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Evidence that pp60^{src}, the Product of the Rous Sarcoma Virus *src* Gene, Undergoes Autophosphorylation

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pp60^{src}, the product of the Rous sarcoma virus *src* gene, was purified greater than 100,000-fold by a combination of ion-exchange and immunoaffinity chromatography. Incubation of pp60^{src} purified in this fashion with [^{32}P-γ]ATP resulted in a single ^{32}P-labeled protein when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Staining of these gels with silver nitrate showed a predominant 60,000-dalton polypeptide which comigrated with the protein labeled with ^{32}P in vitro. Partial digestion of this protein with V8 protease after in vitro iodination indicated that it was pp60^{src}. These results suggest that pp60^{src} is able to autophosphorylate.

The product of the Rous sarcoma virus (RSV) *src* gene is a 60,000-dalton protein termed pp60^{src}, which is phosphorylated in vivo (6, 18). A preliminary function as a protein kinase was assigned to pp60^{src} on the basis of its ability to phosphorylate the heavy chain of immunoglobulin G (IgG) in immune complexes (5). Purification of this activity several thousand-fold by either ion-exchange or immunoaffinity chromatography has indicated that pp60^{src} either is itself a protein kinase or is tightly associated with a protein kinase (9, 10). In addition, this kinase activity has unusual specificity for tyrosine residues (8, 12).

Structural analysis of pp60^{src} by use of V8 protease indicated that this enzyme could cleave pp60^{src} into two large fragments, a 34,000-dalton amino fragment and a 26,000-dalton carboxy fragment. Located on the 34,000-dalton amino-terminal fragment is at least one phosphoserine residue which, in cell-free lysates, is phosphorylated in a cyclic AMP-dependent fashion; located on the 26,000-dalton carboxy fragment is a phosphotyrosine residue which is phosphorylated in a cyclic AMP-independent manner (6, 8).

An earlier observation concerning pp60^{src} was that the enzyme was able to undergo apparent autophosphorylation. This was based on the fact that the addition of [^{32}P-γ]ATP to pp60^{src} purified by ion-exchange or immunoaffinity chromatography resulted in the phosphorylation of pp60^{src} itself (9, 10). This phosphorylation occurred exclusively on tyrosine residues (8), and it has recently been determined by both two-dimensional analysis of tryptic phosphopeptides (A. F. Purchio and R. L. Erikson, submitted for publication) and direct sequencing of these peptides (20) that the same tyrosine site phosphorylated in vivo on pp60^{src} is also labeled in vitro.

There is precedent, however, for protein kinases copurifying with their substrates as in the case of cyclic AMP-dependent protein kinase being present in purified preparations of phosphorylase kinase (21). In addition, Levinson et al. (16) reported that pp60^{src}, purified by immunoaffinity chromatography from avian sarcoma virus-transformed rat kidney fibroblasts (T2 NRK cells), would not undergo autophosphorylation.

To determine more definitively whether pp60^{src} is capable of autophosphorylation, we combined ion-exchange and immunoaffinity chromatography in purifying pp60^{src} to achieve a purification greater than 100,000-fold. Electrophoresis of this material on sodium dodecyl sulfate (SDS)-polyacrylamide gels and staining with silver nitrate indicated that little contaminating protein was present. Addition of [^{32}P-γ]ATP to these enzyme preparations still resulted in the labeling of pp60^{src}, suggesting that this enzyme does undergo autophosphorylation.

MATERIALS AND METHODS

Cells and viruses. The cells used in these experiments were European field vole (*Microtus agrestis*) transformed by subgroup D of the Schmidt-Ruppin strain of RSV (clone 1T; provided by A. J. Faras, Department of Microbiology, University of Minnesota Medical School) and rat kidney fibroblasts transformed by the Schmidt-Ruppin strain of avian sarcoma virus (T2 NRK cells; obtained from H. Oppermann, Department of Microbiology, University of California, San Francisco, originally provided by L. Turek, National Cancer Institute). Cells were grown in Dulbecco modified Eagle medium (H21) containing 5% calf serum and 10% tryptose phosphate.

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Kinase assays. Soluble kinase assays were performed in a total volume of 30 μl containing 5 mM $MgCl_2$, 10 mM Tris, pH 7.2, 0.04 μM [^{32}P-γ]ATP (prepared as described by Johnson and Walseth [13]), and 5 to 10 μl of pp60^{src} (1 to 4 ng) for 40 min at 23°C. Reactions were terminated by adding 0.25 volume of 5× electrophoresis buffer and were fractionated on 10% SDS-polyacrylamide gels as described (15). Gels were dried and autoradiographed with the use of Cronex 4 X-ray film.

Silver staining. Proteins were fractionated on 10% polyacrylamide-SDS gels (15) and stained with silver nitrate exactly as described (17).

Purification of pp60^{src}. pp60^{src} was purified from 1T and T2 NRK cells by immunoaffinity chromatography as described (9, 10) except that enzyme activity was eluted with 1 M KSCN (1T) or with 0.8 M KSCN (T2). For ion-exchange and immunoaffinity chromatography purification, 1T cells (15 g) were lysed in 75 ml of buffer A (0.02 M potassium phosphate, pH 7.2, 0.001 M EDTA, 0.5% deoxycholate, 1.0% Nonidet P-40, and 10% glycerol), clarified for 30 min at 100,000 × *g*, and applied to a 2-cm^3 hexylamine-agarose column equilibrated with buffer A. The column was washed with 0.25 M KCl in buffer A, and pp60^{src} was eluted with 1.0 M KCl in buffer A. This material was dialyzed against 100 volumes of buffer A and immediately applied to a 5-ml Cibacron blue agarose column equilibrated with buffer A. The column was developed with a 0.0 to 0.7 M KCl gradient in buffer A, and fractions were assayed for IgG kinase activity as described (5). Peak fractions were dialyzed against 75 volumes of buffer A and immediately applied to a 5-cm^3 heparin-agarose column equilibrated with buffer A. The column was developed with a 0.0 to 0.7 M KCl gradient in buffer A, and fractions were assayed for IgG kinase activity. Peak fractions were immediately applied to an immunoaffinity column, and pp60^{src} was eluted with 1.0 M KSCN as described (9, 10), dialyzed against 50% glycerol, and stored at −20°C.

The purification was 130,000-fold and was based on the following data. A known amount of extract from 1T cells was chromatographed on an immunoaffinity column, and the enzymatic activity of pp60^{src} (based on casein kinase activity) was taken as 100% since all the pp60^{src} bound to the column. The amount of activity recovered from the ion-exchange and immunoaffinity columns was 10% of that obtained from immunoaffinity chromatography alone when the same amount of protein was used in the starting lysates. The total protein purification was 1.3×10^6 to give an overall enzyme purification of 130,000. This is the best estimate of the fold purification that could be obtained.

Iodination and partial protease analysis of pp60^{src}. pp60^{src} was iodinated with ^{125}I in vitro as described (11), fractionated on a 10% SDS-polyacrylamide gel (15), and subjected to partial protease analysis with V8 protease as described previously (3).

Preparation of antisera. Serum from tumor-bearing rabbits was prepared as described (1).

RESULTS

Figure 1A (track 1) shows a polyacrylamide gel analysis of pp60^{src} purified by immunoaffinity chromatography from [^{35}S]methionine-labeled lysates of 1T cells. In agreement with previous results (9, 10), almost all the methionine label was contained in pp60^{src}. Addition of [^{32}P-γ]ATP to immunoaffinity-purified pp60^{src} (from unlabeled lysates of 1T cells) resulted in the phosphorylation of pp60^{src} itself (Fig. 1A, track 2). We have referred to this phenomenon as autophosphorylation (8–10), and this phosphorylation takes place exclusively on tyrosine residues (8). Figure 1B shows that this phosphorylation was inhibited by tumor-bearing rabbit IgG but not by normal IgG.

To determine whether this phosphorylation was peculiar to pp60^{src} purified from 1T cells, we purified pp60^{src} by immunoaffinity chromatography from T2 NRK cells exactly as described by Levinson et al. (16) and attempted to detect autophosphorylation. Figure 1A, track 3, shows that pp60^{src} prepared from these cells was indeed able to undergo autophosphorylation. In addition, we have observed this phenomenon with pp60^{src} purified from chicken fibroblasts transformed with subgroup D of the Schmidt-Ruppin strain of RSV and rat kidney cells transformed with the Bratislava strain of RSV (data not shown).

Although the results of the methionine labeling indicated that we were dealing with a reasonably pure preparation of pp60^{src}, staining of this same enzyme preparation with silver nitrate (17) showed that a large number of proteins were present which were undetectable by metabolic labeling with methionine (Fig. 1C), and the possibility remained that perhaps one of these proteins was responsible for the phosphorylation of pp60^{src}.

To address this question, we combined ion-exchange and immunoaffinity chromatographic techniques for purifying pp60^{src}. Lysates of 1T cells were chromatographed on hexylamine-agarose, Cibacron blue-agarose, and heparin-agarose. Figure 2 shows the elution profile of the pp60^{src}-associated IgG kinase activity from the latter two columns. The peak fractions from the heparin-agarose column were applied to an immunoaffinity column, and pp60^{src} was eluted as described in Materials and Methods. Figure 3A (track 2) shows that pp60^{src} prepared in this fashion was able to undergo autophosphorylation; silver staining of this preparation indicated that only two major bands were present (Fig. 3, track 1), one which comigrated with pp60^{src} and a second 56,000-dalton protein which was probably a proteolytic cleavage product of pp60^{src} described previously (10, 16).

To demonstrate the relationship of the 56,000-dalton protein to pp60^{src}, both were iodinated in vitro and fractionated on an SDS-polyacrylamide gel. The bands were localized by autoradiography, excised from the wet gel and subjected to limited proteolysis by use of *Staph-*

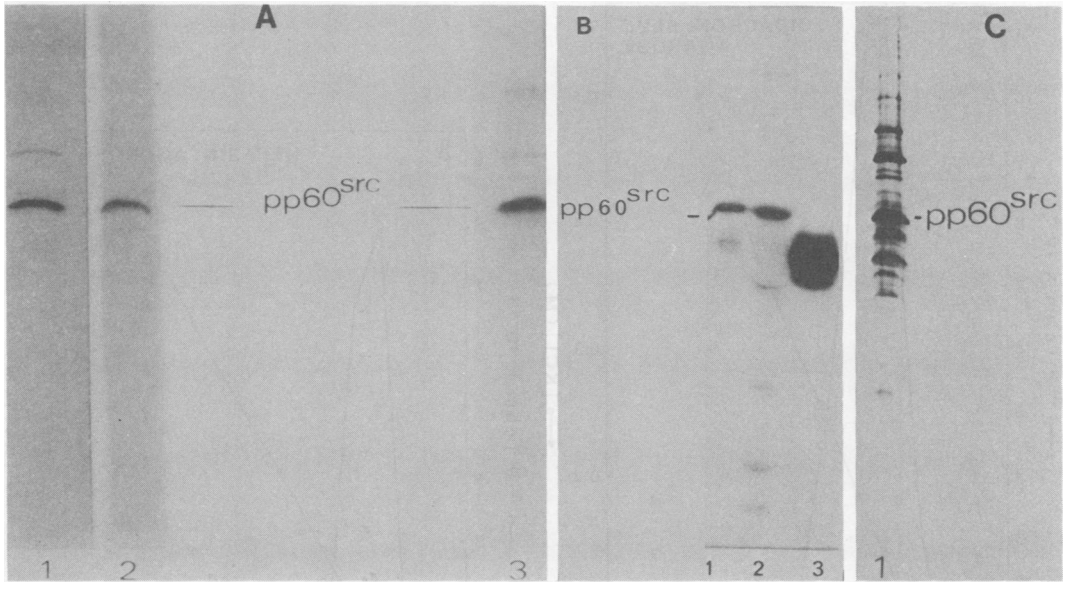

FIG. 1. Autophosphorylation of pp60^{src} purified by immunoaffinity chromatography. (A) pp60^{src} was purified by immunoaffinity chromatography from lysates of 1T cells labeled with [^{35}S]methionine as described in Materials and Methods, and a sample of this material was electrophoresed on a 10% SDS-polyacrylamide gel. The gel was soaked in sodium salicylate as described (2), dried and autoradiographed (track 1). pp60^{src} was prepared from unlabeled lysates of 1T cells and T2 cells by immunoaffinity chromatography, autophosphorylated, fractioned on an SDS-polyacrylamide gel, and autoradiographed as described in Materials and Methods. Track 2, pp60^{src} from 1T cells; track 3, pp60^{src} from T2 cells. (B) pp60^{src}, prepared from 1T cells by immunoaffinity chromatography, was allowed to autophosphorylate after prior incubation with: track 1, nothing; track 2, normal IgG (30 μg); track 3, tumor-bearing rabbit IgG (30 μg). (C) pp60^{src} was purified from lysates of 1T cells by immunoaffinity chromatography. A sample of this material was electrophoresed on a 10% SDS-polyacrylamide gel and stained with silver nitrate as described (17).

ylococcus aureus V8 protease as described (3). Previous results indicated that V8 protease could cleave pp60^{src} into an amino-terminal 36,000-dalton fragment and a carboxy-terminal 24,000-dalton fragment (6). Figure 3B shows that pp60^{src} and p56 are related by this method of analysis and the p56 probably arose by proteolytic cleavage of the amino terminus of pp60^{src} (10). It is interesting to note that although p56 was present in the autophosphorylation reaction (Fig. 3A, track 1) it was not phosphorylated (Fig. 3A, track 2) even though pp60^{src} was present.

When one is performing an enzyme preparation involving several columns, it is customary to allow column fractions to sit overnight on ice and assay for activity the next morning. In the above purification, column fractions spent approximately 24 h on ice, and the entire preparation took about 60 h. If p56 arose as a result of proteolytic cleavage of pp60^{src}, then one might hope at least to increase the ratio of pp60^{src} to p56 by not allowing the enzyme to sit on ice, thereby shortening the time of the entire enzyme preparation. Therefore, lysates of 1T cells were chromatographed on each of the above columns immediately after IgG kinase assays and dialysis were completed. The preparation took about 32 h (from cell lysis to dialysis into 50% glycerol).

Figure 4A, track 2, shows that pp60^{src} prepared in this fashion was able to undergo autophosphorylation; silver staining of this preparation indicated that almost all the protein was pp60^{src}, with p56 barely detectable. To further characterize this protein, it was iodinated in vitro, fractionated on an SDS-polyacrylamide gel, and subjected to limited proteolysis by use of *S. aureus* V8 protease as described (3). Figure 4B shows that this protein has a V8 cleavage pattern identical to that described for pp60^{src} (6). The above data suggest, therefore, that pp60^{src} does undergo autophosphorylation. Tenfold dilutions of pp60^{src} did not result in any decrease in labeling of pp60^{src}, suggesting that this phosphorylation is an intramolecular rather than an intermolecular event (Table 1).

DISCUSSION

Previous observations regarding pp60^{src} indicated that the addition of [^{32}P-γ]ATP to partially purified preparations of the enzyme resulted in the phosphorylation of pp60^{src} itself (9, 10). This

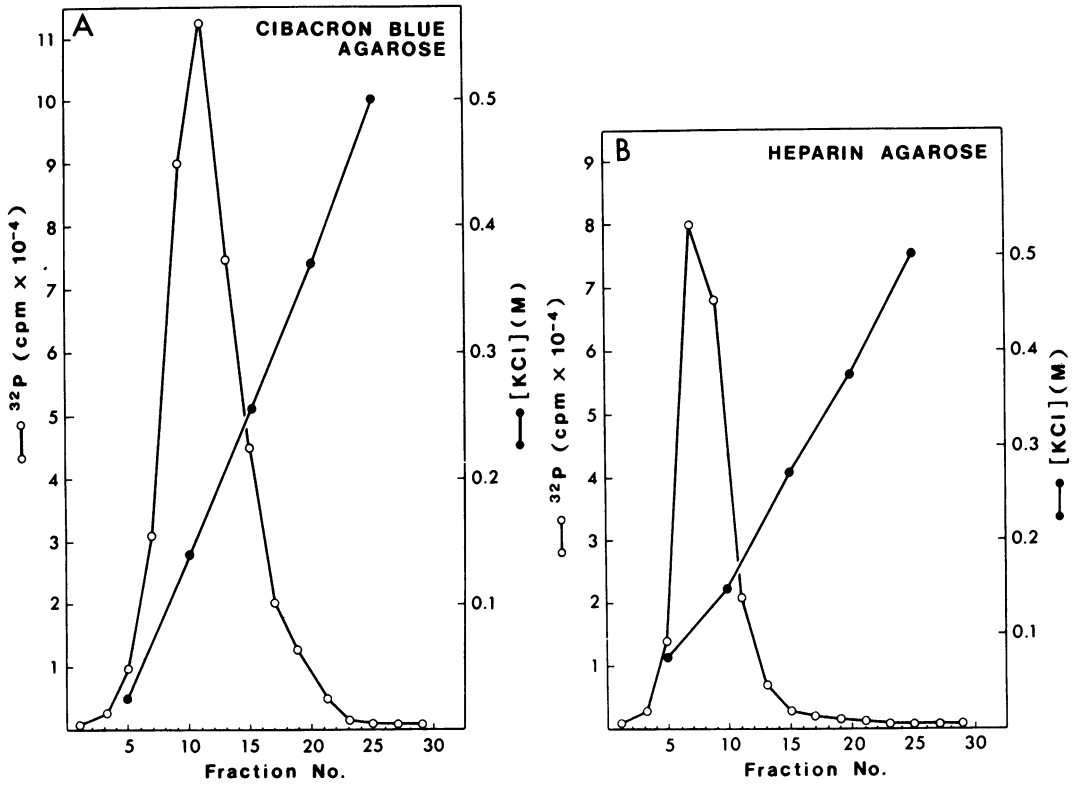

FIG. 2. Ion-exchange chromatography of pp60^{src} on Cibacron blue-agarose and heparin-agarose. Lysates of 1T cells were applied to a hexylamine agarose column, and pp60^{src} was step eluted between 0.25 and 1.0 M KCl. After dialysis, it was applied to columns of Cibacron blue-agarose (A) and heparin-agarose (B) and was eluted with a 0.0 to 0.7 M KCl gradient as described in Materials and Methods. Fractions were assayed for IgG kinase activity as described (5). The reaction products were fractionated on a 10% polyacrylamide gel, and the heavy chain of the IgG band was localized by staining with Coomassie blue. The stained bands were excised from the wet gel, dried, and counted in a Beckman liquid scintillation counter. The peak fractions from the heparin-agarose column were applied to an immunoaffinity column and eluted as described in Materials and Methods.

phenomenon was referred to as autophosphorylation, although the possibility remained that a second kinase which copurified with pp60^{src} was responsible for this phosphorylation.

Although preparations of pp60^{src} purified by immunoaffinity chromatography from lysates of [^{35}S]methionine-labeled 1T cells indicated that a rather homogeneous protein preparation existed when analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 1A), staining of these same gels with silver nitrate (Fig. 1C) showed that several other protein bands were present that were not detected by methionine label. Therefore, ion-exchange and immunoaffinity chromatography were combined to determine whether it was possible to separate this apparent autophosphorylating activity from pp60^{src}.

Lysates of 1T cells were applied to columns of hexylamine-agarose, Cibacron blue-agarose, and heparin-agarose before chromatography on immunoaffinity columns. This procedure resulted in a 100,000-fold purification; pp60^{src} purified in this fashion was still able to undergo autophosphorylation (Fig. 3A). Silver staining of these preparations (Fig. 3C) indicated that the major components were pp60^{src} and p56, a protease cleavage product of pp60^{src} which has been found in partially purified preparations of the enzyme (9, 10); p56 neither is capable of being labeled by autophosphorylation (Fig. 3A) nor is a substrate for the catalytic subunit of cyclic AMP-dependent protein kinase (Purchio and Erikson, unpublished data).

To demonstrate more clearly the autophosphorylating ability of pp60^{src}, the time required for the enzyme preparation was diminished by about 50% with the idea of increasing the ratio of pp60^{src} to p56. This procedure resulted in an enzyme preparation which consisted almost entirely of pp60^{src}, with p56 barely detectable (Fig. 4); still, pp60^{src} prepared in this fashion showed autophosphorylation approximately 40% better than that of the preparation shown in Fig. 3.

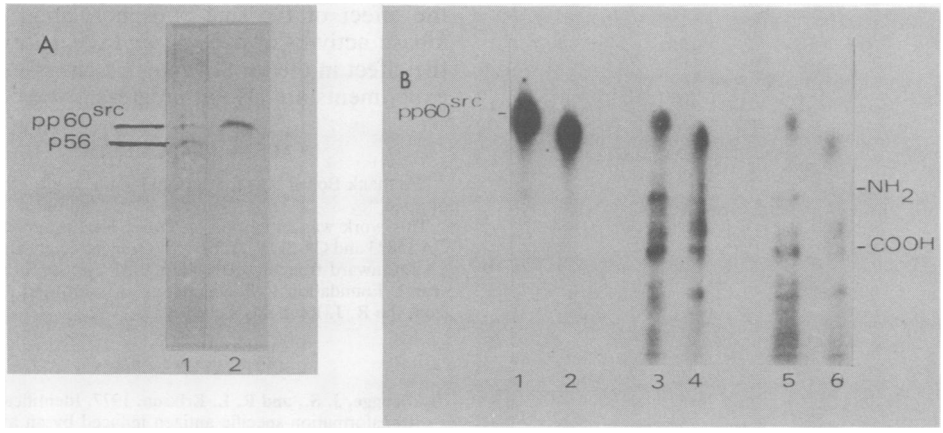

FIG. 3. Analysis of pp60^{src} purified by ion-exchange and immunoaffinity chromatography: 60-h preparation. (A) pp60^{src} was purified by ion-exchange and immunoaffinity chromatography as described in Materials and Methods. The entire preparation took approximately 60 h. A sample of this material was fractioned on an SDS-polyacrylamide gel and stained with silver nitrate (track 1) or allowed to autophosphorylate in the presence of [^{32}P-γ]ATP and analyzed by autoradiography as described in Materials and Methods (track 2). (B) pp60^{src}, purified as described above, was iodinated in vitro and fractionated on a 10% polyacrylamide gel. The bands corresponding to pp60^{src} and p56 were localized by autoradiography of the wet gel, excised, and subjected to limited proteolysis by using *S. aureus* V8 protease during reelectrophoresis as described (3). Track 1, pp60^{src}, no enzyme; track 3, pp60^{src}, 0.005 μg of enzyme; track 5, 0.05 μg of enzyme; track 2, p56, no enzyme; track 4, p56, 0.005 μg of enzyme; track 6, p56, 0.05 μg of enzyme. The notations $-NH_2$ and $-COOH$ mark the position of the 34,000-dalton amino-terminal fragment and the 26,000-dalton carboxy-terminal fragment described previously (6). There is about a 10% cross-contamination between pp60^{src} and p56 due to the fact that neither runs as sharp bands (track 1 and 2) when labeled with ^{125}I and the two do not separate well even after reelectrophoresing on a second polyacrylamide gel.

These data suggest that pp60^{src} does undergo autophosphorylation; 10-fold dilutions of pp60^{src} did not result in any decrease in labeling (data not shown) suggesting that this phosphorylation is an intramolecular event, although further experiments are necessary to confirm this. As a result of the very low amounts of protein in our enzyme preparations, plus the fact that some of the pp60^{src} is already in a phosphorylated state, we have not yet been able to determine the stoichiometry of this reaction.

Recently, Levinson et al. (16) reported that pp60^{src} purified by immunoaffinity chromatography from ASV-transformed rat fibroblasts (T2 NRK cells) did not undergo autophosphorylation; however, in the present study pp60^{src} isolated from these cells did undergo autophosphorylation (Fig. 1A, track 3). This discrepancy may be due to the different manner in which the enzyme was treated after elution from the immunoaffinity column with KSCN. We routinely dialyze the KSCN eluate immediately against 50% glycerol and store it at −20°C; Levinson et al. diluted their KSCN eluates 1:80 and used this as their source of enzyme, which may have accounted for their inability to detect autophosphorylation.

The conclusion that pp60^{src} undergoes autophosphorylation is essentially based on our inability to separate this activity from pp60^{src}. Although labeling with [^{35}S]methionine and silver staining (Fig. 1 and 4) indicated that little contaminating protein was present, it is still possible that other proteins are present which are undetectable by these methods. If such a protein exists, it must be very tightly bound to pp60^{src}.

Several enzymes have been described whose activity is regulated by phosphorylation-dephosphorylation reactions (14). We have recently found that phosphorylation at serine residues of pp60^{src} by the catalytic subunit of cyclic AMP-dependent kinase results in a net increase in the specific activity of the enzyme (Purchio and Erikson, submitted for publication). Experiments are in progress to determine the effect of tyrosine phosphorylation on the kinase activity of pp60^{src}.

A normal cellular protein that has a molecular weight of approximately 60,000 daltons and is similar in structure and function to pp60^{src} has been described (4, 7). It has been termed pp60^{sarc} and is present at one-fiftieth to one-hundredth the concentration of the viral enzyme in infected cells (4). We previously reported that the addition of [^{32}P-γ]ATP to pp60^{sarc} purified by immunoaffinity chromatography also resulted in the phosphorylation of pp60^{sarc} (19). It will be

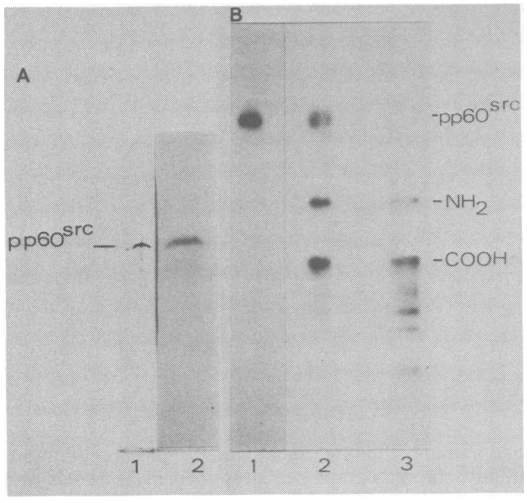

FIG. 4. Analysis of pp60^{src} purified by ion-exchange and immunoaffinity chromatography: 32-h preparation. (A) pp60^{src} was purified by ion-exchange and immunoaffinity chromatography as described in Materials and Methods. The entire preparation took approximately 32 h. A sample of this material was fractioned on an SDS-polyacrylamide gel and stained with silver nitrate (track 1) or allowed to autophosphorylate in the presence of [^{32}P-γ]ATP and analyzed by autoradiography as described in Materials and Methods (track 2). (B) pp60^{src}, purified as described above, was iodinated in vitro and fractionated on a 10% polyacrylamide gel. The band was localized by autoradiography of the wet gel, excised, and subjected to limited proteolysis by using *S. aureus* V8 protease during reelectrophoresis as described (3). Track 1, pp60^{src}, no enzyme; track 2, pp60^{src}, 0.005 μg of enzyme; track 3, 0.05 μg of enzyme. The notations $-NH_2$ and $-COOH$ mark the position of the 34,000-dalton amino-terminal fragment and the 26,000-dalton carboxy-terminal fragment described previously (6).

interesting to determine whether this autophosphorylating activity will remain associated with pp60^{sarc} when purified as described above for pp60^{src}; it will also be of interest to determine the effect of tyrosine phosphorylation on the kinase activity of pp60^{sarc} and compare it with the effect in the virally encoded enzyme. These experiments are also in progress.

TABLE 1. Evidence for intramolecular autophosphorylation of pp60^{src} [a]

Vol (μl)	cpm in pp60^{src}
7	10,136
70	9,486

[a] One microliter of pp60^{src} (prepared as shown in Fig. 4) containing approximately 1 ng of protein was allowed to autophosphorylate in a total volume of 7 or 70 μl for 15 min under conditions described in Materials and Methods (autophosphorylation is linear for 30 min under these conditions [Purchio and Erikson, unpublished data]). The reaction products were fractionated on a 10% polyacrylamide gel; pp60^{src} was localized on the dried gel by autoradiography, cut out, and counted by liquid scintillation spectrometry.

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