cells were returned to the 6% CO_2 incubator at 37°C for 50 min. At the end of the incubation period, the cells were fixed with 3.7% formaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 3 min, and stained first with antibodies to chicken gizzard actin raised in rabbits and then with a 1:15 dilution of fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) (Miles Laboratories). Actin antibodies were affinity purified on alkylated actin-Sepharose. These antibodies greatly enhanced the resolution of individual stress fibers and permitted the visualization of microfilament bundles that were smaller than the large stress fibers seen with conventional actin antisera. The microinjected cells were viewed by epifluorescence microscopy and scored for whether they had acquired a diffusely staining actin matrix. Cells were scored blind, with a stringent criterion used for determining the loss of actin stress fibers; that is, a cell was scored as not having acquired a diffusely stained appearance if one actin stress fiber was seen extending the length of the cell. Uninjected cells on the same cover slip but outside the square containing injected cells were also scored. The $pp60^{src}$-containing fractions were freed from components in the purification buffers by dialysis and concentration with a collodion bag apparatus (Schleicher and Schuell, Inc., Keene, N.H.), with the buffered saline solution used for microinjection surrounding the nitrocellulose bag. Ultrafiltration or standard cellulose membranes could not be used at this stage, as $pp60^{src}$ tended to adhere to them.

Gel assay for protein kinase activity. In a total volume of 30 μl, the assay mixture contained 20 mM Tris-hydrochloride (pH 7.1), 5 mM $MgCl_2$, 5 mM dithiothreitol, 2.5 μCi of [γ-^{32}P]ATP (New England Nuclear; 1,000 to 3,000 Ci/mmol), phosphoacceptor substrate (2 to 50 μg), and $pp60^{src}$. For autophosphorylation assays, no phosphoacceptor substrate was included in the assay. After 10 min at 37°C the reaction was terminated by being boiled for 2 min in sample buffer before being analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Filter assay for IgG kinase activity. The filter assay has been described previously (22). Briefly, $pp60^{src}$-containing samples were immunoprecipitated with tumor-bearing rabbit (TBR) IgG prepared by the method of Brugge and Erikson (2) and protein A-Sepharose (Pharmacia). Immunoprecipitates were washed extensively, and the reaction was carried out at 37°C with [γ-^{32}P]ATP added to the immunoprecipitate under the conditions described for the gel assay. The reaction was stopped after 10 min by boiling the mixture in 5% SDS–1% β-ME. Protein A-Sepharose was removed by centrifugation, and the ^{32}P-labeled IgG was precipitated from the supernatant with trichloroacetic acid, collected on glass-fiber filters, and quantitated by liquid scintillation counting. For quantitating activity in the pooled fractions obtained from various stages of the purification, each fraction was assayed in duplicate at three concentrations to ensure conditions of antibody excess. Only the data points that were linear with respect to $pp60^{src}$ concentration were used in computing the activity of the fraction. The final computed activity represents the extent rather than the rate of reaction due to its exceptionally rapid kinetics.

Phosphoamino acid analysis. ^{32}P-labeled proteins were precipitated in the presence of 1 mg of bovine serum albumin carrier protein with 20% (wt/vol) trichloroacetic acid, washed three times with 5% (wt/vol) trichloroacetic acid and once with ethanol-ethyl ether (1:1), and air dried overnight. Proteins were partially hydrolyzed at 120°C in 6 N HCl for 3 h under vacuum. Phosphoamino acids in the samples were analyzed on cellulose thin-layer sheets by electrophoresis (pH 1.9) in one dimension, followed by ascending chromatography in the second dimension, as previously described (18). Commercially available phosphoserine and phosphothreonine were used as markers and visualized by ninhydrin staining. Phosphotyrosine was a gift from Shelton Earp, University of North Carolina.

RESULTS

Purification of $pp60^{src}$. As source material for $pp60^{src}$ we used RR1022 rat tumor cells, a cell line originally established in culture from tumors induced in the Amsterdam rat by the Schmidt-Ruppin strain of RSV. Our previous purification method for $pp60^{src}$ depended on preparing aqueous extracts of these cells, followed by ammonium sulfate fractionation and chromatography on ω-aminohexyl agarose and phosphocellulose (22, 23). We have now modified and extended the procedure to obtain greater purification of the protein kinase activity of $pp60^{src}$ while preserving the native molecular weight of the protein. In our revised procedure, RR1022 cells were lysed in detergent to extract both soluble and membrane-associated $pp60^{src}$ and to minimize cleavage by proteases during homogenization. To block the action of proteases it was necessary to add to the lysis buffer the protease inhibitors diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and aprotinin at the time of homogenization. The cell lysate was centrifuged at 100,000 × *g* and fractionated immediately on ω-aminohexyl agarose and then on phosphocellulose. The purification was extended to include hydrophobic chromatography on phenyl-Sepharose and affinity chromatography on Cibacron blue-agarose and casein-Sepharose. Details of the fractionation procedure are given above. It should be noted that altering the sequence of chromatographic steps resulted in a lower degree of purity than that attained by the prescribed method. In addition, immunoaffinity chromatography of $pp60^{src}$ with

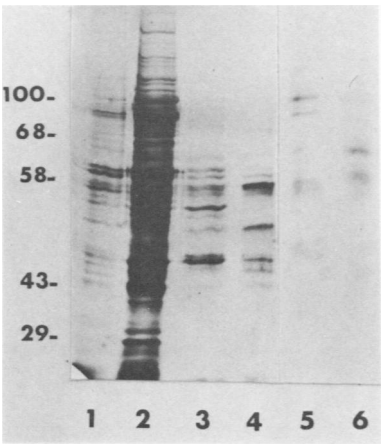

FIG. 1. Protein profiles during purification of pp60^{src}. Samples of active pp60^{src} fractions from the purification were electrophoresed on 8.5% SDS-polyacrylamide gels. Marker proteins are indicated on the left (molecular weight $\times 10^3$). Lanes: 1, extract; 2, ω-aminohexyl agarose; 3, phosphocellulose; 4, phenyl-Sepharose; 5, Cibacron blue-agarose; 6, casein-Sepharose. Lanes 1 through 4, Coomassie blue staining. Lanes 5 and 6, silver staining.

TBR IgG coupled to Sepharose or Affigel resulted in considerable loss of activity.

Pooled fractions from each of the purification steps were assayed for IgG kinase activity in immunoprecipitates with TBR serum, which contains antibodies that react with pp60^{src} (2). Overall, the fractionation resulted in approximately a 7,000-fold enrichment of IgG kinase activity, with a 1% yield from 10^{10} cells (Table 1). The IgG kinase activity was very labile during much of the purification procedure but was stable after chromatography on casein-Sepharose when stored at −20°C in 50% glycerol.

SDS-polyacrylamide gel electrophoresis was carried out on samples of pooled fractions from each step of the purification, and protein profiles were visualized by silver staining (Fig. 1). The casein-Sepharose fraction (lane 6) contained two major proteins, with molecular weights of 60K and 65K, and minor bands in the 100K and 60K regions. The major 60K protein was identified as pp60^{src} by immunoprecipitation and peptide mapping. For immunoprecipitation, the 60K protein was labeled with ^{32}P in an autophosphorylation reaction by incubating the casein-Sepharose fraction with [γ-^{32}P]ATP, as shown below (see Fig. 4). The ^{32}P-labeled 60K protein was immunoprecipitated with TBR antibodies but not with nonimmune rabbit IgG. For peptide mapping, the 60K protein was radioiodinated in SDS gel slices (13) and then subjected to limited proteolysis and analysis of peptide fragments by the procedure of Cleveland et al. (5). Limited cleavage of the 60K species by *Staphylococcus aureus* V_8 protease revealed two major cleavage products in the molecular weight ranges of 34K to 37K and 22K to 27K (Fig. 2, lane b). It has been reported elsewhere that cleavage of pp60^{src} with the V_8 protease results in two major fragments of about 34K and 26K (7). Cleavage of the 65K protein with V_8 protease produced a different fragmentation pattern, with cleavage products in the molecular weight ranges of 46K to 52K and 27K to 35K (Fig. 2, lane d). Attempts to separate the 60K and 65K proteins were unsuccessful. Absorption and elution of pp60^{src} from affinity resins containing covalently bound tubulin or microtubule-associated proteins (MAPs) failed to remove the 65K protein. These proteins may be very similar in their chromatographic behavior or physically associated, perhaps located in the same detergent micelles and therefore not easily separable.

The virtual absence on silver-stained SDS gels of major protein species with molecular weights lower than 60K shows that significant cleavage of pp60^{src} was avoided. Proteolytic cleavage of

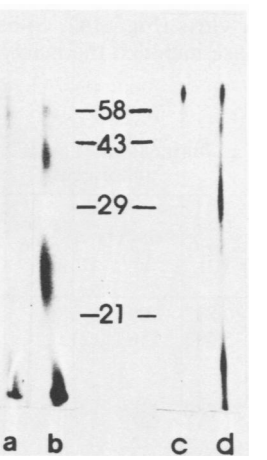

FIG. 2. Peptide mapping of pp60^{src}. Purified protein from the casein-Sepharose step was run on an 8.5% SDS-polyacrylamide gel. The 60K and 65K bands were excised from the gels, radioiodinated in the gel slices (13), and subjected to limited proteolysis with *S. aureus* V_8 protease by the procedure of Cleveland et al. (5). Iodinated peptide fragments were separated on a second gel (12% acrylamide) and visualized by autoradiography. The dye front was allowed to run off the gel to remove free ^{125}I, which tended to obscure the fragmentation pattern. Lanes: a, 60K protein not treated with protease; b, 60K protein treated with protease; c, 65K protein not treated with protease; d, 65K protein treated with protease. Marker proteins are indicated in the center (molecular weight $\times 10^3$).

pp60^{src} to the 52K fragment described by others (1, 20) occurred when application of the cell extract to ω-aminohexyl agarose was delayed or when diisopropyl fluorophosphate was omitted from the cell lysis buffer. For studies employing microinjection of the purified protein it was essential to minimize proteolysis, because the 52K fragment appears to be unable to associate with the plasma membrane (19) and thus may not be in position to phosphorylate appropriate cellular targets.

The total amount of the 60K protein recovered after purification was consistent with the known amount of pp60^{src} in the RR1022 cells. In vivo radiolabeling studies with [^{35}S]methionine have shown that pp60^{src} comprises 0.01 to 0.1% of the total protein of RR1022 cells (22). Accordingly, 100 to 1,000 μg of pp60^{src} should be present in the RR1022 cell extract (about 1,000 mg of total protein). Assuming that the observed 1% recovery of protein kinase activity during the purification (Table 1) reflects the recovery of pp60^{src} protein, 1 to 10 μg of pp60^{src} would be expected in the casein-Sepharose fraction. We recovered 1.4 μg of protein, of which about 50% was concentrated in the 60K band.

In vitro substrates of the pp60^{src} protein kinase activity. Purified pp60^{src} was able to phosphorylate a limited number of protein substrates when assayed in vitro (Fig. 3A). Substrates for the pp60^{src} kinase included the heavy chain of TBR IgG (lane 2), α-casein (lane 3), histone H1 (lane 4), and histone H2B (lane 5). Nonimmune rabbit IgG heavy chain was not a substrate (lane 1), nor were histones H2A, H3, or H4, calmodulin, or concanavalin A.

TABLE 1. Purification of pp60^{src} from 10^{10} rat tumor cells[a]

Purification step	Total activity (U)	Recovery (%)	Total protein (mg)	Sp act (U/mg)	Purification (fold)
Extract	20,832	100	998	20.87	1
ω-Aminohexyl agarose	35,592	156	213	153.0	7
Phosphocellulose	24,080	116	68	354.1	17
Phenyl-Sepharose	9,318	45	14	665.6	32
Cibacron blue-agarose	876	4.2	0.023	38,100	1,839
Casein-Sepharose	198	1.0	0.0014	141,400	6,780

[a] One unit of activity is defined as the amount of pp60^{src} that catalyzes the transfer of 1 fmol of phosphate from ATP to anti-pp60^{src} antibody heavy chains present in excess amounts in 10 min at 37°C under the standard conditions of the filter assay. Total protein in the last two steps was estimated from the intensity of silver staining, using the phenyl-Sepharose fraction as standard. Total protein in other fractions was assayed by the Lowry method.

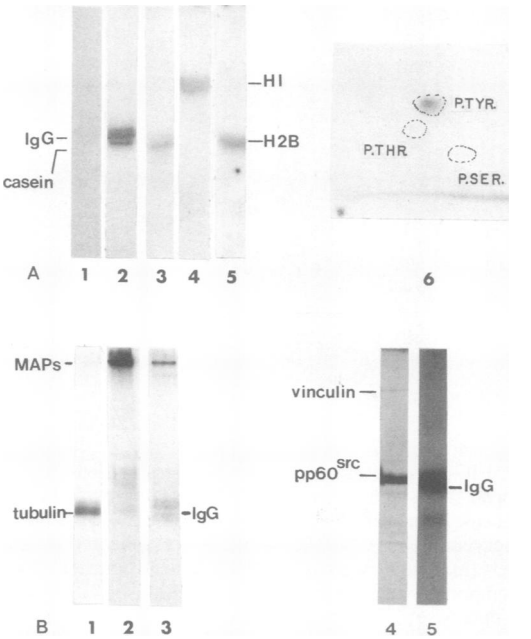

FIG. 3. In vitro substrates of the pp60^{src} kinase. (A) Casein-Sepharose-purified pp60^{src} was assayed for protein kinase activity by gel assay with the following substrates: nonimmune rabbit IgG (lane 1), TBR IgG (lane 2), α-casein (lane 3), calf thymus histone H1 (lane 4), and histone H2B (lane 5) (0.6 mg/ml each). Endogenous kinase activity in the histone samples was inactivated before assay by heating them for 5 min at 90°C. Autoradiographed gels were electrophoresed on 8.5% (lanes 1 and 2) or 12% (lanes 3, 4, and 5) SDS-polyacrylamide gels. The position of the IgG heavy chain and other proteins is indicated. Lane 6, Phosphoamino acid analysis of ^{32}P-labeled TBR IgG phosphorylated by pp60^{src}. The origin is at the lower left. Electrophoresis was carried out in the first dimension toward the anode at the right. Ascending chromatography was carried out in the second dimension toward the top. The dotted circles show the position of ninhydrin-stained phosphotyrosine (P.TYR), phosphothreonine (P.THR), and phosphoserine (P.SER). (B) Casein-Sepharose-purified pp60^{src} was assayed for protein kinase activity by gel assay, with the following cytoskeletal proteins used as substrates: porcine brain tubulin (lane 1), porcine brain MAPs (lane 2), and chicken gizzard vinculin (lane 4) (0.6 mg/ml each). Endogenous kinase activity in the tubulin and MAPs preparations was inactivated before assay by heating them for 5 min at 90°C. TBR IgG (0.06 mg/ml) was included in the assay with MAPs (lane 3) and vinculin (lane 5). Autoradiographed gels were electrophoresed on 8.5% (lanes 1, 4, and 5) or 5% (lanes 2 and 3) SDS-polyacrylamide gels.

Several cytoskeletal proteins were also examined as pp60^{src} kinase substrates (Fig. 3B). In agreement with the results of others (17, 20), porcine brain tubulin was phosphorylated by pp60^{src} (lane 1). Porcine brain MAPs (high-molecular-weight MAPs and smaller "tau" proteins) (lane 2) were highly effective substrates. Vinculin purified from chicken gizzards was also phosphorylated (lane 4), but to a much lesser extent than were other substrates. The phosphorylation of vinculin was also considerably less extensive than the apparent autophosphorylation of pp60^{src} seen in the same lane. It was surprising that the phosphorylation of vinculin was not extensive, since phosphorylation of vinculin at tyrosine residues occurs in vivo and is increased eightfold during cell transformation with RSV (18). This did not appear to be due to the lack of available phosphorylation sites on the vinculin molecule, because pretreatment of vinculin with alkaline phosphatase did not stimulate its phosphoacceptor potential. The phosphorylation of MAPs and vinculin was inhibited by including TBR IgG as an inhibitor in the assays (Fig. 3, lanes 3 and 5), indicating that phosphorylation was due to the action of pp60^{src}. Other cytoskeletal proteins that did not serve as phosphoacceptors were myosin, tropomyosin, purified myosin light chain (L2), and rabbit muscle actin. The amino acid residue phosphorylated in casein, histones, tubulin, and vinculin was phosphotyrosine.

The phosphoamino acid analysis of ^{32}P-labeled TBR IgG is shown in Fig. 3A. MAPs were the only exception, for phosphoamino acid analysis showed that both tyrosine and serine residues in the MAPs were phosphorylated in the presence of pp60^{src}. It should be noted that the MAPs preparation contained endogeneous protein kinase activity that was inactivated by being heated for 5 min at 90°C before the assay. Since pp60^{src} phosphorylated tyrosine residues exclusively in the other proteins, it is possible that the serine residues phosphorylated in the MAPs derived from trace amounts of protein kinase activity remaining in the MAPs. The tyrosine-specific phosphorylation of MAPs was undoubtedly due to pp60^{src}, as it was blocked by inclusion of TBR IgG in the assay.

Phosphatase activity. The purified pp60^{src} fraction contained no demonstrable phosphatase activity. Phosphatases, as measured by hydrolysis of *p*-nitrophenyl phosphate, were abundant in the RR1022 cell extract (717 nmol of *p*-nitrophenyl phosphate hydrolyzed per min per mg of protein) but were virtually absent from the casein-Sepharose fraction containing pp60^{src} ($<1.6 \times 10^{-5}$ nmol of *p*-nitrophenyl phosphate hydrolyzed per min per mg of protein). In a more sensitive assay, the addition of casein-Sepharose-purified pp60^{src} to tubulin prelabeled with ^{32}P by the action of pp60^{src} did not result in tubulin dephosphorylation (data not shown). In addition, incubation of purified pp60^{src} with [γ-^{32}P]ATP for 10 min at 37°C and separation of ATP and P_i by cellulose thin-layer chromatography on polyethyleneimine-cellulose did not reveal significant hydrolysis of ATP.

Phosphorylation of pp60^{src}. Incubation of casein-Sepharose-purified pp60^{src} with [γ-^{32}P]ATP-Mg in the absence of exogeneous phosphoacceptor substrates resulted in the phosphorylation of pp60^{src} (Fig. 4A, lane 1). Immunoprecipitation of the ^{32}P-labeled protein with TBR IgG (lane 3) but not with nonimmune rabbit IgG (lane 4) confirmed its identity as pp60^{src}. The phosphorylation of pp60^{src} was not stimulated by cAMP (lane 2), and phosphoamino acid analysis showed that only the tyrosine residues in pp60^{src} were radiolabeled (Fig. 4B). These results provide evidence that phosphorylation of pp60^{src} was not due to the presence of cAMP-dependent protein kinase, as serine residues in the amino-terminal portion of pp60^{src} have been shown to be susceptible to phosphorylation by cAMP-dependent protein kinase in vitro (7, 17). Tyrosine-specific phosphorylation of pp60^{src} may be due to either an autophosphorylation reaction or a distinct cAMP-independent protein kinase with specificity for tyrosine residues. In support of the latter possibility, we observed that the tyrosine-specific phosphorylation of pp60^{src} declined relative to the IgG kinase activity during purification of pp60^{src}. Equal amounts of pp60^{src} kinase in the phenyl-Sepharose and casein-Sepharose fractions were incubated with [γ-^{32}P]ATP in the presence of the heat-stable protein inhibitor of cAMP-dependent protein kinase and then immunoprecipitated with TBR serum in the presence of sodium EDTA and electrophoresed on SDS gels. Autoradiography of the gels showed that phosphorylation of pp60^{src} in the phenyl-Sepharose fraction (Fig. 4A, lane 5) was considerably greater than it was in the casein-Sepharose fraction (lane 6).

Microinjection of pp60^{src}. We showed previously that extracts of RSV-transformed chicken embryo fibroblasts disrupt the actin microfilament organization when microinjected into living 3T3 mouse fibroblasts (25). To determine whether this activity resulted from the direct action of pp60^{src} in the extract rather than from a substance induced secondarily by the action of pp60^{src}, we microinjected fibroblasts with 7,000-fold-purified pp60^{src} and monitored changes in actin microfilament organization. Casein-Sepharose-purified pp60^{src} in buffer was microinjected into the cytoplasm of 3T3 cells growing on glass cover slips. Control cells were microinject-

ed with buffer alone. The cells were returned to the incubator for 50 min and stained to visualize actin structure by indirect immunofluorescence microscopy with affinity-purified actin antibodies. The actin cytoskeleton of cells that had been microinjected with buffer alone appeared normal (Fig. 5A). Thick bundles of actin microfilaments could be seen coursing through the cell at its lower surface, more or less parallel to its long axis. In contrast, the actin bundles in cells microinjected with purified pp60^{src} had largely disappeared and were replaced by a diffusely stained actin matrix (Fig. 5B). Disruption of microfilament bundles persisted after the injection of pp60^{src} for 2 h. When examined 12 h after injection, actin cytoarchitecture had returned to normal. The decay kinetics of cytoskeletal changes observed by microinjection were consistent with the known in vivo half-life of pp60^{src} (Schmidt-Ruppin D strain of RSV) of 7 to 11 h in chicken embryo fibroblasts at 41°C (31).

To quantitate the dissolution of actin stress fibers, we calculated the percentage of cells in the injected population that exhibited a diffusely stained actin cytoskeleton. For cellular actin structure in a cell to be scored as diffuse, the cell could not present a single stress fiber that traversed the length of the cell. This stringent requirement made scoring more objective, particularly for those cells that displayed an intermediate type of actin cytoarchitecture. The percentage of cells diffusely stained after microinjection of purified pp60^{src} was significantly greater ($P > 0.005$) than that observed after injection of buffered saline alone (Table 2). Other experiments carried out with different preparations of pp60^{src} purified through the casein-Sepharose chromatographic step confirmed this observation; after microinjection of the casein-Sepharose fraction into a total of 218 cells, an average of 55% of the injected cells acquired the diffusely stained actin phenotype. The percentage of cells diffusely stained after microinjection of buffered saline was not significantly different from an uninjected population, indicating that physical damage to the cytoskeleton did not result from the mechanics of microinjection or from the introduction of buffer components into the cell. Diffusely stained cells comprised an appreciable fraction (17%) of the uninjected population and may have arisen from cell cycle variation or cell heterogeneity or as artifacts of fixation and staining. The apparent inability of pp60^{src} to generate the diffusely stained appearance in 100% of the cells was partly due to our strict definition of diffuse; however, other factors, such as inconsistencies in the microinjection technique, may have contributed. In a concurrent experiment, the actin-disrupting activity in the purified pp60^{src} fraction was completely

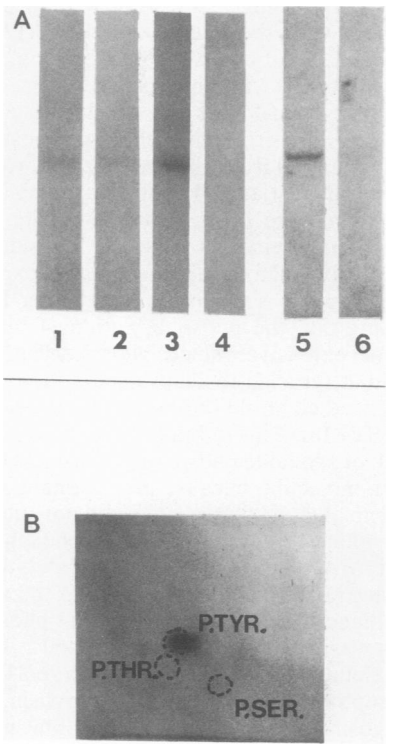

FIG. 4. Tyrosine-specific phosphorylation of pp60^{src}. (A) pp60^{src}, purified through the casein-Sepharose chromatographic step, was incubated with [γ-^{32}P]ATP-Mg under the conditions described for the gel assay and analyzed by autoradiography on 8.5% SDS-polyacrylamide gels. Autophosphorylation of casein-Sepharose-purified pp60^{src} (lane 1) and of casein-Sepharose-purified pp60^{src} in 1.9 μM cAMP (lane 2). Casein-Sepharose-purified pp60^{src} labeled with ^{32}P by autophosphorylation was immunoprecipitated in the presence of 30 mM sodium EDTA with TBR IgG (lane 3) or incubated with nonimmune rabbit IgG (lane 4), and the immune complexes were precipitated with protein A-Sepharose and electrophoresed on 8.5% SDS-polyacrylamide gels, and gels were autoradiographed. In a separate experiment, equal amounts of pp60^{src} kinase obtained from the phenyl-Sepharose (lane 5) and casein-Sepharose (lane 6) fractions were incubated with [γ-^{32}P]ATP-Mg under conditions described for the gel assay in the presence of 1 mg/ml of the heat- and acid-stable protein inhibitor of cAMP-dependent protein kinase, 1% aprotinin, and 30 mM NaF. pp60^{src} was immunoprecipitated from each sample with TBR IgG and protein A-Sepharose in the presence of 30 mM sodium EDTA and electrophoresed on an 8.5% SDS-polyacrylamide gel, and the gel was autoradiographed. (B) Phosphoamino acid analysis was carried out with ^{32}P-labeled pp60^{src} obtained by incubation of the casein-Sepharose fraction with [γ-^{32}P]ATP-Mg under conditions described for the gel assay. Details are given in the legend to Fig. 3A.

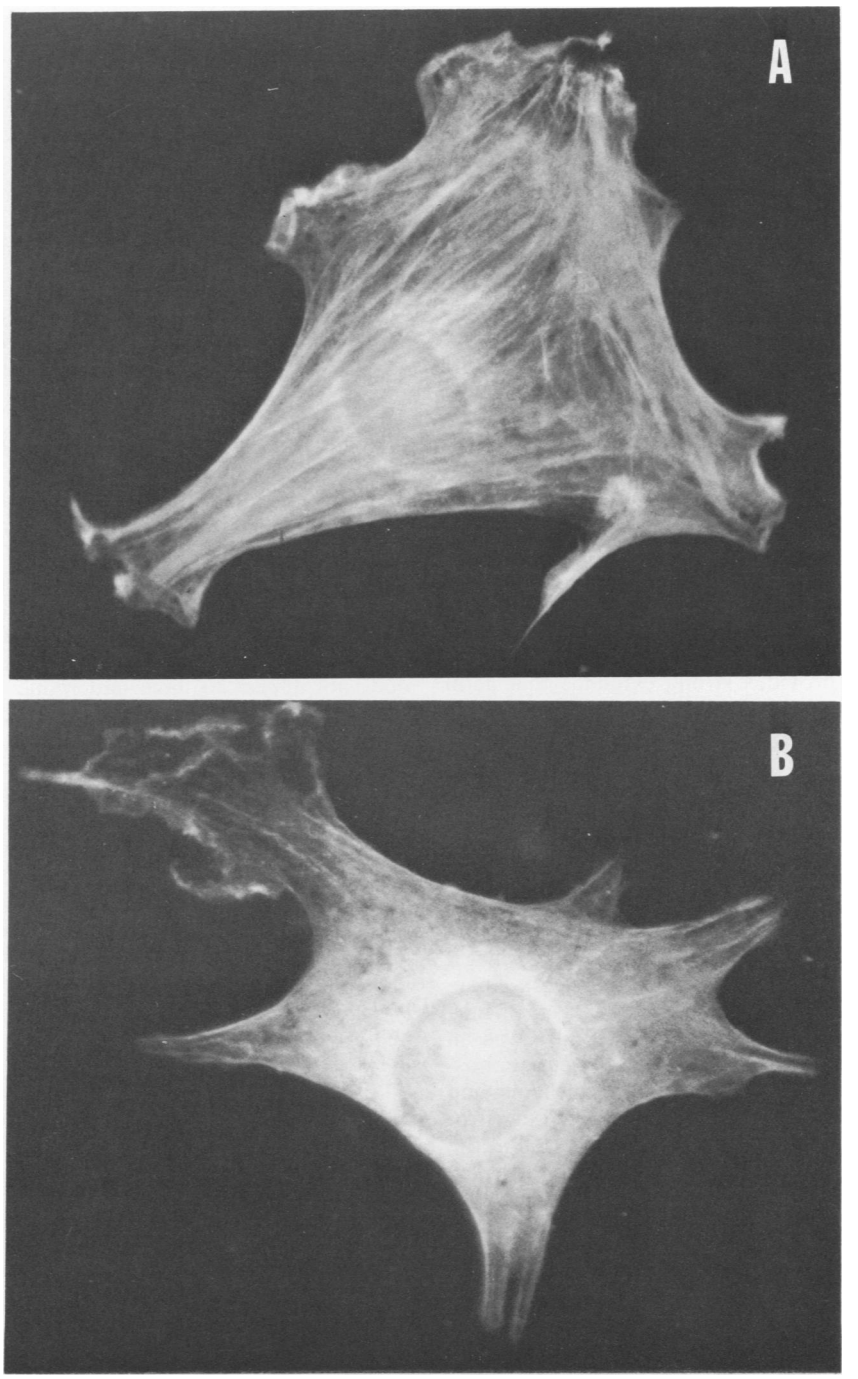

FIG. 5. Microinjection of purified pp60^{src} induces the actin transformation. Mouse 3T3 fibroblasts were microinjected with a buffered physiological saline solution as a control (A) and with casein-Sepharose-purified pp60^{src} in the same buffered solution (B) and stained to reveal actin cytoarchitecture by indirect immunofluorescence with affinity-purified actin antibodies.

inactivated by heat treatment for 2 min at 90°C, thus providing additional assurance that buffer components not removed by dialysis did not contribute to the cytoskeletal changes observed.

In a separate experiment, purified pp60^{src} was incubated with TBR IgG or nonimmune rabbit IgG (0.1 mg/ml) for 1 h at 4°C before microinjection into cells. Actin-disrupting activity in the

sample incubated with TBR IgG was significantly reduced compared with the sample incubated with nonimmune IgG (Table 2). These results support the conclusion that the actin-disrupting activity in the purified fraction is due to pp60^{src}.

To determine whether actin-disrupting activity and IgG kinase activity copurified, we compared specific activities in the extract and casein-Sepharose fractions. The microinjection assay, although limited in sensitivity, can be used in a semiquantitative way to compare the actin-disrupting activity in different fractions. Specific activity was determined from injection experiments in which stress fibers became disrupted in 40 to 50% of the microinjected cells. When a greater fraction of the cells acquire a disrupted actin structure, the assay is less sensitive (25). The specific activity of actin stress fiber disruption increased approximately 8,400-fold through the purification (Table 3). Given the limitations of the microinjection assay, this value roughly paralleled the 6,780-fold increase in specific activity of the IgG kinase (Table 1). The copurification of the two activities, together with the inhibition of actin cable dissolution by TBR IgG, provides strong evidence that actin disruption activity in the purified fraction is brought about by pp60^{src} and furthermore shows that the protein retained a native physiological function despite extensive purification.

TABLE 2. Loss of actin stress fiber-disrupting activity in casein-Sepharose-purified pp60^{src} by heat and TBR IgG treatments

Substance microinjected (treatment)	% Diffusely stained cells (no. of cells injected)
None	17 (148)
Buffered saline	16 (102)
pp60^{src}	52 (42)
pp60^{src} (heated, 90°C, 2 min)	10 (83)
pp60^{src} plus nonimmune rabbit IgG	77 (34)
pp60^{src} plus TBR IgG	35 (54)

TABLE 3. Purification of actin stress fiber-disrupting activity

Fraction	Total activity (U)[a]	Sp act (U/mg)[b]	Purification (fold)
Extract	2.5×10^{12}	2.5×10^{9}	1
Casein-Sepharose	3.0×10^{10}	2.1×10^{13}	8,400

[a] One unit of activity is defined as the amount which disrupted actin stress fibers in 40 to 50% of the 3T3 cells microinjected.

[b] Approximately 4×10^{-10} mg of protein in the extract fraction and 4.7×10^{-14} mg of protein in the casein-Sepharose fraction was injected per cell.

DISCUSSION

In this work we purified a physiologically active form of pp60^{src} 7,000-fold from RSV-transformed mammalian cells. Analysis of the purified pp60^{src} by microinjection showed that it was capable of disrupting actin stress fibers when microinjected into the cytoplasm of normal fibroblasts. Highly purified pp60^{src} was an active tyrosine-specific protein kinase that phosphorylated selected cytoskeletal proteins and other protein substrates when assayed in vitro. In addition, pp60^{src} itself became labeled at the tyrosine residues when incubated with [γ-^{32}P]ATP. These results provide evidence that the native functional properties of pp60^{src} were preserved.

In investigating the mechanism by which pp60^{src} initiates and maintains the transformed phenotype, it is important to have available purified pp60^{src} that retains as many native properties as possible. Our preparation is suitable for this purpose, as it is active in both the phosphorylation and microinjection assays and does not appear to have undergone significant proteolytic cleavage during purification. A drawback is that only small quantities of protein can be obtained. As an alternative route to producing pp60^{src}, plasmids containing the RSV *src* gene have been introduced into bacteria (17, 26). Large amounts of the *src* gene product have been made this way, but the protein kinase activity is only 10% as active as that of pp60^{src} purified from eucaryotic cells (17). This may be due to a lack of appropriate post-translational modification (bacterially produced p60^{src} is not phosphorylated) or to the presence of additional amino acids at the amino terminus. The use of pp60^{src} purified from eucaryotic cells is therefore essential not only for investigating transformation mechanisms but also for providing a standard of comparison with the more easily obtained bacterial protein.

One criticism of this work is that we have not purified pp60^{src} to homogeneity. In addition to pp60^{src}, a major 65K protein unrelated to pp60^{src} and minor bands in the 100K and 60K regions were apparent on silver-stained gels of the most highly purified fraction. Nonetheless, most of the functional properties we have ascribed to pp60^{src} appear to be intrinsic. For example, TBR antibodies effectively inhibited both actin disruption and phosphorylation activities. On the other hand, the apparent autophosphorylation of pp60^{src} at tyrosine may be due to a distinct tyrosine-specific protein kinase. In support of the latter possibility, it was observed that autophosphorylation of pp60^{src} declined relative to IgG kinase activity during purification. It is

possible that an additional kinase activity present in the preparation will prove to be responsible, but further work is required to resolve this question.

Characterization of the protein kinase activity of pp60^{src} purified by the method described here showed that the cytoskeletal proteins (tubulin, MAPs, and vinculin) can serve as substrates for the pp60^{src} kinase when assayed in vitro. Tubulin and MAPs are not likely to be relevant substrates in vivo, as the heat treatment required to inactivate associated kinase activity could have exposed phosphorylation sites not available in the native proteins. In support of this, we did not detect phosphotyrosine in MAPs isolated from RSV-transformed cells (unpublished data). The observed phosphorylation of vinculin by pp60^{src} was in agreement with in vivo labeling studies showing that vinculin is phosphorylated at increased levels on tyrosine residues during cell transformation with RSV (18). Observations that vinculin is a relatively poor substrate in vitro (11, 29; this report) and in vivo (only 1% phosphorylated [18]) raise concern about the functional significance of such phosphorylation. Nonetheless, the cellular location of vinculin within adhesion plaques at which microfilament bundles appear to insert (4, 16) would seem to be strategic for effecting the actin transformation, particularly since pp60^{src} is also concentrated there (32).

It is now essential to learn whether phosphorylation of one or more proteins by pp60^{src} leads to actin stress fiber dissolution. Our microinjection assay showing that microinjection of purified pp60^{src} generates actin cytoskeletal changes phenotypically indistinguishable from those in RSV-infected cells provides a tool for addressing this question. For example, it should be possible to evaluate the effect of phosphorylation of individual proteins on the actin phenotype by extending the microfilament bundle disruption assay to the injection of cytoskeletal proteins modified in their degree of phosphorylation by purified pp60^{src} and other kinases and phosphatases. The availability of purified pp60^{src}, as reported here, should prove to be useful for these and other transformation studies.

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