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Evidence that the avian sarcoma virus transforming gene product is a cyclic AMP-independent protein kinase

(*src* gene product/protein phosphorylation)

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ABSTRACT The avian sarcoma virus transforming gene product pp60^{src} has been partially purified by using ion exchange or immunoaffinity chromatography. These preparations contain a cyclic AMP-independent protein kinase activity capable of transferring radiolabel from [γ-^{32}P]ATP to immune rabbit IgG, casein, and the heavy chain purified from normal rabbit IgG. The casein kinase activity is specifically inhibited by anti-pp60^{src} IgG. Comparison of the pp60^{src}-protein kinase isolated from cells transformed by a wild-type ASV or by an ASV temperature-sensitive transformation mutant revealed that the latter product had a half-life of 3 min at 41°C whereas that of the wild-type product was 20 min.

Genetic evidence clearly indicates that cell transformation and fibrosarcoma induction by avian sarcoma viruses (ASVs) results from the expression of a single viral gene termed *src* (1, 2). The product of the *src* gene is a phosphoprotein with an apparent molecular weight of 60,000, termed pp60^{src} (3–6). A function was provisionally ascribed to pp60^{src} when it was discovered that specific immunoprecipitates formed with anti-pp60^{src} rabbit serum and extracts of ASV-transformed avian or mammalian cells containing pp60^{src} catalyzed the transfer of radiolabel from [γ-^{32}P]ATP to the heavy chain of rabbit IgG. Immunoprecipitates formed with anti-pp60^{src} serum and extracts from normal cells or cells infected with ASV mutants lacking a *src* gene showed no activity in this assay, nor did complexes formed with normal rabbit serum and extracts of normal or transformed cells (7–9). Moreover, a similar enzymatic activity is associated with pp60^{src} synthesized in cell-free extracts programmed by subgenomic viral RNA which contains the *src* gene (10, 11). These results demonstrate that the presence of pp60^{src} correlates with the presence of a phosphotransferase activity (ATP:protein phosphotransferase, EC 2.7.1.37), suggesting that the ASV *src* gene product is, or is closely associated with, a protein kinase.

Because of the well-recognized role of protein phosphorylation in the functional regulation of various cellular processes, these results had obvious implications concerning molecular mechanisms of ASV-induced oncogenesis. Consequently, characterization of the pp60^{src}-protein kinase activity along with identification of possible cellular substrates of this activity would be important to better definition of the biochemical pathways leading to the transformed phenotype. Toward this end, we present in this communication additional conventional biochemical analyses of pp60^{src} which serve to demonstrate more directly that the ASV transforming protein is a cyclic AMP-independent protein kinase.

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MATERIALS AND METHODS

Cells and Viruses. Cells used in these studies as a source of pp60^{src} included chicken embryo fibroblasts transformed by Schmidt–Ruppin strain of ASV (SR-ASV) or by a temperature-sensitive transformation mutant of this strain, NY68 (12), SR-ASV-transformed BALB/c mouse cells, and SR-ASV-transformed *Microtus agrestis* cells (13). Cells were grown to confluency in culture and usually labeled with [^{35}S]methionine (700 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; Amersham) at 100 μCi/ml in methionine-free medium as described (6), washed twice with 0.15 M NaCl/50 mM Tris·HCl, pH 7.2/1 mM EDTA, scraped from the dishes, pelleted by centrifugation, quickly frozen, and stored at −70°C until used. All subsequent procedures were at 4°C and all buffers contained 100 kallikrein inactivator units (KIU)/ml of aprotinin (FBA Pharmaceuticals, New York). Cells were disrupted in 20 mM potassium phosphate, pH 7.1/1 mM EDTA/1 mM 2-mercaptoethanol/0.5% sodium deoxycholate/1% Nonidet P40 (NP40) with 10–15 strokes in a Dounce homogenizer. Homogenates were clarified at $100,000 \times g$ for 30 min.

Ion Exchange and Immunoaffinity Chromatography. The supernatants were adjusted to 10% (vol/vol) glycerol and applied to a 2×7 cm hexylamine-agarose column (P-L Biochemicals) equilibrated with lysis buffer containing 10% glycerol (buffer A). After application of the sample, the column was washed with 2 column volumes of 250 mM KCl in buffer A followed by a 100-ml linear gradient to 1500 mM KCl in buffer A. pp60^{src} eluted between 700 and 1000 mM KCl. Fractions containing pp60^{src} were pooled and dialyzed for 4 hr against 50 vol of buffer A and then applied to a 2×5.5 cm column of phosphocellulose equilibrated with buffer A. The column was eluted with an 80-ml linear gradient 0–700 mM KCl in buffer A. Fractions containing pp60^{src} (50–100 mM KCl) were dialyzed against 10 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol/0.05% NP40 (buffer B) and applied to a 1.2×5 cm column of DEAE-Sephacel equilibrated with the same buffer at pH 7.2. A linear (0–600 mM) gradient of NaCl in buffer B was used to elute pp60^{src} which appeared between 150 and 200 mM. Fractions with activity were pooled and dialyzed against 50% glycerol/20 mM potassium phosphate, pH 7.1/100 mM KCl/1 mM EDTA/1 mM 2-mercaptoethanol and could be stored at −20°C for at least 3 months with little loss of protein kinase activity.

Serum, containing anti-pp60^{src} antibody, from tumor-bearing rabbits (TBR serum) (3) was used to detect methionine-labeled

Abbreviations: ASV, avian sarcoma virus; *src*, designation for the ASV gene responsible for transformation of fibroblasts; pp60^{src}, the phosphoprotein product of the ASV *src* gene; TBR serum, serum from rabbits bearing ASV-induced tumors; SR-ASV, Schmidt–Ruppin strain of ASV; NP40, Nonidet P40; NaDodSO$_4$, sodium dodecyl sulfate; KIU, kallikrein inactivator units.

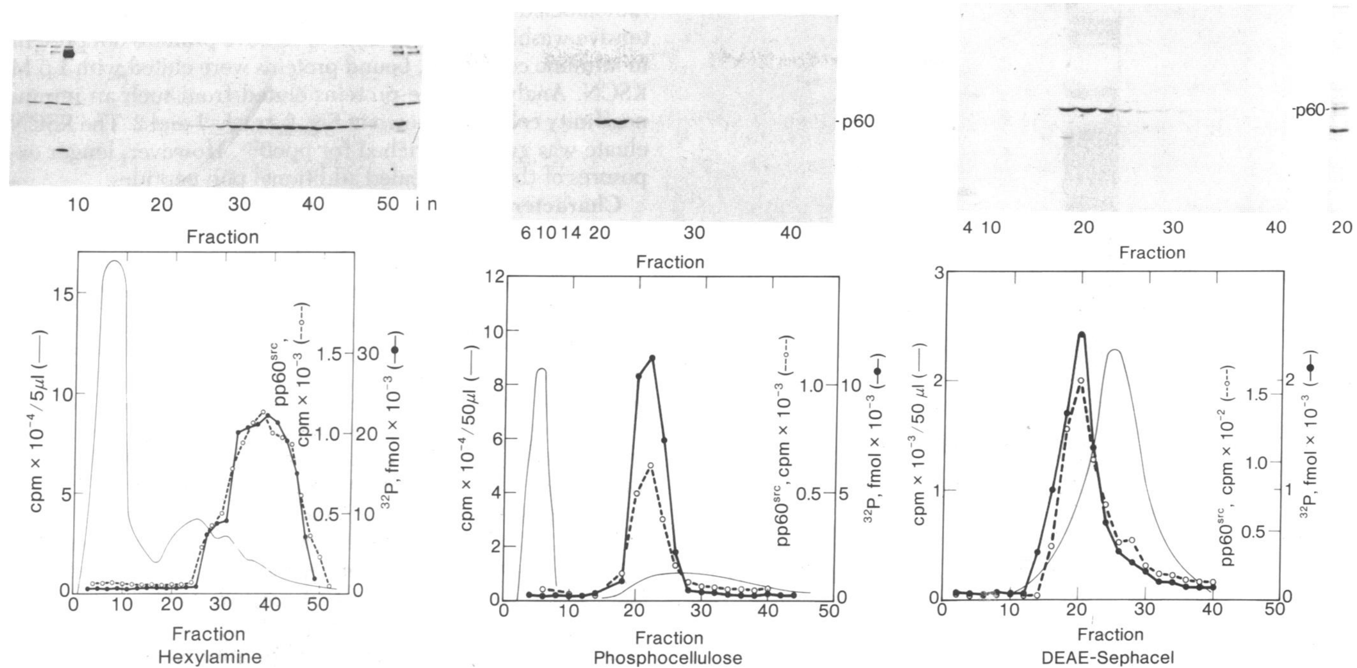

FIG. 1. Fractionation of pp60^{src} and IgG kinase activity by ion exchange chromatography. An extract of [^{35}S]methionine-labeled SR-ASV-transformed mouse cells (approximately 200 mg of protein) was fractionated sequentially through hexylamine-agarose (*Left*), phosphocellulose (*Center*), and DEAE-Sephacel (*Right*). From appropriate fractions, samples were analyzed for total radioactivity (—), 50 μl was used to assay for IgG kinase activity (— ● —), and 500 μl was used to quantify pp60^{src} content (- - O - -). The top half of each panel shows the elution of pp60^{src} as determined by immunoprecipitation of samples from various fractions and polyacrylamide gel analysis. Samples of the starting lysate were immunoprecipitated with normal rabbit (lane n) and TBR (lane i) serum to indicate the position of pp60^{src}. Direct polyacrylamide gel analysis, without immunoprecipitation, of a sample from fraction 20 of the DEAE column is shown at the far right.

antigen or IgG kinase activity. To measure pp60^{src}, 2.5 μl of antiserum was added to samples of appropriate fractions, the mixtures were incubated for 30 min, and the antigen–antibody complexes were collected by using the protein A-containing bacterium *Staphylococcus aureus* (14) and assayed by sodium dodecyl sulfate (NaDodSO$_4$)/polyacrylamide gel electrophoresis. After fluorography (15) and location of pp60^{src}, slices were cut from the gel and ^{35}S was quantified by liquid scintillation spectrometry. IgG kinase activity was detected by incubating samples of appropriate fractions with 2.5 μl of antiserum for 30 min. The immune complexes were collected and assayed for protein kinase activity as described (7) except that, after polyacrylamide gel electrophoresis, the heavy chain of IgG was located by staining with 0.2% Coomassie blue for 5 min and cut from the gel, and ^{32}P was quantified by liquid scintillation spectrometry.

Immunoaffinity columns containing anti-pp60^{src} IgG were prepared by coupling the immunoglobulin fraction of TBR serum obtained by ammonium sulfate precipitation to Affi-Gel 10 (*N*-hydroxysuccinimide derivative of Bio-Gel A support; Bio-Rad). Cellular extracts containing pp60^{src}, prepared as described above, were loaded onto the immunoaffinity column and the column was washed with: 4 column volumes of 0.1 M NaCl/10 mM Tris·HCl, pH 7.2/2 mM EDTA/1% NP40/0.5% sodium deoxycholate/100 KIU of aprotinin per ml; 3 column volumes of 1 M NaCl/10 mM Tris·HCl, pH 7.2/0.1% NP40; 3 column volumes of RIPA buffer [0.15 M NaCl/10 mM Tris·HCl, pH 7.2/1% sodium deoxycholate/1% Triton X-100/0.1% NaDodSO$_4$ (3)]; 2 column volumes of 0.15 M NaCl/10 mM Tris·HCl, pH 7.2. The column finally was eluted with 1.5 M KSCN. Fractions of the KSCN eluate containing radioactivity were pooled and dialyzed against 50% glycerol/40 mM NaCl/10 mM potassium phosphate, pH 6.8/1 mM EDTA/1 mM 2-mercaptoethanol and stored at −20°C.

Assay of Phosphotransferase Activity. Reactions were carried out in a total volume of 25 μl of 20 mM potassium phosphate, pH 7.1/100 mM KCl/1 mM EDTA/1 mM 2-mercaptoethanol. Reactions were initiated by the addition of MgCl$_2$ and [γ-^{32}P]ATP (400–600 Ci/mmol) to a final concentration of 5 mM and 1 μM, respectively, incubated at 22°C for 15 min, and terminated by the addition of 0.2 vol of 5-times concentrated electrophoresis sample buffer followed by heating at 95°C for 1 min and subsequent analysis by polyacrylamide gel electrophoresis. All substrates used here were purchased from Sigma unless otherwise noted.

Heat-inactivation studies were performed by preincubation of the enzyme preparations at 41°C in the buffer described above which also contained 100 μg of bovine serum albumin per ml. After heat treatment, the samples were placed on ice, the pertinent substrates and MgATP were added, and the samples were incubated at 22°C for 15 min and analyzed as described above. Under these conditions, incorporation of ^{32}P is linear for at least 30 min.

Polyacrylamide Gel Analyses. Samples were analyzed by electrophoresis through a discontinuous slab gel system (10% acrylamide, 0.26% bisacrylamide) as described (5). Prior to electrophoresis samples were heated at 95°C for 1 min in electrophoresis sample buffer (70 mM Tris·HCl, pH 6.8/11% glycerol/3% NaDodSO$_4$/0.01% bromophenol blue/5% 2-mercaptoethanol). Gels were stained in 0.1% Coomassie blue/50% trichloroacetic acid, destained in 10% acetic acid/5% methanol, and dried onto Whatman 3MM paper. ^{32}P-Labeled proteins were located by autoradiography with the aid of DuPont Lightning Plus intensifying screens. Fluorography (15) was carried out to enhance detection of ^{35}S-labeled proteins.

Pertinent bands were cut from the gels, and radioactivity was quantified by liquid scintillation spectrometry.

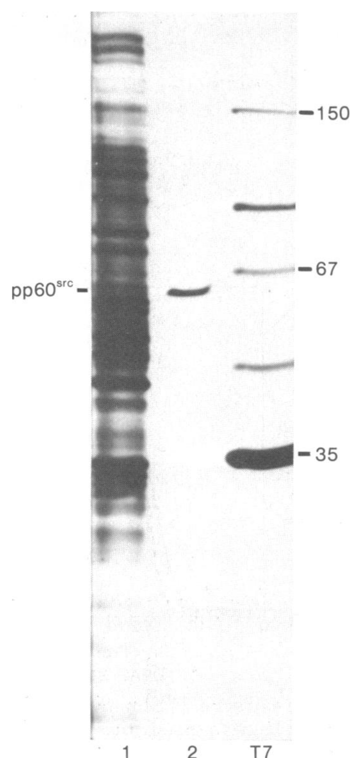

FIG. 2. Purification of pp60^{src} by immunoaffinity chromatography. An immunoaffinity matrix was used for the purification of pp60^{src} from SR-ASV-transformed vole cells (clone 1-T). Cultures were radiolabeled with [^{35}S]methionine for 2 hr, and a cell extract was prepared in 0.1 M NaCl/10 mM Tris·HCl, pH 7.2/2 mM EDTA/1% NP40/0.5% sodium deoxycholate/100 KIU of aprotinin per ml. This extract was then applied to the immunoaffinity column and the column was washed with various buffers. Samples of the starting material applied to the column (track 1) and of the pooled KSCN eluate (track 2) were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis along with [^{35}S]methionine-labeled phage T7 virion proteins as molecular weight markers (displayed as $\times 10^{-3}$).

RESULTS

Ion Exchange and Immunoaffinity Chromatography. In order to determine more directly the significance of the IgG kinase activity associated with pp60^{src} in immunoprecipitates, the partial purification of pp60^{src} from cellular extracts was undertaken by conventional biochemical techniques. Transformed cells were labeled with [^{35}S]methionine to permit detection of biosynthetically radiolabeled pp60^{src}, and the distribution of pp60^{src} and of the IgG kinase was determined upon fractionation through a series of ion exchange columns. The results shown in Fig. 1 reveal that pp60^{src} and the immune IgG kinase coeluted from hexylamine-agarose, phosphocellulose, and DEAE-Sephacel and, as shown below, cosedimented upon glycerol gradient centrifugation. It is important to note that under these conditions no IgG kinase activity was detectable in the absence of pp60^{src}, nor did forms of pp60^{src} appear without IgG kinase activity. Direct analysis, without immunoprecipitation, of fraction 20 from the DEAE column showed only one major protein in addition to pp60^{src}. Because the quantitation of [^{35}S]methionine-labeled pp60^{src}, as assayed by immunoprecipitation, is not directly comparable to our assay for total methionine-labeled protein, it is not possible to judge the amount of the *src* gene product in the polypeptide marked p60.

The availability of anti-pp60^{src} antibody suggested that pp60^{src} could be greatly purified by immunoaffinity chromatography. Therefore, an immune IgG column was prepared and radiolabeled transformed cell extracts were applied. After extensive washing of the column to remove proteins not present in immune complexes, bound proteins were eluted with 1.5 M KSCN. Analysis of the proteins eluted from such an immunoaffinity column is shown in Fig. 2, tracks 1 and 2. The KSCN eluate was greatly enriched for pp60^{src}. However, longer exposures of this gel revealed additional polypeptides.

Characterization of pp60^{src}-Specific Protein Kinase. By using either of the partially purified pp60^{src} preparations described above, phosphorylation of commonly used protein kinase substrates was investigated. Among those tested, histones (both arginine-rich and lysine-rich), phosvitin, and protamine were not phosphorylated (data not shown) whereas casein was phosphorylated (Fig. 3, track 4). As expected, normal rabbit IgG was not phosphorylated (track 2) whereas anti-pp60^{src} IgG was phosphorylated (track 3). However, purified heavy chains from both normal (track 8) and immune rabbit IgG could be phosphorylated, which shows that a site is available but not exposed in the intact immunoglobulin molecule. The phosphorylation of casein could be completely inhibited by preincubation of the enzyme fraction with immune, but not normal, IgG, thus demonstrating the pp60^{src}-specific nature of the casein phosphorylation (tracks 5 and 6). Our interpretation of this result is that, when pp60^{src} is sequestered by antibody, it is no longer able to phosphorylate casein. A similar result can be obtained with the phosphorylation of the heavy chain, but in this case the pp60^{src}–IgG complex must first be removed with protein

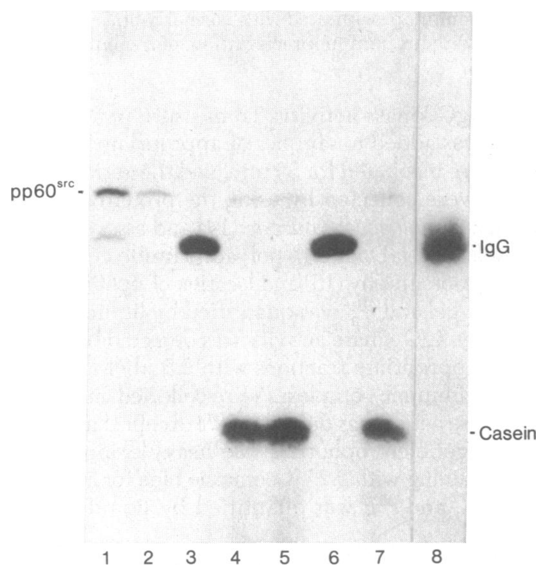

FIG. 3. Protein phosphorylation by preparations of pp60^{src}. A preparation of pp60^{src} purified through the DEAE-Sephacel step as shown in Fig. 1 was used in phosphotransferase reactions. The enzyme preparation was added to reaction mixtures containing the indicated additions and incubated at 4°C for 30 min, MgATP was added, and the samples were then incubated at 22°C for 15 min and analyzed as described. Tracks: 1, pp60^{src} preparation alone; 2, pp60^{src} with normal rabbit IgG; 3, with TBR IgG; 4, with casein; 5, with casein and normal rabbit IgG; 6, with casein and TBR IgG; 7, with casein and 10 μM cyclic AMP; 8, with normal rabbit IgG heavy chains. Casein was present at 1 mg/ml, the IgGs at 360 μg/ml, and heavy chains at 50 μg/ml. No radioactivity was detectable when any of the substrates shown here was incubated without the addition of the enzyme preparation. To prepare normal rabbit IgG heavy chains, IgG was collected from serum with protein A (*S. aureus*), eluted in sample buffer, and fractionated on a NaDodSO$_4$/polyacrylamide gel. The IgG heavy chain was located by use of ^{32}P-labeled markers, eluted (without staining) into NH_4HCO_3, pH 8.5/0.1% NaDodSO$_4$, precipitated with 5 vol of ethanol, dissolved in 1 M acetic acid, and dialyzed against H_2O.

A-bearing *S. aureus* (data not shown). Furthermore, as shown in track 7, the addition of cyclic AMP to the reaction does not stimulate the phosphorylation of casein. It should be noted that $pp60^{src}$ itself becomes phosphorylated in this reaction mixture (track 1). This apparent autophosphorylation activity will be the subject of a separate communication.

The sedimentation rate of the $pp60^{src}$-specific protein kinase activity eluted from the DEAE-Sephacel column (Fig. 1) was determined (Fig. 4 *Left*). The IgG and casein kinase activities cosedimented in a glycerol gradient at approximately the position expected for the monomer size of $pp60^{src}$. By the *S. aureus*-bound IgG kinase assay, recovery from such a gradient was about 70%, showing that very little $pp60^{src}$ could be associated with itself or with other proteins into higher molecular weight forms. Furthermore, all casein kinase activity could be inhibited by preincubation, as described above, with immune IgG. The fractions that contained IgG and casein kinase activities also contained radiolabeled $pp60^{src}$ as determined by immunoprecipitation (Fig. 4 *Right*).

Thermolability of $pp60^{src}$-Protein Kinase from Temperature-Sensitive Transformation Mutant-Infected Cells. Because trace amounts of a cellular protein kinase, undetectable by our methods of analysis, could remain associated with $pp60^{src}$ purified to a much greater degree than described here, the origin of the enzymatic activity was investigated by using mutants temperature-sensitive in the transformation function. Extracts of NY68-transformed chicken cells grown at 35°C and of the wild-type parent SR-ASV subgroup A-transformed cells also grown at 35°C were fractionated in parallel by ion exchange chromatography through the DEAE-Sephacel step where the antibody-inhibited casein kinase activity was evident. However, recovery of mutant enzymatic activity was only about 20% that of wild-type under these conditions.

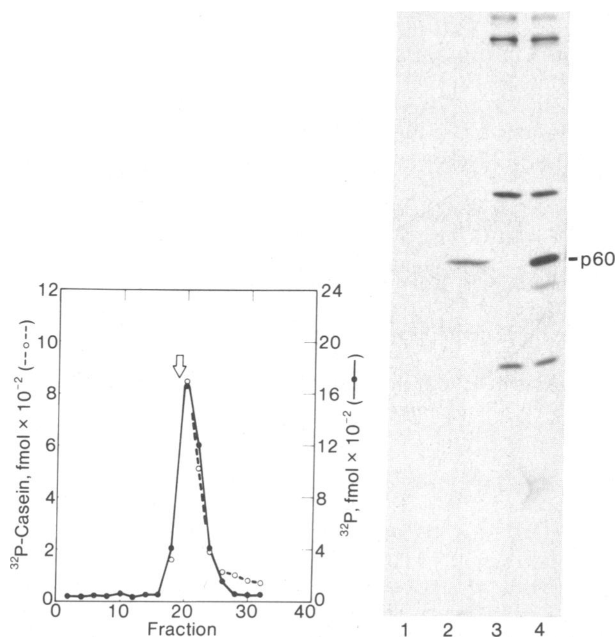

FIG. 4. Sedimentation of kinase activity and of $pp60^{src}$. (*Left*) The fractions from the DEAE-Sephacel column containing IgG kinase activity were pooled, and a 350-μl sample was layered onto a 30–10% glycerol gradient containing 20 mM potassium phosphate/150 mM KCl/1 mM EDTA/1 mM 2-mercaptoethanol/0.05% NP40. Hemoglobin (M_r, 66,000; 0.5 A_{403} unit) was added to the sample as a sedimentation rate marker (arrow). Centrifugation was at 49,000 rpm at 3°C for 17 hr in a SW 50.1 rotor. Fractions (0.16 ml) were collected and 20-μl samples of the indicated fractions were analyzed for IgG kinase (— ● —) and casein kinase (-- ○ --) activities for 30 min at 22°C. Sedimentation was from right to left. (*Right*) Fractions with kinase activity were pooled, and 500 μl was immunoprecipitated with normal rabbit (track 1) and TBR (track 2) serum. Tracks 3 and 4 show samples of the starting lysate immunoprecipitated with normal rabbit and TBR serum, respectively.

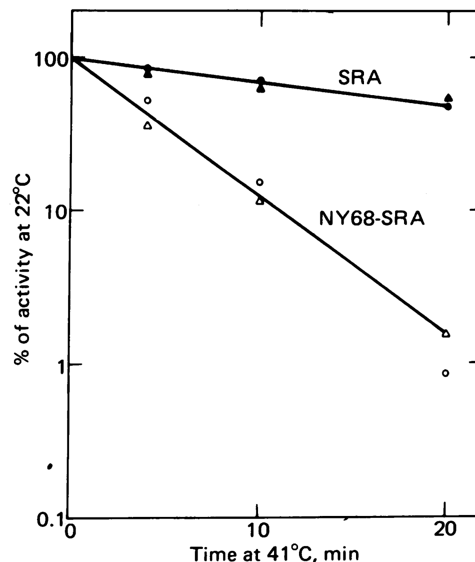

FIG. 5. Thermolability of $pp60^{src}$ protein kinase. $pp60^{src}$ was prepared from both NY68- and SR-ASV-transformed chicken embryo fibroblasts through the DEAE-Sephacel step (Fig. 1). These preparations were heat-inactivated at 41°C and then phosphotransferase reactions were carried out with casein (1 mg/ml) or TBR IgG (100 μg/ml) as substrates. After polyacrylamide gel electrophoresis, the phosphorylated proteins were located by staining and autoradiography, the bands were excised, and radioactivity was determined by liquid scintillation spectrometry. Percentage activity was calculated from the amount of radioactivity in the bands corrected for radioactivity present when substrate had been incubated in the reaction mixture without enzyme. △, ▲, IgG; ○, ●, casein.

In order to test the *in vitro* thermolability of the enzymatic activity, samples were preincubated at 41°C, the nonpermissive temperature for transformation, for the times shown in Fig. 5, prior to determination of protein kinase activity on immune IgG and casein. The latter substrate served as a more conventional soluble substrate for protein kinase activities than immune IgG. Wild-type protein kinase activity was considerably more stable, with a half-life of 20 min, than was that prepared from NY68-transformed cells, which had a half-life of about 3 min. Furthermore, the phosphorylation of both substrates showed similar thermolabilities.

DISCUSSION

In this communication we describe additional features of the ASV *src* gene product with regard to its associated protein kinase activity. The *src* gene product, $pp60^{src}$, and its associated immune IgG kinase activity have been partially purified by two independent approaches—ion exchange chromatography and immunoaffinity chromatography—yielding preparations that are capable of phosphorylating $pp60^{src}$ itself, casein, anti-$pp60^{src}$ immunoglobulin, and purified heavy chains of rabbit IgG. All of these activities are apparently independent of cyclic AMP and are specifically inhibited by rabbit anti-*src* IgG.

Because protein kinase assays are very sensitive, biochemical evidence alone does not exclude the possibility that even a very highly purified preparation could be contaminated with trace amounts of a normal cellular enzyme. Therefore, to demonstrate more directly the origin of the protein kinase activity, $pp60^{src}$ was purified from chicken cells transformed with a *src* gene temperature-sensitive mutant of ASV (NY68) and from

cells transformed by its wild-type parent. Comparison of the thermostability of the protein kinase activities on casein and immune IgG revealed that the wild-type phosphotransferase activity was nearly 7-fold more stable than that of the mutant. We think that it is compatible with these data to conclude that the ASV *src* gene encodes a protein kinase.

Other considerations make it unlikely that the enzymatic activity is due to an associated protein and not to $pp60^{src}$. The IgG kinase activity and $pp60^{src}$ coelute from three different types of ion exchange columns. They also cosediment upon glycerol gradient sedimentation in a manner consistent with a native molecular weight of about 60,000 which corresponds to the apparent molecular weight of $pp60^{src}$ judged by analysis on denaturating gels. These results suggest that $pp60^{src}$ cannot be associated with a very large protein because if it were its sedimentation rate would be altered; however, the resolution of glycerol gradients is such that smaller proteins could still be associated and not reveal their presence. It is worth noting that this sedimentation behavior is exhibited by $pp60^{src}$-specific protein kinase activity at any point in the purification scheme. This result is also relevant to the cyclic AMP-independent nature of the phosphotransferase reactions observed because it suggests that $pp60^{src}$ is not associated, under these conditions, with a protein that could be regarded as a regulatory subunit.

A major issue raised by the ion exchange chromatography results pertains to the fact that $pp60^{src}$ is a phosphoprotein (6, 16), and it might be anticipated that more than one form could be resolved by the techniques used here; however, this was not the case. It is of considerable interest to investigate this issue because the precedent set in studies with other phosphoenzymes suggests that their state of phosphorylation influences their enzymatic activity (17). The results obtained here may be explained if we have isolated a form of $pp60^{src}$ that is either nearly completely phosphorylated or dephosphorylated or if the conditions selected for fractionation were unable to resolve different forms of $pp60^{src}$. Direct analysis of ^{32}P-labeled $pp60^{src}$ should resolve this question.

Despite the conclusion that $pp60^{src}$ is a cyclic nucleotide-independent protein kinase, the possibility exists that it has additional functions not revealed by the studies carried out to date. Consequently, additional investigation into other potential functions will be necessary. Still, the capacity of protein kinases to influence the function of other proteins via phosphorylation is well recognized and this activity alone may be responsible for the multiplicity of phenotypic alterations found in the transformed cell (1). It is obvious that the phosphorylation of casein and the heavy chain of rabbit IgG studied here do not reflect physiologically significant substrates. Some preliminary results regarding several cellular proteins that serve as substrates for $pp60^{src}$ protein kinase have recently been presented (18).

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