The Purified Product of the Transforming Gene of Avian Sarcoma Virus Phosphorylates Tyrosine*

(Received for publication, May 28, 1980)

Arthur D. Levinson‡, Hermann Oppermann§, Harold E. Varmus, and J. Michael Bishop
From the Department of Microbiology, University of California, San Francisco, California 94143

The product of the avian sarcoma virus transforming gene (src) is a phosphoprotein of 60,000 daltons (pp60\textsuperscript{src}) which is responsible for the oncogenic potential of the virus. Recent findings indicate that this protein possesses an affiliated protein kinase activity. We have determined by hydrodynamic measurements and gel filtration that this kinase activity tracks with a highly asymmetric molecule of 60,000 daltons, strengthening the idea that pp60\textsuperscript{src} alone (as opposed to a complex) possesses the enzymatic activity.

To more fully characterize the properties of this kinase activity, we undertook its purification by two independent methods. In each case, a protein related to pp60\textsuperscript{src} was extensively purified from contaminating cellular proteins. The yields from one of the procedures were sufficient to induce high titer monospecific antibodies against pp60\textsuperscript{src} in mice. We have shown that purified pp60\textsuperscript{src} is able to phosphorylate several protein substrates other than IgG. The conclusion that pp60\textsuperscript{src} possesses the responsible enzymatic activity was strengthened by demonstrating that a temperature-sensitive conditional mutation in src affects the thermal stability of the purified protein.

It has recently been shown that the protein kinase activity affiliated with pp60\textsuperscript{src} phosphorylates tyrosine residues on IgG. We have examined the target specificity of the purified protein on several substrates other than IgG, and show that in every case, the phosphorylation occurs exclusively at a tyrosine residue; it therefore appears that tyrosine phosphorylation is not an artifact of phosphorylation in the immunoprecipitate, but instead represents the general substrate specificity of pp60\textsuperscript{src}.

Genetic evidence indicates that the oncogenic potential of avian sarcoma virus resides in a single viral gene termed src (Vogt, 1977; Hanafusa, 1977). The product of this gene is a 60,000-dalton phosphoprotein (Brugge and Erikson, 1977; Purchase et al., 1978; Levinson et al., 1978; Collett et al., 1979) which is located in the plasma membrane of ASV\textsuperscript{+} cells (Willingham et al., 1979; Krueger et al., 1980; Courtneidge et al., 1980). A closely related protein, pp60\textsuperscript{proto-src}, occurs in uninfected cells from a wide variety of vertebrates (Collett et al., 1978; Oppermann et al., 1979; Karess et al., 1979; Collett et al., 1979) and is presumed to be encoded by a cellular gene (src or proto-src).

Recent work has prompted the conclusion that pp60\textsuperscript{src} may function as a protein kinase: immunoprecipitates prepared with extracts of ASV-transformed cells and anti-pp60\textsuperscript{src} serum catalyzed the transfer of labeled phosphate from \[^{32}P\]ATP to the heavy chain of IgG (Collett and Erikson, 1978; Levinson et al., 1979). Chemical analysis of the IgG phosphorylated in the immune complex kinase reaction has shown the site of phosphorylation to be a tyrosine residue (Hunter and Setton, 1980), an unexpected result in view of the apparently low incidence of such a modification (Taborsky, 1974; Uy and Wold, 1977). Moreover, cells transformed by ASV contain 7-fold more phosphotyrosine than do their untransformed counterparts (Hunter and Setton, 1980). As a result of these observations, it was suggested that pp60\textsuperscript{src} may function in vivo as a phosphotyrosine kinase (Hunter and Setton, 1980). However, since phosphorylation of IgG in the I.C. assay is an in vitro reaction occurring under unusual conditions, we cannot be certain that the newly described phosphorylation of tyrosine accurately reflects the activity of pp60\textsuperscript{src} in infected cells. Indeed, because the I.C. kinase displays properties unlike those of known protein kinases (Richert et al., 1979), it has been suggested that the observed activity may not reflect the action of an authentic protein kinase, but rather may represent an in vitro anomaly which obscures a more fundamental function (Richert et al., 1979).

Furthermore, while the above experiments clearly demonstrate a relationship between I.C. kinase and the presence of pp60\textsuperscript{src} in the immunoprecipitate, it has not been conclusively established that the activity is an inherent property of the pp60\textsuperscript{src} molecule itself, as opposed perhaps to such an activity binding tightly and specifically to pp60\textsuperscript{src}.

In an attempt to address these issues, we determined by hydrodynamic measurements and column chromatography that the kinase is associated with a highly asymmetric molecule of 60,000 daltons. Furthermore, we undertook the purification of pp60\textsuperscript{src} by two separate and independent methods. In each case, the protein was extensively purified. It could be demonstrated that the I.C. kinase activity copurified with a protein related to pp60\textsuperscript{src}, a result also reported recently by Erikson et al. (1979). The purified protein could phosphorylate several exogenously added substrates while in solution, and in each instance, phosphorylation occurred exclusively at tyrosine residues.

**Experimental Procedures**

**General Procedures**—In two previous publications (Levinson et al., 1978; Oppermann et al., 1979), we have described our procedures for the propagation and isotopic labeling of cultured cells, the preparation of antisera from rabbits bearing tumors induced by the...
Characterization of the ASV Transforming Gene Product

Schmidt-Kupoin strain of ASV (TBR serum), the immunoprecipitation of virus-specific proteins, the assay of pp60"-associated immune complex kinase activity, and the fractionation of proteins by SDS-polyacrylamide gel electrophoresis. T2 NRK cells (rat kidney fibroblasts transformed by SR-AV) were kindly provided by L. Turek (National Cancer Institute). NRK cells transformed by SR-ALnY68 (National Cancer Institute, 1971) and pBR322 (Pharmacia) were kindly provided by S. Kawai (Institute of Medical Science, University of Tokyo). All reagents were from Sigma unless otherwise indicated.

Determination of Sedimentation Coefficients—2 × 106 T2 NRK cells were lysed in 0.4 ml of Buffer A (0.5 M NaCl, 10 mM EDTA, 2 mM diithiothreitol (Bio-Rad), 1% NP40 (Particle Data Laboratories), 20% ethylene glycol, 50 mM Tris, pH 8.1) and centrifuged for 30 min at 15,000 × g to remove insoluble material. Sedimentation markers internally added to the clarified lysate included bovine IgG (7.0 S), bovine serum albumin (4.3 S), ovalbumin (3.6 S), and cytochrome c (1.9 S). The lysate was applied to 15% to 20% linear sucrose gradient in Buffer B in an SW41 rotor at -2°C. Twenty-one fractions were collected and analyzed for I.C. kinase activity. Marker proteins were visualized by SDS-polyacrylamide gel electrophoresis of samples from each fraction.

Determination of Stokes Radius—Stokes radius was determined by gel filtration using Sephadex G-150 superfine (Pharmacia). A clarified lysate of T2 NRK cells was prepared in the same way as for determination of sedimentation coefficients. Standards used included: catalase (62 kDa), bovine serum albumin (68 kDa), ovalbumin (28 kDa), and cytochrome c (12 kDa) (Arrighi et al., 1977). The extract was applied to and eluted from a packed column (1 × 60 cm) of G-150 superfine Sephadex equilibrated in Buffer B. Thirty-five fractions were collected and alternate fractions assayed for I.C. kinase activity and for markers by SDS-polyacrylamide gel electrophoresis. The elution of catalase was monitored by direct enzyme assay using hydrogen peroxide.

Purification of I.C. Kinase Activity by Ion Exchange Chromatography—T2 NRK cells (-1 × 107 from 50 roller bottles were collected, washed with phosphate-buffered saline (0.15 M KCl, 10 mM NaPO4, pH 7.2), and frozen at -70°C until needed. Extracts were prepared by lysing the packed cells in sufficient Buffer C (0.25 M NaCl, 10 mM EDTA, 0.1% hexamethylphosphoramide, 0.1% diithiothreitol, 1% NP40, 25 mM 2-(N-morpholino)ethanesulfonic acid (Calbiochem), pH 6.2) to maintain protein at ~15 mg/ml. Insoluble material was removed by centrifugation at 20,000 × g for 1 h at 2°C. The clarified lysate was batch processed on DEAE-52 (Whatman) (equilibrated in Buffer C). Material not binding to and eluted from a hydroxylapatite (Clarkson Chemicals, Inc.) column (1 × 15 cm) was adjusted to 10% ethylene glycol and loaded onto a hydroxyapatite (Clarkson Chemicals, Inc.) column (2 × 20 cm) pre-equilibrated with Buffer C. Following loading, the column was washed with Buffer C (190 ml) and eluted with a 0-ml linear salt gradient (0-0.5 M NaCl, 10 mM NaPO4, 25 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.2). This material was then chromatographed on a carboxymethyl cellulose 500 (Pharmacia) column (1 × 15 cm) pre-equilibrated with Buffer D. Elution was performed with a 250-ml gradient (0.04 to 0.5 M KCl) in Buffer D. Fractions containing the peak of I.C. kinase activity were pooled and dialyzed overnight against Buffer D (0.04 M KCl, 1 mM MgCl2, 10 mM NaPO4, 0.1% hexamethylphosphoramide, 0.1% diithiothreitol, 1% NP40, 25 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.2)). This material was then chromatographed on a carboxymethyl cellulose 500 (Pharmacia) column (1 × 15 cm) pre-equilibrated with Buffer D. Elution was performed with a 250-ml gradient (0.04 to 0.5 M KCl) in Buffer D. Fractions containing the peak of I.C. kinase activity were pooled and dialyzed overnight against Buffer D. This material was then chromatographed on a Bio-Rad blue agarose (Bethesda Research Laboratories) column (1 × 5 cm) pre-equilibrated with Buffer D. Elution was performed with a 100-ml linear gradient (0.04 to 0.45 M KCl) in Buffer D. All chromatographic steps were performed at 3°C. Selected fractions from all 3 columns were monitored for conductivity. Protein was monitored by the Bio-Rad protein assay (Bio-Rad, Hercules, Calif.). Proteins were eluted from appropriate fractions using iodogen as described (Levinson et al., 1978), using limiting amounts of pp60". Under these conditions, incorporation of radio-labeled phosphate into IgG is linear with respect to pp60" concentration (Richert et al., 1978). For soluble protein kinase assays with purified pp60" substrates (usually 1% pp60"), we incubated in 0.1 ml of reaction mixture (0.1 M KCl, 10 mM MgCl2, 1% ATP, 20 mM buffer [piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.8]) containing purified pp60" and 1 × 105 cpm of [γ-32P]ATP (40 Ci/mmol) (New England Nuclear). The reaction was typically terminated by dilution into SDS sample buffer (Laemml, 1970) and then heated to 90°C for 10 min prior to analysis by SDS-gel electrophoresis. Tubulin (chicken), kindly provided by D. Cleveland, was heated before use to 80°C for 5 min to eliminate endogenous protein kinase activity.

Identification of Phosphorylated Proteins—Proteins to be analyzed were typically obtained from SDS-gels and visualized as described (Beeman and Hunter, 1978). Amino acids were separated by thin layer chromatography at pH 3.5 (Hunter and Sefton, 1980). Phosphoserine and phosphothreonine (Sigma) were run as internal standards and visualized by ninhydrin staining. We hydrolyzed "P-labeled IgG of TBR serum phosphorylated with pp60" in order to obtain phosphothreonine as an additional standard (Hunter and Sefton, 1980).

RESULTS

Properties of the I.C. Kinase Activity—To assess the size and shape of the protein responsible for I.C. phosphorylation, we subjected clarified lysates of ASV-infected rat cells to rate-zonal centrifugation and gel filtration. Fig. 1A illustrates that the activity which phosphorylates IgG from tumor-bearing rabbit serums on a sucrose gradient as a sharp symmetrical peak with a sedimentation coefficient of 4.0 S. By gel filtration in Sephadex G-150, we determined that the I.C. kinase activity has a Stokes radius of 37 A (Fig. 1B). Using the sedimentation coefficient and the Stokes radius, we computed the molecular

Fig. 1. Determination of the sedimentation coefficient and Stokes radius of the I.C. kinase activity. Clarified lysates were subjected to rate-zonal centrifugation and gel filtration. I.C. kinase activity was monitored as described under "Experimental Procedures." A. Rate-zonal centrifugation. Lysate was layered on a 0 to 20% sucrose gradient and centrifuged. IgG (7.0 S) and catalase (5.4 S) were used as internal standards. B. Gel filtration on Sephadex G-150 superfine. Lysate was applied to a column (30 manipulations involving gel filtration) and eluted with 0.7 cm) of Sephadex. Catalase (Cat), bovine serum albumin, ovalbumin, and cytochrome c c were included as internal standards. Fractions were collected and assayed for I.C. kinase activity.

11974
weight of the protein to be 62,000 (Siegal and Monty, 1966). The frictional coefficient was calculated similarly ($f_f / f_0 = 1.40$), indicating the protein to be highly asymmetric. Because the calculated molecular weight of the I.C. kinase (62,000) agrees well with the molecular weight of pp60$^{src}$ (60,000 as determined by independent methods), we attribute the I.C. kinase activity to pp60$^{src}$ alone, rather than to a complex between pp60$^{src}$ and a cellular enzyme. In addition, it is clear that the enzymatically active pp60$^{src}$ behaves largely as a monomer in solution, in agreement with the data obtained by Maness et al. (1979).

**Purification of the I.C. Kinase Activity by Ion Exchange Chromatography**—Our purpose in extensively purifying the I.C. kinase activity was 2-fold. We first sought to characterize the protein(s) which copurified with the kinase. Second, we required a sufficient supply of purified material for more thorough in vitro analysis of this enzymatic activity.

We chose as starting material a continuous line of ASV-transformed T$_2$ NRK cells. Extracts were prepared from 50 g of these cells. Since the I.C. kinase assay is the most sensitive and simplest method of detection of pp60$^{src}$ (Levinson et al., 1978), the purification was monitored with this procedure. The extract was fractionated according to the scheme outlined under "Experimental Procedures": the results of sequential chromatography on hydroxylapatite, carboxymethyl cellulose, and Cibacron blue agarose are shown in Fig. 2. Column chromatography fractions were analyzed for total protein and I.C. kinase activity. We succeeded in purifying the I.C. kinase activity ~650-fold (this calculation was based on the increase of specific activity of the enzyme during purification and could not allow for any presently unrecognized activation or inactivation that might have occurred).

Fractions from the Cibacron blue agarose column were iodinated to visualize the proteins by SDS-polyacrylamide gel electrophoresis. Fig. 3A illustrates that while a heterogeneous collection of proteins was applied to the Cibacron blue agarose column, a prominent protein of 52,000 daltons (p52) was specifically bound. To determine if this protein bore any relationship to pp60$^{src}$, the bound fraction was immunoprecipitated with control serum, serum directed against ASV structural proteins, and serum from two rabbits bearing ASV-induced tumors (TBR serum). Fig. 3B demonstrates that at least a portion of $^{[35S]}$-labeled p52 is immunoprecipitated by TBR serum (Lanes c and d), but not by either normal or anti-ASV serum (Lanes a and b).

Because of the specific reactivity of p52 with anti-pp60$^{src}$ sera, this 52,000-dalton protein seemed likely to represent a proteolytic digestion product of pp60$^{src}$. To test this hypothesis, 6 BALB/c mice were immunized with the pooled fractions containing p52 from the Cibacron blue agarose column (see "Experimental Procedures"). Preimmune and immune sera were then tested for their ability to react with pp60$^{src}$. To optimize the detection of antibodies directed against contaminating proteins, we tested the antiserum with labeled extracts prepared from the same cell line as was used for the purification of the I.C. kinase activity. Serum from mice immunized with the purified protein was capable of specifically reacting with a protein of 60,000 daltons (Fig. 4, Lanes c, d, and e) that co-migrated with pp60$^{src}$ immunoprecipitated with conventionally prepared TBR serum (Fig. 4, Lane g). In addition, the titer of the mouse sera to this 60,000-dalton protein increased dramatically following multiple injections of the purified protein (Fig. 4, Lanes a to c).

To establish unambiguously the nature of the protein precipitated by the mouse serum, we compared its peptide map with that of pp60$^{src}$ by limited proteolysis using Staphylococcus aureus V8 protease (Cleveland et al., 1978). Fig. 5 illustrates that the 60,000-dalton protein immunoprecipitated by the mouse sera is identical with pp60$^{src}$. Moreover, the V8 protease map of total p52 was indistinguishable from that of the immunoprecipitated material (data not shown). We are thus confident that at least a major portion of p52 is indeed related to pp60$^{src}$, generated most likely from the latter by proteolysis. Because p52 co-purifies with I.C. kinase activity through fractionations on 4 columns, it appears that the kinase activity resides on p52$^{src}$.

**Purification of pp60$^{src}$ by Affinity Chromatography**—We have repeatedly been unable to achieve the large scale purification of intact pp60$^{src}$ by conventional methods, even when any of a wide variety of protease inhibitors were included throughout the manipulations. While the purification of p52$^{src}$ addresses several fundamental issues (see "Discussion"), the in vitro properties of p52$^{src}$ may not faithfully reflect those of pp60$^{src}$. We therefore sought an alternate method of purification which would minimize proteolytic degradation of pp60$^{src}$.

We attempted this purification of pp60$^{src}$ by immunoaffinity
Characterization of the ASV Transforming Gene Product

A labeled protein could be immunoprecipitated by TBR, but not by normal serum (Fig. 6, Lanes e and f). We thus conclude that immunoaffinity chromatography provides a one-step procedure resulting in extensive purification of pp60<sup>src</sup>. Presumably because of the rapidity of this method, little degradation of pp60<sup>src</sup> to pp52<sup>src</sup> occurs.

**Phosphoprotein Kinase Activity of Purified pp60<sup>src</sup>**—To determine whether purified pp60<sup>src</sup> can function as a protein kinase in a soluble assay system, the kinase activity of affinity-purified pp60<sup>src</sup>- was measured using the method described above.

**SDS-gel electrophoresis of protein fractionated by ion exchange chromatography.** A, samples from the Cibacron blue agarose column were iodinated as described under “Experimental Procedures” and analyzed by SDS-polyacrylamide gels. Input, proteins present in the material applied to column; unbound, proteins not retained by the column resin (fraction 10); bound, proteins binding to column resin and eluted with KCl (fraction 28). B, proteins retained by the column (fraction 28) were immunoprecipitated with normal rabbit serum (a), serum directed against ASV-structural proteins (b), or TBR serum from two different rabbits (c and d).

**Immunoprecipitation of pp60<sup>src</sup>** by serum from mice immunized with p52. Clarified lysates were prepared from ASV-transformed rat cells labeled with [35S]methionine as described under “Experimental Procedures.” Samples were then immunoprecipitated with preimmune serum from mouse 5 (a), serum from mouse 5 obtained after first immunization (b), serum from mouse 5 after second immunization (c), serum from mouse 4 obtained after second immunization (d), serum from mouse 6 obtained after second immunization (e), normal rabbit serum (f), and TBR serum (g). Immunoprecipitated proteins were then visualized by SDS-polyacrylamide gel electrophoresis and autoradiography.

**Purification of pp60<sup>src</sup> by immunoaffinity chromatography.** Lysates of T<sub>a</sub> NKRK cells labeled with either [32P] or [35S]-methionine (Lane c) or [32P] (lane d) were passed over the column, a phosphoprotein of 60,000 daltons was retained which could be eluted with 0.8 M NaSCN. When an extract labeled with [35S]methionine was passed over a column containing normal IgG coupled to Sepharose, no such protein bound to the resin (Fig. 6, Lane b). The affinity purified [32P]-labeled proteins binding to normal IgG-Sepharose, d, [32P]-labeled proteins binding to TBR IgG-Sepharose, e, proteins immunoprecipitated from sample d with normal rabbit serum; f, proteins immunoprecipitated from sample d with TBR serum.
purified pp60<sup>src</sup> was tested with a variety of protein substrates. Phosphorylation of TBR IgG was observed, but not normal IgG (Fig. 7A). In contrast to the immunoprecipitated activity observed with crude extracts (Collett and Erikson, 1978), however, the purified enzyme was now able to phosphorylate exogenously added substrates, presumably because pp60<sup>src</sup> was not immobilized by antibody. Of 14 proteins tested, we found only 3 that served as substrates for this kinase activity. The most active acceptor among the proteins tested was tubulin, a cytoplasmic protein which is the major structural element of microtubules. Fig. 7A illustrates that while both the α and β subunits of tubulin served as substrates, the β subunit was preferred. Also effective, although less so, was casein. Proteins which did not detectably serve as substrates included bovine serum albumin, actin, tropomyosin, myosin, cytochrome c, ovalbumin, phosvitin, and any of the histones (data not shown).

To determine whether phosphorylation of substrate proceeded with the kinetics expected of a conventional enzyme-substrate reaction, we determined the time course of phosphorylation of tubulin under standard conditions using purified pp60<sup>src</sup>. In contrast to the virtually instantaneous reaction observed during I.C. phosphorylation (Levinson et al., 1978; Rubsam et al., 1979), phosphorylation of tubulin proceeded in a time-dependent fashion for periods up to 1 h (Fig. 7B). Analysis of this reaction has permitted determination of kinetic parameters: a $K_m$ for ATP of 12 μM and a $V_{max}$ of 40 nmol of tubulin phosphorylated/min/mg of pp60<sup>src</sup> were observed. The $K_m$ approximates a previous value obtained with the I.C. kinase assay (Rubsam et al., 1979). By contrast, the $V_{max}$ is appreciably lower than might be expected from the properties of other, better characterized protein kinases (Kemp et al., 1977; Bylund and Krebs, 1975).

The phosphotransfer activity in the I.C. reaction can use both ribo- and deoxyribonucleoside triphosphates as donors of phosphate (Richert et al., 1979). Our purified enzyme displayed the same unusual property, transferring phosphate from dATP with an efficiency comparable to that found with ATP (data not shown). The activity of the purified protein was unaffected by cAMP, in agreement with the report of Erikson et al. (1979).

To more directly implicate pp60<sup>src</sup> in the phosphorylation of soluble substrates, immunoaffinity purified pp60<sup>src</sup> was prepared from clones of NRK cells transformed with either wild type virus (SR-A) or tsNY68 SR-A virus. The latter contains a mutation in src, and thus appears that phosphorylation at tyrosine residues is not an artifact of the I.C. kinase assay but rather is representative of the properties of the viral src gene product. As mentioned above, pp60<sup>src</sup> is itself phosphorylated at tyrosine and serine residues in vivo (Hunter and Sefton, 1980). It has therefore been proposed that pp60<sup>src</sup> may phosphorylate itself unencumbered by antibody immobilization. To test this, we acid-hydrolyzed proteins that had been phosphorylated by purified pp60<sup>src</sup> and then fractionated the resulting free amino acids by thin layer chromatography (Hunter and Sefton, 1980). Using IgG labeled in the I.C. kinase reaction as the source of phosphotyrosine for reference (Fig. 9, Lane b) (Hunter and Sefton, 1980), we found that purified pp60<sup>src</sup> phosphorylated TBR IgG exclusively at tyrosine residues (Lane c). Lanes d to f in Fig. 9 illustrate that all other exogenously added substrates of pp60<sup>src</sup> which were tested also become phosphorylated at tyrosine residues. In addition, we confirmed the recent report that pp60<sup>src</sup> labeled in vivo with <sup>32</sup>P contains both phosphoserine and phosphotyrosine residues (Lane a) (Hunter and Sefton, 1980). Pr76, the polyprotein precursor of ASV structural proteins, apparently is phosphorylated in vivo only at serine residues (Lane g). It thus appears that phosphorylation at tyrosine residues is not an artifact of the I.C. kinase assay but rather is representative of the substrate specificity displayed by pp60<sup>src</sup> in vitro.

As mentioned above, pp60<sup>src</sup> is itself phosphorylated at tyrosine and serine residues in vivo (Hunter and Sefton, 1980); moreover, mutations in src that affect its kinase activity also affect phosphorylation of the tyrosine residue (Collett et al., 1979; Oppermann et al., 1979; Hunter and Sefton, 1980). It has therefore been proposed that pp60<sup>src</sup> may phosphorylate itself...
by means of either an intra- or intermolecular reaction (Erikson et al., 1979; Hunter and Sefton, 1980). Using partially purified pp60\(^{ss}\), Erikson et al. (1979) have observed phosphorylation of pp60\(^{ss}\) which they believe to be autocatalytic. We, by contrast, have failed to detect self-phosphorylation of pp60\(^{ss}\), either in the absence of exogenous substrates (Fig. 7, Lane 1) or in their presence (Lanes b, f, and h). We cannot presently account for these conflicting results.

DISCUSSION

Is the I.C. Kinase Activity an Inherent Property of pp60\(^{ss}\)?—Previous evidence has implicated pp60\(^{ss}\) in the I.C. kinase reaction (Collett and Erikson, 1978; Levinson et al., 1978: Erikson et al., 1979), but the possibility remained that pp60\(^{ss}\) tightly and specifically bound a cellular enzyme responsible for phosphorylation of IgG. If this latter possibility were correct, the active complex between pp60\(^{ss}\) and the cellular enzyme would display an aggregate molecular weight greater than 60,000. Since the size of proteins can be accurately determined from a knowledge of their sedimentation coefficient and Stokes radius (Siegel and Monty, 1966), we determined these values for the I.C. kinase activity by rate-zonal centrifugation and gel filtration. In both cases, the activity behaved as a sharply migrating species (Fig. 1), allowing accurate determination of the necessary parameters. From this knowledge, a M, = 62,000 was calculated for the I.C. kinase activity. We therefore conclude that pp60\(^{ss}\) is alone responsible for I.C. phosphorylation. This conclusion is consistent with data obtained by purification of the I.C. kinase activity.

Purification by ion exchange chromatography yields a protein of 52,000 daltons (p52) (Fig. 3). Much of p52 seems to be related to pp60\(^{ss}\) by several criteria. First, it could be immunoprecipitated by TBR sera, but not by control serum. Anti-ASV serum also fails to precipitate p52, ruling out the possibility that p52 bears any relationship to virion structural proteins. Second, the V8 protease maps of immunoprecipitated and total p52 demonstrated a chemical relationship of these proteins to pp60\(^{ss}\). Third, the injection of as little as 5 \(\mu\)g of p52 into mice resulted in the production of apparently monospecific antibodies which immunoprecipitated pp60\(^{ss}\) (Figs. 4 and 5). There is no evidence in any of the immunized mice for production of antibody against proteins unrelated to pp60\(^{ss}\) (Fig. 4). It should be emphasized that this experiment was performed using p52 purified from rat cells, serum from mice injected with p52, and \(^{35}\)S-labeled extracts prepared from the rat cells used originally for purification of p52. This experimental design maximizes the opportunity to detect antibodies against proteins unrelated to pp60\(^{ss}\) that might contaminate the purified material.

By peptide map analysis, we have recently established that p52 is derived from pp60\(^{ss}\), most likely by proteolytic removal of 8000 daltons of protein from the NH\(_2\) terminus of pp60\(^{ss}\). Since p52 can be generated by prolonged incubation of cell lysates (Courtneidge et al., 1980), it appears that the bulk of purified p52 results from proteolysis of pp60\(^{ss}\) during the course of purification.

An apparently analogous protein is usually recovered from extracts of infected cells by immunoprecipitation with TBR serum. The recoveries of p52 in this manner are erratic and often quite low. We therefore suspect that the presence of p52 in both crude and purified preparations is an artifact, although the protein might also be an intermediate in the physiological breakdown of pp60\(^{ss}\).

Purification by immunofinity chromatography yields pp60\(^{ss}\) which is active in the I.C. kinase assay (Figs. 6 and 7), a result also reported recently by Erikson et al. (1979). Presumably because purification in this fashion is much less time consuming than purification by ion exchange chromatography, preparations of pp60\(^{ss}\) can be obtained which contain little or no p52. Since pp60\(^{ss}\) purified in this manner is not significantly contaminated with cellular proteins (Fig. 6), this provides an independent demonstration of the affiliation of pp60\(^{ss}\) with I.C. kinase activity.

It should be noted that the availability of purified pp60\(^{ss}\) (or p52) in quantities sufficient for immunization allows the preparation of monospecific antibodies directed against pp60\(^{ss}\) in a variety of animals. The use of mice for the immunizations will facilitate the production of hybridoma clones producing antibody specific for pp60\(^{ss}\). Monoclonal antisera should prove to be invaluable reagents for detailed analysis of the structure and function of pp60\(^{ss}\).

Purified pp60\(^{ss}\) Phosphorylates Substrate Proteins at Tyrosine Residues—Recent evidence from Hunter and Sefton (1980) has established that the phosphorylation of TBR IgG by extracts containing pp60\(^{ss}\) occurs exclusively at tyrosine residues. This observation was surprising, since protein kinases typically phosphorylate proteins at the hydroxyl groups of serine and threonine residues (Rubin and Rosen, 1975; Krebs and Beavo, 1979). One could conclude from this result that either pp60\(^{ss}\) is a novel protein kinase, or that I.C. phosphorylation, representing a highly unusual reaction, was failing to faithfully reflect the in vivo properties of 60\(^{ss}\). We have shown that purified preparations containing pp60\(^{ss}\) are able to phosphorylate a variety of protein substrates, and that this phosphorylation invariably occurs at tyrosine residues (Figs. 7 and 9).

To more directly implicate pp60\(^{ss}\) in these reactions, pp60\(^{ss}\) was purified from cells infected with wild type ASV, and from cells infected with a strain of ASV carrying a temperature-sensitive lesion in pp60\(^{ss}\) (tsNY68 ASV). It is clear that the phosphorylation of substrate (tubulin) is much more thermodatable when pp60\(^{ss}\) is encoded by tsNY68 ASV than when it is encoded by wild type ASV. Since the only identified lesion in tsNY68 ASV is located in src, these findings strongly implicate pp60\(^{ss}\) in the kinase reaction. This conclusion, in turn, lends support to the claim that pp60\(^{ss}\) may be functioning in vivo as a phosphotyrosine kinase. It should be emphasized, however, that the possibility the protein possesses ad-
ditional enzymatic properties, or displays additional specific-
ities, has not been excluded.

We have compared published amino acid sequences of
proteins (Dayhoff, 1979) with their ability to serve as sub-
strates for pp60
\(^{c-src}\). In our experience to date, no direct rela-
tionship seems to exist between tyrosine content and acceptor
ability. In general, acidic proteins seem to serve as more
efficient substrates than basic proteins. A more thorough
analysis of various acceptors will be required to extend these
generalizations.

**Implications of in Vitro Phosphorylation by pp60
\(^{c-src}\)**—The observation that denatured tubulin serves as a relatively effi-
cient substrate for src kinase may have little or no physiolo-
gical significance. In accord with previous reports (Sloboda et
al., 1975), our tubulin contains an endogenous protein kinase
activity which must be heat-inactivated before use as sub-
strate. Since proteins are often more active as substrates for
protein kinases following their denaturation (Bylund and
Krebs, 1975), it is not clear at this time whether native tubulin
would even serve as substrate for pp60
\(^{c-src}\). For example, we
have failed to detect acceptor activity in other cytoskeletal
elements such as actin, myosin, and tropinin. Since protein
kinases are typically somewhat indiscriminant in their sub-
strate specificity in vitro, attempts to predict specific in vitro
targets from results in vitro must be regarded with caution.

The phosphorylation of exogenous substrates by pp60
\(^{c-src}\) is linear with respect to time for periods of at least 1 h (Fig. 7), as
expected for a conventional enzyme-substrate reaction. Our
results indicate that, as a kinase, pp60
\(^{c-src}\) is much less efficient at phosphor-ylyzing substrates than are other protein kinases. The
significance of this is presently uncertain; perhaps pp60
\(^{c-src}\) has an unusually strict substrate specificity.

Our initial search for kinase activity associated with pp60
\(^{c-src}\) was prompted by the hypothesis that pp60
\(^{c-src}\) was phos-
phorylating itself in a crude in vitro assay system (Levinson et
al., 1978). We have, however, consistently failed to observe any phosphorylation of pp60
\(^{c-src}\) in soluble kinase assays in-
volving the purified protein, in contrast to the results obtained
by Erikson et al. (1979). We cannot presently account for this
discrepancy.

The protein kinase activity associated with pp60
\(^{c-src}\) has greatly enhanced our ability to analyze the nature and func-
tion of the protein. We (Levinson et al., 1978) and Collett and
Erikson (1979) have suggested that phosphorylation of cellular
proteins might be responsible for neoplastic transformation by
ASV. The present report and the results of others (Erikson et
al., 1979; Hunter and Sefton, 1980) are in accord with this
proposal, but by no means constitute definitive evidence for
the hypothesis. Moreover, the number and nature of in vivo
targets of pp60
\(^{c-src}\) remain enigmatic, although recent work
indicates that a cellular protein of 36,000 daltons (pp36) may
be phosphorylated by pp60
\(^{c-src}\) (Radke and Martin, 1979).

Although pp60
\(^{c-src}\) is affiliated with the plasma membrane in
infected cells (Willingham et al., 1979; Kreuger et al., 1980;
Courtneidge et al., 1980), its targets in vivo may not be so
associated. Our findings indicate that pp60
\(^{c-src}\) is a highly elon-
gated molecule (see "Results") which is apparently anchored
to the plasma membrane through an NH
\(_2\)-terminal domain, the
remainder of the molecule being exposed to the cyto-
plasm. The exposed domain seems to represent most of p52,
which we have purified and shown to have protein kinase
activity. Thus, the otherwise unwanted breakdown of pp60
\(^{c-src}\) during large-scale purification has fortuitously provided us
with evidence that the enzymatically active domain of pp60
\(^{c-src}\) may function by affecting substrates located in the cytoplasm

\(^4\) S. Courtneidge and A. D. Levinson, unpublished observations.

rather than within the substance of the plasma membrane.

We conclude that pp60
\(^{c-src}\) has the properties expected of a
cyclic nucleotide-independent phosphotyrosine kinase, al-
though its activity on the arbitrary substrates which we have
used does not approach conventional levels for protein
kinases. The kinase activity of pp60
\(^{c-src}\) is unusual in at least two
regards: both ribo- and deoxyribonucleoside triphosphates can
serve as donors of phosphate; and the recipient of the phos-
phate is tyrosine. Phosphorylation of tyrosine has also been
attributed to proteins encoded by the transforming genes of
at least two other tumor viruses: Abelson murine leukemia
virus (Witte et al., 1980) and polyoma virus (Eckhart et
al., 1979). These findings raise the possibility that neoplastic
transformation by different viruses may follow a common
design. Rigorous characterization of the enzymatic properties
of purified pp60
\(^{c-src}\) should assist in the test of this hypothesis.

**Acknowledgments**—We thank T. Hunter for candid discussions
regarding work in progress, and L. Levintow for assistance and advice.

**Note Added in Proof**—Following submission of this manuscript for
publication, M. Collett, A. Purcho, and R. Erikson also reported that
purified pp60
\(^{c-src}\) phosphorylates proteins at tyrosine residues (1980)
*Nature* 285, 167-169.

**REFERENCES**

Beemont, K., and Hunter, T. (1978) *J. Virol.* 28, 551-566.

Brugge, J. S., and Erikson, R. L. (1977) *Nature* 269, 246-248.

Bylund, D. B., and Krebs, E. G. (1975) *J. Biol. Chem.* 250, 6355-6361.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* 252, 1102-1106.

Collett, M. S., Brugge, J. S., and Erikson, R. L. (1978) *Cell* 15, 1363-1369.

Collett, M. S., and Erikson, R. L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 2021-2024.

Collett, M. S., Erikson, E., and Erikson, R. L. (1979) *J. Virol.* 29, 770-781.

Collett, M. S., Erikson, E., Purcho, A. F., Brugge, J. S., and Erikson, R. L. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 3159-3163.

Courtneidge, S. A., Levinson, A. D., and Bishop, J. M. (1980) *Proc. Natl. Acad. Sci. U. S. A.* 77, 3783-3787.

Dayhoff, M. O. (1979) *Atlas of Protein Sequence and Structure, Vol.* 5, National Biomedical Research Foundation, Silver Spring, Mar-

Davare, S. G., and Stephenson, J. R. (1977) *J. Virol.* 23, 443-447.

Eckhart, W., Hutchinson, M. A., and Hunter, T. (1979) *Cell* 18, 925-933.

Erikson, R. L., Collett, M. S., Erikson, E., and Purcho, A. F. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 6260-6264.

Hankisson, H. (1977) in Comprehensive Virology Fraenkel-Conrat, (Fraenkel-Conrat, H., and Wagner, R. P., eds) Vol. 10, pp. 401-483, Plenum Press, New York.

Hunter, T., and Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U. S. A.* 77, 1311-1315.

Karess, R. E., Hayward, W. S., and Hanafusa, H. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 3154-3158.

Kawai, S. W., and Hanafusa, H. (1971) *Virology* 46, 470-479.

Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977) *J. Biol. Chem.* 252, 4888-4894.

Krebs, E. G., and Beavo, J. A. (1979) *Annu. Rev. Biochem.* 48, 923-960.

Krueger, J. G., Wang, E., and Goldberg, A. R. (1980) *Virology* 101, 25-40.

Laemmli, U. K. (1970) *Nature (Lond.)* 227, 680-684.

Levinson, A. D., Oppermann, H., Levinow, L., Varmus, H. E., and Bishop, J. M. (1978) *Cell* 15, 561-572.

Linblad, B., Lindstedt, G., Lindstedt, S., and Rundgren, M. (1977) *J. Biol. Chem.* 252, 5073-5084.

Maness, P. F., Engeser, H., Greenberg, M. E., O'Farrell, M., Gall, W. E., and Edelman, G. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 5028-5032.

Oppermann, H., Levinson, A. D., Varmus, H. E., Levintow, L., and Bishop, J. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 1804-1808.

Purcho, A. F., Erikson, E., Brugge, J. S., and Erikson, R. L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 1567-1571.

Purcho, A. F., Erikson, E., and Erikson, R. (1977) *Proc. Natl. Acad.*
Characterization of the ASV Transforming Gene Product

Radke, K., and Martin, G. S. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 5212–5216
Richert, N. D., Davies, P. J. A., Jay, G., and Pastan, I. H. (1979) *J. Virol.* 31, 695–706
Rubin, C. S., and Rosen, O. M. (1975) *Annu. Rev. Biochem.* 44, 831–887
Rubsamen, H., Friis, R. R., and Bauer, H. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 967–971
Siegal, I. M., and Monty, K. J. (1966) *Biochim. Biophys. Acta* 112, 346–362
Sloboda, R. D., Rudolph, S. A., Rosenbau, J. L., and Greengard, P. (1973) *Proc. Natl. Acad. Sci. U. S. A.* 72, 177–181
Taborsky, G. (1974) *Adv. Protein Chem.* 28, 1–187
Vogt, P. K. (1977) in *Comprehensive Virology* (Fraenkel-Conrat, H., and Wagner, R., eds) Vol. 10, pp. 341–455, Plenum Press, New York
Willingham, M. C., Jay, G., and Pastan, I. (1979) *Cell* 18, 125–134
Witte, O. N., Dasgupta, A., and Baltimore, D. (1980) *Nature* 283, 826–831
Uy, R., and Wold, F. (1977) *Science* 198, 890–896