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Purification and Activity of the PP60SRC Protein Kinase

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PURIFICATION AND ACTIVITY OF THE $PP60^{SRC}$ PROTEIN KINASE

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ABSTRACT

Transformation of cells by Rous Sarcoma virus results from the action of a single virally coded phosphoprotein with a molecular weight of 60,000 daltons ($pp60^{src}$). The expression of $pp60^{src}$ results in many significant changes in the cell's phenotype which include disruption of the cytoskeleton and the deregulation of cell growth.

During the last few years significant progress has been made in many laboratories towards understanding the structure and function of $pp60^{src}$ (reviewed by Erikson et al., 1980). This thesis work describes procedures that were developed for purifying and characterizing $pp60^{src}$ and some of its target proteins.

The src protein associated kinase activity was purified several thousand fold, in detergent-free solution. Analysis of the purified protein by immunoprecipitation indicated that the $pp60^{src}$ had degraded during the purification to a 52,000 dalton species. Detergents were found to be necessary to extract $pp60^{src}$ from cell membranes in an undegraded form. A second reproducible procedure was developed to purify $pp60^{src}$ as an intact protein in the presence of detergents. The purified protein migrated as a monomer with an approximate molecular weight of 60,000 daltons when analyzed on glycerol gradients or by gel filtration.

The purification schemes successfully removed phosphatases and inhibitors so that it was possible to study the kinase activity of $pp60^{src}$ in solution under controlled conditions. Both casein and TBR IgG were phosphorylated at tyrosine residues by partially purified $pp60^{src}$. The src protein did not

phosphorylate histones or phosvitin and the phosphorylation of casein was not enhanced in the presence of cAMP.

The interaction of $pp60^{src}$ with more physiological substrates was also examined. No direct interaction of $pp60^{src}$ with purified cytoskeletal components was detected but several of these proteins were phosphorylated at tyrosine residues by $pp60^{src}$ *in vitro* including tubulin, actin, vinculin, myosin, and filamin. Other proteins including the 34 kD phosphoprotein identified by Radke and Martin (1979) and cytosolic malate dehydrogenase were also phosphorylated by $pp60^{src}$.

Further biochemical characterization of the partially purified $pp52^{src}$ and $pp60^{src}$ preparations resulted in the identification of a 50 kD protein that appears to interact with the *src* protein. The 50 kD protein was found to be phosphorylated in a Ca^{2+}-calmodulin dependent manner and this phosphorylation was inhibited by the calmodulin inhibitor chlorpromazine. The Ca^{2+}-calmodulin activated phosphorylation of the 50 kD protein is not $pp60^{src}$-induced since it occurs to a significant extent in samples prepared from non-transformed chick embryo cells. Several experiments suggested that $pp52^{src}$ binds to the 50 kD protein. Ca^{2+}-calmodulin regulation was further implicated in the *in vivo* action of $pp60^{src}$. The calmodulin inhibitor chlorpromazine was observed to inhibit the $pp60^{src}$-induced deregulation of DNA synthesis. The drug had no detectable *in vivo* effect on the $pp60^{src}$-associated kinase activity.

While a variety of *in vitro* and *in vivo* experiments from different laboratories (Sefton et al., 1981a; Radke and Martin, 1979; Brugge et al., 1981) have identified several $pp60^{src}$ substrates, the role of these proteins

in cell transformation is still not understood. To begin characterization of the src targets, monoclonal antibodies were prepared against vinculin and the 34 kD protein. Four different monoclonal antibodies were raised which immunoprecipitated a 130,000 dalton phosphoprotein from both normal and transformed cells. By immunofluorescence the antibodies were found to stain chick embryo fibroblasts at sites of cell contact with the substratum. The immunoprecipitation and immunofluorescence data demonstrated that the four antibodies recognized vinculin.

One monoclonal antibody was identified which immunoprecipitated a 34 kD phosphoprotein from normal and transformed cells. A second antibody was isolated which recognized a non-phosphorylated 34 kD protein. It was determined that the 34 kD phosphoprotein was phosphorylated at a tyrosine residue in a transformation specific manner and it is probably the same protein originally identified by Radke and Martin (1979).

The tissue distribution and subcellular localization of the two 34 kD proteins was examined using the monoclonal antibodies. The 34 kD phosphoprotein was localized, by indirect immunofluorescent staining, to the plasma membrane. The antibody to the non-phosphorylated protein specifically stained the nucleoli.

The monoclonal antibodies to vinculin and the 34 kD phosphoprotein allowed the rapid purification and characterization of these substrates from normal and transformed cells. The purified target proteins and the specific antibodies should make it possible to further probe the biochemical mechanisms involved in cellular transformation by $pp60^{src}$.

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I. INTRODUCTION

The controlled growth and division of cells is necessary for the development and survival of multicellular organisms. A cell can exist in a proliferative state, during which it grows and divides; but it can also survive in a quiescent state during which its division is inhibited and its overall metabolism is slowed down. Whether a cell will be quiescent or proliferative is controlled in part by the cells extracellular milieu. Biological molecules such as hormones present in the cell's external environment can have stimulatory or inhibitory effects on growth. Other factors that influence cell growth or division are cell density and, perhaps, cell-cell contact.

The biochemical mechanisms by which extracellular growth factors stimulate a cell to divide or inhibit a cell so it becomes quiescent are not understood. Signals, generated by factors in the cell's external environment, must be transmitted across the cell membrane to the cytoplasm and eventually to the nucleus where DNA synthesis occurs. An hypothesis was proposed by Edelman et al. (1973), suggesting that interactions between the cell surface and the cytoskeleton were crucial in the propagation of signals across the plasma membrane. The state of the cytoskeleton has been correlated with the cellular growth state in a variety of cells but a causal connection has not been established.

In an effort to elucidate some of the detailed biochemical mechanisms by which the cytoskeleton might regulate cell growth I chose to study transformation of cells by Rous sarcoma virus (RSV). This sytem is attractive because a single virally coded protein (the src protein, or src gene product) has been shown to cause the disruption of the cytoskeleton

and the deregulation of cell growth. By analyzing the way this protein perturbs these cellular processes it might be possible to determine if the state of the cytoskeleton is biochemically linked to the state of cell growth.

The src protein was identified biochemically only four years ago. Since then significant progress towards understanding its mechanisms of action has been made in many laboratories. The goal of this thesis work was to purify the src protein and to at least in part identify some of its substrates. Purified src substrates could be used to study cytoskeletal alterations as they relate to cell growth under well-defined conditions.

The first section of this thesis briefly describes the genetic structure of Rous sarcoma virus and the ways in which the virus is known to affect the cell. The present understanding of the structure and function of the src protein and its potential substrates is described in detail.

The experimental sections of this thesis describe two different purification schemes which have enabled us to study activities associated with the src protein in vitro under controlled conditions. Experiments are presented which identified several in vitro src targets. One of the src protein targets identified interacts with the src protein and is regulated by the calcium binding protein calmodulin. Two other target proteins were studied using monoclonal antibodies. The preparation and characterization of the monoclonal antibodies, one specific for vinculin and one for a 34,000 dalton protein of unknown function, are described. The antibodies and purified proteins have made it possible to study the roles these proteins play in the regulation of cell growth.

II. HISTORY AND BACKGROUND

Rous Sarcoma Virus

Rous sarcoma virus was isolated in 1911 by Peyton Rous at The Rockefeller Institute and was shown by him to cause sarcomas in chickens (Rous, 1911). More recently Temin and Rubin (1958) showed that the virus can also infect and transform chick cells in tissue culture. By transforming cells in culture it has been possible to study Rous sarcoma virus infection under well-defined conditions.

RSV is a member of the class of RNA tumor viruses called retroviruses (reviewed by Bishop, 1978). These viruses are composed of a single stranded RNA genome which is a dimer, surrounded by a nuclear capsid and enclosed in an outer envelope made up of lipid and protein (reviewed by Bader, 1974). When the retrovirus infects a cell, the viral RNA is transcribed into double stranded DNA by the viral enzyme reverse transcriptase. The double stranded DNA is circularized and incorporated into the cellular genome. Transcription of the incorporated DNA into RNA and translation of the RNA into proteins results in cellular transformation and also in the production of progeny virus. For comprehensive reviews of RNA tumor viruses, see Bader (1974) and Hanafusa (1977).

The Rous sarcoma virus genome consists of four genes (Fig. 1) (Wang et al., 1976 a,b). Starting at the 5' end of the RNA, are found the genes for the capsid antigen (*gag*), the RNA dependent DNA polymerase (*pol*), the envelope glycoprotein (*env*) and the transforming protein

Fig. 1. Genetic map of Rous sarcoma virus.

(src). The c region is a noncoding region which is required for propagation of the virus. Expression of the first three genes gag, pol, and env is necessary for virus production. By isolation of both non-conditional (Goldé, 1970; Toyoshima et al., 1970; Graf et al., 1971; Vogt, 1971) and conditional (Martin, 1970; Biquard and Vigier, 1970; Kawai and Hanafusa, 1971) transformation defective mutants it has been shown that the fourth viral gene src is necessary for cellular transformation but not for virus production. The non-conditional transformation defective mutants have a deletion in the src gene and do not transform cells.

The conditional mutants have significantly aided the study of the mode of action of the src gene product. These temperature sensitive mutants transform cells at permissive (37°C) but not at nonpermissive (42°C) temperatures. Viral infection and replication proceeds equally well at both temperatures. It is possible to shift cells infected with temperature sensitive virus from the transformed to normal phenotype and vice versa by changing the temperature at which the cells are incubated. For example, the mutant virus, tsNY68-SRA RSV (Kawai and Hanafusa, 1971) has been used to study some of the biochemical changes that occur upon transformation by RSV. Cells infected with tsNY68-RSV are grown at the nonpermissive temperature so that they are phenotypically normal. When these cells are then shifted to the permissive temperature the src gene product becomes active and the sequence of temporal events that occur as a result of src expression can be studied. In this way it has been possible to distinguish between cellular changes that occur early and may be direct effects of src gene product action and those that are secondary changes occuring later in the sequence of events.

Cellular Transformation

Expression of the src gene in cells transformed by Rous sarcoma virus results in many significant changes in the cell's phenotype. For example, transformed cells will grow under conditions of nutrient deprivation or serum starvation that will not support the growth of normal cells. Transformed cells also do not exhibit the same degree of density dependent inhibition of growth as do normal cells. Significant changes also occur in cellular morphology (Fig. 2). Whereas normal fibroblasts are flattened and grow in orderly arrays, transformed cells become round or spindle shaped and pile up on top of one another in culture.

Both the changes in cell morphology and cell growth in transformed cells have been found to correlate with a dramatic change in the cellular cytoskeleton. Edelman and Yahara (1976) have shown by immunofluorescence microscopy that the microfilament and microtubule networks are disrupted when cells are transformed by RSV. Similar results have been obtained by Wang and Goldberg (1976) using immunofluorescence and electron microscopy to visualize the microfilaments.

In addition to the morphological and cytoskeletal changes, many other changes in cellular properties occur upon transformation. The levels of some cell surface components increase while others decrease in amount. A drastic decrease in the content of fibronectin (Hynes and Destree 1978; Hogg, 1974) occurs upon transformation. Fibronectin, a protein with a molecular weight of 240,000 daltons present on the cell surface, is a component of the extracellular matrix that may play a role in cell adhesion. Unkeless et al. (1973 a,b) discovered that the level

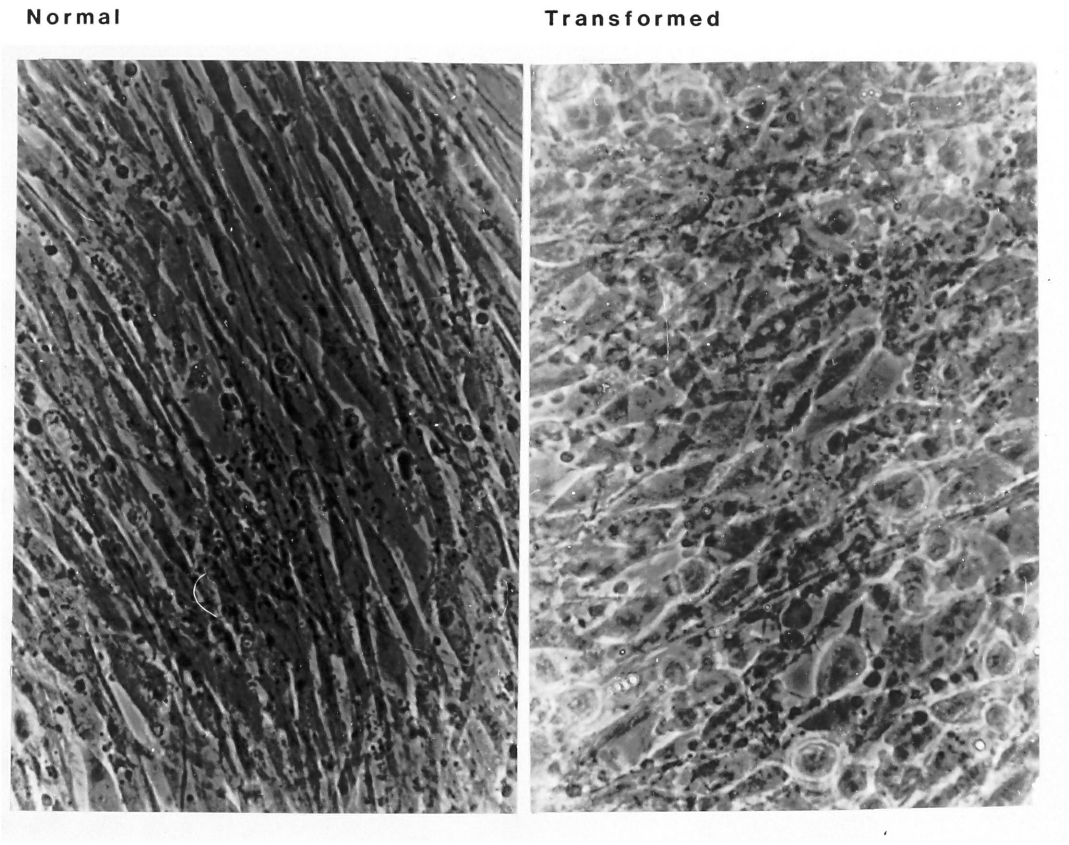


Fig. 2. Transformation of chick embryo fibroblasts with SR-RSV. (A) uninfected cells; (B) SR-RSV-infected cells, 48 h after infection.

of the protease plasminogen activator is also significantly increased when cells are transformed by RSV. In addition, novel tumor specific antigens can be detected on the surface of the transformed cells (Rohrschneider et al., 1975). Functions associated with the plasma membrane are also affected by transformation with RSV. Transformed cells exhibit an increase in the rate of sugar transport across the plasma membrane (reviewed by Hatanaka, 1974) and increased agglutinability by plant lectins (Burger and Goldberg, 1967; Aub et al., 1965). The latter property may be related to alterations in the carbohydrate portions of glycoproteins or in these proteins' location or mobility within the plasma membrane.

Compared with normal cells, RSV infected cells also have an increased rate of aerobic glycolysis, consistent with Warburg's (1931) hypothesis that increased glycolysis was an important change common to all tumor cells. Although RSV transformed cells have been shown to have increased levels of some of the glycolytic enzymes (Singh et al., 1974), other studies have determined that aerobic glycolysis is not necessarily correlated with malignancy (Steck et al., 1968; Temin, 1968).

It is clear that the changes that occur in the cell during transformation are complex (reviewed by Tooze, 1973 and Hanafusa, 1977). It was possible, using the temperature sensitive virus infected cells, in downshift experiments, to study the kinetics of cellular transformation. While cytoskeletal changes occur rapidly as a result of src expression after temperature shift from the nonpermissive to the permissive temperature, the transport and metabolic changes occur more slowly. The extensive list of RSV-induced changes and a knowledge of the kinetics

by which these events occur still did not point clearly to the key early biochemical events that are necessary and sufficient to cause transformation.

Identification of the Src Gene Product

In recent years significant progress has been made towards defining the molecular basis of cellular transformation by studying the src gene product and its activities. By 1972, studies using temperature sensitive viral mutants (Martin, 1970; Kawai and Hanafusa, 1971) had suggested that the product of the src gene was likely to be a protein. The identification and biochemical characterization of the src gene product is clearly of great interest. This area of research is one of intense activity, with many different laboratories attempting to understand the biochemical mechanisms of src action. The most direct approach would be to purify the src protein so that it would be possible to study its activity in vitro under controlled conditions. In order to do this an assay for detecting the src protein in the extracts of transformed cells was needed.

By 1978, several assays had been developed which could be used to detect the src protein. McClain, Maness and Edelman (1978) developed a cell microinjection assay for the src gene product designed to probe its effects on the cytoskeleton. They showed that extracts containing the src protein caused disruption of cytoskeletal structures when microinjected into normal cells. The disruption appeared to result from the action of the src gene product since the activity was temperature labile in extracts from cells transformed with a mutant virus temperature sensitive for transformation. The microfilament disrupting activity was inactivated by treatment with trypsin but not by treatment with RNase, indicating that

the activity was due to a protein in the extract rather than to a small molecule or to a protein synthesized from injected RNA (Stacey and Hanafusa, 1978). Disruption of the cytoskeleton was not inhibited by puromycin or cycloheximide, suggesting that ongoing protein synthesis is not required for dissolution of the microfilaments. Together, these results suggest that the changes in microfilament morphology resulted from the direct or indirect action of the src gene product itself.

Around the same time, Brugge and Erikson (1977, 1978a) developed a direct immunological assay for the src gene product using an antibody raised by injecting Rous sarcoma virus into newborn rabbits. The rabbits subsequently developed tumors at the site of injection. Serum obtained from tumor bearing rabbits (referred to as TBR serum) immunoprecipitated a phosphoprotein with a molecular weight of 60,000 daltons from transformed cells but not from normal cells. The TBR serum specifically immunoprecipitated the 60 kD protein as well as structural proteins of Rous sarcoma virus from transformed cell extracts. Absorption of the antiserum with disrupted unlabelled RSV decreased the precipitation of the viral structural proteins, but did not diminish the amount of the 60 kD protein immunoprecipitated. This suggested that the 60 kD protein was not a structural protein of Rous sarcoma virus. The 60 kD protein was later shown to be the src gene product and not a cellular protein whose synthesis is induced during cell transformation. This was accomplished by cell free translation experiments using src specific RNA (Purchio et al., 1978). The 60 kD protein synthesized in vitro by translation of the viral RNA was immunoprecipitated by TBR serum. In vitro translation experiments

in other laboratories (Beemon and Hunter, 1978; Kamine et al., 1978) corroborated the results of Purchio et al., showing that the viral RNA coded for a 60 kD protein. The 60 kD proteins synthesized by cell-free translation and the cell transformation specific antigen yielded identical tryptic maps indicating that they are the same protein (Purchio et al., 1978). The src protein is designated $pp60^{src}$ because it is a phosphoprotein with a molecular weight of 60,000 daltons.

A significant breakthrough by Collett and Erikson (1978) gave a clue to an enzymatic activity associated with the src gene product. $pp60^{src}$ in immunoprecipitates catalyzed the transfer of radioactive phosphate from [γ-^{32}P]ATP to the heavy chain of TBR specific IgG (diagrammed in Fig. 3). It was subsequently shown (Maness et al., 1980) that the site of phosphorylation on the IgG heavy chain is in the variable region, indicating that the reaction occurs in the antigen binding site. $pp60^{src}$ did not catalyse the transfer of phosphate from ATP to kinase substrates such as casein or histones added exogenously to the immunoprecipitates. No phosphotransferase activity was detected in non-immune serum immunoprecipitates from transformed cell extracts. In addition Collett and Erikson detected only very low levels of kinase activity in TBR immunoprecipitates from normal chick fibroblasts. Measuring the kinase activity found in immunoprecipitates proved to be a useful assay for the presence of the src protein. Levinson et al. (1978) have independently observed that $pp60^{src}$ in immunoprecipitates is associated with a kinase activity.

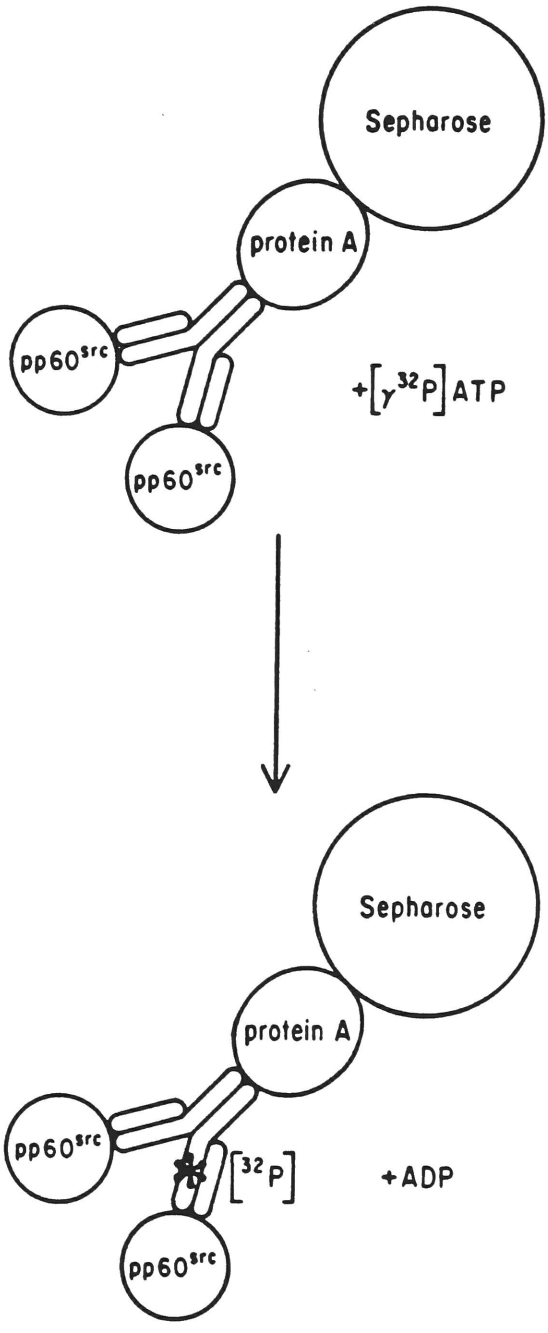


Fig. 3. Diagram of the immune complex kinase assay of pp60^{src} activity. (Collett and Erikson, 1978; Levinson et al., 1978). Cell extracts containing pp60^{src} are incubated with TBR serum followed by the addition of protein A-Sepharose which binds to the Fc region of the IgG molecule. Immune complexes are washed and incubated with [γ-^{32}P]ATP and Mg^{2+}. In this reaction radioactive phosphate is transferred from [γ-^{32}P]ATP to the heavy chain of the pp60^{src} specific antibody.

Assaying for pp60^{src} kinase activity in immunoprecipitates is more rapid and quantitative than the microinjection assay of the src gene product, and the TBR serum and kinase assay is generally used to detect pp60^{src} during fractionation and other in vitro experiments. In the last few years, largely due to the availability of the TBR antibody, significant progress has been made in many laboratories towards understanding the structure and function of pp60^{src} (for review see Erikson et al., 1980). In the first section of this thesis I will summarize what is currently known about pp60^{src} and its substrates as a result of work done in other laboratories.

A Normal Cell Protein Similar to pp60^{src}

When pp60^{src} was first identified by Brugge and Erikson (1977) in extracts of transformed chick embryo fibroblasts (CEFs) using TBR serum, no comparable protein was detected in extracts of normal uninfected cells. However, normal uninfected cells do contain a src gene, as shown by Stehelin et al. (1976) using a DNA probe complementary to viral src sequences. This work raised the possibility that the src gene is actually a normal cellular gene that has become associated with the Rous virus. In the virus the gene may be deregulated resulting in the deregulated growth of infected cells. Since the normal src sequences are transcribed into polyadenylated polyribosome associated RNA (Spector et al., 1978 a,b) it seemed likely that the protein product of these sequences would be present in normal cells.

Workers in a number of different laboratories have recently identified a normal cell protein similar in structure and function to pp60^{src}. Such a protein is present in a variety of different species. This normal

cellular protein is designated $pp60^{c\text{-}src}$ and the viral analogue is designated $pp60^{v\text{-}src}$. $pp60^{c\text{-}src}$ has been identified by immunoprecipitation of ^{32}P labelled cell extracts from frog, chicken, rat, and human cells but was not detected in Drosophila cells (Collett et al., 1978; Brugge et al., 1979; Oppermann et al., 1979; Rohrschneider et al., 1979; Sefton et al., 1980a). $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ from chick cells are similar but not identical by peptide analysis, and analyses of peptide maps of $pp60^{c\text{-}src}$ from different species indicate that the protein is highly conserved. The level of $pp60^{v\text{-}src}$ found in RSV transformed CEF is 100-fold greater than the amount of $pp60^{c\text{-}src}$ found in normal chick cells, consistent with the hypothesis that it is the deregulation of the protein's expression that causes transformation.

$pp60^{c\text{-}src}$ also has an associated kinase activity in TBR immunoprecipitates (Oppermann et al., 1979; Rohrschneider et al., 1979; Karess et al., 1979; and Collett et al., 1979a). The $pp60^{c\text{-}src}$-associated kinase activity in normal cells is 100-fold less than the amount of activity detected in RSV transformed cells. Since the enzyme concentration in normal cells is also 100-fold lower, the specific activities of $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ are probably similar. Karess et al. (1979) however have reported a significantly lower specific activity for the $pp60^{c\text{-}src}$ kinase.

Studies using recovered transforming viruses (Karess et al., 1979; Karess and Hanafusa, 1981) further support the possibility that $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ have similar functions in the cell. These viruses are transforming viruses that have been recovered from tumors arising in

chickens infected with transformation defective src gene deletion mutants of RSV (Hanafusa et al., 1977). The recovered viruses are apparently generated by recombination between viral and cellular src sequences to form a functional src gene (Wang et al., 1978). 75% of the src gene in the recovered viruses is derived from cellular sequences and the $pp60^{src}$ produced by these viruses is similar to $pp60^{c\text{-}src}$. The $pp60^{src}$ from cells infected with recovered viruses, however, has a level of associated kinase activity similar to $pp60^{v\text{-}src}$, and the expression of $pp60^{src}$ in these cells results in transformation (Karess and Hanafusa, 1981).

It has been proposed (reviewed by Erikson et al., 1980) that $pp60^{c\text{-}src}$ may function in the normal cell as a protein kinase and that overproduction of this kinase in the cell after infection with RSV is the cause of cellular transformation (Karess et al., 1979). The other possibility that has been suggested is that small differences in $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ structure may actually be significant, resulting in altered activity for $pp60^{v\text{-}src}$. Such a structural alteration in $pp60^{v\text{-}src}$ might be responsible for cellular transformation. Current evidence favors the possibility that transformation is the result of overproduction of the src kinase; however, at this time, the data is insufficient to conclude definitively which of these hypotheses is correct.

In a recent report it was shown (Shealy and Erickson, 1982) by immunoprecipitation with TBR serum that cultured human cells contain two forms of $pp60^{c\text{-}src}$; one of molecular weight 59,000 daltons, the other 60,000 daltons. The significance of the two forms has not yet been determined.

Subcellular Localization of $pp60^{src}$

The TBR serum proved to be a useful reagent for localizing $pp60^{v\text{-}src}$ in the cell. Brugge and her collaborators (1978b) and Rohrschneider (1979) localized $pp60^{src}$ to the cell cytoplasm of transformed cells by crude cell fractionation and immunofluorescence staining experiments. More detailed analysis in several laboratories has suggested that $pp60^{v\text{-}src}$ may interact with the plasma membrane. It was shown by cell fractionation and immunofluorescence microscopy (Krueger et al., 1980b) that $pp60^{v\text{-}src}$ in RSV transformed chick cells is associated with the plasma membrane. By subcellular fractionation Courtneidge et al. (1980) also localized both $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ to the inside of the plasma membrane. No $pp60^{src}$ antigenic determinants were detected on the outside of the cell either by fluorescence microscopy (Brugge et al. 1978b; Rohrschneider, 1979) or cell surface iodination (Sefton et al., 1978). Using electron microscopic immunocytochemistry Willingham et al. (1979) found that in ASV transformed rat kidney cells the src protein is concentrated on the inner surface of the plasma membrane near sites of cell-cell contact. They have also detected small amounts of $pp60^{src}$ in the cytoplasm and in the perinuclear Golgi region of the cell. Rohrschneider (1980) reported a more specific membrane localization for $pp60^{src}$. By immunofluorescence and interference-reflection microscopy he has localized $pp60^{src}$ to cell membrane adhesion plaques in RSV transformed rat kidney cells, transformed chick cells, and tsNY68-infected chick cells at the permissive temperature. At the nonpermissive temperature, when cells are phenotypically normal, $pp60^{src}$ was not detectable in adhesion plaques. Rohrschneider has proposed that, since adhesion plaques are the points of microfilament

attachment to the plasma membrane, the pp60^{src} might interact directly with both the cytoskeleton and the cell membrane.

In the rat tumor cell line RR1022 it was found that pp60^{src} is associated with intracellular membranes (Krueger et al., 1980b). In these cells pp60^{src} was localized to the nuclear envelope and juxtanuclear reticular membrane structures by immunofluorescence microscopy and subcellular fractionation. Their data suggest that pp60^{src} association with the plasma membrane may not be a requirement for transformation. Further experiments are needed to address this question rigorously.

The nature of the association of pp60^{src} with the plasma membrane has been characterized. pp60^{src} was found to be synthesized on soluble polyribosomes (Lee et al., 1980) and pulse chase experiments have shown that pp60^{src} is first synthesized in a soluble form which rapidly becomes associated with the plasma membrane (Levinson et al., 1981). In the pulse chase experiments no high molecular weight pp60^{src} precursor was detected. The data suggests that pp60^{src} is synthesized without a hydrophobic signal sequence (Blobel and Dobberstein, 1975) and that it becomes localized at the plasma membrane after synthesis. An earlier report by Kamine and Buchanan (1978), indicating that pp60^{src} is synthesized with a signal sequence that is subsequently cleaved, has not been substantiated.

Incubation of membranes from transformed cells with reagents such as EDTA or 0.5 M KCl does not release pp60^{src} from the membrane, suggesting that pp60^{src} may be an integral membrane protein (Levinson et al., 1981; Krueger et al., 1980a). Several different experiments indicate that pp60^{src} is linked to the plasma membrane via a sequence at its amino terminus. It

was observed that a 52K proteolytic fragment of pp60^{src} which has lost an 8,000 dalton fragment from its amino terminus no longer binds to membranes. The studies suggest that this amino terminal portion is involved in anchoring pp60^{src} to the membrane (Levinson et al., 1981; Krueger et al., 1980b).

Most of the data support the conclusion that pp60^{src} interacts with the inside of the plasma membrane, perhaps at the points where the cytoskeleton is linked to the membrane. It is not yet clear if pp60^{src} interacts directly with the lipid bilayer or indirectly by binding to another membrane associated protein. The possibility that pp60^{src} interacts with both the plasma membrane and the cytoskeleton is especially intriguing since it could explain the observed correlations of cellular growth control with cytoskeletal states and membrane phenomena discussed earlier.

pp60^{src} is a Tyrosine Kinase

Hunter and Sefton (1980) have found that the pp60^{src} kinase activity seen in TBR serum immunoprecipitates is novel in that it phosphorylates a tyrosine residue on the TBR IgG heavy chain. All previously described protein kinases have been found to phosphorylate either serine or threonine residues. The site of phosphorylation on the TBR IgG heavy chain, in pp60^{src} immunoprecipitates, was originally identified by Collett and Erikson (1978) as a threonine residue. This error was due to the fact that classical one dimensional phosphoamino acid analysis by electrophoresis at pH 1.9 does not distinguish phosphothreonine from phosphotyrosine; two dimensional electrophoresis is required. The discovery that pp60^{src} phosphorylates tyrosine in TBR immunoprecipitates has aided significantly

in the effort to understand the biochemical mechanisms of $pp60^{src}$ action since src induced phosphorylation can be distinguished from the large background of normal cellular phosphorylations at serine and threonine residues.

Hunter and Sefton (1980) have further observed that the level of phosphotyrosine in RSV transformed cells is increased 10-fold over the levels detected in normal chick cells. This increase is partly accounted for by the fact that $pp60^{src}$ itself was found to be phosphorylated at a tyrosine residue. Hunter and Sefton have hypothesized that the overall increase in phosphotyrosine levels in transformed cells results from the phosphorylation of substrate proteins at tyrosine residues by $pp60^{src}$.

The phosphorylation of tyrosine in RSV transformed cells was shown to be an early effect of $pp60^{src}$ expression (Sefton et al., 1980b). Normal levels of phosphotyrosine were detected in cells infected with a mutant RSV, temperature sensitive for transformation, grown at the non-permissive temperature. One hour after downshift to the permissive temperature, however, the levels of phosphotyrosine incorporated into protein had increased 3-5 fold. The amount of phosphotyrosine present in protein decreased rapidly if the cells were shifted back up to the nonpermissive temperature. These results suggest that $pp60^{src}$ phosphorylation of tyrosine residues in substrate proteins may be critical for transformation. Subsequently, studies on other RNA tumor viruses have established that phosphorylation of tyrosine may be a generalized mechanism by which avian sarcoma viruses mediate transformation (Cooper and Hunter, 1981b). So far, the retroviruses can be grouped into four classes which contain structurally similar transforming proteins (Table I). Little or no structural homology is detected between the transforming

TABLE I

Classes of Retroviruses that Code for Tyrosine Kinases

Class	Virus	Transforming Protein
I	Rous sarcoma virus (RSV) (various strains)	p60
II	Fujinami sarcoma virus (FSV)	p140
	PRC II sarcoma virus (PRC II)	p105
	Snyder-Theilen feline sarcoma virus (ST-FeSV)	p85
	Gardner-Arnstein feline sarcoma virus (GA-FeSV)	p90
III	Yamaguchi 73 avian sarcoma virus (Y73)	p90
	Esh avian sarcoma virus (ESV)	p80
IV	Abelson murine leukemia virus (Ab-MuLV)	p120

The retroviruses, in most cases, each code for a phosphoprotein which is actually a polyprotein composed of virus gag protein sequences fused to nonstructural transformation specific sequences. $Pp60^{src}$ is an exception in that it is composed only of transformation specific sequences. The different viral phosphoproteins are each associated wtih a tyrosine kinase activity in immunoprecipitates. Furthermore, the level of phosphotyrosine increases 5-10 fold when normal cells are infected by any one of these viruses. The transforming sequences of the proteins within a virus class exhibit structural homology but no homology exists between the transforming sequences of proteins from different classes. The data compiled in the table is reviewed (Neil et al., 1981; Ghysdael et al., 1981 a,b; Beemon, 1981).

proteins from different classes (Neil et al., 1981; Beemon, 1981); however, all of the proteins are similar in that each one is coded for by a viral gene that has a normal cell homologue (Shibuya et al., 1980; Yoshida et al., 1980). Rous sarcoma virus, Yamaguchi 73 avian sarcoma virus (Y73) (Kawai et al., 1980), Fujinami sarcoma virus (FSV) (Hanafusa et al., 1980; Lee et al., 1980) and Abelson murine leukemia virus (Ab-MuLV) (Abelson and Rabstein, 1970) are representative viruses from each class. The transforming protein coded for by each virus gene has been shown to be associated with protein kinase activity in immunoprecipitates (Collett and Erikson, 1978; Kawai et al. 1980; Feldman et al., 1980; Witte et al., 1980). These kinases specifically phosphorylate tyrosine residues on substrate proteins and the level of phosphotyrosine is increased approximately 10-fold when cells are transformed by any one of these viruses (Hunter and Cooper, 1981; Sefton et al., 1981b; Beemon, 1981).

Increased tyrosine phosphorylation is not, however, a general phenomenon in all transformed cells. No increase in the abundance of phosphotyrosine in protein is detected when mouse cells are transformed by Kirsten sarcoma virus, Moloney sarcoma virus, or SV-40 virus, when chick embryo fibroblasts are transformed with avian myelocytomatosis virus MC29, or in rat cells transformed by polyoma virus (Sefton et al., 1980b).

Structural Characterization of pp60^{src}

The phosphorylation sites of both pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ have been characterized (Collett et al., 1979b). Cells were radiolabelled with [^{32}P]orthophosphate, extracts immunoprecipitated with TBR serum, and precipitates digested with V8 protease from Staphylococcus aureus. Two large phosphopeptide fragments of pp60^{src} were obtained after V8 protease digestion. Phosphoamino acid analysis showed that the amino terminal fragment contained a phosphoserine residue. The carboxy terminal fragment was originally reported to be phosphorylated at a threonine residue. However, after the discovery (Hunter and Sefton, 1980) that pp60^{src} is a tyrosine kinase, more careful analysis revealed that the C-terminal fragment was actually phosphorylated at a tyrosine residue. The same amino-terminal serine residue is phosphorylated on pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ (Collett et al., 1979b) but the viral and cellular src proteins are phosphorylated at different tyrosine residues (Collett et al., 1979a; Smart et al., 1981; Karess and Hanafusa, 1981). Possibly this difference in phosphorylation will be reflected in differences in the kinase activity of the two proteins.

pp60^{src} was shown to be phosphorylated at the serine residue by a cAMP-dependent protein kinase in cell-free extracts (Collett et al., 1979b). Since the activity of an enzyme can be influenced by phosphorylation (Krebs and Beavo, 1979), the activity of pp60^{src} may be regulated by the cAMP-dependent protein kinase. In contrast, the phosphorylation of pp60^{src} at the tyrosine residue is cAMP-independent (Collett et al., 1979b). Interestingly, pp60$^{v\text{-}src}$ extracted from tsNY68-infected cells

grown at the nonpermissive temperature is not phosphorylated at the tyrosine residue, whereas pp60^{src} extracted from cells transformed at the permissive temperature is phosphorylated at a tyrosine residue. In parallel with the decreased amount of phosphotyrosine, the kinase activity in tsNY68 cells grown at the nonpermissive temperature is significantly lower than the activity found in these cells grown at the permissive temperature (Levinson et al., 1978; Rübsamen et al., 1979). Thus, phosphorylation at tyrosine may activate the pp60^{src} kinase, resulting in cellular transformation at the permissive temperature.

More detailed analysis has recently revealed the sequence of amino acids surrounding the phosphotyrosine residue on pp60$^{v\text{-}src}$. Phosphorylation occurs at residue 419 in the amino acid sequence of the protein (Smart et al., 1981). The surrounding sequence was determined to be Leu-Ile-Glu-Asp-Asn-Glu-Tyr(P)-Thr-Ala-Arg. Three of the four amino acids that precede phosphotyrosine in the sequence are acidic residues and it has been proposed (Smart et al., 1981) that the acidic residues may be a common aspect of acceptor sites for tyrosine phosphorylation in proteins. The site of tyrosine phosphorylation on pp60$^{c\text{-}src}$ *in vivo* is different from the site phosphorylated in pp60$^{v\text{-}src}$, but because pp60$^{c\text{-}src}$ is present in such small amounts in the cell it has not yet been possible to determine the sequence surrounding the phosphotyrosine in the normal protein.

Patchinsky et al. (1982) have reached somewhat different conclusions by analyzing the sequence around the site of phosphorylation on pp60^{src} and several other tyrosine kinases and their substrates. The sequences around the site of phosphorylation on pp60^{src} and p90, the transforming

protein of the avian sarcoma virus Y73, are identical. In addition, these two kinases and two other transforming proteins that are phosphorylated at tyrosine residues, p105 of PRCII virus and p85 of Snyder-Theilen feline sarcoma virus (ST-FeSV), all have a lysine or arginine seven residues to the NH_2-terminal side of the phosphotyrosine residue. All four sites also have at least one acidic amino acid among the residues between the phosphotyrosine and the NH_2-terminal lysine or arginine. Patchinsky et al. have preliminary data on the acceptor sites of several other tyrosine kinases and substrates that suggest that the location of the basic amino acid and the acidic amino relative to the phosphotyrosine residue is not always constant.

Wong and Goldberg (1981) recently synthesized a peptide fragment of $pp60^{src}$ which contains the phosphotyrosine. They have found that the peptide inhibits the kinase activity of $pp60^{src}$ in vitro. These experiments are consistent with the hypothesis that the sequence around the tyrosine residue in $pp60^{src}$ may be a widely used signal site for tyrosine phosphorylation and may be involved in the regulation of cellular function.

It has not yet been determined how phosphorylation at serine and tyrosine residues affects the enzymatic activity associated with wild type $pp60^{v\text{-}src}$. This is because it is difficult to obtain the various phosphorylated forms of $pp60^{src}$ in pure form and in large enough quantities to do the experiments. The src gene was recently cloned and could be expressed in Escherichia coli (Gilmer and Erikson, 1981; McGrath and Levinson, 1982). $pp60^{src}$ synthesized in bacteria is present as 5% of the bacterial cell protein; therefore, in the future, it should be

possible to obtain large quantities of this protein. Furthermore, E. coli have been reported to exhibit very low levels of protein kinase activity and the pp60^{src} isolated from these cells does not appear to be phosphorylated (McGrath and Levinson, 1982). This bacterial source of pp60^{src} should make it possible to answer some of the questions concerning the effect of phosphorylation on pp60^{src} activity.

Identification of a Potential pp60^{src} Substrate, a 34,000 Dalton Phosphoprotein

Cellular transformation after infection with Rous sarcoma virus results in a pleiotypic response. There is considerable evidence, presented above, suggesting that transformation is mediated via the action of a special kind of kinase, the tyrosine kinase. Several other types of protein kinase, such as the cAMP-dependent protein kinase (reviewed by Greengard, 1978) have already been well characterized. This kinase regulates a variety of different cellular enzymes by phosphorylation, and analogously pp60^{src} could cause a drastic alteration in many cell properties by phosphorylating different substrate proteins at tyrosine residues. In order to better understand the mechanisms of pp60^{src} action there has been considerable effort in the last few years aimed at defining its substrates.

The first potential substrate was identified indirectly by the experiments of Radke and Martin (1979). They compared [^{32}P]orthophosphate labelled extracts of normal and transformed cells using two dimensional polyacrylamide gel electrophoresis. A protein with a molecular weight of 36,000 daltons was found to be phosphorylated in transformed cells but not in normal cells. Using a temperature sensitive RSV mutant the

36,000 dalton protein was found to be phosphorylated within 30 minutes of downshift from the nonpermissive to the permissive temperature, marking this phosphorylation as an early event in transformation. Radke and Martin have suggested that the 36 kD might be a direct target of $pp60^{src}$. Erikson and Erikson (1980) also identified a protein, with a molecular weight of 34,000 daltons, that is phosphorylated in a transformation specific manner. The 34 kD protein has the same isoelectric point as Radke and Martin's 36 kD protein, suggesting that the two proteins may be identical. Erikson and his colleagues have partially purified the 34 kD protein and have raised a rabbit antibody to the purified protein. Further characterization of this protein (Erikson and Erikson, 1980) has shown that it is phosphorylated at a tyrosine and a serine residue in transformed cells but only at the serine residue in normal cells. They have found that partially purified $pp60^{src}$ will phosphorylate the 34 kD protein *in vitro* at the same tyrosine residue that is phosphorylated *in vivo*. These data suggest that the 34 kD protein might be an actual $pp60^{src}$ substrate, although the function of this 34 kD protein is virtually unknown. Unconfirmed results by Rübsamen et al. (1982) showed that the 34 kD protein copurifies with cytosolic malate dehydrogenase. These workers have suggested that the two proteins may be identical and that $pp60^{src}$ phosphorylation of malate dehydrogenase may regulate this enzyme which functions as a link between the tricarboxylic acid cycle and glycolysis.

Phosphorylation of the 34 kD protein also occurs in cells transformed by several unrelated avian sarcoma viruses which code for other tyrosine kinase transforming proteins (Erikson et al., 1981). The 34 kD protein

is phosphorylated at the same tyrosine residue in cells transformed by Fujinami, PRCII, or Rous sarcoma virus. These data suggest that phosphorylation of the 34 kD protein may be intrinsic to the mechanism of oncogenesis by avian sarcoma viruses. Further analysis of this protein will be presented in the Results section.

The Cytoskeleton as a Target of $pp60^{src}$

Another possible direct target of $pp60^{src}$ is the cytoskeleton. Transformed cells differ in morphology from normal cells and specific cytoskeletal alterations are an early event in cellular transformation (Edelman and Yahara, 1976; Wang and Goldberg, 1976). The effects of transformation on the cytoskeleton are of particular interest in view of the proposed relationships between cell surface receptor mobility, the cytoskeleton and cell growth (Edelman, 1976). The cytoskeleton is known to be composed of three major filamentous systems: the microfilaments, the microtubules, and the intermediate filaments.

The microfilaments can be seen in electron micrographs as a thin fibrous network of 5-7 nm filaments arrayed both in a reticular meshwork and in bundles extending across the length of the cell. The microfilaments can be visualized by indirect immunofluorescent microscopy of cultured cells (Fig. 4A). Actin and myosin are the major constituents of the microfilament bundles (reviewed in Pollard and Weihing, 1974). Actin is a monomer (G-actin) with a molecular weight of 42,000 daltons. It polymerizes to form double helical filaments (F-actin) which interact with myosin. Other proteins such as filamin (Wang and Singer, 1977), α-actinin (Lazarides, 1976), tropomyosin (Lazarides, 1975) and vinculin (Geiger, 1979) have been found to interact with F-actin both *in vitro* and *in vivo*.

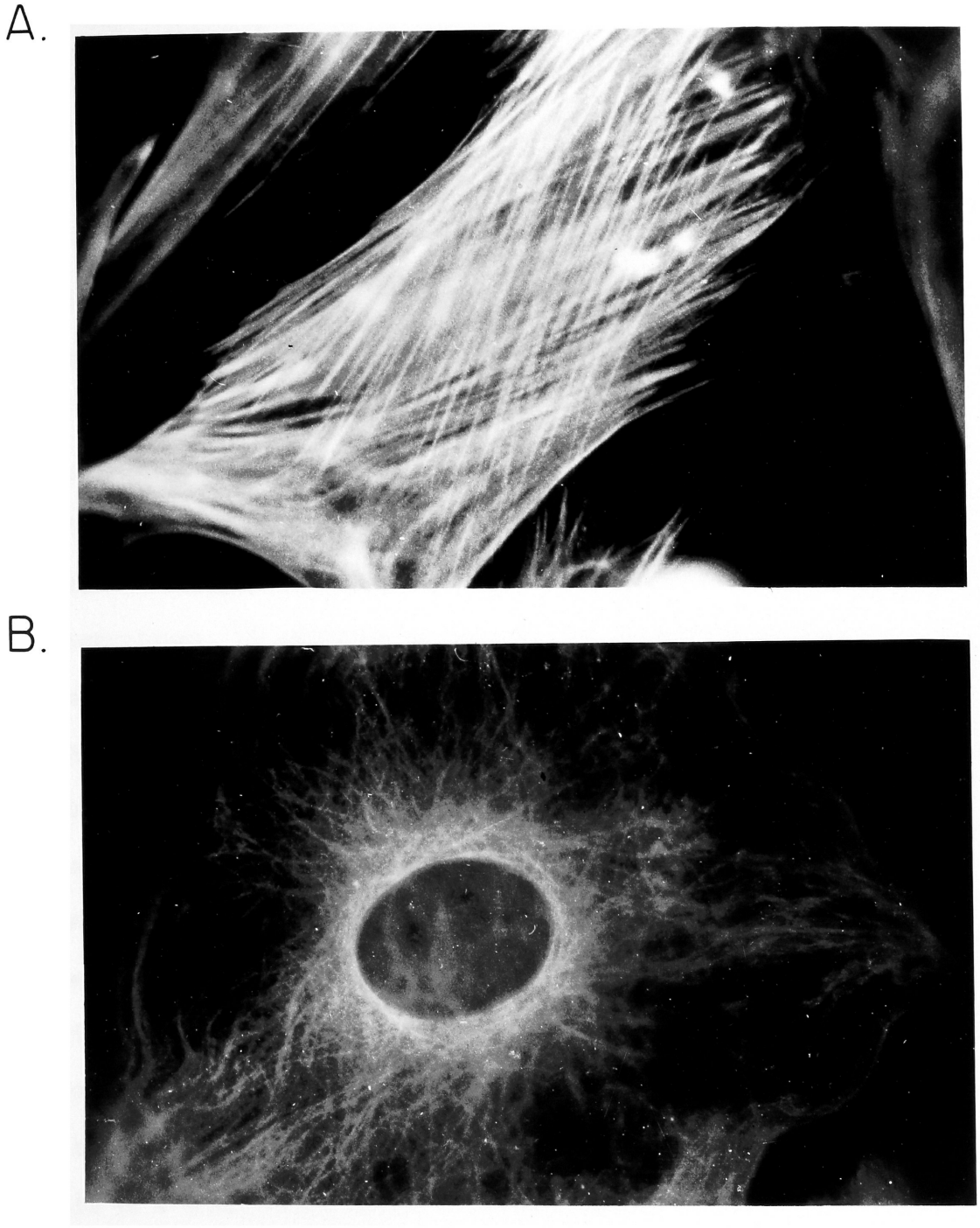


Fig. 4. Indirect immunofluorescence microscopy of cultured cells. (A) chick embryo fibroblasts; fixed, permeabilized and stained with rabbit antibody to actin. (B) mouse embryo fibroblasts; fixed, permeabilized and stained with rabbit antibody to tubulin.

By analogy to skeletal muscle the microfilaments were postulated to play a role in cell motility (Pollard and Weihing, 1974). This acto-myosin system has been implicated in such processes as cell surface receptor mobility (Taylor et al., 1971; Yahara and Edelman, 1972) and phagocytosis (Stossel and Hartwig, 1976). When cells are transformed by RSV the actin cables dissolve (Edelman and Yahara, 1976; Wang and Goldberg, 1976) (Fig. 5). The distributions of the microfilament associated proteins α-actinin, myosin, tropomyosin, and vinculin are also altered (David-Pfeuty and Singer, 1980; Boschek et al., 1981). These cytoskeletal alterations occur as early as 15 min. after downshift of cells infected with tsNY68 virus from the nonpermissive to the permissive temperature, suggesting that the changes are an early effect of $pp60^{src}$ action (Fig. 6). These observations, in addition to subcellular localization and microinjection experiments, support the hypothesis that $pp60^{src}$ acts directly on the microfilaments.

The microtubules are large cylindrical structures 25 nm in diameter, and are composed of tubulin dimers and associated proteins. Tubulin polymerization can be studied in vitro (Weisenberg, 1972) and the microtubules can be visualized within the cell by immunofluorescence microscopy using antibodies to tubulin (Weber et al., 1975) (Fig. 4B). The assembly of microtubules in vitro is Ca^{2+}-dependent and is also regulated by the action of the cAMP-dependent protein kinase (Gillespie, 1974). Tubulin is a dimer composed of similar subunits (α and β) of molecular weights 54,000 and 56,000 daltons (Shelanski and Taylor, 1967; Weisenberg et al., 1968; Bryan and Wilson, 1971; Lee et al., 1973). A wide variety of non-tubulin proteins have been reported to copurify

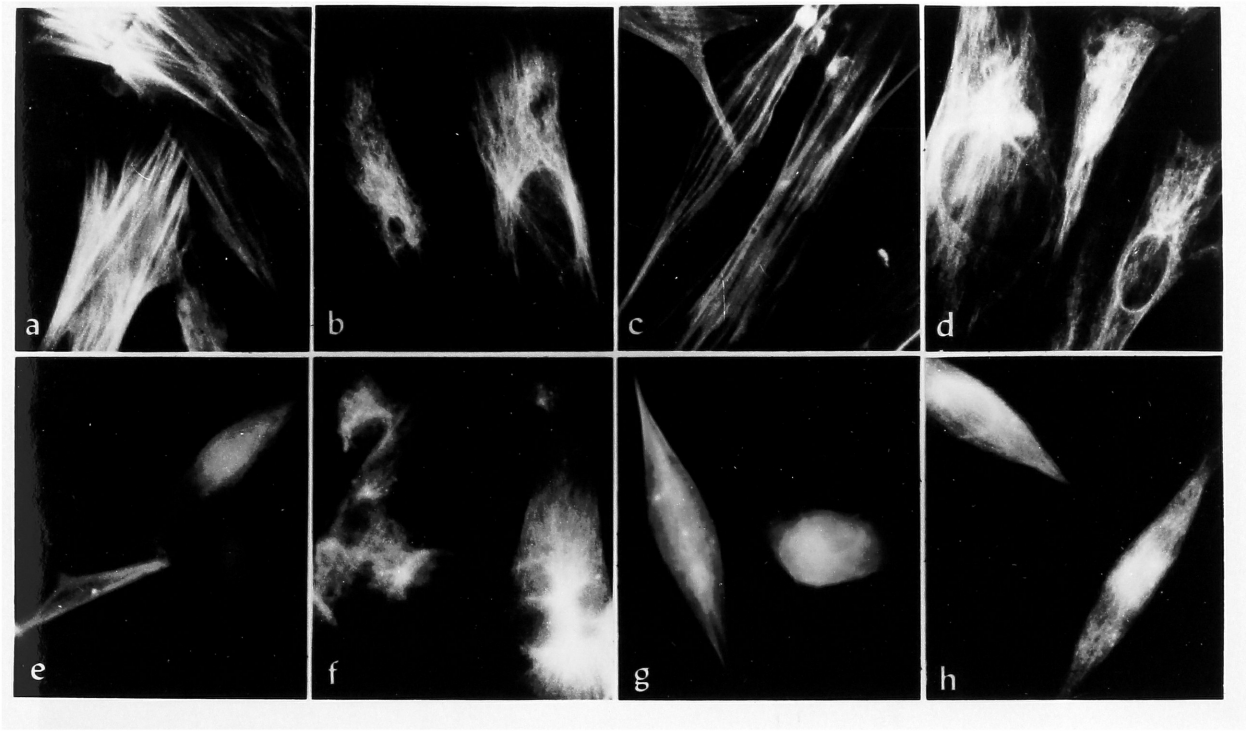


Fig. 5. Comparison of immunofluorescence staining patterns with anti-actin and anti-tubulin in chick fibroblasts and SR-RSV-A infected fibroblasts at 41°C and 37°C: (a-d) chick fibroblasts: (a) 41°C, anti-actin, (b) 41°C, anti-tubulin, (c) 37°C, anti-actin; (d) 37°C, anti-tubulin; (e-h) SR-RSV-A-infected cells: (e) 41°C, anti-actin; (f) 41°C, anti-tubulin; (g) 37°C, anti-actin; (h) 37°C, anti-tubulin (magnification x800). From Edelman and Yahara, 1976.

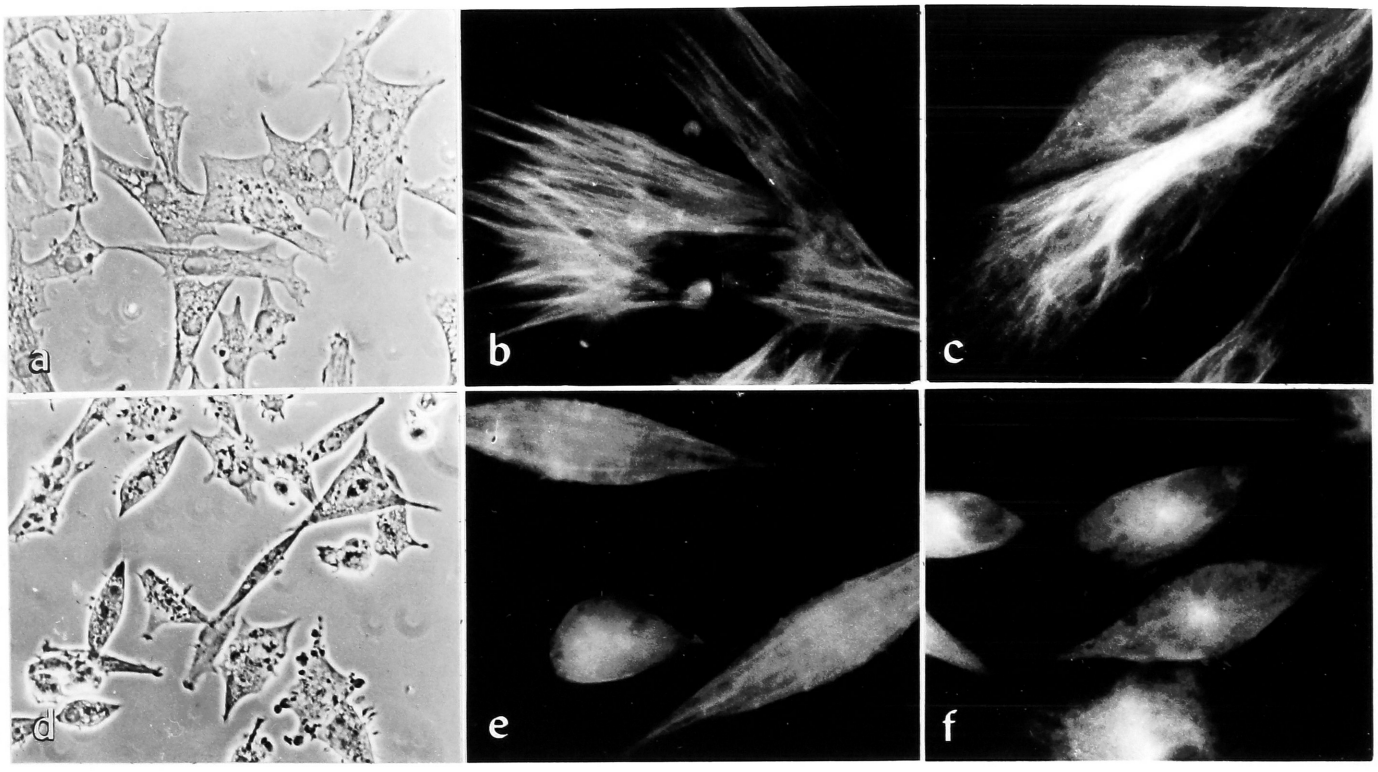


Fig. 6. Comparison of tsNY68-RSV infected cells at restrictive (41°C) and permissive (37°C) temperatures after binding of fluorescein-labelled antibodies to actin and tubulin: (a) 41°C, phase contrast; (b) 41°C, anti-actin; (c) 41°C, anti-tubulin; (d) 37°C, phase contrast; (e) 37°C, anti-actin; (f) 37°C, anti-tubulin (magnification a and d, x360; b,c,e, and f, x900). From Edelman and Yahara, 1976.

with the microtubules (Borisy et al., 1975; Gaskin et al., 1974; Burns and Pollard, 1974). These microtubule associated proteins, or MAPs, include a spectrum of high molecular weight proteins and a protein of 70,000 molecular weight. The association of several of the MAPs with microtubules has recently been found to be regulated by Ca^{2+} and protein phosphorylation (Schliwa et al., 1981; Burke and DeLorenzo, 1981).

There has been some controversy over the effect of Rous sarcoma virus transformation on the state of the microtubules. Edelman and Yahara (1976) reported that the microtubules are disrupted when cells are transformed by RSV (Figs. 5,6). It was later suggested by Osborn and Weber (1977) that this difference in the microtubules was not real but instead was a visualization artifact resulting from cell shape changes during transformation. However, Edelman and Yahara (1976) observed disruption of microtubules 1 hour after a temperature shift of tsNY68 -infected CEFs from 42°C to 37°C, well before a detectable change in cell shape has occurred (Fig. 6). The visualization of the microtubules in transformed cells is known to be affected by the method of extraction prior to immunofluorescence staining, and certain extraction and fixation procedures may not detect the difference in microtubular structure that exists between normal and transformed cells (McClain, 1978).

Further evidence that microtubules are altered in transformed cells comes from the work of Edelman and Yahara (1976). They showed that the ability of plant lectins to induce anchorage of cell surface receptors was reduced in transformed cells. This anchorage modulation has been shown to require intact microtubules and is thought to reveal interactions

of surface receptors with the submembranous cytoskeleton. Lectins in doses that induce anchorage modulation also prevent $pp60^{src}$-induced changes in cytoskeletal morphology as well as $pp60^{src}$-induced growth stimulation. Microtubules are implicated in the positive and negative control of normal cell growth in a variety of different cell types (Wang et al., 1975; Baker, 1976; Walker et al., 1977; McClain et al., 1977; Friedkin et al., 1979; McClain and Edelman, 1980; Crossin and Carney, 1981). All of these data are consistent with the hypothesis (Edelman et al., 1973) that the microtubules function as key regulators of cellular division.

The third cytoskeletal system seen in cells consists of filaments 8-10 nm in diameter. The filaments are intermediate in size between microfilaments and microtubules and are thus often referred to as intermediate filaments (reviewed by Lazarides, 1980). These filaments exist in fibroblasts as radial arrays similar to the microtubules. The protein composition of the intermediate filament varies depending on the cell type. In chick embryo fibroblasts they are composed of two proteins; vimentin and desmin, each with an approximate molecular weight of 52,000 daltons. The intermediate filament subunits are phosphorylated *in vivo* and can be phosphorylated *in vitro* (O'Connor et al., 1981). The role of phosphorylation in the regulation of assembly or other functions of the filaments is unknown.

The state of the intermediate filaments is dramatically altered in cells transformed by RSV (Hynes and Destree, 1978; Ball and Singer, 1981). In cells transformed by temperature sensitive transformation defective mutants, the intermediate filaments retract around the nucleus

within one hour of downshift from the nonpermissive to permissive temperature (Ball and Singer, 1981). Thus, the change in intermediate filament structure is a direct and rapid result of transformation. Ball and Singer (1981) have demonstrated by indirect immunofluorescence microscopy that the intermediate filaments and the microtubules are often coincident, and they have proposed that this may reflect an interaction between these two filamentous systems. In fact, dissolution of microtubules with drugs such as colchicine also causes a retraction of the intermediate filaments (Hynes and Destree, 1978). A consequence of transformation by RSV could be that the two filamentous systems no longer interact normally, perhaps due to phosphorylation by $pp60^{src}$ of an as yet unidentified protein that links the microtubules and intermediate filaments.

Identification of a Cytoskeletal $pp60^{src}$ Substrate, Vinculin

Vinculin is a 130,000 dalton protein reported by Geiger (1980) to be found at sites of cell-cell and cell-substratum contact. It has been localized by immunofluorescence and electron microscopy to focal adhesion plaques (Geiger, 1979; Geiger et al., 1980) and is thought to be involved in linking the microfilaments to the plasma membrane. An additional pool of vinculin has been detected in the cytoplasm by immunofluorescence microscopy. Normal cellular vinculin has been purified (Feramisco and Burridge, 1980) and has been shown (Wilkins and Lin, 1982) to interact with the ends of actin filaments *in vitro*.

The distribution of vinculin in the cell is altered after transformation with Rous sarcoma virus. In transformed cells less vinculin is present in focal adhesion plaques and sites of cell-cell contact and

instead, vinculin is clustered in small patches near the ventral surface of the cell (David-Pfeuty and Singer, 1980; Shriver and Rohrschneider, 1981). Shriver and Rohrschneider (1981) have localized $pp60^{src}$ to the adhesion plaques in transformed cells and have suggested that $pp60^{src}$ might specifically phosphorylate vinculin resulting in release of the vinculin from the adhesion plaques with the concomitant disruption of the cytoskeleton. The observation that vinculin in RSV transformed cells contains eight-fold more phosphotyrosine than vinculin from normal cells (Sefton et al., 1981a) supports this hypothesis. No significant change in phosphotyrosine content in several other cytoskeletal proteins (α-actinin, filamin, vimentin and myosin) was detected as a result of transformation.

Vinculin was also specifically phosphorylated at a tyrosine residue when cells were transformed by two other viruses: the Abelson virus and avian sarcoma virus Y73, but not when cells were transformed by the avian sarcoma virus PRCII (Sefton et al., 1981a). This is intriguing because cells transformed by PRCII differ in cellular morphology from the cells transformed by the other three viruses. Cells infected with PRCII are not round but are elongated or fusiform and are morphologically more like normal cells than RSV transformed cells. The lack of tyrosine phosphorylation on vinculin in PRCII infected cells offers support to the hypothesis that phosphorylation of vinculin at tyrosine by $pp60^{src}$ can mediate cytoskeletal and morphological changes.

It is not clear from the experiments described above whether $pp60^{src}$ phosphorylates vinculin directly or if it activates an intermediate kinase(s). <u>In vitro</u> experiments using purified components might be a

useful way of addressing the question of direct interaction between pp60^{src} and vinculin.

Other pp60^{src} Targets

Recent work (Brugge et al., 1981) has identified two other potential pp60^{src} substrates. They demonstrated that two cellular proteins having molecular weights of 90,000 and 50,000 daltons coimmunoprecipitate with pp60^{src}. The two proteins were also found to copurify with pp60$^{v\text{-}src}$ on glycerol gradients. 5% of pp60^{src} from RSV transformed cells sediments as a high molecular weight species apparently complexed with the 90 kD and 50 kD proteins. The remaining 95% of the pp60^{src} sediments on glycerol gradients as a monomer with a molecular weight of 60,000 daltons. The three proteins pp60^{src}, 50 kD, and 90 kD were present in the complex in equimolar amounts. Interestingly, the pp60^{src} associated with these two proteins has no detectable kinase activity and was found to be phosphorylated only at a serine residue, again suggesting that tyrosine phosphorylation may be necessary for the activity of pp60^{src} as a kinase. Complex formation is dependent on the presence of salt, and no complex was detected if salt was absent during cell lysis. In chick cells infected with tsNY68 RSV, significantly more of the pp60^{src} was associated with the 50 kD and 90 kD proteins than in cell infected with wild type virus.

The 90 kD protein that interacts with pp60^{src} is a major cellular protein in normal and transformed cells. Its synthesis has been shown to be induced as a response of fibroblasts to heat shock (Oppermann et al., 1981). Brugge et al. (1981) and Hunter and Sefton (1980) have

found that this protein is not phosphorylated at a tyrosine residue in vivo. The 50 kD protein that interacts with pp60^{src} is a protein of unknown function that has recently been found to be phosphorylated at a tyrosine residue in a transformation-dependent manner (Brugge and Darrow, 1982; Gilmore et al., 1982) and thus it may be a substrate protein that mediates some of the pp60^{src} induced changes that occur during cell transformation.

Cooper and Hunter (1981a) have developed a technique which has aided in the search for pp60^{src} substrates. They first compared [^{32}P] labelled transformed and normal cell extracts by two dimensional polyacrylamide gels. Treating these gels with alkali hydrolyzed phosphoserine bonds, thus reducing the number of protein spots visualized due to phosphoserine. Cooper and Hunter observed that, out of a total of approximately 200 alkali-resistant phosphoproteins, seven proteins had increased levels of phosphotyrosine in transformed cells. These proteins may be substrates of pp60^{src} or of other tyrosine-specific kinases activated by pp60^{src}. Currently the function of these proteins in the cell is not known. In vivo phosphorylation of these same seven proteins was detected in cells infected with avian sarcoma viruses of the other three classes. Perhaps these proteins are also important in the general mechanism of oncogenesis by avian sarcoma viruses.

Ross et al. (1981) have taken an alternative approach in a general search for substrates of the pp60^{src} kinase. They developed a synthetic hapten analogue to phosphotyrosine and raised specific antibodies to the hapten which specifically immunoprecipitated phosphotyrosine containing proteins.

The search for targets of $pp60^{src}$ kinase continues. Most of the targets identified so far are proteins whose function is unknown. Of special interest is the way in which tyrosine phosphorylation induces the deregulation of cell growth. A clue may be found in recent experiments that suggest that the tyrosine kinases also play a role in the control of normal cell growth (Ushiro and Cohen, 1980; Hunter and Cooper, 1981).

Activation of Tyrosine Kinases by Growth Factors

Epidermal growth factor (EGF) binds with high affinity to cell surface receptors and stimulates the growth and replication of a number of normal cell types (reviewed by Carpenter and Cohen, 1979; Gospodarowicz et al., 1978). In addition to the stimulation of growth, EGF has a variety of effects on the cell, such as increases in the rate of ion flux and hexose transport across the plasma membrane, rapid morphological changes, and disruption of the cytoskeleton. Many of the changes are similar to those seen in cells transformed by oncogenic viruses.

It was recently shown that *in vitro* EGF stimulates a membrane-associated protein kinase which catalyzes the phosphorylation of the EGF receptor (Cohen et al., 1980; Ushiro and Cohen, 1980). The EGF receptor, a 150-170 kD glycoprotein, was found to be phosphorylated *in vitro* in the presence of EGF at a tyrosine residue both in a crude membrane preparation and in a purified form (Carpenter et al., 1978, 1979; Cohen et al., 1980; King et al., 1980). The tyrosine kinase activity co-purifies with the EGF receptor but it is not clear whether it is an intrinsic property of the receptor or a tightly bound associated kinase (Cohen et al., 1980).

Hunter and Cooper (1981) recently observed that the level of phosphotyrosine in the human epithelioid tumor cell line A431 is increased approximately four-fold when these cells are treated with EGF. This increase in the level of phosphotyrosine detected in cellular protein was complete within one minute of the addition of EGF and is the earliest known cellular response to this hormone. Most of the increase in phosphotyrosine in vivo after treatment with EGF was not due to phosphorylation of the EGF receptor, but was a result of the phosphorylation of two other cellular proteins with molecular weights of 39 kD and 81 kD (Hunter and Cooper, 1980). The 39 kD protein appears to be homologous to the 34 kD $pp60^{src}$ substrate.

The effect of EGF on the properties of A431 cells is unusual in that the hormone inhibits rather than stimulates the growth of these cells (Gill and Lazar, 1981) and this inhibition of proliferation in EGF treated A431 cells is correlated with an increased phosphotyrosine content. It would be interesting to know how the levels of phosphotyrosine are affected when other cell types are stimulated to proliferate by EGF addition.

Ek et al. (1982) observed that another growth factor, platelet derived growth factor (PDGF) also stimulates the phosphorylation of tyrosine residues. PDGF is a 30,000 molecular weight polypeptide which stimulates the division of connective tissue cells and glial cells in culture (Ross and Vogel, 1978; Scher et al., 1979; Heldin et al., 1979). PDGF causes changes such as membrane ruffling, reduction in cell adhesion, as well as the disruption of the cytoskeleton (Chinkers et al., 1979). The addition of PDGF to plasma membranes from human fibroblasts or glial

cells resulted in the phosphorylation of tyrosine residues on two membrane proteins with apparent molecular weights of 175,000 and 130,000 daltons (Ek et al., 1982). The effect of PDGF on the phosphotyrosine content of intact cells was not determined. Hunter and Cooper (1981) have not detected in vivo increases in phosphotyrosine levels when A431 cells were treated with a variety of other peptide hormones including nerve growth factor, fibroblast growth factor, or insulin, supporting the conclusion that the mechanism of action of these growth factors is different from that of EGF (Rudlund and Jimenez de Asua, 1979; Halegoua and Patrick, 1980). Further in vitro and in vivo studies of the action of a variety of growth factors on normal cells should help to unravel the role played by tyrosine kinases in the control of cell growth.

III. PURIFICATION OF THE PP60^{src} PROTEIN KINASE

The availability of an antibody to the src gene product has made it possible in the last few years to identify and characterize this transforming protein. Collett and Erikson (1978) discovered that pp60^{src} in TBR immunoprecipitates is associated with a protein kinase activity. Hunter and Sefton (1980) later found that the pp60^{src} associated kinase is unique in that it phosphorylates a tyrosine residue on TBR IgG *in vitro* and possibly on other pp60^{src} protein substrates *in vivo*. Purification of pp60^{src} was necessary in order to better characterize its associated protein kinase activity in solution under well-defined conditions. The antiserum was used to screen a variety of tumor cell lines for pp60^{src} kinase activity. The RR1022 rat cell line was chosen as a source of pp60^{src} and two different purification protocols were developed for purifying pp60^{src} from the RR1022 cells. The first purification procedure was developed in collaboration with several colleagues (Maness et al., 1979).

Materials and Methods

Cell Cultures and Viruses. Chicken embryo fibroblasts (CEFs) were cultured as described (Hanafusa, 1969) except that medium DMEM:NCTC 109 (1:1) was used for primary cultures and Ham's F-10 medium containing 5% calf serum and 20 μg/ml Gentamycin was used for secondary cultures. Chick fibroblasts were transformed as described (Hanafusa, 1969) with Schmidt-Ruppin strain of Rous sarcoma virus subgroup A (SR-RSV-A) and with a temperature sensitive mutant tsNY68, both kindly provided by Dr. H. Hanafusa (Rockefeller University, New York, N. Y.). The RR1022 cell

line, derived from a tumor induced in the inbred Amsterdam rat after injection of Schmidt-Ruppin strain ASV (Ahlström and Jonsson, 1961) was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium containing 10% calf serum. Cells were grown in plastic roller bottles and were harvested when the cultures were approximately 80% confluent. Harvested cells were washed three times with ice cold Dulbecco's phosphate buffered saline (PBS) and stored at -70°C.

Preparation of Antiserum and Immunoprecipitation. Serum from tumor bearing rabbits (TBR serum) was prepared as described (Brugge and Erikson, 1977) by injecting newborn New Zealand white rabbits with 0.2 ml of 12 hour culture medium from a confluently transformed culture of SR-RSV-D infected CEFs. Serum was collected six weeks after innoculation at the point that the RSV induced tumors had begun to regress.

Uninfected and SR-RSV infected cells grown in 60 mm tissue culture plates were incubated for 10 hours in 4 ml DMEM medium minus leucine (GIBCO, Grand Island, N.Y.) containing 0.25 mCi/ml L-[^{3}H]-leucine (40-60 Ci/mmole), Amersham, Arlington Heights, Ill.) with 10% dialyzed fetal calf serum or for 4 hours with 1.0 mCi of carrier-free [^{32}P]-phosphate (Amersham) in phosphate-free DMEM containing 10% dialyzed fetal calf serum. After labelling, the culture medium was removed and the cells cooled on ice. Cells were washed three times with ice cold STE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA) and lysed in 1 ml modified RIPA buffer (Brugge and Erikson, 1977) containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet-40, 0.1% SDS, 1 mM EDTA, 10 mM KCl, 1% trasylol (FBA Pharmaceutical, New York, N.Y.). Cell lysates were vortexed vigorously for 30 sec and then

centrifuged at 100,000 g for 30 min at 4°C. Protein determinations were done on the clarified supernatants by the method of Bradford (1976). The protein concentration of the transformed extract was diluted to that of the normal extract by addition of RIPA buffer. The protein concentration of the transformed extract prior to dilution was usually twice that of normal extracts. Extracts of labelled cells were incubated with TBR or preimmune serum (10 µl serum per 100 µl extract) for 60 min on ice in Beckman 1.4 ml microfuge tubes. 50 µl protein A Sepharose CL-4B (50% W/V slurry, Pharmacia) were added and mixed for 60 min to absorb immune complexes by the method of Kessler (1975). Immunoprecipitates were washed four times with modified RIPA buffer, two times with modified RIPA/1.0 M NaCl, and one additional time with modified RIPA. During the last wash immunoprecipitates were transferred to clean microfuge tubes.

SDS-Polyacrylamide Gel Electrophoresis. Washed immunoprecipitates were resuspended in 100 µl SDS-sample buffer (Laemmli, 1970). Samples were boiled for 5 min, pelleted, and the supernatants were electrophoresed on SDS polyacrylamide gels by the method of Laemmli (1970). The stacking gel was 5% acrylamide, and the separation gel was 8.5% acrylamide. The ratio of acrylamide to bisacrylamide was 30:0.8 and the gels were 10 cm. long. Gels were run at 20 mAmp constant current until the bromphenol blue tracking dye reached the bottom of the gel. Gels were stained in 0.1% Coomassie brilliant blue, 8% acetic acid, 20% methanol for 1 hr, destained overnight in 8% acetic acid, 20% methanol. Gels containing [^{3}H]leucine labelled proteins were prepared for fluorography by treatment with sodium salicylate according to the procedure of Chamberlain (1979). After drying gels were exposed at -70°C to Kodak XAR-5 X-ray film with a Cronex lightning plus intensifier screen.

Protein Kinase Assay. Extracts to be assayed for pp60^{src} kinase activity were immunoprecipitated and washed as described above. The immune complex-protein A sepharose was washed two times with 20 mM Tris-HCl, (pH 7.2), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) (kinase buffer), and resuspended in 25 µl 10^{-7} M γ-[^{32}P]-ATP (3000 Ci/mmole, Amersham) dissolved in kinase buffer. The reaction mixture was incubated at 37°C for 10 min with occasional mixing. The reaction was quenched by the addition of 25 µl 2x concentrated SDS gel sample buffer. Samples were boiled 5 min and applied to 8.5% SDS-polyacrylamide gels. Gel procedures and autoradiography were described above.

Filter Assay for pp60^{src}-Associated Protein Kinase. Aliquots of cell extracts to be assayed for enzymatic activity were incubated with 10 µl of TBR serum on ice for 1 hr. Protein A-Sepharose was added to absorb the immune complexes formed. The antigen-antibody complexes were washed three times with 200 µl of 50 mM Tris-HCl, (pH 7.2), 0.15 M NaCl; once with 2.5 M KCl, 60 mM Tris-HCl, (pH 7.2); and once with 40 mM Tris-HCl, (pH 7.2), 10 mM $MgCl_2$. The reaction was started by the addition of 2.5 µCi of [γ-^{32}P]-ATP (Amersham, 1000-3000 Ci/mmol; 1 Ci = 3.7 x 10^{10} becquerels) in 20 mM Tris-HCl, (pH 7.2), 5 mM $MgCl_2$ and, after 10 min at 37°C, was stopped by washing the immunoprecipitates three times with 200 µl of 20 mM Tris-HCl, (pH 7.2), 5 mM $MgCl_2$ and boiling for 2 min in 5% $NaDodSO_4$, 1% 2-mercaptoethanol. After centrifugation to remove protein A-Sepharose, the supernatant was brought to 10% in trichloroacetic acid and heated to 90°C for 15 min to hydrolyze ATP. After the precipitates were cooled, they were collected on Whatman glass fiber

filters. The filters were washed with 0.1 M Na pyrophosphate, 5% trichloroacetic acid and with methanol before drying. ^{32}P was quantitated by liquid scintillation counting. This filter assay is a modification of the $NaDodSO_4$/polyacrylamide gel assay developed by Collett and Erikson (1978). The recovery of radioactivity was comparable in both assays.

V8 Protease Digestion Analysis. Proteins were excised from gel bands and digested with V8 protease as described by Levinson and Levine (1977). Gel bands were swelled in V8 sample buffer (0.0625 M Tris, (pH 6.8), 0.15% SDS, 20% glycerol, 20 mM DTT, 0.01% bromphenol blue) and loaded onto a 10% polyacrylamide gel. 1.0 mg/ml of S. aureus V8 protease (Miles Biochemicals) in V8 sample buffer was loaded into the stacking gel slots on top of the gel slices. The electrophoresis was run at 10 mA constant current until the dye front had run through the stacking gel. The current was turned off for 15 min and then increased to 20 mA. The gel was processed for autoradiography as described above.

Preparation of Casein-Sepharose. Casein-Sepharose was prepared by the method of Garvey et al. (1977). To 60 ml packed volume of Sepharose CL-4B (Pharmacia) equilibrated with 2 M sodium carbonate, pH 11, was added dropwise 9 ml of 0.8 mg cyanogen bromide/ml acetonitrile. The mixture was stirred on ice for 5 min. CNBr-activated Sepharose was washed with 2 l. ice cold water followed by 2 l. 0.2 M sodium bicarbonate, pH 9.5. The washed Sepharose was resuspended to a total volume of 70 ml with sodium bicarbonate buffer. 60 ml of casein 5 mg/ml bicarbonate were added dropwise to the Sepharose. The mixture was incubated overnight with shaking at $4^{o}C$. The casein-Sepharose was

washed with 2 l. ice cold water followed by 60 ml 2 M ethanolamine-HCl, pH 8.0. Casein-Sepharose was then incubated with an additional 60 ml 2 M ethanolamine-HCl (pH 8.0) with shaking for 2 hours at 4°C and was washed with 2 l. ice cold water, 500 ml 0.1 M sodium acetate (pH 4.5), 1 liter water and stored in 500 ml 20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 1 mM sodium azide.

Partial Purification of $pp52^{src}$-Associated Protein Kinase. Frozen RR1022 cells (30 ml packed volume) were suspended in 60 ml of 10 mM potassium phosphate, (pH 7.0), 5 mM $MgCl_2$, 10 mM diothiothreitol, 0.1 mM EDTA, 0.34 M sucrose, 0.5 mM ATP, 1% (vol/vol) Trasylol, 2 mM phenylmethylsulfonyl fluoride and cells were lysed by homogenization for 5 min in a Potter-Elvejhem homogenizer followed by 40 strokes in a Dounce homogenizer. The homogenate was centrifuged for 2 hr at 100,000 x g. Nucleic acids were removed from the supernatant by the addition of 1.25% (wt/vol) streptomycin sulfate and centrifugation. Material precipitating from this supernatant between 20 and 40% saturation with ammonium sulfate contained approximately 90% of the $pp60^{src}$ kinase activity. The ammonium sulfate precipitate was dialyzed against buffer A (10 mM potassium phosphate, (pH 7.0), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.34 M sucrose, 10 mM dithiothreitol) and chromatographed on a column (1.5 x 10 cm) of ω-aminohexylagarose (Miles) previously equilibrated with buffer A. The column was washed with 100 ml of buffer A, and a 500-ml linear gradient of NaCl (0-1.0 M) in buffer A was applied. The active fractions, eluting between 0.6 M and 0.7 M NaCl, were pooled and dialyzed against buffer B (buffer A containing 1 mM dithiothreitol) and chromatographed on a column (1.2 x

16 cm) of phosphocellulose equilibrated with buffer B. The column was washed with 60 ml of buffer B, and a 200-ml linear gradient of potassium phosphate, pH 7.0, (0.01-0.4 M) in buffer B was applied. The active fractions, eluting at 0.15 M potassium phosphate, were pooled and dialyzed against buffer A. The dialyzed pool was chromatographed on a casein-Sepharose affinity column (1 x 12 cm) equilibrated with buffer A. The column was washed with 50 ml buffer A and a 100-ml linear gradient of NaCl (0-1.5 M) in buffer A was applied. The active fractions eluting at 0.50 M NaCl were pooled and dialyzed against buffer A. All procedures were carried out at 4°C.

Partial Purification of $pp60^{src}$. RR1022 cells (6 ml packed volume) were suspended in 60 ml 20 mM Tris-maleate (pH 8.0), 1 mM EDTA, 0.5% deoxycholate (DOC), 1% Nonidet P-40 (NP40), 1 mM β-mercaptoethanol, 2 mM iodoacetamide, 1 mM phenylmethylsulfonylfluoride (PMSF), 1% (v/v) Trasylol and homogenized by 20 strokes in a Dounce homogenizer with a tight fitting plunger. The homogenate was centrifuged for 45 min at 100,000 x g. To 60 ml of supernatant were added 6.6 ml glycerol and the sample was chromatographed on a column of affigel blue (1.2 x 16 cm) equilibrated with the initial extraction buffer/10% glycerol (buffer C). The column was washed with 150 ml of buffer C and a 150-ml linear gradient of NaCl (0-1 M NaCl) in buffer C was applied. The active fractions eluting at 0.75 M NaCl were pooled and dialyzed against buffer C, and chromatographed on a column (1.2 x 10 cm) of ω-aminohexylagarose equilibrated with buffer C. The column was washed with 50 ml buffer C and a 100 ml linear gradient of NaCl (0-1.5 M) in buffer C was applied. The active fractions eluting at 0.7 M NaCl were pooled, dialyzed overnight and applied to a column (1.2 x 8 cm) of phosphocellulose. This column was

washed with 20 ml buffer C, followed by 60 ml 20 mM Tris-maleate (pH 8.0), 1 mM EDTA, 1 mM β-mercaptoethanol, 0.05% NP-40, 1% trasylol (buffer D) and eluted with a linear gradient of KCl (0-1.0 M) in buffer D. The active fractions eluting at 0.4 M KCl were dialyzed against buffer D overnight and chromatographed on a casein-Sepharose column (1.2 x 16 cm) equilibrated with buffer D. The casein-Sepharose column was washed with 50 ml buffer D and eluted with a linear gradient of NaCl (0-1.5 M). The active fractions eluted in two peaks. Pool A was loosely bound to the casein-Sepharose column and eluted during the column wash. Pool B was eluted at 0.6 M NaCl. Both pools were dialyzed against buffer D/50% glycerol and stored at -20^{o}C. All procedures were carried out at 4^{o}C.

Results

Preparation and Characterization of Tumor Bearing Rabbit Serum. Newborn rabbits were injected with Schmidt-Ruppin D strain Rous sarcoma virus (Brugge and Erikson, 1977). The rabbits developed palpable tumors after approximately four weeks and were bled for serum two weeks later when the tumors had begun to regress. The serum immunoprecipitated $pp60^{src}$ and its associated kinase activity from detergent extracts of transformed cells (described below). The antibody titer to $pp60^{src}$ remained high for 10 weeks. Serum was collected from rabbits every ten days during this time period. The serum was tested for antibodies to $pp60^{src}$.

Chick embryo fibroblasts and SR-A RSV infected CEFs were labelled with [^{3}H]-leucine and immunoprecipitated with tumor bearing rabbit serum. Immunoprecipitates were washed and analyzed by SDS-polyacrylamide gel electrophoresis. The TBR serum immunoprecipitated $pp60^{src}$ as well as the structural proteins of Rous sarcoma virus, Pr180, Pr76 and p27, from extracts of SR-A RSV infected CEFs (Fig. 7, lane 1). The precipitation of viral structural proteins was diminished by the addition of unlabelled detergent disrupted RSV to the TBR immunoprecipitates but the amount of the 60 kD protein precipitated did not decrease (Fig. 7, lanes 2 and 3). This indicates that the 60 kD protein immunoprecipitated by the TBR serum is not a structural protein of Rous sarcoma virus. Preimmune serum did not precipitate the 60 kD species from transformed cell extracts (Fig. 7, lane 4) and the TBR serum did not immunoprecipitate significant

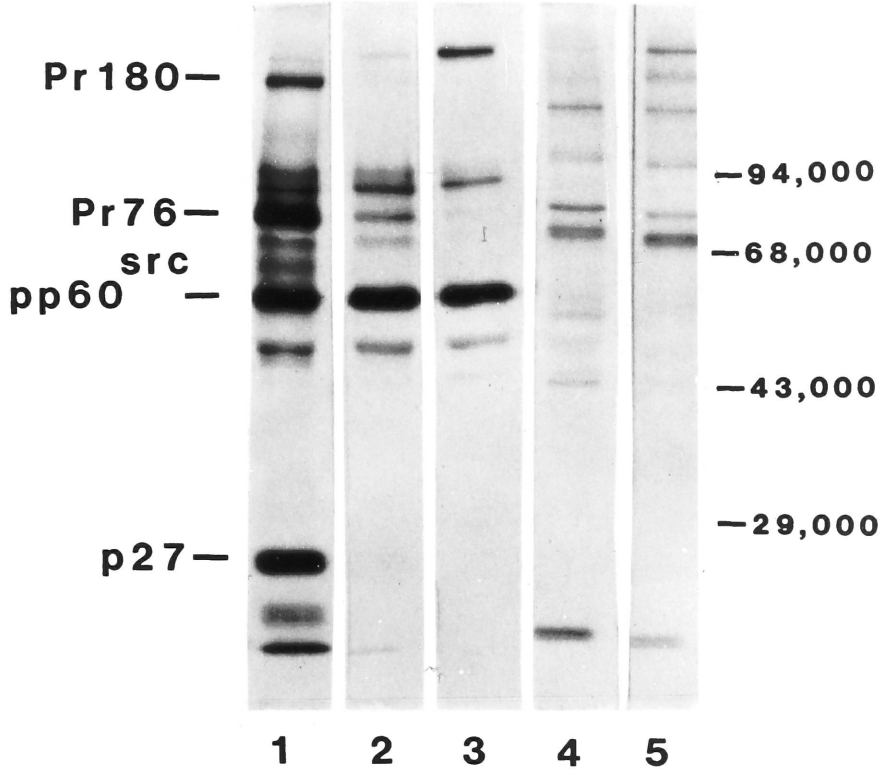


Fig. 7. Autoradiogram of immunoprecipitates of [^{3}H]leucine-labelled normal and transformed CEFs after electrophoresis on a 8.5% SDS-polyacrylamide gel (see Materials and Methods). (1-4) SR-A RSV infected cells immunoprecipitated with (1) TBR serum, (2) TBR serum preabsorbed with 25 μg disrupted RSV, (3) TBR serum preabsorbed with 250 μg disrupted RSV, (4) preimmune serum, (5) uninfected CEFs immunoprecipitated with TBR serum. SDS gel standards are indicated in the right margin in daltons: phosphorylase a, 94 kD; bovine serum albumin, 68 kD; ovalbumin, 43 kD; carbonic anhydrase, 29 kD.

amounts of the 60 kD protein from radiolabelled extracts of normal chick cells (Fig. 7, lane 5). Similar results were obtained in immunoprecipitates from cells radiolabelled with [^{32}P]orthophosphate indicating that the transformation specific 60 kD protein is a phosphoprotein. These data as well as V8 protease peptide analysis of the 60 kD protein established that the TBR serum specifically recognizes pp60^{src} as originally described by Brugge and Erikson (1977).

The TBR serum was tested as a substrate of pp60^{src}-associated kinase activity in immunoprecipitates (Collett and Erikson, 1978). Significant amounts of radioactive phosphate were incorporated into IgG heavy chain when γ^{32}P-ATP was incubated with TBR serum immunoprecipitates of SR-A transformed cell extracts (Fig. 8, lane 1). Very little phosphotransferase activity was detected in TBR immunoprecipitates of normal cell extracts (Fig. 8, lane 2). In addition, very little transfer of phosphate occurred when non-immune serum was used to immunoprecipitate extracts of normal or transformed cells (Fig. 8, lanes 3 and 4). These results further indicate that the TBR serum recognizes pp60^{src}.

Characterization of pp60^{src} in the RR1022 Tumor Cell Line. Using the TBR serum a variety of cell lines were screened for pp60^{src} kinase activity. The rat cell line RR1022 was chosen as a source of kinase activity for fractionation experiments. This permanently transformed cell line was easy to grow in culture and also had a high level of kinase activity in TBR immunoprecipitates. The RR1022 rat cell line used was originally derived from a tumor induced in the inbred Amsterdam rat after injection of the Schmidt-Ruppin strain of ASV (Ahlström and Jonsson, 1961). The cells exhibit typical properties of transformation:

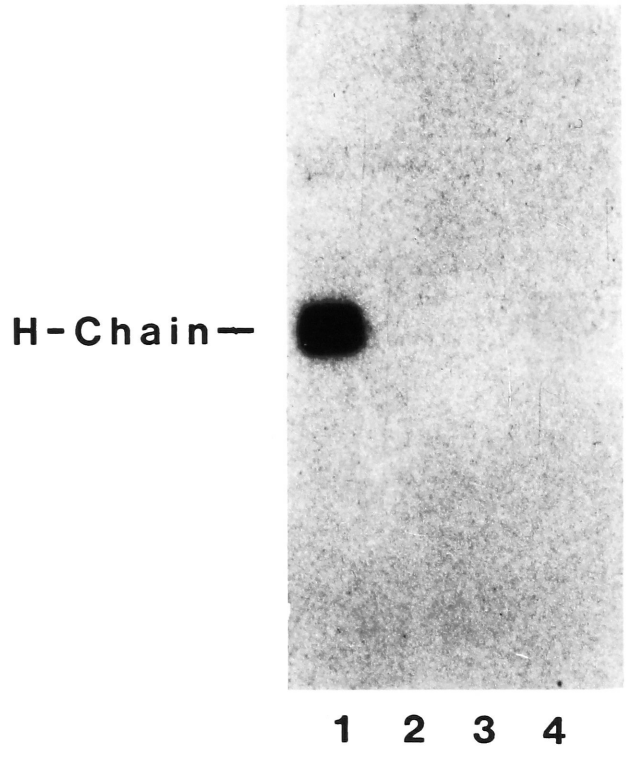


Fig. 8. Autoradiogram of the products of the immune complex kinase reaction after electrophoresis on an 8.5% SDS-polyacrylamide gel. (1 and 3) SR-A RSV-infected cell extracts were immunoprecipitated with (1) TBR serum, (3) preimmune serum; (2 and 4) uninfected CEF cell extracts were immunoprecipitated with (2) TBR serum, (4) preimmune serum. The reaction was performed as described in Materials and Methods.

diffuse immunofluorescence staining for actin, rounded morphology, reduced adhesion to substratum, and lack of density-dependent inhibition of growth. The cells produce tumors when transplanted into susceptible rats, but do not produce a detectable titer of infectious viral particles.

It was first necessary to verify that the kinase activity detected in TBR immunoprecipitates of rat cell extracts was actually due to the presence of $pp60^{src}$. TBR serum immunoprecipitated a 60,000 dalton phosphoprotein from the rat cells. The 60 kD protein is immunoprecipitated from [^{3}H]leucine labelled rat cell extracts by TBR serum (Fig. 9, lane 1) but not by non-immune rabbit serum (Fig. 9, lane 2). A phosphoprotein of the same molecular weight is the major protein immunoprecipitated with TBR serum from [^{32}P] labelled extracts of the rat cells (Fig. 9, lane 3). This 60 kD phosphoprotein is not detected in immunoprecipitates of [^{32}P] labelled rat cell extracts using an unrelated antibody raised against the cell plasma membrane (Fig. 9, lane 4). The amount of the 60 kD protein precipitated by the TBR serum was not diminished if the serum was preabsorbed with disrupted unlabelled SR-D RSV, indicating that the 60 kD protein is not a structural protein of Rous sarcoma virus. Other evidence verified that the RR1022 60 kD protein with associated kinase activity is related to $pp60^{src}$. The 60 kD phosphoprotein from the rat cells and $pp60^{src}$ yield two peptide fragments of 34 kD and 26 kD after V8 protease cleavage. Fragments generated after cleavage of the RR1022 60 kD protein are shown in Fig. 9, lane 5. More extensive V8 protease maps of the rat cell 60 kD protein and $pp60^{src}$ labelled with tritiated leucine are also similar. These results established that the 60 kD protein immunoprecipitated by TBR serum from the RR1022 cells is $pp60^{src}$.

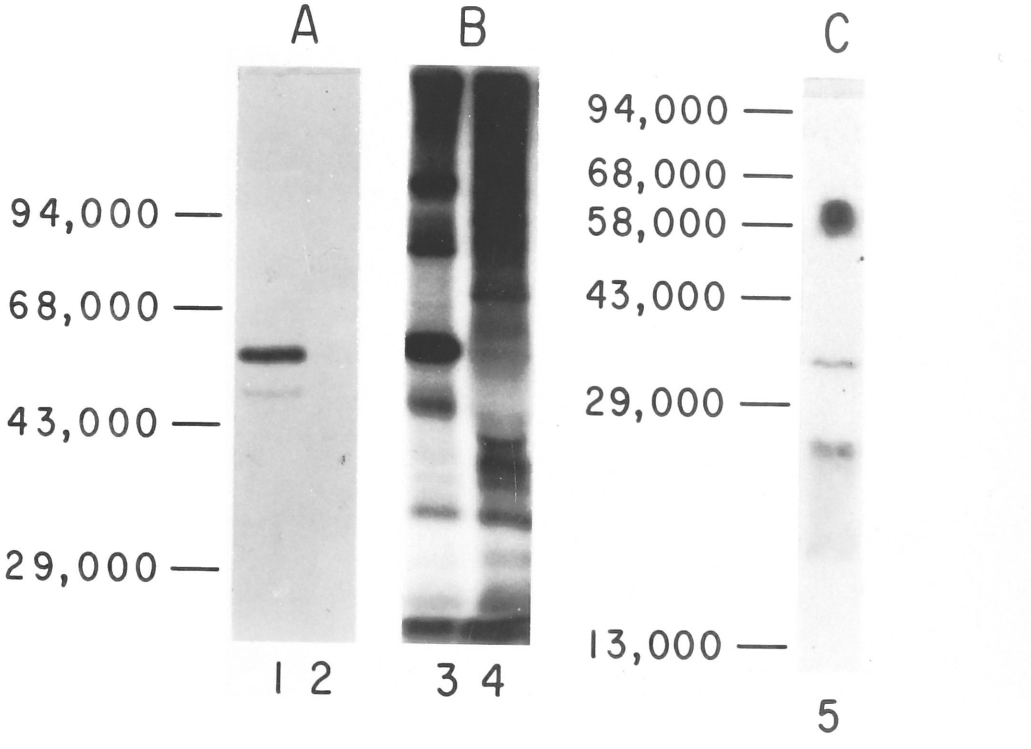


Fig. 9. Autoradiogram of immunoprecipitates of RR1022 cell extracts analyzed by SDS-polyacrylamide gel electrophoresis. (A) [^{3}H]-leucine-labelled cells, (B) [^{32}P] labelled cells; immunoprecipitated with (1) TBR serum, (2) preimmune serum, (3) TBR serum, (4) anti-plasma membrane serum. (C) V8 protease cleavage analysis of [^{3}H]leucine-labelled 60 kD protein immunoprecipitated by TBR serum from RR1022 cell extracts (described in Materials and Methods).

Filter Assay for pp60^{src}-Associated Protein Kinase Activity. Extracts of RR1022 cells stimulated phosphorylation of antibody heavy chains in immunoprecipitates with TBR serum but not with normal rabbit serum, as determined using the autoradiographic SDS-polyacrylamide gel assay for the pp60^{src}-associated protein kinase described by Collett and Erikson (1978). This assay has been modified to provide a faster quantitative method which could be used to rapidly screen column fractions for pp60^{src} kinase activity during fractionation experiments. Instead of analyzing the products of the kinase reaction by SDS-polyacrylamide gel electrophoresis, the ^{32}P-labelled IgG heavy chains in immunoprecipitates were released from protein-A-Sepharose beads by boiling in SDS and mercaptoethanol, precipitated with hot TCA, and collected on glass-fiber filters. As shown in Fig. 10, the recovery of radioactivity was comparable in both assays. Increasing the amount of rat cell extract increased the amount of ^{32}P incorporated into IgG heavy chains. When large volumes of extract were used, inhibition of the pp60^{src}-associated kinase activity was observed. Similar levels of inhibition were detected in both systems; this inhibition may result from nonspecific competition by other proteins during immunocomplex formation.

Partial Purification of the pp60^{src}-Associated Kinase Activity. A purification protocol was developed by which it was possible to purify the pp60^{src}-associated kinase activity several thousand fold. The filter assay described above was used to screen column fractions rapidly. Cells were extensively homogenized in detergent free buffers and centrifuged at 100,000 g. Up to 50% of the kinase activity was recovered in the cell supernatant. The rest of the activity was in the membrane containing pellet.

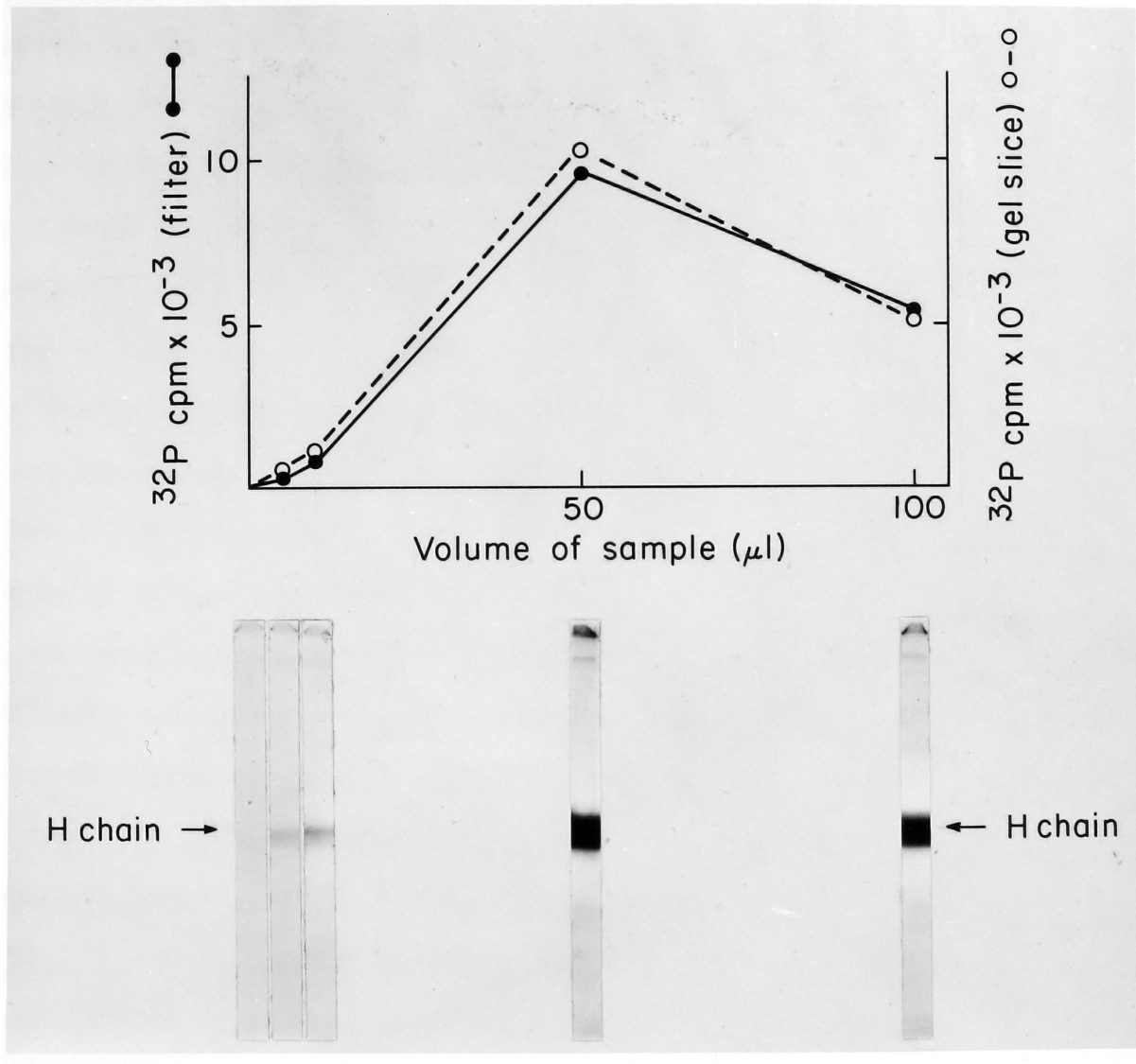


Fig. 10. Comparison of filter assay with SDS-polyacrylamide gel assay. Increasing amounts of rat cell extract were assayed by the filter assay (●) and by running the samples on SDS-polyacrylamide gels, cutting out the IgG heavy chain and quantitating by liquid scintillation counting (o). The phosphorlyation of the IgG heavy chain was detected by autoradiography.

The soluble fraction was fractionated by ammonium sulfate precipitation, followed by sequential column chromatography on ω-aminohexylagarose, phosphocellulose, and casein-Sepharose. The results of the chromatographic steps are shown in Fig. 11 A, B and C. A good separation of the pp60^{src} kinase activity from the bulk of the contaminating protein was achieved during each fractionation step. The results of the purification scheme are summarized in Table II. The overall fractionation resulted in greater than a 1000 fold purification of pp60^{src} kinase activity with a 5% yield. The protein obtained after the casein-Sepharose column was, however, not homogeneous when analyzed by SDS gels stained with silver nitrate (Merrill et al., 1980) to detect proteins.

In order to verify that pp60^{src} actually copurified with the kinase activity, rat cells were radiolabelled with tritiated leucine and the same purification scheme was followed. TBR serum immunoprecipitated pp60^{src} from detergent extracts of rat cells (Fig. 12, lane 1). When rat cells were extracted without detergent as in the purification scheme, the TBR serum immunoprecipitated a 52,000 molecular weight protein (Fig. 12, lane 3). This 52 kD protein was also present in TBR immunoprecipitates of the most purified column fractions (Fig. 12, lane 5). Lanes 2, 4 and 6 in Fig. 12 are comparable immunoprecipitates using preimmune rabbit serum. The 52 kD protein was shown to be similar to pp60^{src} by peptide analysis and most likely arises by proteolytic cleavage of pp60^{src} during cell lysis.

Krueger et al. (1980b) have reported that under certain conditions of cell lysis, pp60^{src} can be cleaved to a smaller molecular weight species designated pp52^{src}. They have shown that pp60^{src} is associated

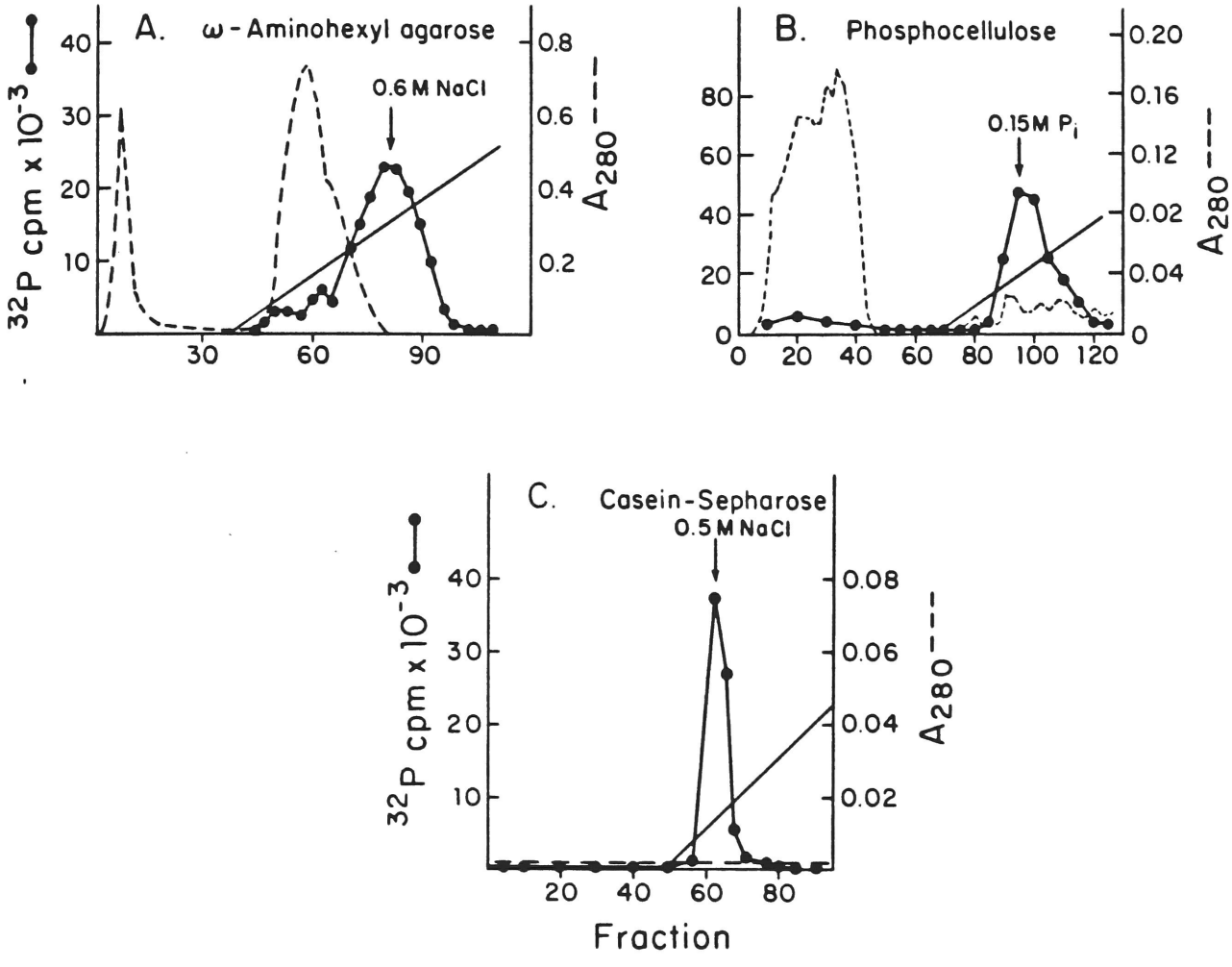


Fig. 11. Purification of pp60^{src} kinase activity by column chromatography (A) Chromatography of the 20-40% ammonium sulfate fraction on ω-aminohexyl agarose. (B) Phosphocellulose chromatography of the active pp60^{src} fractions obtained from ω-aminohexyl agarose chromatography. (C) Casein-Sepharose chromatography of the active pp60^{src} fractions obtained from the phosphocellulose column. (●—●) represents the pp60^{src}-associated protein kinase activity in TBR serum immunoprecipitates of column fractions. (---) designates the absorbance of column fractions at A_{280} nm.

TABLE II

Purification of pp52^{src} from RR1022 Tumor Cells

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Extract	1625	280	0.17
20-40% Ammonium Sulfate	235	180	0.77
ω-Aminohexyl Agarose	15	80	5.3
Phosphocellulose	0.19	21	110
Casein-Sepharose	< 0.03	15	500

One unit of activity incorporates $1X10^6$ cpm into IgG heavy chains in 10 min at 37°C under the conditions described for the filter assay in Materials and Methods.

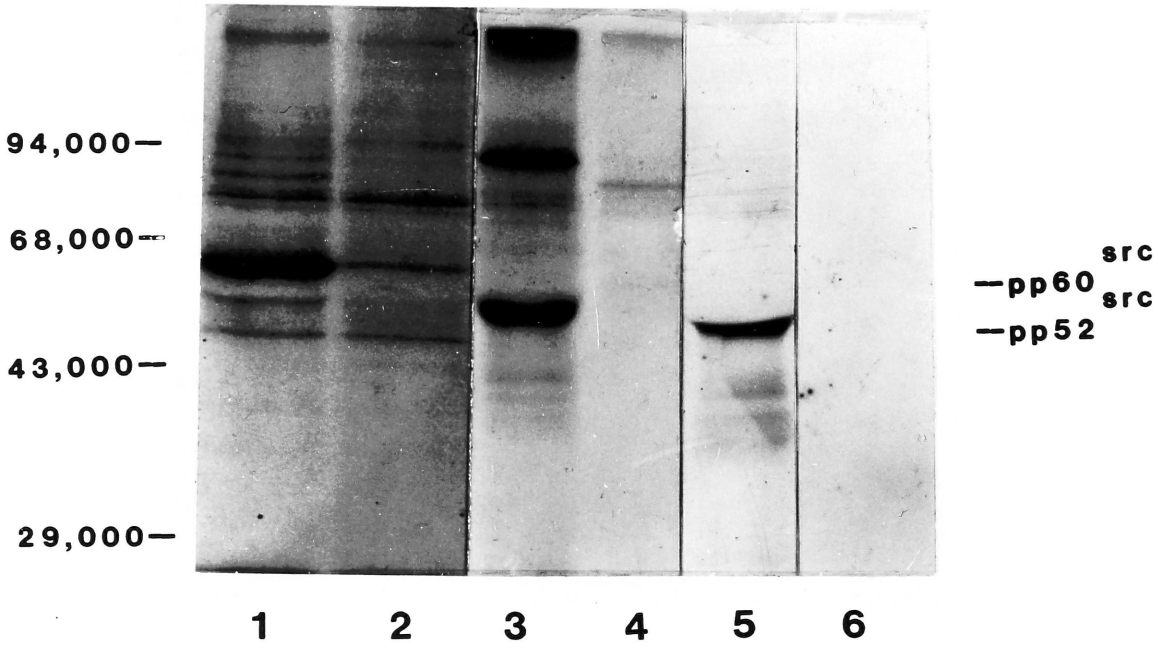


Fig. 12. Autoradiogram of immunoprecipitates of partially purified $pp60^{src}$ kinase activity analyzed by SDS-polyacrylamide gel electrophoresis. (1 and 2) are detergent extracts of RR1022 cells, (3 and 4) detergent free extracts of RR1022 cells, (5 and 6) partially purified $pp60^{src}$ kinase activity; (1,3 and 5) were immunoprecipitated with TBR serum, (2,4, and 6) with preimmune serum.

with cell membranes and have proposed that cleavage to the 52 kD form results in the release of $pp52^{src}$ into the cytoplasmic fraction. If RR1022 cells are lysed in the cold, in the presence of detergents, no $pp52^{src}$ is detected in immunoprecipitates, only $pp60^{src}$. This suggests that the 52 kD protein does not exist in an intact cell.

Partial Purification of $pp60^{src}$ as an Intact Species. $pp60^{src}$ was purified in the absence of detergents, since detergents could interfere with *in vitro* and *in vivo* assays of the enzymes activity. Purified $pp52^{src}$ proved to be useful for studying the protein's activity in detergent free solution (see sections IV and V). Detergents are, however, necessary to extract $pp60^{src}$ from membranes in high yield in an undegraded form. Since it is possible that degradation of $pp60^{src}$ to the 52 kD protein might result in a protein with an altered enzymatic activity, it would be useful to purify $pp60^{src}$ as an intact species.

Erikson et al. (1979) reported the partial purification of $pp60^{src}$ in an undegraded form. An alternative protocol has been devised for the purification of intact $pp60^{src}$. In this new protocol, which gives more reproducible results, cells are lysed in dilute suspension in the presence of high concentrations of detergents and protease inhibitors. The extracted $pp60^{src}$ was found to be stable as a 60 kD species in crude cell extracts incubated at $4^{o}C$ for at least a week. $pp60^{src}$ was purified from [^{3}H]leucine labelled rat cells by sequential column chromatography on affi-gel blue, ω-aminohexylagarose, phosphocellulose, and casein-Sepharose affinity supports. Column fractions were analyzed for $pp60^{src}$-associated kinase activity by the filter assay procedure. The results

of purification scheme are shown in Fig. 13 A, B, C and D. $pp60^{src}$ was located by immunoprecipitation of radiolabelled column fractions with TBR serum and was found in all cases to co-migrate exclusively with the kinase activity.

In some preparations as much as 20% of the $pp60^{src}$ degraded during the purification to the 52 kD species; however, the two src species eluted in separate fractions (Fig. 13, panel D), pools A and B, from the casein-Sepharose column. The 52 kD species was found in TBR immunoprecipitates of pool A (Fig. 14, lane 3). $pp60^{src}$ was present in TBR immunoprecipitates of pool B (Fig. 14, lane 4). The partially purified $pp60^{src}$ was not homogeneous when analyzed by SDS gel electrophoresis and visualized by autoradiography (Fig. 14, lane 2). The partially purified $pp52^{src}$ was also not homogeneous (Fig. 14, lane 1). In the partially purified $pp60^{src}$ pool a major band migrating with an apparent molecular weight of 60,000 daltons was present. Other proteins in the preparation of 47,000 and 50,000 daltons (Fig. 14, lane 2) may be substrate proteins that interact with and copurify with $pp60^{src}$. Proteins of similar molecular weight were found by Purchio (1982) to copurify with $pp60^{src}$ under somewhat different conditions.

Molecular Characteristics of Partially Purified $pp60^{src}$. The native molecular weight of the partially purified proteins was estimated. $pp52^{src}$, detected by its associated kinase acitivity, sedimented on glycerol gradients at a rate similar to that of bovine serum albumin (Fig. 15A) and migrated with an apparent molecular weight of approximately 60,000 daltons during gel filtration on Sephacryl S-200 (Fig. 15B). Partially purified $pp60^{src}$ also cosedimented on glycerol gradients with bovine serum albumin. These results indicate that the partially purified

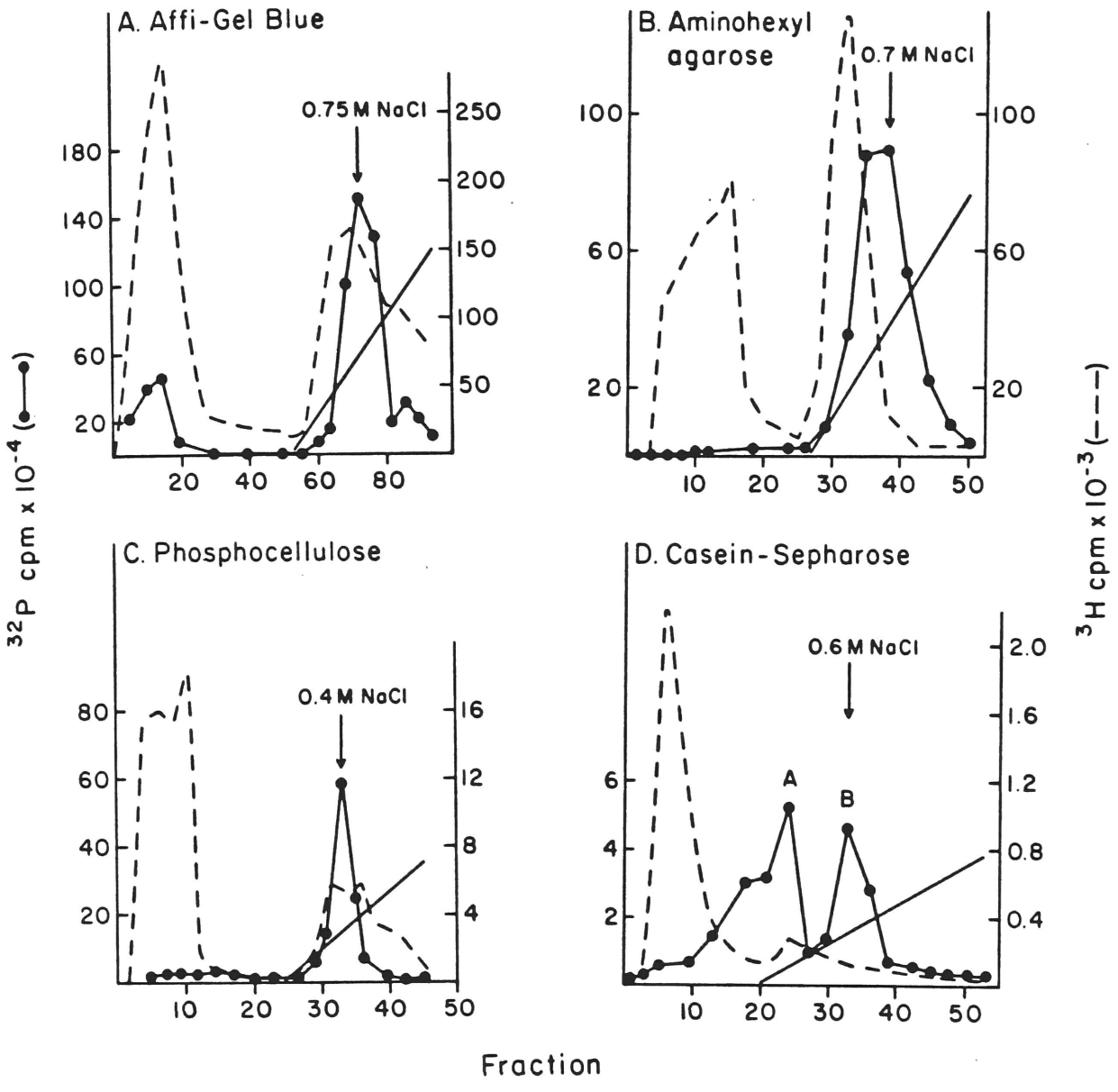


Fig. 13. Purification of intact pp60^{src} in the presence of detergents. Pp60^{src} was purified by sequential column chromatography on (A) affi-gel blue, (B) ω-aminohexyl agarose, (C) phosphocellulose, and (D) casein-Sepharose. (---) designates the total protein profile measured by counting column fractions for [^{3}H]. (●—●) represents the pp60^{src}-associated protein kinase activity in TBR serum immunoprecipitates of column fractions.

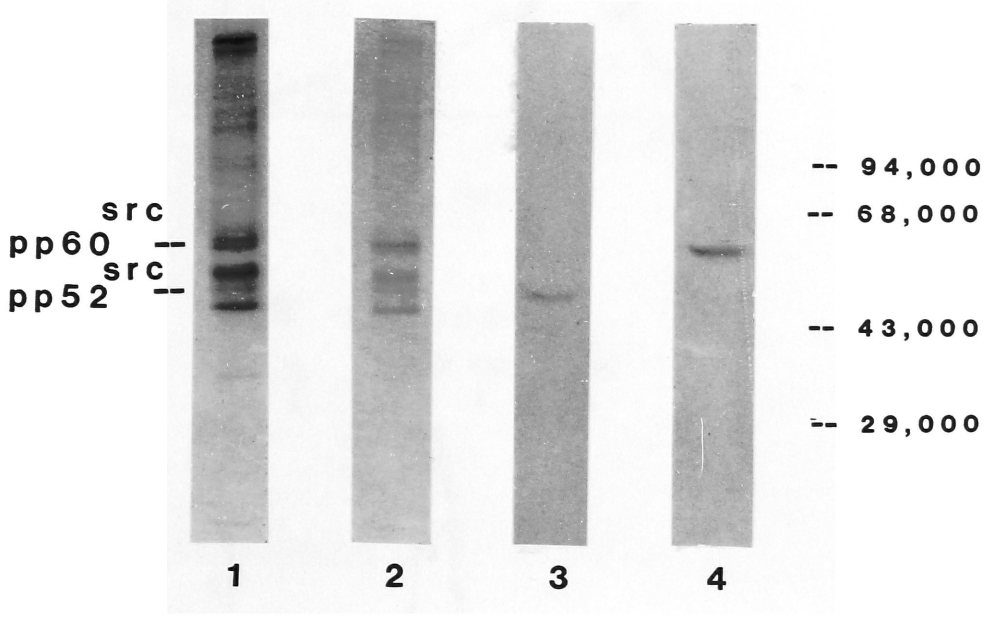


Fig. 14. Autoradiogram of partially purified pp60^{src} analyzed by SDS-polyacrylamide gel electrophoresis. (1) Pool A, (2) pool B, (3) pool A immunoprecipitated with TBR serum, (4) pool B immunoprecipitated with TBR serum.

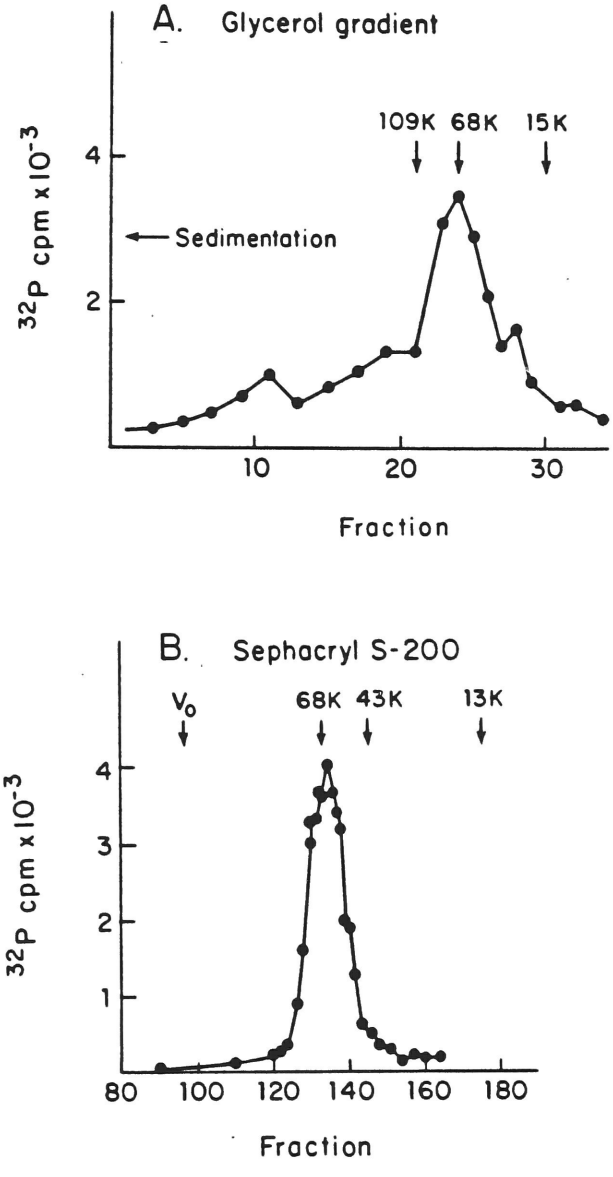


Fig. 15. (A) Native molecular weight determination of $pp52^{src}$. Glycerol gradient centrifugation. Partially purified $pp52^{src}$ fractions were layered on a linear gradient of 10-30% (wt/vol) glycerol in 10 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 1 mM $MgCl_2$ and centrifuged for 40 hr at 40,000 rpm in an SW 40 Beckman rotor (Freifelder, 1973). The gradient was calibrated with E. coli DNA polymerase I (109 kD), bovine serum albumin (68 kD) and lysozyme (15 kD). (B) Sephacryl S-200 chromatography. Partially purified $pp52^{src}$ was applied to a column of Sephacryl S-200 (1.4x100 cm) previously equilibrated in 10 mM potassium phosphate, (pH 7.0), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.34 M sucrose, 0.5 M NaCl, 1 mM diothiothreitol. The column was calibrated with blue dextran (Vo), bovine serum albumin (68 kD), ovalbumin (43 kD), and cytochrome c (13 kD).

src proteins are monomers. They have a native molecular weight of approximately 60,000 daltons similar to the weight determined on SDS gels.

When the partially purified $pp52^{src}$ was analyzed by isoelectric focusing under non-denaturing conditions (Holtlund and Kristenson, 1978), two peaks of src kinase activity were detected with apparent pIs of 5.0 and 6.0 (Fig. 16). The relative size of the two peaks varied in different preparations. Possibly the two peaks represent $pp52^{src}$ in different states of phosphorylation or $pp52^{src}$ interacting with other protein components present in the preparation.

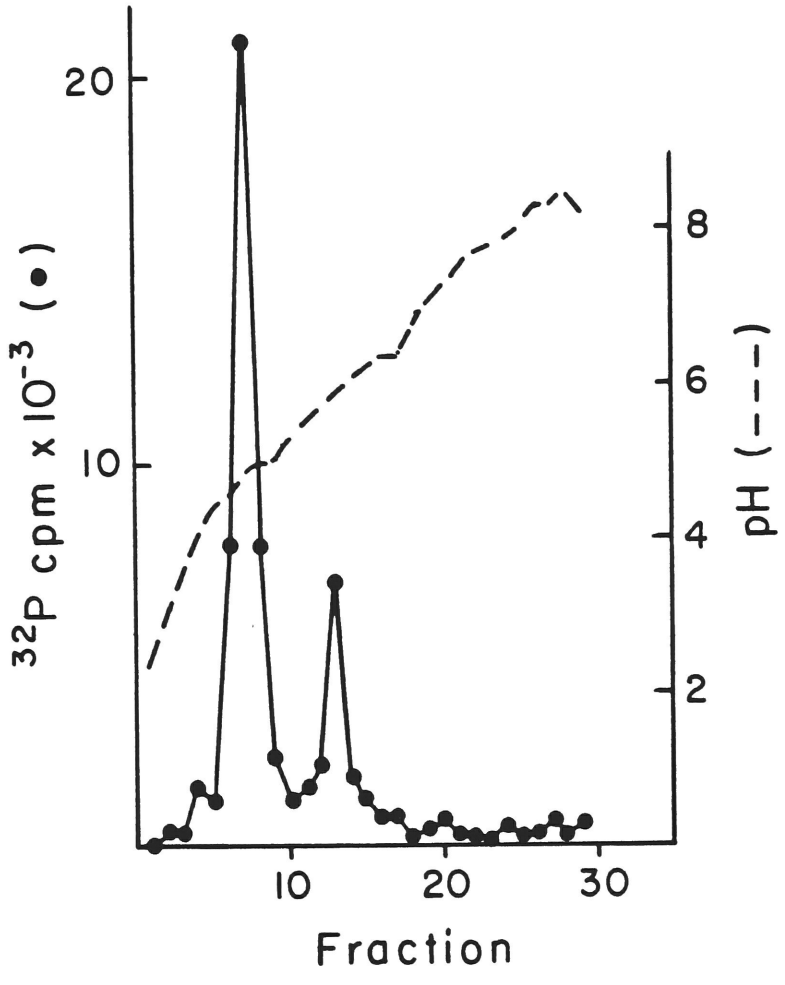


Fig. 16. Isoelectric focusing of $pp52^{src}$ was by the method of Holtlund and Kristensen (1978). $Pp52^{src}$ was dialyzed into 20% sucrose, 1 mM DTT, 0.1 mM EDTA, and focused for 20 hr in a 20-40% (6 ml) sucrose density gradient containing LKB ampholytes pH 5-8. Gradient fractions were collected (0.3 ml/tube), immunoprecipitated with TBR serum and assayed for transfer of phosphate from [γ-^{32}P]ATP to IgG heavy chain (•—•). The pH of the column fractions was recorded (---).

Conclusions

TBR serum prepared by injection of newborn rabbits specifically recognized $pp60^{src}$ as well as structural proteins of Rous sarcoma virus in extracts of transformed cells. $pp60^{src}$ and viral structural proteins were not detected in normal cell extracts. A significant amount of transfer of phosphate from $\gamma[^{32}P]$-ATP to IgG heavy chain was observed in TBR immunoprecipitates of transformed chick cell extracts.

TBR immunoprecipitates of extracts from the rat tumor cell line RR1022 contained a high level of phosphotransferase activity. The TBR serum immunoprecipitated a 60 kD phosphoprotein from these cells which was shown to be $pp60^{src}$. The $pp60^{src}$-associated kinase activity was purified from the RR1022 cells over a thousand-fold in detergent free solution. Analysis of the purified protein indicated that it was degraded to a smaller species with an apparent molecular weight of 52,000 daltons. It was determined that detergents are required to extract $pp60^{src}$ from cell membranes in high yield in an undegraded form. A second reproducible protocol was developed which allows the purification of $pp60^{src}$ as an intact species.

The partially purified src proteins will be useful for functional microinjection studies and also for *in vitro* assays of $pp60^{src}$ function.

IV. BIOCHEMICAL ACTIVITIES OF PP60^{src}

Assays of pp60^{src} activity in TBR serum immunoprecipitates suggested that pp60^{src} was tightly associated with an unusual kinase activity, but little was known about the activity of pp60^{src} in solution. pp60^{src} phosphorylates TBR IgG in immunoprecipitates, but does not catalyze the transfer of phosphate from ATP to classical kinase substrates such as casein or histones added exogenously to immunoprecipitates. It is likely that pp60^{src} sequestered by the antibody in immunoprecipitates is unable to interact with substrates, and therefore no phosphorylation occurs. In solution, however, pp60^{src} is accessible to interaction with substrates; but in crude cell extracts containing pp60^{src} no IgG heavy chain kinase activity is observed. It is likely that the kinase activity is obscured by competing kinases, phosphatases or inhibitors present in the extract.

This chapter describes experiments which examined the activity of partially purified pp60^{src} in solution. In particular the substrate specificity of the pp60^{src} kinase was tested. First, the ability of partially purified pp60^{src} to catalyze the phosphorylation of classical kinase substrates was measured. Later, additional experiments examined the affect of pp60^{src} on cytoskeletal proteins and other potential *in vivo* substrates.

Materials and Methods

Reagents. Histones type IIA, phosvitin, casein, avidin, bovine serum albumin, phosphorylase a, alcohol dehydrogenase, lactate dehydrogenase and malate dehydrogenase (cytosolic) were purchased from Sigma (St. Louis, Mo.). Malate dehydrogenase (mitochondrial) was purchased from Boehringer-Mannheim (Indianapolis, Ind.). Concanavalin A was a gift from Dr. John Hemperley (The Rockefeller University). β_2 microglobulin was a gift of Dr. Jack Ziffer (The Rockefeller University). Phosphotyrosine was synthesized from tyrosine, phosphoric acid, and phosphorous pentoxide as described by Rothenberg et al. (1978).

Assay of Phosphotransferase Activity. The reaction solution contains 10 mM Tris-HCl (pH 7.5), 1 mM β-mercaptoethanol, 1 mM EDTA, 0.05% NP-40, 20% glycerol, 5 mM $MgCl_2$ and 0.1-1 μM [γ-^{32}P]ATP (800-1000 Ci/mmole, Amersham). The kinase reaction was initiated by the addition of [γ-^{32}P]ATP and $MgCl_2$ to the solution containing pp60^{src} and protein substrate in a total volume of 50 μl. After incubation for 20 min at 30°C the reaction was quenched by the addition of 25 μl 3-times concentrated electrophoresis sample buffer, heated to 95°C for 5 min, and analyzed by SDS polyacrylamide gel electrophoresis. Gels were stained, destained, dried and radiolabelled proteins were visualized by autoradiography.

Phosphoamino Acid Analysis. Proteins were processed for phosphoamino acid analysis by a modification of the procedure of Hunter and Sefton (1980). SDS gels were dried without fixing and the position of the ^{32}P band to be excised for phosphoaminoacid analysis was detected by

autoradiography. The gel band was cut out and swollen for 5 min in 25 mM $(NH_4)HCO_3$, pH 7.0 and then digested in 0.5 ml 0.1 mg proteinase K per ml 25 mM $(NH_4)HCO_3$, overnight with shaking at 37°C. The digested protein mixture was centrifuged in a Beckman microfuge, the supernatant was removed and saved and the gel pellet was reextracted with 0.5 ml 25 mM $(NH_4)HCO_3$, pH 7.0, for five hours. The mixture was microfuged again and the two supernatants combined. The extracted material was lyophilized, the pellet resuspended in H_2O and relyophilized several times. For acid hydrolysis the residue was dissolved in 1 ml 6 M HCl by heating to 100°C under vacuum. The HCl was removed by repeated lyophilization and the hydrolysate was dissolved in a marker mixture containing phosphoserine, phosphothreonine, and phosphotyrosine. The acid hydrolysates with standards were analyzed by one dimensional thin layer electrophoresis on 100 μm cellulose plates. Electrophoresis was at pH 3.5 for 90 min at 1 KV in glacial acetic acid/pyridine/H_2O, 5:5:945 (vol/vol). Frequently the hydrolysates were analyzed by two dimensional thin layer electrophoresis. In this case electrophoresis in the first dimension was at pH 1.9 for 90 min at 1 KV in glacial acetic acid/formic acid (98% by vol)/H_2O, 78:25:297 (vol/vol) and in the second dimension at pH 3.5 as described above. Phosphoamino acid standards were detected by staining with ninhydrin and the radioactive amino acids were detected by autoradiography.

Purification of Cytoskeletal Proteins. Tubulin was purified according to the procedure of Bloodgood and Rosenbaum (1976). Fresh calf brain was homogenized 2 gm/ml PM buffer (50 mM Pipes [pH 6.9], 0.5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM GTP) in a Waring blender. The homogenate was centrifuged at 100,000 g for 1 hour at 4°C. The supernatant was mixed with an

equal volume of PM buffer with 8 mM glycerol, incubated at 37°C for 40 min, and centrifuged at 100,000 g for 1 hour at 30°C. The microtubular pellet was resuspended in 1/5 the original volume of PM buffer, homogenized and incubated for 30 min at 4°C to redissolve the tubulin dimers. After centrifugation at 4°C the supernatant was again mixed with an equal volume of PM-glycerol buffer and left at 37°C for 40 min. The microtubules were centrifuged, resuspended in PM buffer at 4°C, centrifuged and the depolymerized tubulin stored in glycerol at -20°C.

Dry acetone powders from freshly excised rabbit skeletal muscle were prepared by the method of Straub (Feuer et al., 1948) and actin was purified according to the procedure of Spudich and Watt (1971). The acetone powder was extracted for 30 min with buffer A (2 mM Tris-HCl, [pH 8.0], 0.2 mM ATP, 0.5 mM β-mercaptoethanol, and 0.2 mM $CaCl_2$). The extract was filtered and the residue re-extracted two times. The filtrates were combined and centrifuged at 10,000 g for 1 hour, 4°C. KCl (to 50 mM) and $MgCl_2$ (to 2 mM) were added slowly to the supernatant and the actin was left to polymerize with slow stirring at 4°C for 2 hours. KCl was then brought to a final concentration of 0.6 M and the solution was stirred for 2 hours at 4°C followed by 2 hours at room temperature and then centrifuged at 100,000 g for 3 hours at 4°C. The actin-containing pellet was resuspended in buffer A and dialyzed against buffer A for 3 days at 4°C. This buffer depolymerizes F-actin to the G-actin monomer. The G-actin was clarified by centrifugation at 100,000 g for 3 hours at 4°C and polymerized to F-actin by the addition of KCl to 50 mM and $MgCl_2$ to 2 mM, followed by stirring for 2 hours at room temperature. KCl was added to a final concentration of 0.5 M and the solution became viscous and cloudy. After stirring for 2 hours at room temperature the F-actin was stored at 4°C and used within a week.

Vinculin was purified by the method of Feramisco and Burridge (1980). Frozen chicken gizzards were thawed, ground up, and homogenized with 10 volumes of distilled water, 0.5 mM PMSF ($4^{o}C$) in a Waring blender. The homogenate was centrifuged 10,000 g, 10 min and the pellet was re-extracted same as above. After centrifugation the pellet was resuspended in 10 volumes of buffer B at $37^{o}C$ (2 mM Tris, 1 mM EGTA, 0.5 mM PMSF, pH 9.0, room temperature). The suspension was stirred 30 min, centrifuged, and the pellet discarded. The pH of the supernatant was adjusted to 7.2 with acetic acid and 1 M $MgCl_2$ was added to a final concentration of 10 mM. A precipitate formed and after stirring for 15 min the suspension was centrifuged at room temperature. The supernatant was cooled to $4^{o}C$ and protein was precipitated by the addition of ammonium sulfate (14.9 gm $(NH_4)_2SO_4$/100 ml supernatant). The suspension was centrifuged 10,000 g for 10 min and additional $(NH_4)_2SO_4$ added to the supernatant (5.6 gm/100 ml supernatant). The suspension was centrifuged, the pellet resuspended in and dialyzed against buffer C (20 mM Tris-acetate (pH 7.6), 20 mM NaCl, 0.1 mM EDTA, 15 mM β-mercaptoethanol. The dialyzed solution was applied to a DEAE-cellulose column (1.2x20 cm) equilibrated in buffer C, washed with buffer C, and eluted with a linear salt gradient of NaCl (0-0.37 M) in buffer C. Three protein peaks were eluted by the salt gradient. The first peak contained highly purified vinculin, the second peak was enriched in filamin and the third peak contained partially purified α-actinin.

Purification of filamin was as described by Shizuta et al. (1976). Briefly, chicken gizzards were minced and then homogenized with 5 mM

EDTA, 5 mM dithiothreitol pH 7.0. The homogenate was centrifuged and the supernatant extracted in high salt buffer, 50 mM potassium phosphate (pH 7.5), 0.6 M KCl, 1 mM EDTA, 1 mM DTT. After centrifugation the high salt extract was dialyzed overnight. Dialyzed protein was precipitated with ammonium sulfate (35% saturation), the pellet resuspended, dialyzed and chromatographed on a Sepharose 6B column. Three major peaks of protein were eluted from the column; filamin was present in the second peak. The filamin peak was applied to a DEAE cellulose column equilibrated with 20 mM potassium phosphate (pH 7.5), 0.5 mM EDTA, 0.5 mM DTT. The column was washed with this buffer and eluted with a linear salt gradient of NaCl (0-0.4 M) in the column buffer. The first peak of protein eluted with the salt gradient contained highly purified filamin when analyzed by SDS polyacrylamide gel electrophoresis.

Myosin was purified from rabbit skeletal muscle as described by Perry (1956). Tropomyosin, prepared by the procedure of Greaser and Gergely (1971), was a gift from Dr. Minnie O'Farrell (The Rockefeller University).

Purification of 34K Phosphoprotein. The 34 kD protein was purified by the procedure of Erikson and Erikson (1980) except for a few modifications. Confluent primary chick embryo fibroblasts were scraped from roller bottles, washed 3 times with 1XPBS, quickly frozen in liquid N_2 and stored at -70°C. 12 ml thawed chick cells were resuspended in 120 ml buffer D (10 mM Tris-HCl (pH 7.2), 1 mM EDTA, 0.05% NP-40, 1 mM β-mercaptoethanol, 1% trasylol) and homogenized with 40 strokes in a Dounce homogenizer with a tight fitting plunger. The homogenate was centrifuged at 100,000 g for 45 min., the supernatant brought to 10%

glycerol and applied to a DEAE cellulose column (2 x 35 cm) equilibrated with buffer D/10% glycerol. The unbound fraction from the DEAE cellulose column was directly applied to a hydroxylapatite column (Bio-Gel HTP, Biorad) equilibrated with buffer D/10% glycerol. The hydroxylapatite column was washed with buffer D/glycerol, washed with buffer E (10 mM Tris-HCl [pH 7.2], 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, 1% trasylol) and eluted with a gradient of potassium phosphate (0-0.5 M) in buffer E. The 34 kD protein eluted at 300-400 mM potassium phosphate, fractions were analyzed by SDS polyacrylamide gel electrophoresis, and proteins visualized by staining with silver nitrate. The 34 kD protein was highly purified at this stage but was purified further by concentration and then fractionation on Sephacryl S-200.

Results

A. Inhibitors of pp60^{src} Kinase Activity in Cell Extracts. Significant amounts of phosphatase activity were detected in crude cell extracts of transformed cells. To demonstrate the presence of phosphatases or other inhibitors in the cell extracts, crude ammonium sulfate fractions and pp60^{src} fractions purified by column chromatography on ω-aminohexyl agarose were added to immunoprecipitates containing pp60^{src} and the kinase assay was initiated with [γ-^{32}P]ATP (Table III). Addition of the crude ammonium sulfate fraction caused a significant reduction in the amount of ^{32}P incorporated into immunoglobulin heavy chains, as measured by the filter assay. However, addition of purified pp60^{src} did not significantly reduce the ^{32}P incorporation. Furthermore, incubation of the ammonium sulfate fraction with immunoprecipitates previously labelled with ^{32}P resulted in marked dephosphorylation of the immunoglobulin heavy chains. In contrast, the purified pp60^{src} fraction contained little dephosphorylating activity. These results suggest that a phosphatase activity acting on phosphorylated IgG was present in crude extracts but was removed by hydrophobic chromatography.

Further evidence for a phosphatase in crude extracts was obtained from studies on the kinetics of heat inactivation of pp60^{src} kinase activity. The phosphatase inhibitor sodium fluoride increased the half-life of pp60^{src}-associated kinase activity in crude cell extracts from 2.5 to 6.3 minutes during incubation at 42^{o}C. Purification of pp60^{src} increased the half-life of kinase activity to 12.8 min, and the addition of sodium fluoride resulted in no further increase in the half-life of

TABLE III

Dephosphorylation of IgG Heavy Chain by Fractionated Cell Extracts

Fraction added	^{32}P incorporation (cpm)	
	added before reaction	added after reaction
Buffer	73,500	84,800
Ammonium sulfate	1,000	500
ω-Aminohexyl agarose	76,200	50,000

Equal amounts of $pp60^{src}$ (0.08 units) were immunoprecipitated from an ω-aminohexyl agarose fraction with TBR serum. Either buffer (20 mM Tris-HCl, [pH 7.2], 5 mM $MgCl_2$, 10 mM DTT), an aliquot of the ammonium sulfate fraction of $pp60^{src}$ (0.0006 units), or an aliquot of the ω-aminohexyl agarose fraction of $pp60^{src}$ (0.0006 units) was added. The kinase reaction was initiated by adding [γ-^{32}P]ATP. After 10 min at 37°C, samples were washed to remove [γ-^{32}P]ATP. An aliquot of either the ammonium sulfate fraction (0.0006 units) or the ω-aminohexyl agarose fraction (0.0006 units) was then added to samples that had received buffer initially. All samples were incubated for a further 10 min at 37°C before assay by the filter method. Results were confirmed by SDS-polyacrylamide gel electrophoresis.

the purified kinase activity. These data are consistent with the possibility that the purification removed a phosphatase that could act on phosphorylated $pp60^{src}$ to render its activity more thermolabile. Similarly, sodium fluoride was found to increase the thermostability of the src kinase activity in extracts of ASV-infected chick embryo fibroblasts made in a different buffer (Rübsamen et al., 1979). However, the identity of the activity sensitive to sodium fluoride and the phosphatase activity has not yet been established.

B. Phosphorylation of Casein and TBR IgG by $pp60^{src}$ in Solution. Removal of the inhibitory activity made it possible to detect src kinase activity in solution. The $pp60^{src}$ purified through the casein-Sepharose step (Chapter III) phosphorylated IgG heavy chains from TBR serum in solution but did not phosphorylate IgG from preimmune rabbit serum. In contrast, addition of [γ-^{32}P]ATP and TBR serum to the ammonium sulfate fraction did not lead to incorporation of ^{32}P into IgG.

Partially purified $pp60^{src}$ phosphorylated α-casein in solution when incubated with [γ-^{32}P]ATP and magnesium, as shown in two experiments (Fig. 17, lanes 2 and 3). A low level of endogenous phosphorylation was observed when casein was incubated with [γ-^{32}P]ATP alone (Fig. 17, lane 1). The ability of partially purified $pp60^{src}$ to phosphorylate casein was inhibited by the addition of TBR IgG to the reaction (Fig. 17, lane 5) but not by incubation with preimmune IgG (Fig. 17, lane 4). The inhibition of casein phosphorylation by TBR IgG indicates that casein is being phosphorylated by $pp60^{src}$ and not by another kinase present as a contaminant in the enzyme preparation.

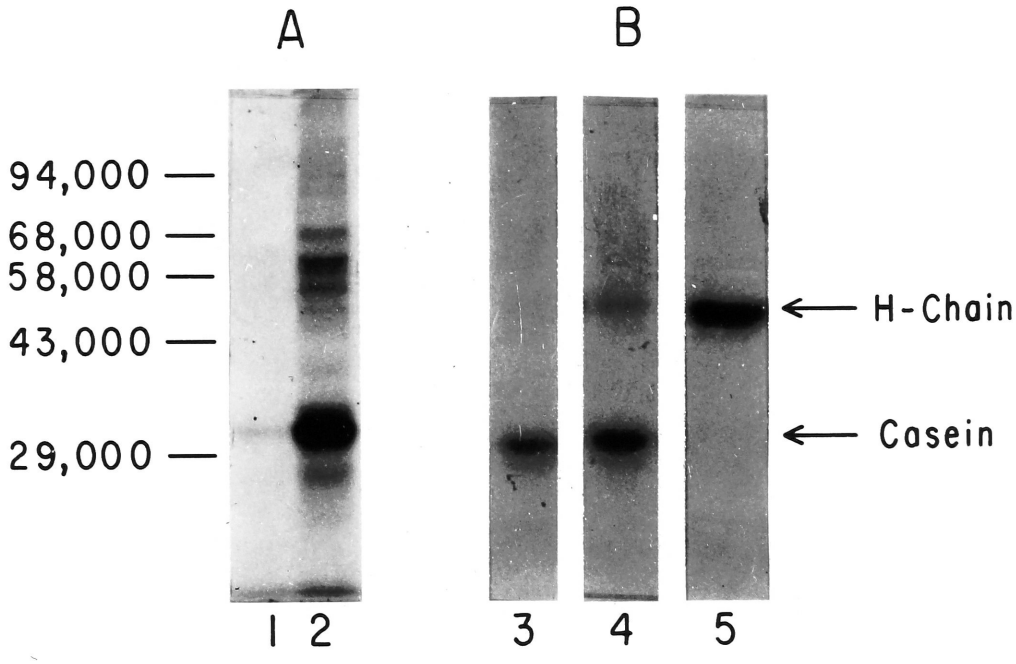


Fig. 17. Phosphorylation of α-casein by partially purified pp60^{src}. In two separate experiments (A) and (B) α-casein was incubated with [γ-^{32}P]ATP and Mg^{2+} in the presence of pp60^{src}, (lanes 2, 3, 4 and 5); or the absence of pp60^{src}, (lane 1). The pp60^{src} specificity of the reaction was tested by adding 10 μl 1.0 mg/ml preimmune rabbit IgG, (lane 4) or 10 μl 1.0 mg/ml TBR IgG, (lane 5) to the reaction solution. The reaction was run as described in Materials and Methods; products were analyzed by SDS-polyacrylamide gels and visualized by autoradiography.

Under similar conditions, histones (Sigma Type IIA) and phosvitin were not phosphorylated by pp60^{src} either in the presence or in the absence of cAMP. Casein phosphorylation by pp60^{src} was unaffected by the addition of cAMP or cGMP, whereas the phosphorylation of both histones and casein by the cAMP-dependent protein kinase was enhanced 10 fold by the addition of cAMP.

The phosphorylation of casein by pp60^{src} exhibits a linear time dependence for at least 30 minutes at 25^{o}C. This is in contrast to phosphorylation of TBR IgG by pp60^{src} in immunoprecipitates which is essentially complete within 5 minutes at 4^{o}C. The casein phosphorylation reaction requires the addition of 10 mM magnesium or manganese.

The nucleotide specificity of pp60^{src} in immunoprecipitates is quite broad. It was found that both CTP and GTP can serve as phosphate donors in the kinase reaction (Richert et al., 1979a; Krueger et al., 1980b). Partially purified pp60^{src} was found to use [γ-^{32}P]GTP as a phosphate donor in the phosphorylation of casein (Fig. 18, lane 1) or TBR IgG (Fig. 18, lane 3) in solution. Preimmune rabbit IgG was not phosphorylated by pp60^{src} under similar conditions (Fig. 18, lane 5). No detectable endogenous phosphorylation of casein, TBR IgG or preimmune IgG occurred when these reagents were incubated with [γ-^{32}P]ATP alone (Fig. 18, lanes 2, 4 and 6).

Hunter and Sefton (1980) have found that in immunoprecipitates the pp60^{src} is novel in that it phosphorylates a tyrosine residue on TBR IgG heavy chain. All previously known protein kinases phosphorylate either serine or threonine residues. The site of phosphorylation of substrates reacted with partially purified pp60^{src} in solution was determined. TBR

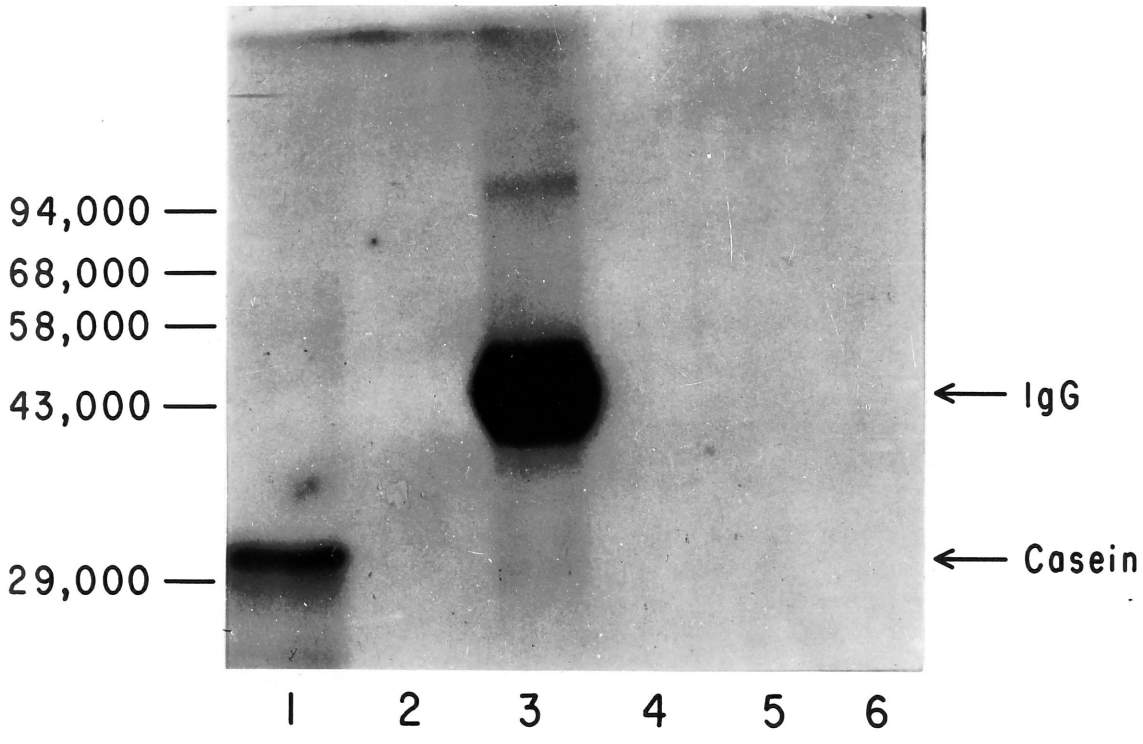


Fig. 18. Phosphorylation of α-casein or TBR-IgG in solution, by partially purified $pp60^{src}$, using GTP as a phosphate donor. The reaction was initiated by the addition of $[\gamma\text{-}^{32}P]GTP$ to solutions containing: lane 1, α-casein and $pp60^{src}$; lane 2, α-casein alone; lane 3, TBR IgG and $pp60^{src}$; lane 4, TBR IgG alone; lane 5, preimmune IgG and $pp60^{src}$; lane 6, preimmune IgG alone. The reaction was run as described in Materials and Methods, analyzed by SDS-polyacrylamide gel electrophoresis, and products visualized by autoradiography.

IgG or casein was incubated in solution with [γ-^{32}P]ATP and partially purified pp60^{src} and the reaction products were analyzed by SDS polyacrylamide gels. The radiolabelled casein and IgG detected by autoradiography were eluted from gel slices, hydrolyzed with HCl, and analyzed for phosphoamino acids by electrophoresis at pH 3.5. Standards of phosphoserine, phosphothreonine, and phosphotyrosine were separated by thin layer electrophoresis at pH 3.5. Casein and TBR IgG were both found to be phosphorylated at tyrosine residues by pp60^{src} in solution (Fig. 19). Casein was also phosphorylated at serine and threonine residues (Fig. 19), however, this phosphorylation was shown in subsequent experiments to be due to endogenous kinase activity in the casein preparation. Collett et al. (1980) have reported similar in vitro phosphorylation results.

Additional experiments were done to further characterize the activity of pp60^{src} in solution. N-α-Tosyl-L-lysyl-chloromethylketone (TLCK) was reported to inhibit the pp60^{src}-associated IgG heavy chain kinase activity in immunoprecipitates (Richert et al., 1979b). TLCK (0.01-1.0 mM) also inhibits TBR IgG and casein phosphorylation by pp60^{src} in solution. The phosphorylation of casein by pp60^{src} is not inhibited by the addition of tyrosine, phenylalanine, or phosphotyrosine to the reaction.

Phosphorylation and Interaction of pp60^{src} with Physiological Substrates. With knowledge gained from studying the phosphorylation of casein and TBR IgG by pp60^{src} in solution it was possible to begin to investigate the physiological phosphorylation events which are responsible for pp60^{src} transforming activity in living cells. One possible cellular

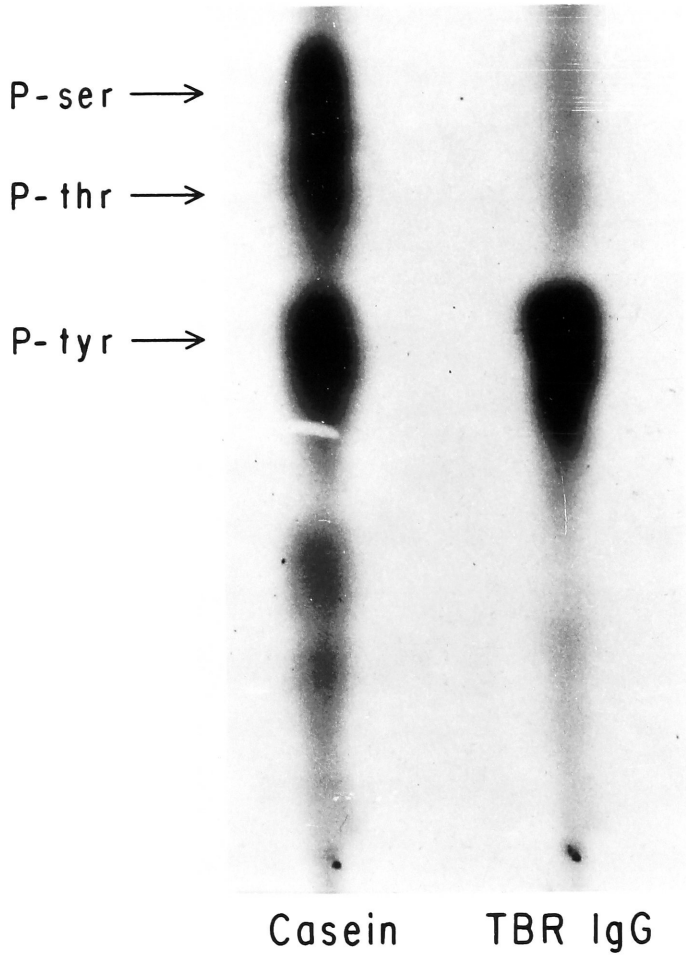


Fig. 19. Phosphoamino acid analysis of casein and TBR IgG phosphorylated *in vitro* by partially purified $pp60^{src}$. Phosphorylated proteins were eluted from gels and hydrolyzed as described in Materials and Methods. Phosphoamino acids were separated by electrophoresis at pH 3.5. Marker phosphoamino acids were co-electrophoresed with the samples and visualized by staining with ninhydrin. Radioactive phosphoamino acids were detected by autoradiography.

target of pp60^{src} action is the cytoskeleton. There is considerable evidence indicating that the disruption of the cytoskeleton is an early effect of RSV transformation and that it might be a result of the direct interaction of pp60^{src} with one or more cytoskeletal components (see Chapter II). Early experiments were designed to test for the direct interaction of partially purified pp60^{src} with purified cytoskeletal components.

pp60^{src} was incubated with purified cow brain microtubules or rabbit skeletal muscle F-actin under a variety of solution conditions that were found to stabilize the polymer and the pp60^{src} kinase activity. The mixture was centrifuged at 100,000 g and the supernatant and pellet were assayed for pp60^{src} kinase activity. No significant amount of pp60^{src} kinase activity was found associated with the microtubule or microfilament pellets even when the actin and tubulin were present in 1000-fold molar excess over pp60^{src}. A large number of experiments failed to show any direct interaction of pp60^{src} with the cytoskeletal proteins actin, tubulin, filamin, and vinculin. Furthermore, a variety of functional assays, including actin gelation and microtubule and microfilament polymerization, were also unaffected by the addition of partially purified pp60^{src}.

The possibility that pp60^{src} might affect components of the cytoskeleton enzymatically was tested. Cytoskeletal proteins purified by published procedures were incubated with the pp60^{src} kinase in the presence of [γ-^{32}P]ATP and magnesium. A number of proteins were found to be phosphorylated by pp60^{src} *in vitro*. For example, partially purified pp60^{src} phosphorylated the α and β subunits of tubulin (Fig. 20, lane

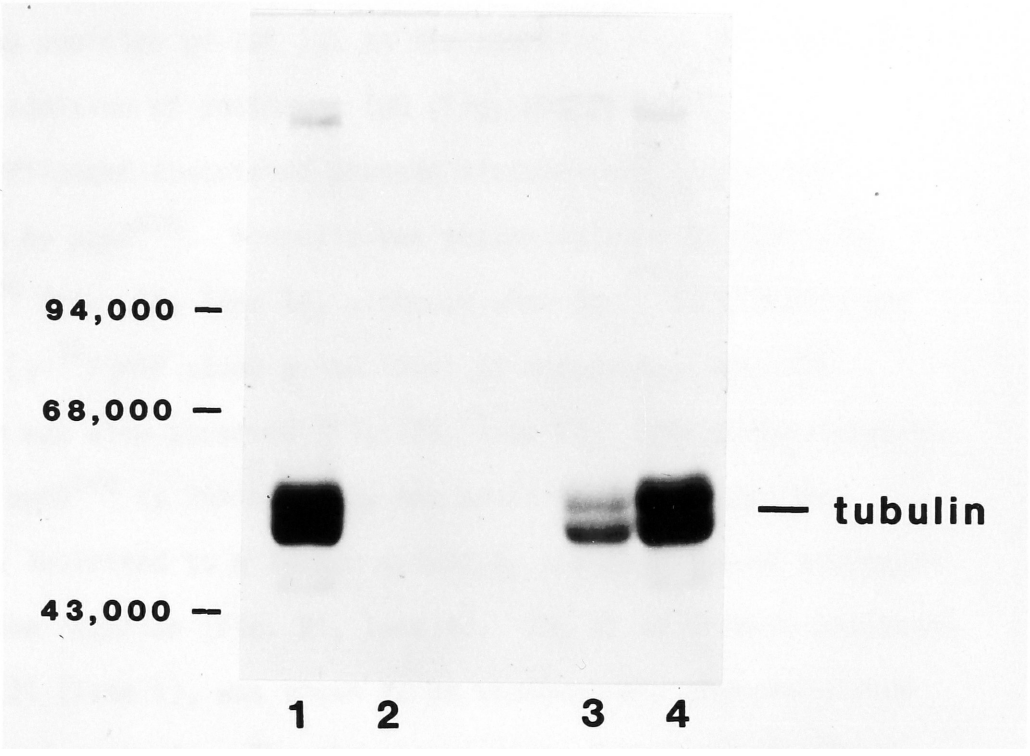


Fig. 20. Phosphorylation of tubulin by partially purified pp60^{src}. Tubulin was incubated with [γ-^{32}P]ATP in the presence of: lane 1, pp60^{src}; lane 2, buffer alone; lane 3, pp60^{src} and 10 μl 1.0 mg/ml TBR IgG; lane 4, pp60^{src} and 10 μl preimmune IgG. Reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

1). In this reaction tubulin was heated to 90°C for 3 min to inactivate high levels of endogenous kinase activity. After such treatment no endogenous phosphorylation occurs when tubulin is incubated with [γ-^{32}P]ATP alone (Fig. 20, lane 2). The phosphorylation of tubulin by pp60^{src} was inhibited by the addition of TBR IgG to the reaction (Fig. 20, lane 3) but not by the addition of preimmune IgG (Fig. 20, lane 4).

The actin filament-associated protein vinculin was tested for phosphorylation by pp60^{src}. Vinculin was phosphorylated by partially purified pp60^{src} (Fig. 21, lane 1), although when purified vinculin was incubated with [γ-^{32}P]ATP alone a low level of endogenous vinculin phosphorylation was also observed (Fig. 21, lane 2). The phosphorylation of vinculin by pp60^{src} is inhibited by the addition of TBR IgG (Fig. 21, lane 3). It is inhibited to a lesser extent by the addition of preimmune rabbit IgG to the reaction (Fig. 21, lane 4). The 60 kD protein phosphorylated in Fig. 21 (lane 1), was shown to be endogenously phosphorylated pp60^{src} by several criteria. The phosphorylation of this 60 kD protein occurs in the absence of vinculin and its phosphorylation is inhibited by the addition of TBR IgG to the reaction. In addition, the phosphorylated protein is immunoprecipitated by TBR serum.

pp60^{src} was found to specifically phosphorylate many cytoskeletal proteins <u>in vitro</u> including actin, filamin, tubulin, and myosin light and heavy chains (Table IV). The phosphorylation of various cytoskeletal proteins by pp60^{src} occurs exclusively at tyrosine residues. Actin or tubulin was incubated with pp60^{src}, [γ-^{32}P]ATP, and magnesium. Reaction products were isolated, hydrolyzed with HCl, and analyzed for phosphoamino acid by two dimensional thin layer electrophoresis. As can be seen in Fig. 22, both actin and tubulin were phosphorylated at tyrosine residues.

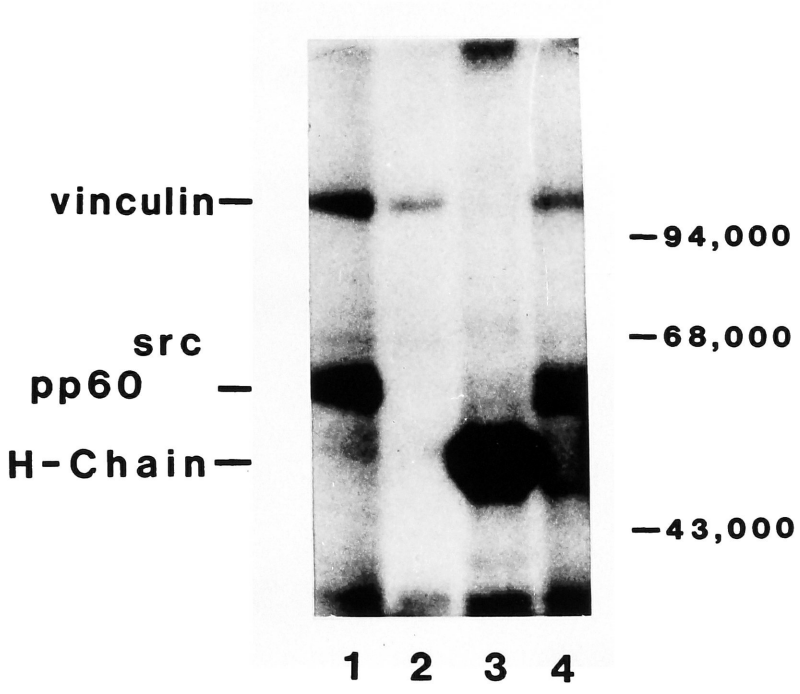


Fig. 21. Phosphorylation of vinculin by partially purified $pp60^{src}$. Vinculin was incubated with [γ-^{32}P]ATP in the presence of: lane 1, $pp60^{src}$; lane 2, buffer alone; lane 3, $pp60^{src}$ and 10 μl 1.0 mg/ml TBR IgG; lane 4, $pp60^{src}$ and 10 μl 1.0 mg/ml preimmune IgG. Reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

TABLE IV

Proteins Tested as In Vitro Substrates of $pp60^{src}$

PHOSPHORYLATED	NOT PHOSPHORYLATED
CYTOSKELETAL PROTEINS	
Vinculin	α-actinin
Actin	Tropomyosin
Filamin	
Myosin	
Tubulin	
MAPs	
OTHER PROTEINS	
34 kD protein	Phosvitin
α-casein	Histones
TBR IgG	IgG
Malate dehydrogenase (cytoplasmic)	Malate dehydrogenase (mitochondrial)
Alcohol dehydrogenase	β_2 microglobulin
Concanavalin A	Lactate dehydrogenase
	Phosphorylase A
	Avidin
	Bovine serum albumin

The in vitro phosphorylation of substrates by $pp60^{src}$ was assayed as described in Materials and Methods.

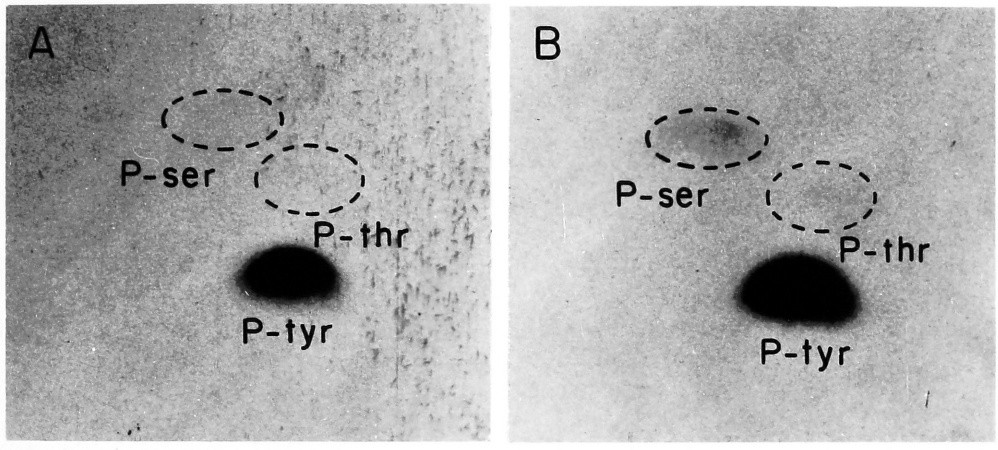


Fig. 22. Phosphoamino acid analysis of actin and tubulin phosphorylated in vitro by partially purified $pp60^{src}$. Phosphorylated actin (A) and tubulin (B) were eluted from gels, hydrolyzed, and phosphoamino acids separated by two dimensional thin layer electrophoresis as described in Materials and Methods. Marker phosphoamino acids were co-electrophoresed with the samples and visualized by staining with ninhydrin. Radioactive phosphoamino acids were detected by autoradiography.

A variety of other non-cytoskeletal proteins were tested as $pp60^{src}$ substrates (Table IV). The 34 kD phosphoprotein was purified according to the procedure of Erikson and Erikson (1980). Partially purified $pp60^{src}$ was found to phosphorylate the 34 kD protein in vitro. This confirms the results reported by Erikson and Erikson (1980). It was recently proposed by RÜbsamen et al. (1982) that the 34 kD protein is the subunit of cytosolic malate dehydrogenase. In vitro $pp60^{src}$ phosphorylated pig heart cytosolic malate dehydrogenase to a significant extent and no phosphorylation of mitochondrial malate dehydrogenase was detected. The partially purified $pp60^{src}$ was also incubated with several other enzymes that are known to be involved in cellular metabolism. Alcohol dehydrogenase but not lactate dehydrogenase was phosphorylated by $pp60^{src}$ to a significant extent. It was demonstrated that in vitro the src protein kinase was not highly specific. Even non-physiological proteins such as the lectin concanavalin A were phosphorylated to a limited extent by $pp60^{src}$ and the in vitro data must therefore be viewed with caution.

Conclusions

The purification of $pp60^{src}$ described in Chapter III successfully removed phosphatases and other inhibitors so that it was possible to study the activity of this enzyme in solution under controlled conditions. While it had proved difficult to study the substrate specificity of $pp60^{src}$ in the immune complex, once purified $pp60^{src}$ was easily tested for its ability to phosphorylate a variety of substrates.

Both casein and TBR IgG were phosphorylated at tyrosine residues by partially purified $pp60^{src}$. The src protein did not phosphorylate histones or phosvitin and the phosphorylation of casein was not enhanced in the presence of cAMP. On the basis of these results $pp60^{src}$ can be categorized as a cAMP-independent protein kinase.

The interaction of $pp60^{src}$ with more physiological substrates was also examined. No direct interaction of $pp60^{src}$ with purified cytoskeletal components could be detected but a number of these proteins were phosphorylated by $pp60^{src}$ *in vitro* including tubulin, actin, vinculin, myosin, and filamin. Other proteins including the 34 kD phosphoprotein identified by Radke and Martin (1979) and cytosolic malate dehydrogenase were also phosphorylated by $pp60^{src}$. The *in vitro* phosphorylation results should be viewed cautiously; the significance of these results is discussed in Chapter VII.

V. Ca^{2+}-CALMODULIN ACTIVATION OF A POTENTIAL $PP60^{src}$ SUBSTRATE

Protein phosphorylation is an important mechanism by which the activity of proteins is regulated. $pp60^{src}$ may cause transformation by phosphorylating tyrosine residues on proteins within the cell. *In vitro* experiments described in Chapter IV as well as *in vivo* experiments detailed in the History and Background Section suggest that one likely target of $pp60^{src}$ phosphorylation is the cytoskeleton. The present chapter describes experiments aimed at identifying other possible targets of the *src* protein which led to the identification of a new protein that is a potential substrate of $pp60^{src}$. Phosphorylation of this protein appeared to be regulated by the calcium binding protein calmodulin.

Calmodulin is a heat-stable calcium binding protein with a molecular weight of 17,000 daltons. It has been found to bind proteins in the presence of calcium and thereby modulate their activities (reviewed by Means and Dedman, 1980; Cheung, 1980). Among these proteins is a class of kinases that are regulated by Ca^{2+}-calmodulin (Cheung, 1980). This chapter describes experiments which examined the interaction of $pp60^{src}$ and $pp60^{src}$ substrates with Ca^{2+}-calmodulin. A protein with a subunit molecular weight of 50,000 daltons that was present in the partially purified *src* preparation was phosphorylated in a Ca^{2+}-calmodulin dependent manner. This protein, which was detected in normal and transformed cells, appeared to interact with $pp60^{src}$. Additional experiments, using the calmodulin inhibitor chlorpromazine, raised the possibility that $pp60^{src}$-induced deregulation of cell growth may involve control by calcium.

Materials and Methods

Purification of Calmodulin. Calmodulin was prepared by a rapid method described by Watterson et al. (1980). 2 g chicken gizzards were homogenized in 3 l. buffer A (50 mM Tris-HCl pH 8.0, 1 mM EGTA, 1 mM β-mercaptoethanol) in a Waring blender at 4°C. The homogenate was centrifuged 10,000 g for 60 min and the supernatant filtered through cheesecloth. The filtrate was adjusted to 55% saturation by the slow addition of ground solid ammonium sulfate (351 g/l). The mixture was stirred for 1 hour and centrifuged 10,000 g for 45 min. The supernatant was adjusted to pH 4.1 by the slow addition of 1 M sulfuric acid and stirred for 2 hours at 4°C. The mixture was centrifuged 10,000 g and the supernatant discarded. The pellet was suspended in buffer B (10 mM Tris-HCl pH 8.0, 1 mM EGTA, 1 mM β-mercaptoethanol, 0.2 M NaCl) and the pH adjusted to 7.4 by the addition of 1 M Tris-base. After overnight dialysis the sample was applied to a DEAE-cellulose column (5.5x25 cm) equilibrated with buffer B. The column was washed with buffer B and eluted with a linear gradient of NaCl (0.2-0.7 M) in buffer B. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis and those containing calmodulin were applied to a column (1.5x15 cm) of phenothiazine-Sepharose equilibrated with buffer C (20 mM Tris-HCl pH 7.4, 1 mM β-mercaptoethanol, 1 mM $CaCl_2$, 0.3 M NaCl) with a flow rate of 10-20 ml per hour at room temperature. The column was washed with buffer C and the calmodulin eluted with 20 mM Tris-HCl (pH 7.4), 1 mM β-mercaptoethanol, 0.3 M NaCl, 10 mM EGTA at a flow rate of 40 ml per hour. Starting with 2 kg gizzards approximately 40 mg of purified calmodulin were obtained.

Preparation of Phenothiazine Sepharose. The phenothiazine Sepharose was prepared from 2-chloro-10-(3-aminopropyl)phenothiazine (CPZ) (a gift of Dr. A. Manian, National Institutes of Health) and CNBr activated Sepharose. The coupling procedure was the same as that already described in Materials and Methods, Chapter III for casein-Sepharose (Garvey et al., 1977). 30 ml packed CNBr activated Sepharose were reacted with 60 mg of CPZ.

Preparation of Calmodulin-Sepharose. The calmodulin affinity column was prepared by a modification of the method of Garvey et al. (1977) as described in Materials and Methods, Chapter III. 20 ml packed CNBr activated Sepharose were reacted with 20 mg calmodulin.

Assay of Calcium-calmodulin Dependent Phosphorylation. A partially purified preparation of $pp52^{src}$ or $pp60^{src}$ was incubated in 90 µl of 20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 0.17 M sucrose, 5 mM DTT, 2 mM potassium phosphate, containing 1 µg calmodulin and either 0.1 mM $CaCl_2$ or 10 mM EGTA. The reaction was initiated by the addition of 0.1 µM [γ-^{32}P]ATP (3000 Ci/mmole, Amersham), incubated for 15 min at 37°C, and quenched by the addition of 50 µl 3-times concentrated electrophoresis sample buffer. Samples were heated to 95°C for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis. Radiolabelled proteins were visualized by autoradiography.

Calmodulin Affinity Column. 10 ml partially purified $pp52^{src}$ was dialyzed overnight against buffer D (75 mM Tris-HCl pH 7.4, 10 mM β-mercaptoethanol, 1 mM $CaCl_2$) and applied to a 5 ml calmodulin-Sepharose column equilibrated with buffer D at a flow rate of 10 ml/hour. The column was washed with buffer A and eluted with 75 mM Tris-HCl (pH 7.4), 10 mM β-mercaptoethanol, 10 mM EGTA. Column fractions were assayed for

Ca^{2+}-calmodulin activation of phosphorylation and for $pp60^{src}$ kinase activity in TBR serum immunoprecipitates as previously described. All procedures were done at $4^{o}C$.

Glycerol Gradient Sedimentation Analysis. SR-A RSV infected CEFs were labelled overnight with [^{3}H]leucine as described in Materials and Methods Chapter III, and extracted with 0.5 ml buffer E (10 mM potassium phosphate pH 7.0, 1 mM EDTA, 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM DTT) per 60 mm dish. After centrifugation at 100,000 g for 30 min, 0.2 ml of supernatant was applied to the top of a 4 ml linear gradient of 10-30% glycerol in buffer E and centrifuged at 56,000 rpm in a SW60Ti rotor for 16 hours. The gradient was fractionated into 0.2 ml fractions and assayed for Ca^{2+}-calmodulin activation of phosphorylation and for $pp60^{src}$ kinase activity in TBR serum immunoprecipitates as previously described. Fractions were also immunoprecipitated with TBR serum and analyzed directly by SDS polyacrylamide gel electrophoresis.

Cell Culture. Primary cell cultures were prepared as described in Materials and Methods, Chapter III. Secondary cultures of chick embryo fibroblasts were infected with tsNY68-SRA RSV (a gift of Dr. H. Hanafusa, The Rockefeller University) at the permissive temperature ($37^{o}C$) and grown in Ham's F-10 medium containing 5% calf serum and 20 μg/ml of Gentamycin. Two days later the cultures were shifted to the nonpermissive temperature ($42^{o}C$). The culture medium on the cells was changed every 24 hours. After two days the tsNY68-infected CEFs were passaged to multi-welled Linbro tissue culture plates, $0.40x10^{5}$ cells/cm^2 (area per well 2 cm^2) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 mM Hepes and 0.25% calf serum. 12 hours later the medium was replaced

with DMEM containing 10 mM Hepes and the cells were starved at the nonpermissive temperature for 48 hours. In many experiments 2-chloro-10-(3-aminopropyl)phenothiazine (CPZ) was added to the cultures to a final concentration of 10 mM in DMEM. At this time the cultures were shifted from 42°C to 37°C.

Assay of DNA Synthesis. The amount of DNA synthesis was estimated from the incorporation of [^{3}H]thymidine (1.9 Ci/mmole; Schwarz/Mann, Div. of Becton, Dickinson and Co., Orangeburg, N.Y.) as detected by bulk counts in acid precipitable material or by autoradiographic analysis of cells grown on coverslips. For bulk counting, cells in multi-well Linbro dishes were incubated in the presence of 2 μCi of [^{3}H]thymidine for various amounts of time. The medium was removed, cells were lysed by the addition of 1 M NaOH, and incubated for 1 hour at room temperature. Cells were pipetted on to glass fiber filters (Whatman GF/A) and washed with 10% TCA and methanol before drying. ^{3}H was quantitated by liquid scintillation counting.

For autoradiographic analysis cells were grown on 18x18 mm coverslips. The cultures were incubated with 6 μCi of [^{3}H]thymidine and fixed in 0.9% saline-3.7% formaldehyde. After rinsing in water and acetic acid:methanol:water (1:89:10), the coverslips were air-dried and mounted cell-side up on glass microscope slides using Permount. The slides were dipped in Kodak NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.), and developed after 36 hours in Kodak D-19 developer for 2 min, fixed in Kodak Rapid Fixer for 4 min, and washed in water for 1 hour. After air drying, the slides were fixed for 30 min in absolute methanol, dried, stained 20 min in Giemsa solution, washed in three changes of water for 30 min, and air dried. Coverslips were applied to the slides with Permount.

Assay of Kinase Activity in TBR Immunoprecipitates. tsNY68-SRA RSV-infected chick cells were lysed with 0.2 ml extraction buffer (10 mM sodium phosphate pH 7.2, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1% trasylol) per well on the multiwell Linbro plates. Extracts were vortexed lightly and centrifuged in a Beckman Microfuge for 10 min. The supernatant was immunoprecipitated with TBR serum and then washed four times with extraction buffer and assayed for phosphotransferase activity as described in Materials and Methods, Chapter III. All operations were done on ice in the cold room up to the point of addition of [γ-^{32}P]ATP.

Results

Ca^{2+}-Calmodulin Dependent Activation of Phosphorylation. A 50,000 dalton protein was present in partially purified preparations of $pp52^{src}$ and $pp60^{src}$ that was phosphorylated in a Ca^{2+}-calmodulin dependent manner. When $pp52^{src}$ (Fig. 23, lane 1) or $pp60^{src}$ (Fig. 23, lane 4) was incubated with [γ-^{32}P]ATP and magnesium in the presence of Ca^{2+}-calmodulin the 50,000 dalton protein was phosphorylated. When calcium was replaced by EGTA, so that calmodulin was in an inactive conformation (Cheung, 1980), significantly less phosphorylation of the 50 kD protein was detected in the $pp60^{src}$ preparation (Fig. 23, lane 3) and in the $pp52^{src}$ sample (Fig. 23, lane 2). $pp52^{src}$ rather than $pp60^{src}$ was used in many of these experiments since the detergent NP-40 present in the $pp60^{src}$ preparation was found to inhibit Ca^{2+}-calmodulin activation. The results of a Ca^{2+}-calmodulin activation experiment using a second preparation of $pp52^{src}$ are shown in Fig. 24. Again phosphorylation of the 50 kD protein occurs when Ca^{2+}-calmodulin is present (lane 1) but not if calcium is replaced by EGTA (lane 2). If calmodulin is omitted from the reaction solution the low level of phosphorylation of the 50 kD protein is not stimulated by calcium compared with EGTA (Fig. 24, lanes 3 and 4).

The Ca^{2+}-calmodulin dependent phosphorylation of the 50 kD protein required micromolar amounts of free calcium. Higher concentrations of free calcium, in the millimolar range, were inhibitory. Furthermore, the calcium triggered phosphorylation of the 50 kD protein occurred linearly for 10 min (Fig. 25), and had a temperature optimum of 30^{o}C (Fig. 26).

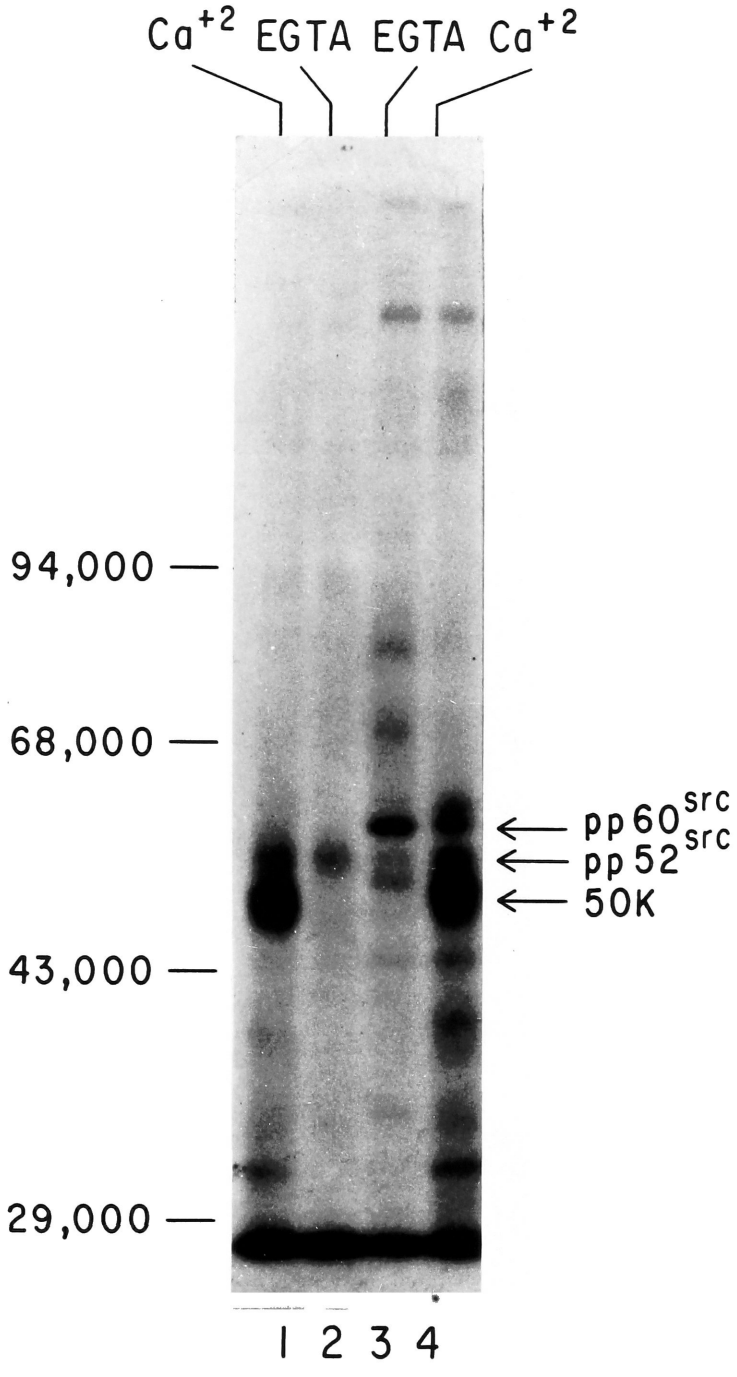


Fig. 23. Ca^{2+}-calmodulin activation of phosphorylation. Partially purified $pp52^{src}$ (lanes 1 and 2), or $pp60^{src}$ (lanes 3 and 4); was incubated with [γ-^{32}P]ATP in the presence of Ca^{2+}-calmodulin (lanes 1 and 4) or calmodulin and EGTA (lanes 2 and 3). The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

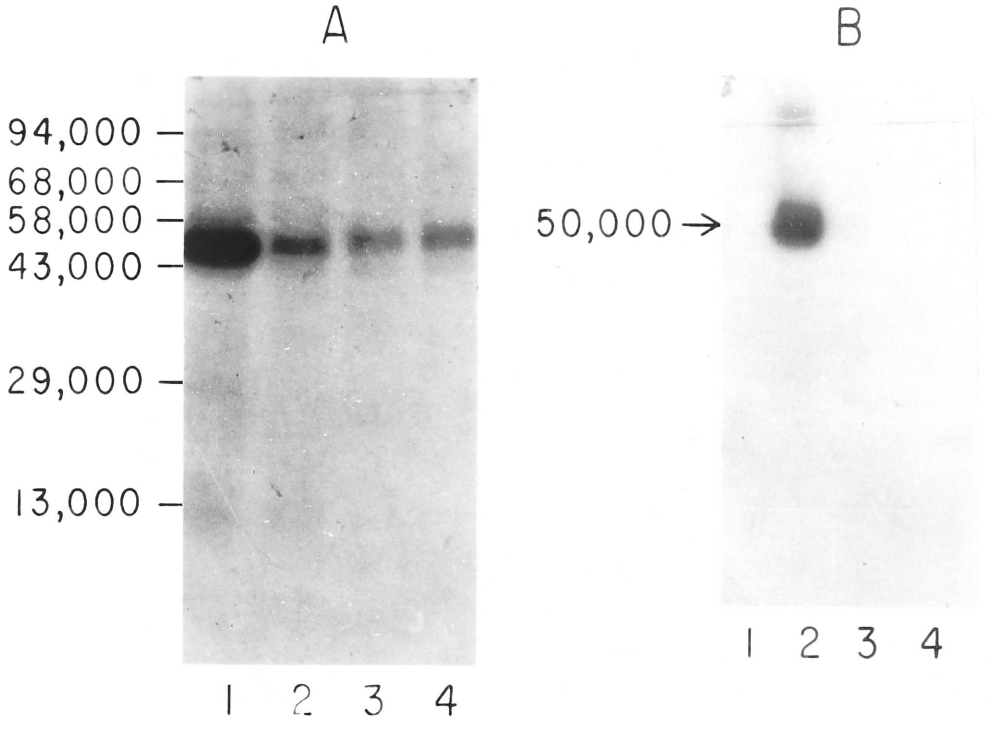


Fig. 24. Ca^{2+}-calmodulin activation of phosphorylation. (A) Partially purified $pp52^{src}$ and [γ-^{32}P]ATP were incubated with: (lane 1) Ca^{2+}-calmodulin; (lane 2) EGTA-calmodulin; (lane 3) Ca^{2+} without calmodulin; (lane 4) EGTA without calmodulin. (B) Partially purified $pp52^{src}$ and [γ-^{32}P]ATP were incubated with Ca^{2+}-calmodulin (lanes 1 and 2), or EGTA-calmodulin (lanes 3 and 4) and the reaction products immunoprecipitated with preimmune serum (lanes 1 and 3), or TBR serum (lanes 2 and 4). The reaction was run and products were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography, as described in Materials and Methods.

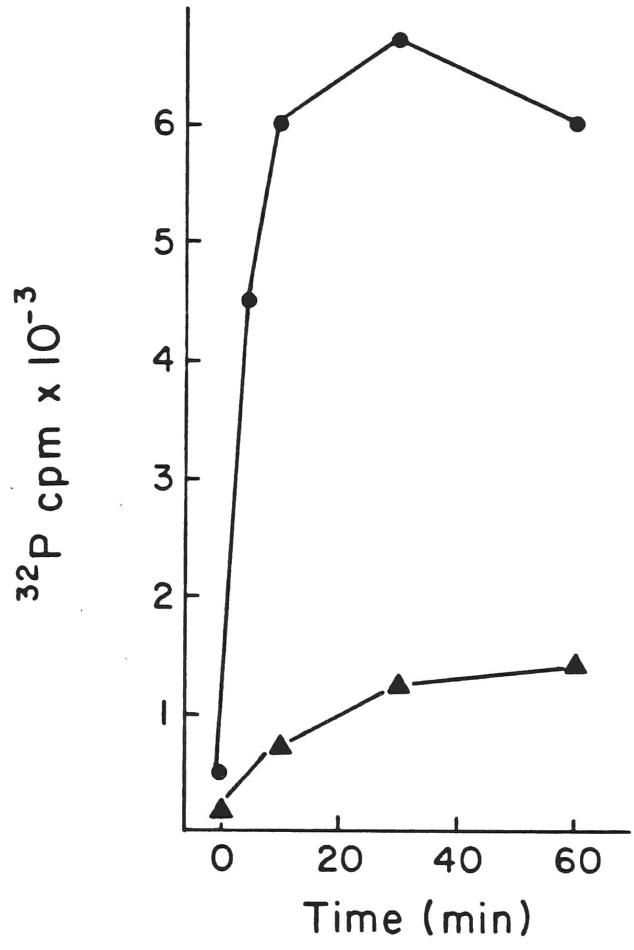


Fig. 25. Time dependence of reaction for Ca^{2+}-calmodulin activation of phosphorylation of the 50 kD protein. Partially purified $pp52^{src}$ and [γ-^{32}P]ATP were incubated with Ca^{2+}-calmodulin (●—●) or EGTA-calmodulin (▲—▲) for varying periods of time. The reaction was quenched by the addition of 10% TCA. [^{32}P] incorporated into TCA precipitable counts is plotted as a function of the time of incubation.

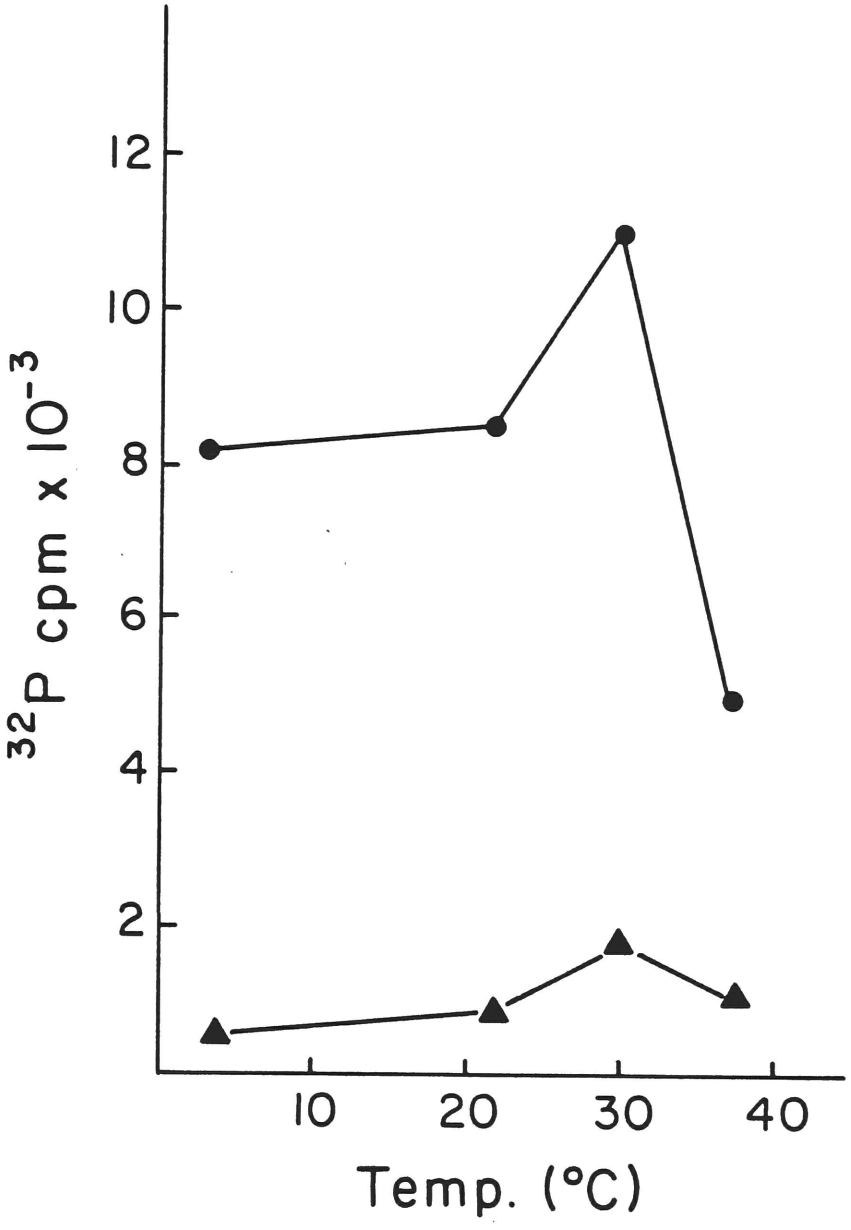


Fig. 26. Temperature dependence of the Ca^{2+}-calmodulin activation of phosphorylation of the 50 kD protein. Partially purified pp52^{src} and [γ-^{32}P]ATP were incubated with Ca^{2+}-calmodulin (●—●) or EGTA-calmodulin (▲—▲) for 20 min, at either 4°, 23°, 30° or 37°C. The reaction was quenched by the addition of 10% TCA. [^{32}P] incorporated into TCA precipitable counts is plotted as a function of the temperature of incubation.

Inhibition of 50 kD Phosphorylation by Chlorpromazine. The drug chlorpromazine binds to calmodulin with high affinity and has been shown to inhibit calmodulin mediated processes (Levin and Weiss, 1977; Weiss and Levin, 1978). Chlorpromazine signficantly reduced the Ca^{2+}-calmodulin activated phosphorylation of the 50 kD protein. In the absence of chlorpromazine Ca^{2+}-calmodulin stimulated phosphorylation of the 50 kD protein approximately 8-fold (Fig. 27). The activation of phosphorylation was inhibited by increasing amounts of chlorpromazine in the micromolar range (Fig. 27). Half maximal inhibition was observed with 10 μM chlorpromazine, a concentration known to inhibit other calmodulin mediated processes (Weiss and Levin, 1978). Increasing concentrations of chlorpromazine had no effect on the low level of phosphorylation observed when the 50 kD protein was incubated with [γ-^{32}P]ATP, EGTA, and calmodulin (Fig. 27).

Presence of the 50 kD Protein in Normal Cells. The Ca^{2+}-calmodulin activated 50 kD protein was found to be present in partially purified preparations of normal cell extracts. Extracts were prepared from normal and SR-A transformed chick embryo fibroblasts in parallel by lysis in the absence of detergents. After ammonium sulfate precipitation the proteins were fractionated by chromatography on ω-aminohexyl agarose as previously described for the purification of $pp52^{src}$. Column fractions were assayed for Ca^{2+}-calmodulin stimulation of phosphorylation of the 50 kD protein. The comparison of the 50 kD protein from transformed and normal cells demonstrated that the phosphorylation of the 50 kD protein was activated to a similar extent in both preparations by Ca^{2+}-calmodulin. A pooled

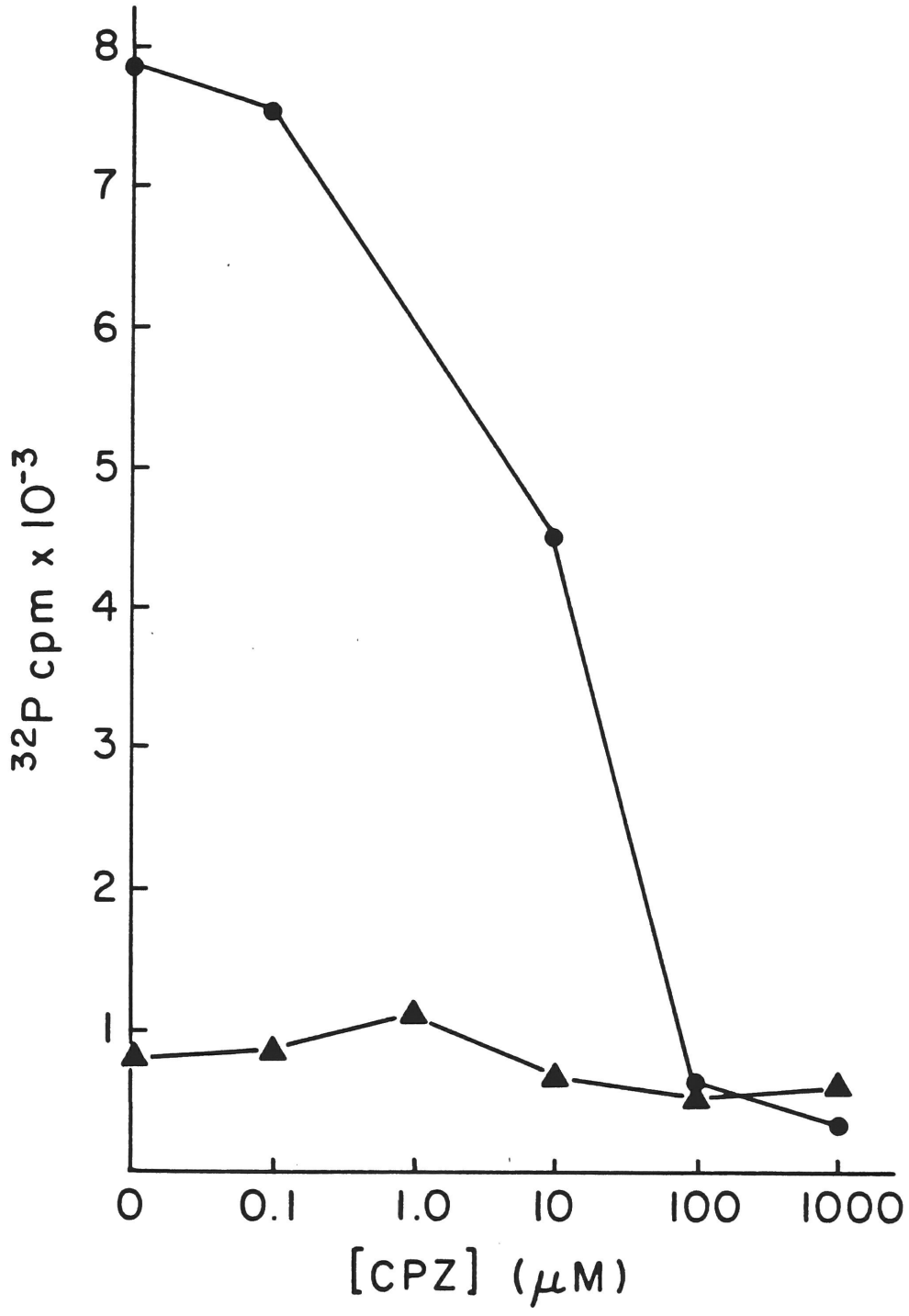


Fig. 27. Chlorpromazine inhibition of Ca^{2+}-calmodulin activated phosphorylation. Partially purified $pp52^{src}$, [γ-^{32}P]ATP and Ca^{2+}-calmodulin (●—●) or EGTA calmodulin (▲—▲) were incubated with varying concentrations of chlorpromazine. The reaction was quenched by the addition of 10% TCA. [^{32}P] incorporated into TCA precipitable counts is plotted as a function of chlorpromazine concentration.

fraction of the 50 kD protein for normal cells was phosphorylated in the presence of [γ-^{32}P]ATP and Ca^{2+}-calmodulin (Fig. 28, lane 3); less phosphorylation occurred when calcium was replaced by EGTA (lane 4). Ca^{2+}-calmodulin activation of phosphorylation of the 50 kD protein from transformed cells is shown in Fig. 28, lane 1; again less phosphorylation was observed when Ca^{2+} was replaced by EGTA (lane 2). These results indicate that the phosphorylation of the 50 kD protein is not, at least for the most part, dependent on the presence of $pp60^{src}$.

Interaction of $pp52^{src}$ with the 50 kD Protein. The fact that $pp52^{src}$ and the 50 kD protein copurified suggested that the 50 kD protein might be a src substrate and that the two proteins might interact with each other. The possibility of a direct interaction of $pp52^{src}$ with the 50 kD protein was tested by three different kinds of experiments. Preliminary experiments supported the conclusion that $pp52^{src}$ and the Ca^{2+}-calmodulin activated 50 kD protein bind to each other.

In the first experiment, the 50 kD protein was phosphorylated *in vitro* by incubation with [γ-^{32}P]ATP in the presence of Ca^{2+}-calmodulin; the reaction was stopped by the addition of EDTA and the products immunoprecipitated with TBR serum or preimmune serum. As can be seen in Fig. 24B (lane 2), TBR serum immunoprecipitates the 50 kD phosphoprotein but this protein is not precipitated by preimmune serum (Fig. 24B, lane 1). When the reaction was run with EGTA instead of calcium, neither the TBR nor the preimmune serum detectably immunoprecipitated a phosphorylated 50 kD protein (Fig. 24B, lanes 3 and 4). This result suggests that the phosphorylated protein interacts with $pp52^{src}$.

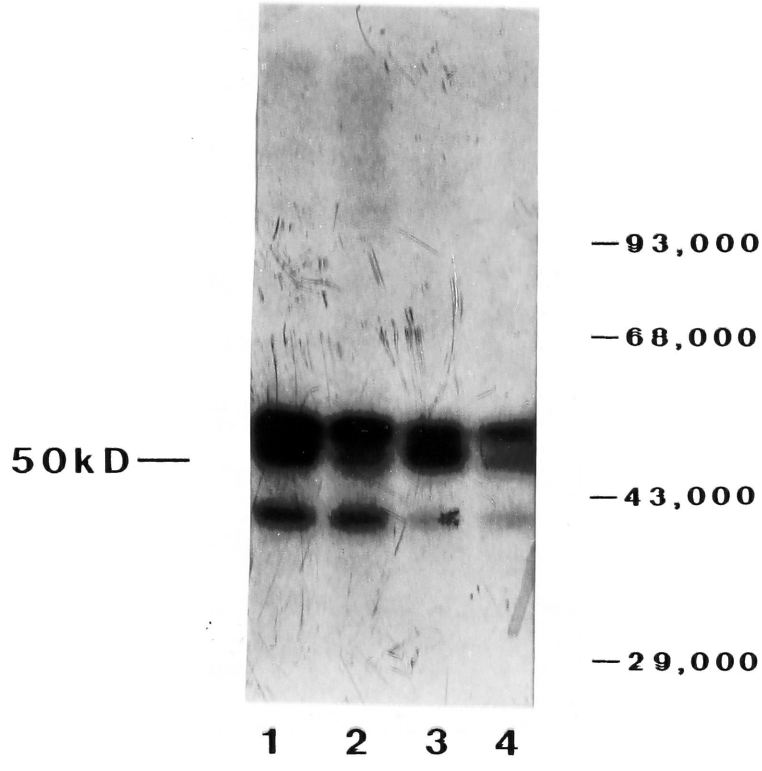


Fig. 28. Autoradiogram of Ca^{2+}-calmodulin activation of phosphorylation of the 50 kD protein from normal and transformed cells. Extracts of uninfected and SR-A RSV-infected CEFs were fractionated by ammonium sulfate precipitation and chromatography on ω-aminohexyl agarose. Column fractions were assayed for Ca^{2+}-calmodulin activation of phosphorylation. Fractions containing the 50 kD protein were pooled. Pooled 50 kD protein from RSV-infected cells were incubated with [γ-^{32}P]ATP, calmodulin and Ca^{2+}, (lane 1); EGTA (lane 2). 50 kD protein from uninfected CEFs incubated with [γ-^{32}P]ATP, calmodulin and Ca^{2+}, (lane 3); EGTA, (lane 4). The reaction was carried out and the products were analyzed as described in Materials and Methods.

An alternative possibility, that the 50 kD protein is a proteolytic cleavage product of $pp60^{src}$ that is immunoprecipitated by TBR serum was ruled out by the observation that the Ca^{2+}-calmodulin activated 50 kD protein is present in similar amounts in both normal and transformed cells. This indicated that the phosphorylated 50 kD protein is not a degradation product of $pp60^{src}$ but is a normal cell protein that may interact with $pp60^{src}$ in transformed cells.

Evidence suggesting an interaction between the src protein and the 50 kD protein was also obtained when these components were chromatographed on a calmodulin affinity support. Partially purified $pp52^{src}$ containing the 50 kD protein was applied to a calmodulin-Sepharose column equilibrated with a $CaCl_2$ buffer; specifically bound proteins were eluted with EGTA (Fig. 29). Column fractions were assayed for $pp52^{src}$ by measuring its kinase activity in TBR serum immunoprecipitates. The 50 kD protein was detected by analyzing fractions for Ca^{2+}-calmodulin activation of phosphorylation. Both $pp52^{src}$ and the 50 kD protein bound to the calmodulin affinity column and were eluted by EGTA. $pp52^{src}$ and the 50 kD protein did not bind to the calmodulin-Sepharose in control experiments during which the column was equilibrated with magnesium instead of calcium. While the 50 kD protein was found in all cases to bind to the calmodulin-Sepharose column, in some preparations the $pp52^{src}$-associated kinase activity did not bind to the column. The conditions for interaction of the two proteins need to be rigorously defined, but preliminary experiments suggest that salt may be necessary to promote the interaction between $pp52^{src}$ and the 50 kD protein, as well

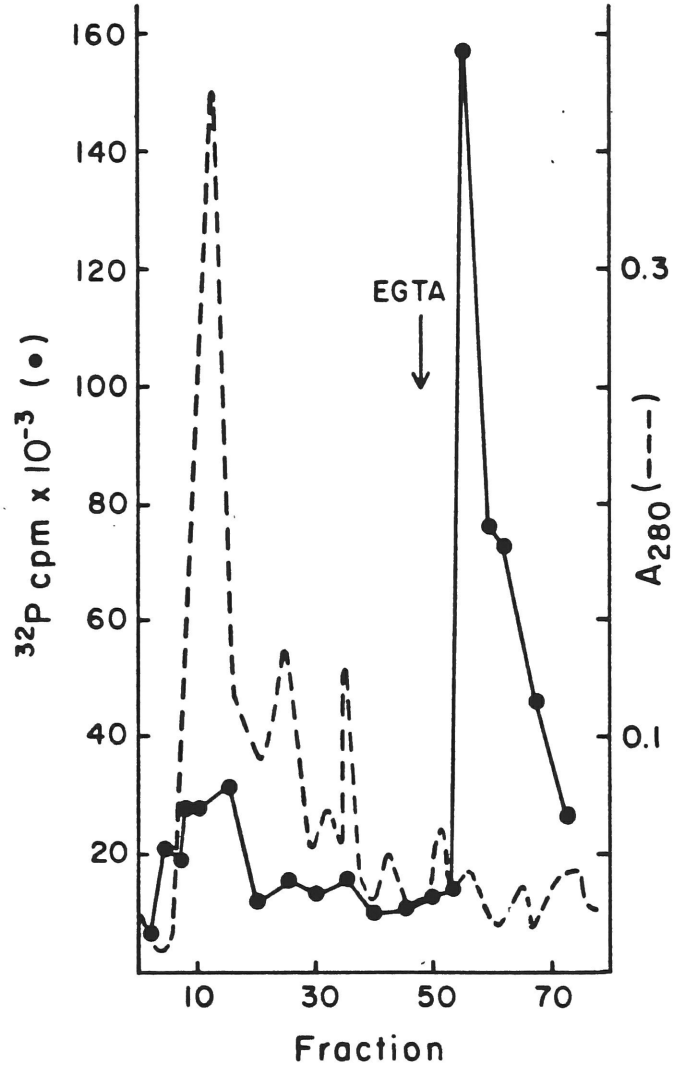
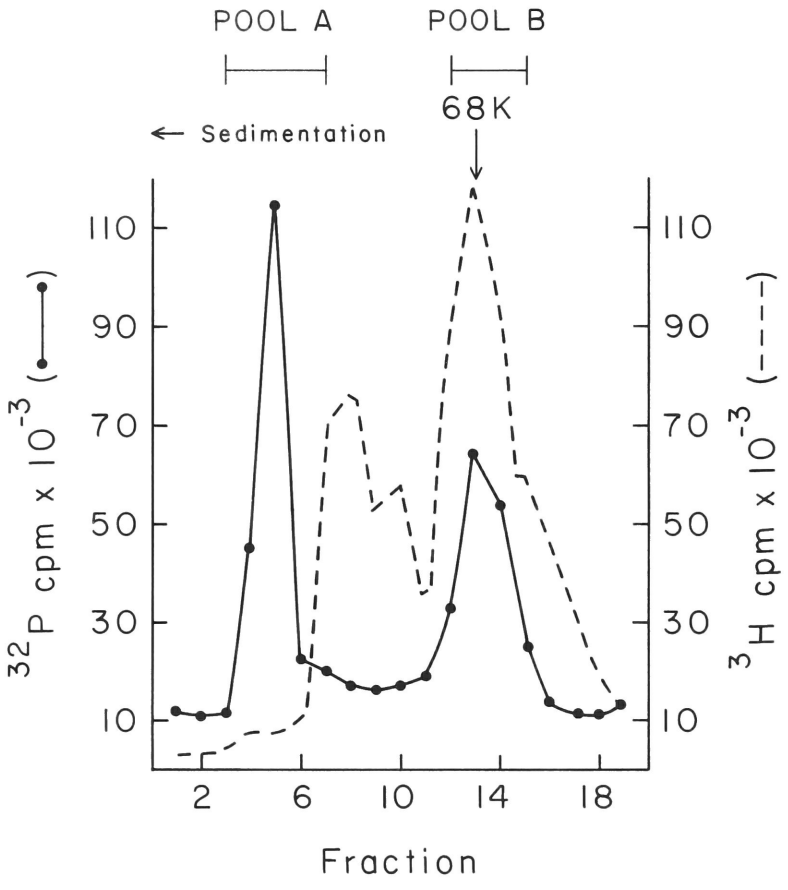


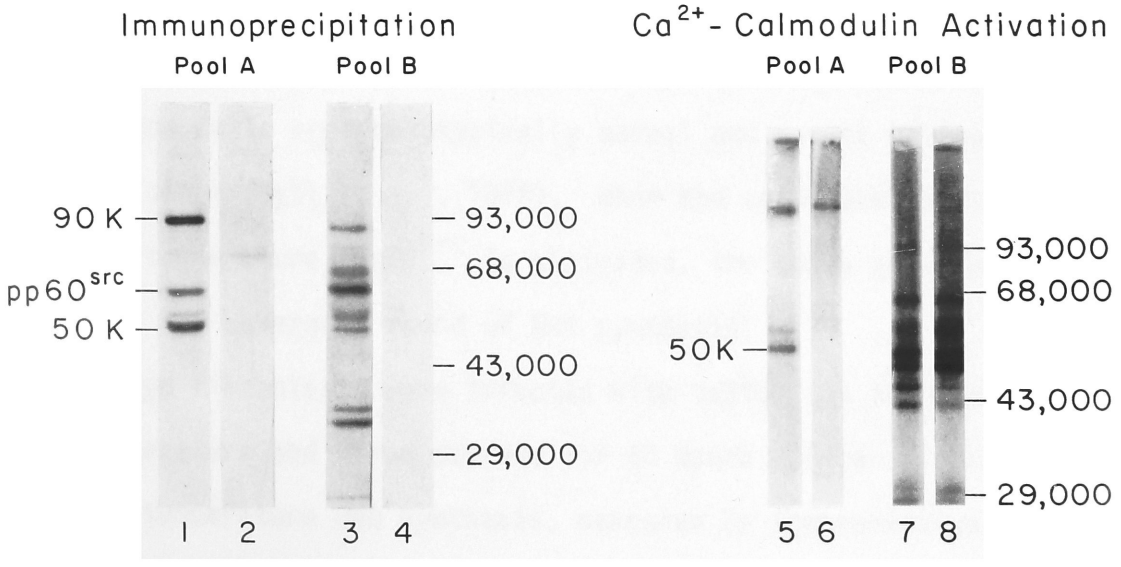
Fig. 29. Calmodulin-Sepharose chromatography of $pp52^{src}$. Partially purified $pp52^{src}$ was applied to a calmodulin affinity column equilibrated with $CaCl_2$ buffer and eluted with EGTA as described in Materials and Methods. The column fractions were immunoprecipitated with TBR serum and assayed for $pp60^{src}$-associated phosphotransferase activity (●—●). (---) designates the absorbance of column fractions at A_{280} nm.

as to promote the binding of $pp52^{src}$ to the calmodulin affinity column. The data are consistent with the possibility that $pp52^{src}$ binds to the calmodulin-Sepharose column via its interaction with the 50 kD protein.

While these experiments were in progress, Brugge and her collaborators (1981) reported that $pp60^{src}$ interacts on glycerol gradients and in immunoprecipitates with a 50 kD and a 90 kD protein of unknown function. In order to compare the findings described above with those of Brugge et al., the interaction of $pp60^{src}$ with the Ca^{2+}-calmodulin activated 50 kD protein was analyzed by sedimentation on glycerol gradients. Radiolabelled crude cell extracts of SR-A RSV transformed CEFs were centrifuged on glycerol gradients and yielded two peaks of kinase activity (Fig. 30). Immunoprecipitation of the radiolabelled gradient fractions with TBR serum demonstrated that $pp60^{src}$ was present in both peaks. The slower sedimenting fraction of activity migrated at a rate similar to that of bovine serum albumin and apparently contained free $pp60^{src}$ monomer. The faster sedimenting fraction contained proteins with molecular weights of 50,000 and 90,000 daltons that coprecipitate with $pp60^{src}$ in TBR serum immunoprecipitates (Fig. 30). These results confirms the findings of Brugge et al. (1981). Fractions from the glycerol gradient were assayed for Ca^{2+}-calmodulin activation of phosphorylation and a 50,000 dalton protein, present in the rapidly sedimenting peak of $pp60^{src}$-associated kinase activity, was phosphorylated in a Ca^{2+}-calmodulin dependent manner (Fig. 30). This result suggests that rapidly sedimenting $pp60^{src}$ may be interacting with the Ca^{2+}-calmodulin activatable 50 kD protein. It has not yet been established if the Ca^{2+}-calmodulin activated 50 kD protein is identical to the 50 kD protein precipitated by the TBR serum.

Fig. 30. Glycerol gradient centrifugation of $pp60^{src}$ from crude cell extracts of SRA-RSV transformed CEFs. The extract was fractionated as described in Materials and Methods. Column fractions were immunoprecipitated with TBR serum and assayed for $pp60^{src}$-associated kinase activity (●—●). The total protein profile was determined by counting gradient fractions for [^{3}H] (---). Fractions across the gradient were immunoprecipitated with TBR serum. Fractions were also assayed for Ca^{2+}-calmodulin activation of phosphorylation as described in Materials and Methods. Immunoprecipitation of Pool A with TBR serum, (lane 1); preimmune serum, (lane 2). Pool B immunoprecipitated with TBR serum, (lane 3), preimmune serum, (lane 4). Ca^{2+}-calmodulin activation of phosphorylation. Pool A was incubated with [γ-^{32}P]ATP, calmodulin and Ca^{2+} (lane 5); EGTA (lane 6). Pool B was incubated with [γ-^{32}P]ATP, calmodulin and Ca^{2+} (lane 7); EGTA, (lane 8). The gradient was calibrated with bovine serum albumin (68 kD) and catalase (240 kD).


POOL A
POOL B
68K
← Sedimentation
110
90
70
50
30
10
^{32}P cpm x 10^{-3} (——)
^{3}H cpm x 10^{-3} (- - - -)
2
6
10
14
18
Fraction


Immunoprecipitation
Pool A
Pool B
90 K
pp60^{src}
50 K
93,000
68,000
43,000
29,000
1
2
3
4
Ca^{2+}- Calmodulin Activation
Pool A
Pool B
50K
93,000
68,000
43,000
29,000
5
6
7
8

These observations must be extended, but they support the conclusion that the Ca^{2+}-calmodulin stimulated 50 kD protein binds to $pp60^{src}$ in both crude and purified preparations. The two proteins copurified on several ion exchange and affinity columns, cosedimented on glycerol gradients and were found to coprecipitate in TBR serum immunoprecipitates. The conditions promoting the interaction of $pp52^{src}$ and the Ca^{2+}-calmodulin activated 50 kD protein need to be rigorously established.

In Vivo Effects of Chlorpromazine. The experiments described above raised the possibility that Ca^{2+}-calmodulin stimulated phosphorylation of the 50 kD src protein substrate might be related to $pp60^{src}$-induced changes during cellular transformation. To test this hypothesis, some in vivo effects of the calmodulin inhibitor chlorpromazine were investigated. In preliminary experiments chlorpromazine had a significant effect on the $pp60^{src}$-induced deregulation of DNA synthesis.

The effect of chlorpromazine was studied under conditions in which activation of $pp60^{src}$ in transformed cells is rapidly followed by the initiation of DNA synthesis. CEFs were infected with tsNY68-SR-A RSV, which is temperature-sensitive for transformation. At the nonpermissive temperature these cells are phenotypically normal and cannot divide in the absence of serum (Bell et al., 1975). When the cells are shifted to the permissive temperature, $pp60^{src}$ is activated, the cells transform morphologically and undergo a round of DNA synthesis.

Chick embryo fibroblasts were infected with tsNY68, at the non-permissive temperature and serum starved for 60 hours. After a shift to the permissive temperature DNA synthesis, measured by incorporation of tritiated thymidine, increased approximately 10-fold (Fig. 31, open circles). Cells maintained at the nonpermissive temperature remained

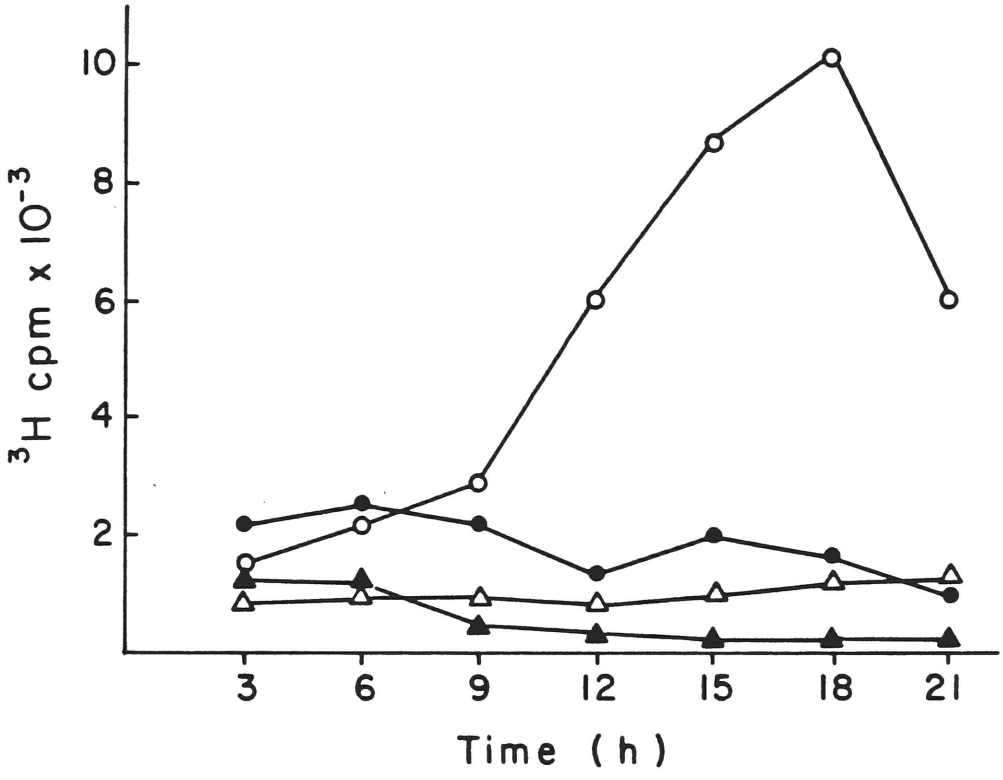


Fig. 31. Inhibition of DNA synthesis by chlorpromazine after temperature shift of serum starved tsNY68-infected CEFs from the nonpermissive to permissive temperature. Cells were starved for 60 hr, as described in Materials and Methods. After 60 hr (time 0 on abscissa) cultures either received no CPZ (○,△) or a [CPZ] of 10 μM (●,▲). One of the sets of drug treated and not treated cultures (●,○) was shifted to the permissive temperature. The other set of cultures (▲,△) was kept at the nonpermissive temperature. At various times 2 μCi of [^{3}H]thymidine were added to the cultures; after 2 hr the incorporation of [^{3}H]thymidine into TCA precipitable counts was determined.

arrested and very little tritiated thymidine was incorporated into DNA (Fig. 31, open triangles). These observations (Bell et al., 1975) established an experimental system in which the effect of a chosen agent on $pp60^{src}$ induced DNA synthesis could be studied. The increase in DNA synthesis observed upon downshift of tsNY68-infected cells was completely abolished if micromolar amounts of the calmodulin inhibitor chlorpromazine were added to the culture prior to the temperature shift (Fig. 31, closed circles). Chlorpromazine also had an inhibitory effect on the low level of DNA synthesis detected in the tsNY68-infected cells serum starved at the nonpermissive temperature (Fig. 31, closed triangles). The stimulation of DNA synthesis upon downshift to the permissive temperature was measured as a function of chlorpromazine concentration. Micromolar concentrations of chlorpromazine effectively inhibited DNA synthesis (Fig. 32). This is similar to the concentration of chlorpromazine that inhibited the in vitro phosphorylation of the 50 kD protein (Fig. 27).

The inhibition of [^{3}H]thymidine incorporation into DNA by chlorpromazine did not result from decreased uptake of thymidine. By autoradiography of labelled cells it was determined that the number of cells incorporating [^{3}H]thymidine had dramatically decreased. The intensity of labelling per nucleus was not affected indicating that chlorpromazine did not alter the cellular uptake of thymidine. The effect of chlorpromazine on $pp60^{src}$-induced DNA synthesis was found to be reversible. If cells were treated with chlorpromazine for 12 hours prior to downshift and the drug washed away before the downshift, much less inhibition of DNA synthesis was observed. The concentrations of chlorpromazine used in these experiments did not have a pronounced effect on cellular morphology.

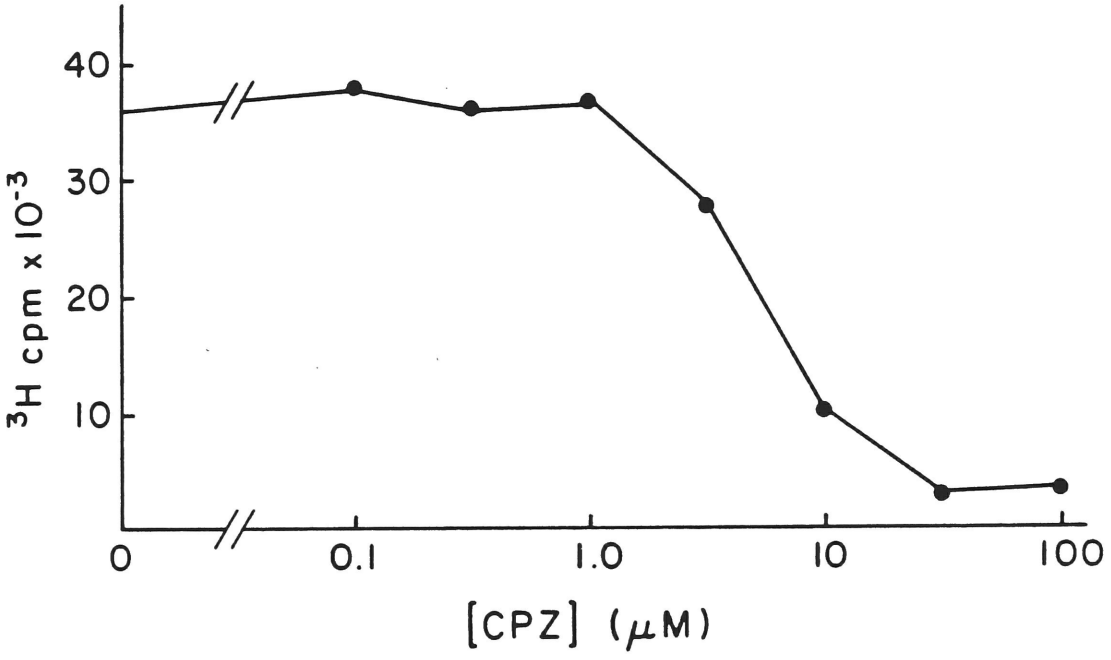


Fig. 32. Dose response curve for chlorpromazine effect on DNA synthesis. Cells were starved for 60 hr at the nonpermissive temperature, as described in Materials and Methods. After 60 hr cultures were treated with a range of concentrations of CPZ (0-100 μM) - 2 μCi of [^{3}H]thymidine was added per dish and the cells were shifted to the permissive temperature. After 18 hr the incorporation of [^{3}H]thymidine into TCA precipitable counts was determined.

Using this experimental system it was also possible to test the effect of chlorpromazine on the activation of $pp60^{src}$ in tsNY68-infected cells after downshift from the nonpermissive to permissive temperature. Extracts of tsNY68-infected CEFs grown chronically at the permissive temperature had a 5-10 fold higher level of $pp60^{src}$-associated kinase activity in TBR immunoprecipitates than extracts from infected cells grown at the nonpermissive temperature (Fig. 33). Immunoprecipitates of radiolabelled cell extracts demonstrated that approximately equal amounts of $pp60^{src}$ are present in tsNY68-infected cells grown at the permissive and the nonpermissive temperature (Levinson et al., 1978). The lower levels of kinase activity measured in cells grown at the nonpermissive temperature probably reflect heat inactivation of the temperature-sensitive $pp60^{src}$ kinase. The kinase activity detected in extracts of wild type SR-A RSV infected cells grown at both temperatures was similar to the high amount of activity measured in tsNY68-infected cells grown at the permissive temperature. When tsNY68-infected cells grown at 42°C were growth arrested and shifted to the permissive temperature 37°C, an increase in the $pp60^{src}$ kinase activity measured in TBR serum immunoprecipitates occurred within one hour of the temperature shift. After four hours the activity detected in the extracts had doubled (Fig. 33). Addition of 10 μM chlorpromazine to the cultures did not affect the increase in $pp60^{src}$ kinase activity after the shift from the nonpermissive to permissive temperature. Chlorpromazine also did not affect the kinase activity found in extracts of cells grown chronically at 37 or 42°C. Even when chlorpromazine was added to the cultures 24 hours prior to temperature shift no effect on $pp60^{src}$ kinase activity was observed (Table V).

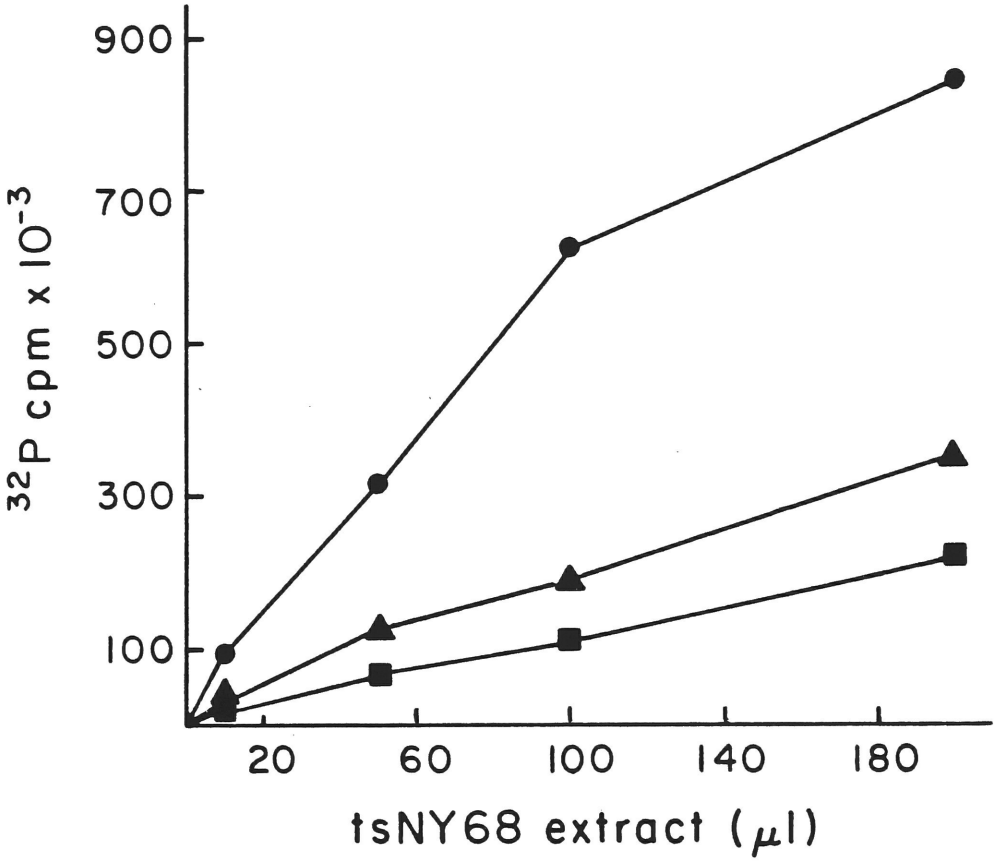


Fig. 33. Quantitation of pp60^{src}-associated kinase activity in tsNY68-infected CEFs. TsNY68 infected CEFs were grown chronically at 42°C (■), chronically at 37°C (●) or at 42°C and shifted to 37°C for 4 hrs (▲). The cells were lysed with detergents as described in Materials and Methods. Increasing amounts of extract were immunoprecipitated with TBR serum and assayed for pp60^{src}-associated kinase activity.

TABLE V

Effect of Chlorpromazine on the pp60^{src} Kinase Activity of tsNY68-RSV Infected Cells*

Condition	Concentration of Chlorpromazine Added (μM)			
	0	0.1	1.0	10
Cells grown at 37°C	55	-	-	54
Cells grown at 42°C	16	-	-	18
Cells grown at 42°C shifted to 37°C	25	26	22	25

*The kinase activity is expressed in terms of ^{32}P cpm x 10^{-3}. The activity is assayed as described in Materials and Methods.

Conclusions

Further characterization of the partially purified pp52^{src} and pp60^{src} preparations resulted in the identification of a 50 kD protein that appears to interact with the src gene product. The 50 kD protein was found to be phosphorylated in a Ca^{2+}-calmodulin dependent manner and this phosphorylation was inhibited by the calmodulin inhibitor chlorpromazine. The Ca^{2+}-calmodulin activated phosphorylation of the 50 kD protein is not pp60^{src}-induced since it occurs to a significant extent in 50 kD samples prepared from uninfected CEFs. It has not yet been determined if the Ca^{2+}-calmodulin activation of phosphorylation is an autophosphorylation event or if the 50 kD protein is phosphorylated by another kinase present in the preparation.

Several lines of experimentation suggested that pp52^{src} and the 50 kD protein bind to each other. The two proteins copurified during the pp52^{src} purification scheme and the 50 kD protein was coprecipitated with pp52^{src} in TBR serum immunoprecipitates. Comparable amounts of the 50 kD protein were detected in normal and transformed cells indicating that this protein is not simply a degradation product of pp60^{src}. Furthermore, under certain conditions the pp52^{src}-associated kinase activity and the 50 kD protein specifically bound to a calmodulin affinity column and were eluted with EGTA. In addition, pp60^{src} from crude cell extracts sedimented on glycerol gradients in two separate fractions. The more rapidly sedimenting form of pp60^{src} comigrated with the Ca^{2+}-calmodulin activated 50 kD protein. This fraction represents pp60^{src} that may be in a complex with substrate proteins (see also Brugge et al., 1981). It is not known if the Ca^{2+}-calmodulin activated 50 kD protein is identical to the 50 kD pp60^{src} substrate identified by

Brugge et al. (1981). Possibly the 50 kD protein is a pp60^{src} substrate that is also regulated or is itself a Ca^{2+}-calmodulin dependent protein kinase. Analysis of the *in vivo* and *in vitro* sites of phosphorylation of the 50 kD protein from normal and transformed cells should aid in understanding the way in which this protein interacts with pp60^{src}.

The possible involvement of Ca^{2+}-calmodulin in pp60^{src}-induced transformation *in vivo* was investigated indirectly using the calmodulin inhibitor chlorpromazine. This drug significantly inhibited the pp60^{src}-induced deregulation of cell growth but had no detectable effect on the pp60^{src}-associated kinase activity. Perhaps, the pp60^{src}-induced deregulation of DNA synthesis is mediated via the interaction of pp60^{src} with the Ca^{2+}-calmodulin activated 50 kD protein *in vivo*.

VI. MONOCLONAL ANTIBODIES TO PP60^{src} SUBSTRATES

Several *in vitro* pp60^{src} targets have been identified which may also mediate the effects of pp60^{src} in the transformed cell. The actin associated protein vinculin is phosphorylated *in vitro* by pp60^{src} (Chapter IV) and has been determined to be phosphorylated at a tyrosine residue *in vivo* in a transformation specific manner (Hunter and Sefton, 1980). Similar observations have been reported for the 34 kD phosphoprotein, although the function and location of this protein within the cell are not known (Radke and Martin, 1979; Erikson and Erikson, 1980). An additional potential protein target of pp60^{src} may be the Ca^{2+}-calmodulin activated 50 kD protein described in Chapter V.

This section describes an approach that has made it possible to better characterize and understand the function of two of the pp60^{src} targets vinculin and the 34 kD protein. Monoclonal antibodies to vinculin and the 34 kD protein were generated and characterized. These specific antibodies were used to study the pp60^{src} substrates in normal and transformed cells.

Materials and Methods

Antigens. Vinculin and the 34 kD protein were purified by published procedures as described in Materials and Methods, Chapter III.

Production of Monoclonal Antibodies. The monoclonal antibodies were produced by hybridomas between spleen cells of immunized mice and an established myeloma line. BALB/c mice received four intraperitoneal injections each with 100 μg of partially purified vinculin or 34 kD

protein at two week intervals, first in complete Freund's adjuvant, then twice in incomplete adjuvant, and once in saline. Spleens were removed from the immunized mice four days after the final immunization. Splenocytes were obtained by pressing the excised mouse spleens through a stainless steel mesh. The red blood cells were lysed by treatment with 0.75% ammonium chloride, on ice for 5 min and the remaining splenocytes (10^8 cells) were mixed with P3U-1 myeloma cells in a 5:1 splenocyte:myeloma cell ratio. The cells were then pelleted and fused by treatment for 8 min with 300 µl of 30% vol/vol polyethylene glycol 1000 (Baker Corp.). The cells were gently washed twice in DMEM and allowed to sit overnight in growth medium (DMEM-10% NCTC 109 with 5% human serum obtained from outdated fresh frozen plasma from the New York Blood Center). The next day cells were resuspended in a selective medium (growth medium plus aminopterin and supplementary hypoxanthine and thymidine) to kill the unfused myeloma cells deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRT). The cells were plated in 96 well flat bottom culture dishes ($5x10^4$ cells/ well) and fed after five and ten days with 65 µl growth medium. The clones were easily visible after 10 days and 50 µl of culture supernatant was removed for assay. Cells from positive wells were cloned by dilution into 96 well plates (at 1 and 4 cells/well) containing feeder layers of UV-irradiated mouse embryo fibroblasts. After approximately ten days supernatants from wells with single clones were reassayed and positives were recloned. Cells were frozen in 95% calf serum-5% DMSO for storage. Cells were also injected (10^8 cells) intraperitoneally into BALB/c mice and after two weeks ascitic fluid was collected. The fluid contained approximately 4-5 mg/ml of monoclonal

antibody. Antibody was precipitated from the ascites fluid by the addition of an equal volume of saturated ammonium sulfate. The precipitated protein was resuspended in 0.1 M sodium bicarbonate (pH 9.5) and was used for coupling to CNBr-activated Sepharose as described by Garvey et al. (1977). 10 mg monoclonal antibody were reacted with 10 ml packed CNBr-activated Sepharose as described in Materials and Methods, Chapter III.

Plate Binding Assay. Antigen-binding activity in hybridoma supernatants was detected as described by McKearn (1980). Antigen, either partially purified vinculin or 34 kD was bound to polyvinylchloride 96-well plates by incubating each well with 50 µl 0.2 mg protein/ml PBS for 4 hours. The liquid was poured off, the antigen fixed to the plate with methanol, and air dried. Culture supernatants were incubated with the antigen coated plates overnight. After washing 5 times with PBS/0.1% BSA, the wells with bound antibody were detected by the addition of a peroxidase-conjugated rabbit antibody to mouse IgG (1:100 dilution, Miles). The second antibody was incubated on the plate for 2 hours, the plate washed 5 times with PBS/BSA and 160 µl 1.0 mg/ml o-Phenylene diamine in 0.1 M citrate pH 4.5, 0.2% H_2O_2 added to the wells. Wells incubated with cell supernatants that contain antibodies positive for binding to the antigen turned brown in this colorimetric assay. Positive clones were screened using additional assays.

Immunoprecipitation. Monoclonal antibodies were used to immunoprecipitate radiolabelled extracts of normal and transformed cells as described in Materials and Methods, Chapter III. In immunoprecipitation experiments using clonal supernatants 500 µl supernatant were incubated

with 50 µl of protein A-Sepharose CL-4B for 1 hour. The mixture was centrifuged and the supernatant removed. The protein A-Sepharose pellet was mixed with the radiolabelled cell extracts and immunoprecipitated as usual. In some experiments prior to the addition of protein A-Sepharose, 25 µl 1.0 mg rabbit anti-mouse IgG, IgM was added to the clonal supernatants. This eliminated the possibility that monoclonal antibodies that do not bind to protein A directly were being passed over in the immunoprecipitation screen. Ascites fluid containing the monoclonal antibodies was used in some immunoprecipitation experiments (5 µl per 200 µl cell extract).

Immunofluorescence Staining. Chick embryo fibroblasts or SR-A RSV transformed CEFs were passaged on to collagen coated coverslips (18x18 mm) in DMEM containing 5% tryptose phosphate broth (GIBCO), 2% calf serum. After the cells had attached the medium was changed to DMEM containing 10 mM Hepes, 0.25% calf serum. Cells were used for immunofluorescence staining experiments 24-48 hours later. For 34 kD immunofluorescence staining cells on coverslips were fixed in PBS/3.7% formaldehyde for 20 min at room temperature. After rinsing 3-times in PBS cells were permeabilized by incubating in water for 1 min, methanol (-20°C) for 4 min, acetone (-20°C) for 3 min and air dried. Alternatively the cells were permeabilized by incubating with 0.2% Triton X100/PBS for 4 min followed by quenching for 5 min in 0.1 M glycine/PBS, and washing 3-times in PBS. Fixed and permeabilized cells were treated with 500 µl monoclonal culture supernatant or 500 µl monoclonal ascites fluid, diluted 1:100 with PBS containing 1 mg/ml goat antibody, for 60 min at room temperature. After washing 3-times with PBS the cells were incubated

with 500 µl 0.04 mg rabbit antibodies to mouse IgG, IgM per ml PBS containing 1 mg/ml goat antibody for 60 min. Cells were again washed 3-times with PBS and then incubated with 500 µl of a 1:50 dilution of fluorescein-conjugated goat antibodies to rabbit IgG (Microbiological Associates, Bethesda, Md.) for 60 min. After washing in PBS, the cells were mounted in 70% glycerol/PBS. Cells were stained with the vinculin antibodies as described above except in some cases the cells were permeabilized before fixation with 50 mM Mes (pH 6.1), 2 mM EGTA, 5 mM $MgCl_2$, 0.8% octylglucoside for 2-3 min followed by rinsing twice in the same buffer without detergents and then fixation with 3.7% formaldehyde/PBS. The formaldehyde was quenched by rinsing in 0.1 M glycine/PBS for 5 min, the cells were rinsed in PBS 3-times and incubated with antibodies as detailed above.

Affinity Purification of 34 kD Protein. Chicken gizzards (Pel-Freez, Rogers, Ar.) were thawed, trimmed, and passed through a meat grinder. 50 gm of gizzards + 500 ml buffer A (10 mM Tris-HCl pH 7.2, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.05% NP-40, 1% trasylol) were homogenized for 30 sec at high speed in a Waring blender. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant centrifuged at 100,000 g, 45 min, 4°C. The supernatant was brought to 10% glycerol and applied to a DEAE-cellulose column (1.8x40 cm) equilibrated with buffer A/10% glycerol. The column was washed with 200 ml buffer A/ 10% glycerol and the unbound fraction from this column was applied to a 30 ml 34 kD monoclonal antibody affinity column equilibrated with buffer A/10% glycerol. The column was washed with 100 ml buffer A/10% glycerol, 100 ml buffer A/1.0 NaCl, followed by 100 ml buffer A. The 34 kD protein

was eluted by washing with 80 ml buffer A containing 0.4 ml diethylamine. The column eluate was collected with stirring into 20 ml 1 M Tris-HCl (pH 6.8) and dialyzed extensively against buffer A. Approximately 1 mg of the 34 kD protein was obtained from 50 gm of gizzards.

The 34 kD protein was also purified by affinity chromatography from chick embryo fibroblasts. 10 ml thawed primary chicken embryo fibroblasts in 90 ml buffer A were lysed with 40 strokes in a Dounce homogenizer with a tight fitting plunger. The homogenate was centrifuged 100,000 g, 30 min, the supernatant brought to 10% glycerol and incubated with the 34 kD monoclonal affinity column by shaking for 2 hours. The resin was washed extensively with 10 volumes of buffer A, buffer A/1.0 M NaCl, again with buffer A and was eluted with buffer A containing diethylamine. The 34 kD protein eluate was collected into 1 M Tris-HCl (pH 6.8) and dialyzed exhaustively against buffer A. Approximately 7 mg of 34 kD protein were obtained from 10 ml chick embryo fibroblasts.

Assay of Malate Dehydrogenase. Malate dehydrogenase (MDH) activity was measured by the spectrophotometric method of Siegel and Bing (1956). MDH was measured by recording the rate at which oxaloacetate was reduced to malate via coupling the reaction to the oxidation of nicotinamide adenine dinucleotide (NADH). Conversion of NADH to NAD^+ results in a measurable reduction in light absorbance at λ=340 nm.

Results

Monoclonal Antibodies to Vinculin. Vinculin purified from chicken gizzards (Fig. 34) was used to immunize mice. Spleen cells from immunized mice were fused with P3U1 cells and cell hybrids were selected and cloned. Approximately 1000 clones were assayed by binding of the cell supernatants containing secreted antibody to partially purified vinculin immobilized on plastic plates. Of the 1000 clones, 70 were positive for binding and were screened further by immunoprecipitation and immunofluorescence assays.

The potential anti-vinculin monoclonals were further used to immunoprecipitate [^{3}H]leucine labelled chick embryo fibroblast cell extracts. Supernatants from four clones immunoprecipitated a 130,000 dalton protein. Immunoprecipitation data for two of the monoclonals is shown in Fig. 35. A 130,000 dalton protein was immunoprecipitated from normal (Fig. 33, lanes 1 and 2) or transformed (Fig. 35, lanes 4 and 5) cell extracts by the monoclonal antibodies but this protein was not precipitated using control antibodies (Fig. 35, lanes 3 and 6). These monoclonal antibodies also immunoprecipitated a 130,000 dalton phosphoprotein from extracts of normal (Fig. 35, lane 7) and transformed (Fig. 35, lane 8) cells labelled with ^{32}P orthophosphate.

The four monoclonal antibodies that immunoprecipitated the 130,000 dalton phosphoprotein were further tested for staining of chick embryo fibroblasts. CEFs were extracted with octylglucoside, fixed, and stained by indirect immunofluorescence. All four monoclonal antibodies showed the characteristic vinculin staining pattern (Fig. 36) (Geiger, 1979; Burridge and Feramisco, 1980). Staining was seen at sites of cell

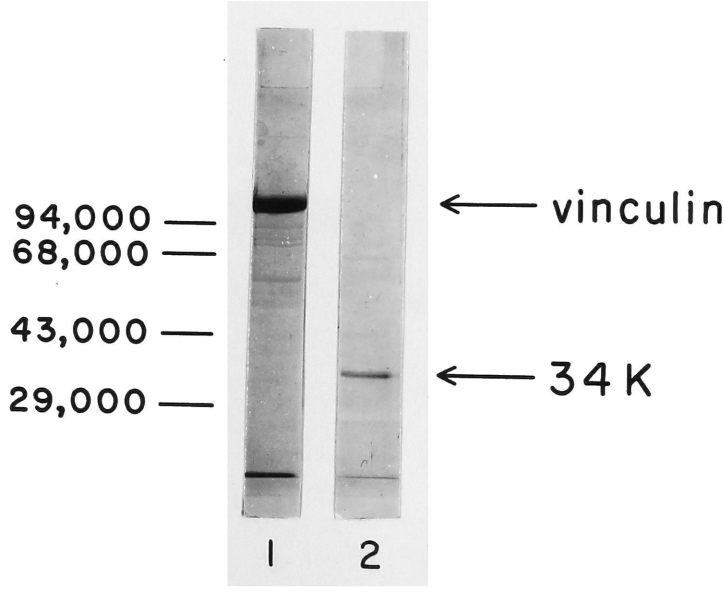


Fig. 34. SDS-polyacrylamide gel analysis of antigens used to generate monoclonal antibodies. Partially purified vinculin (lane 1), 34 kD protein (lane 2). The gel was stained with Coomassie brilliant blue to detect proteins.

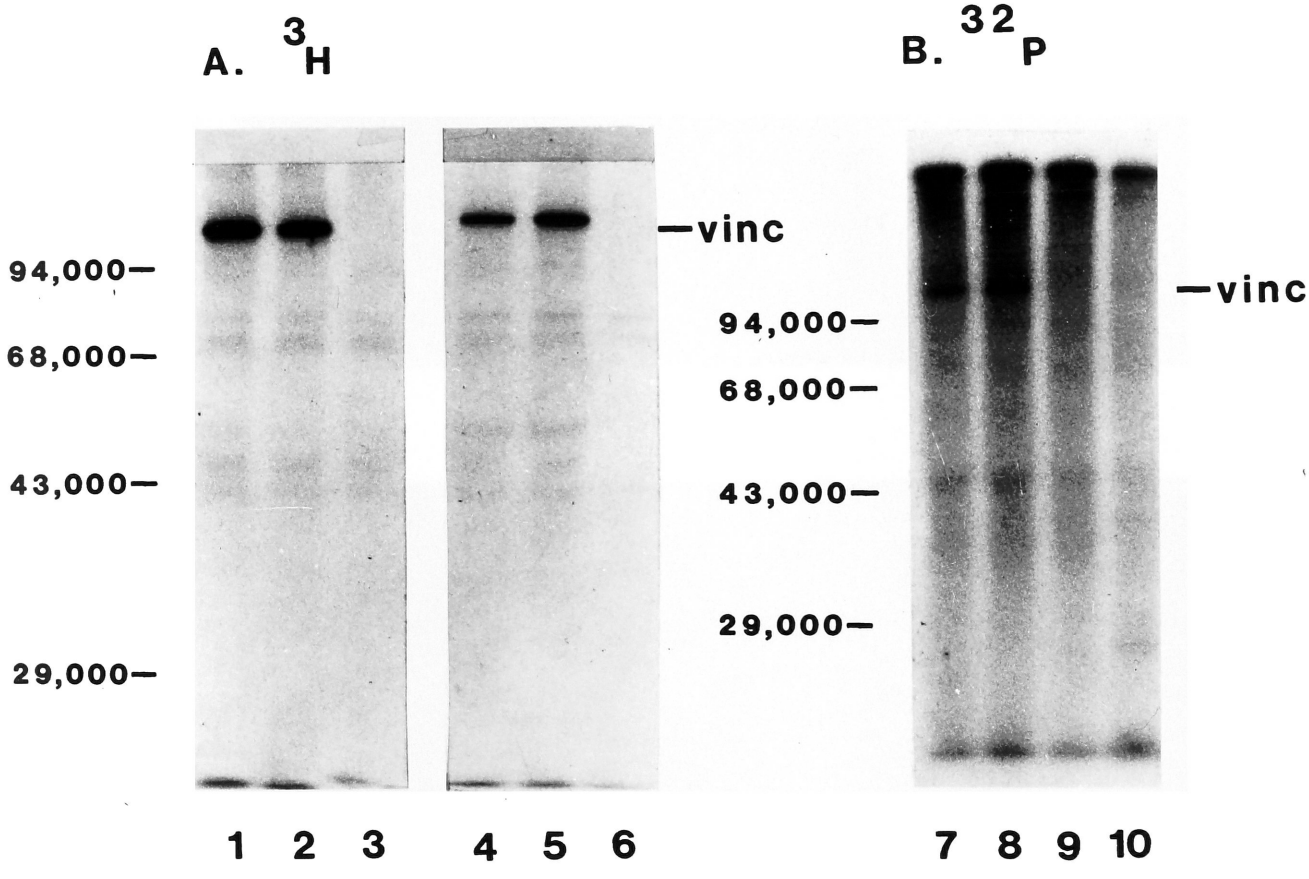


Fig. 35. Autoradiogram of immunoprecipitates using monoclonal antibodies generated against vinculin. (A) [^{3}H]leucine labelled CEFs, (lanes 1-3); SR-A RSV infected CEFs, (lanes 4-6). [^{3}H] labelled extracts were immunoprecipitated with monoclonals, 2A2 (lanes 1 and 4); 2C2, (lanes 2 and 5); control antibody, (lanes 3 and 6). (B) [^{32}P] labelled cell extracts; uninfected CEFs immunoprecipitated with monoclonal 2A2, (lane 7); control antibody, (lane 9); SR-A RSV infected CEFs immunoprecipitated with monoclonal 2A2, (lane 8); control antibody, (lane 10).

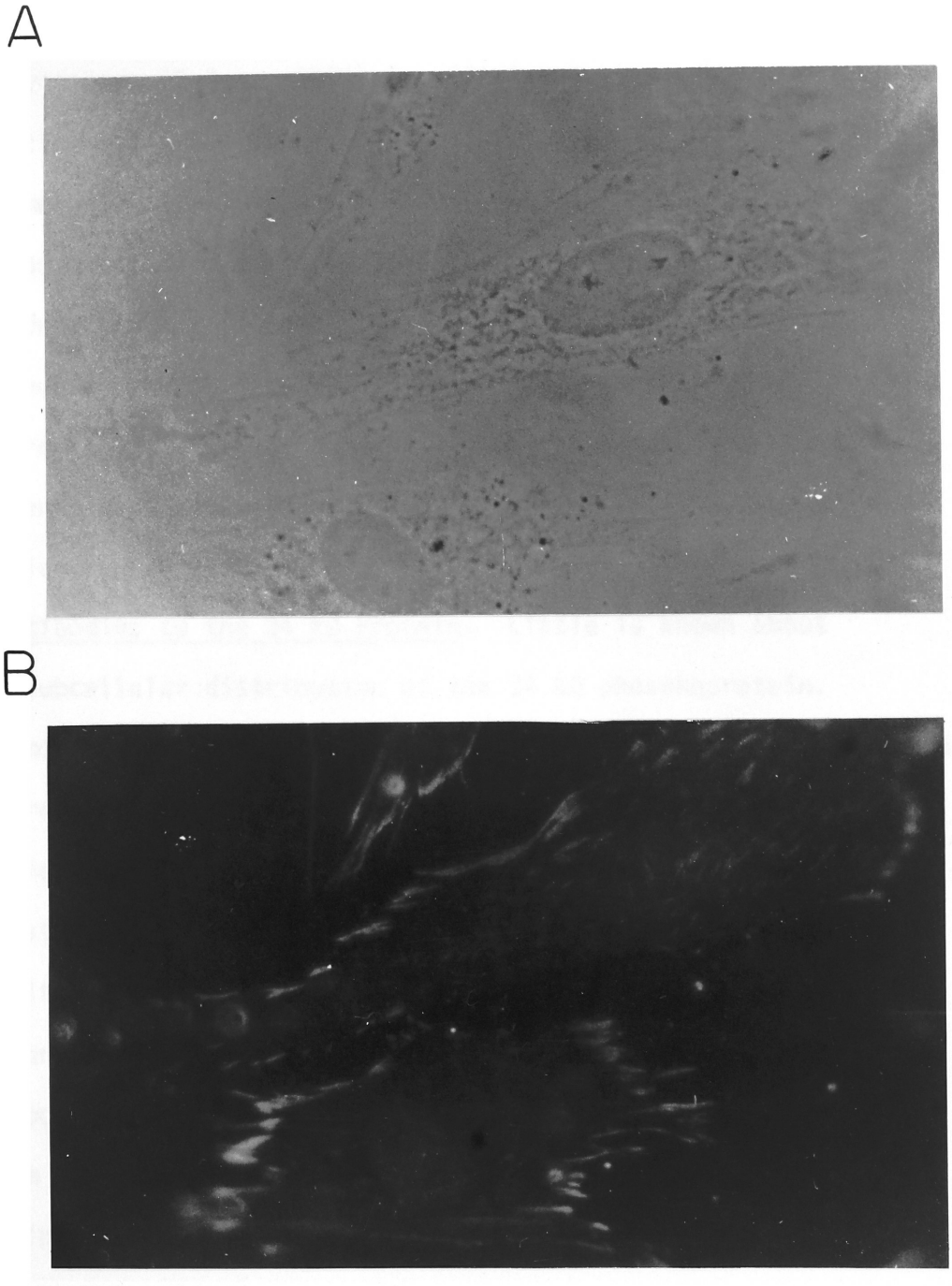


Fig. 36. Indirect immunofluorescence staining of cultured cells with a monoclonal antibody generated against vinculin. (A) Phase contrast illumination. (B) Staining, with anti-vinculin monoclonal 2A2. Cells were permeabilized with octyl-glucoside as described in Materials and Methods.

contact with the substratum which was often coincident with the ends of microfilaments. In cells fixed before permeabilization, vinculin antibodies stain the cytoplasm (Geiger, 1979) in addition to the adhesion plaques. The monoclonal antibodies also stained the cytoplasm in cells fixed prior to permeabilization. No staining of either cytoplasm or focal adhesion plaques was detected using control monoclonal antibodies. In the absence of detergents to permeabilize the cell no staining was seen with any of the four monoclonal antibodies, indicating that the antibodies do not recognize cell surface antigens. The immunoprecipitation and immunofluorescence data were sufficient to conclude that the four monoclonal antibodies recognized vinculin.

Monoclonal Antibodies to the 34 kD Protein. Little is known about the structure and subcellular distribution of the 34 kD phosphoprotein. Nevertheless, two monoclonal antibodies were identified that immunoprecipitated proteins with apparent molecular weights of approximately 34,000 daltons on SDS polyacrylamide gels. Immunoprecipitation data for one of the monoclonals designated 5E1 is shown in Fig. 37. The antibody immunoprecipitated a 34,000 dalton protein from [^{3}H]leucine labelled extracts of normal (Fig. 37, lane 1) and transformed (Fig. 37, lane 2) cells. Only one of the two monoclonal antibodies (5E1) immunoprecipitated a 34 kD phosphoprotein from ^{32}P labelled transformed cell extracts. The 34 kD protein immunoprecipitated by the second monoclonal antibody (12E3) was not detectably phosphorylated. The 34 kD phosphoprotein immunoprecipitated by 5E1 was determined by phosphoamino acid analysis to be phosphorylated at both a serine and a tyrosine residue in transformed cells (Fig. 38), however, in the normal cell the protein was phosphorylated

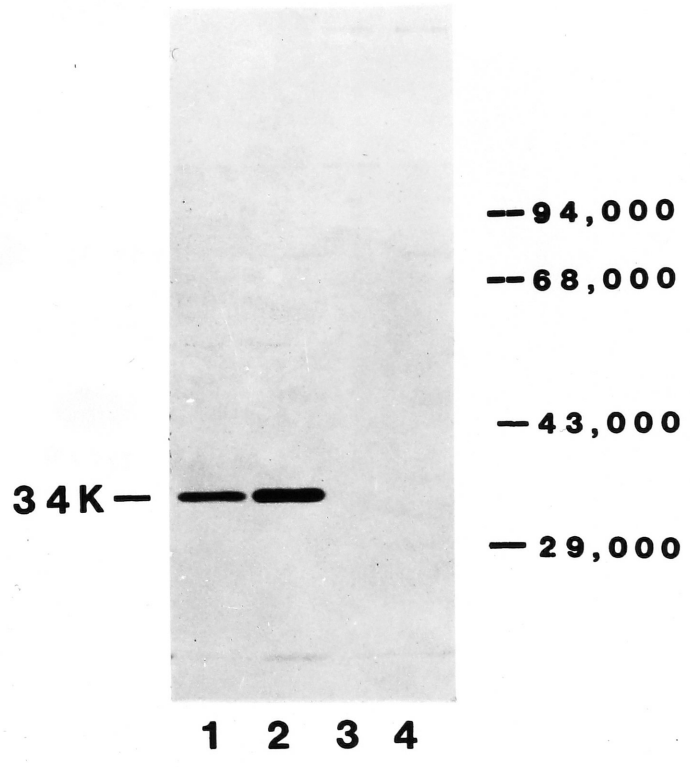


Fig. 37. Autoradiogram of immunoprecipitates using a monoclonal antibody generated against the 34 kD protein. Uninfected CEFs (lanes 1 and 3), and SR-A RSV infected CEFs (lanes 2 and 4), were labelled with [^{3}H]leucine. Cells were extracted and immunoprecipitated with the 34 kD monoclonal (lanes 1 and 2), or a control antibody (lanes 3 and 4).

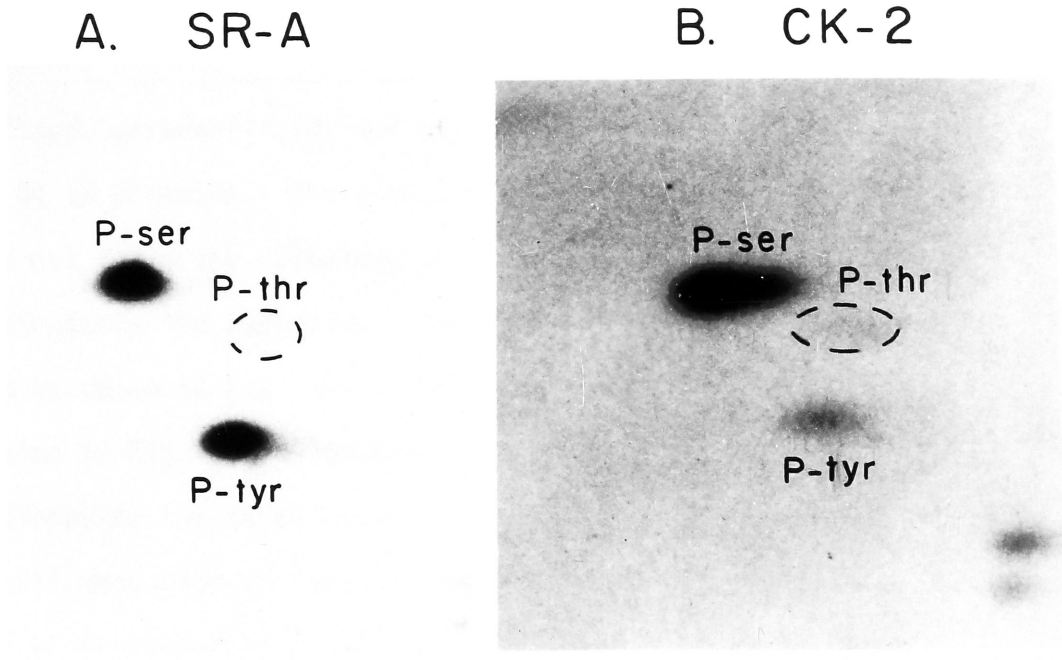


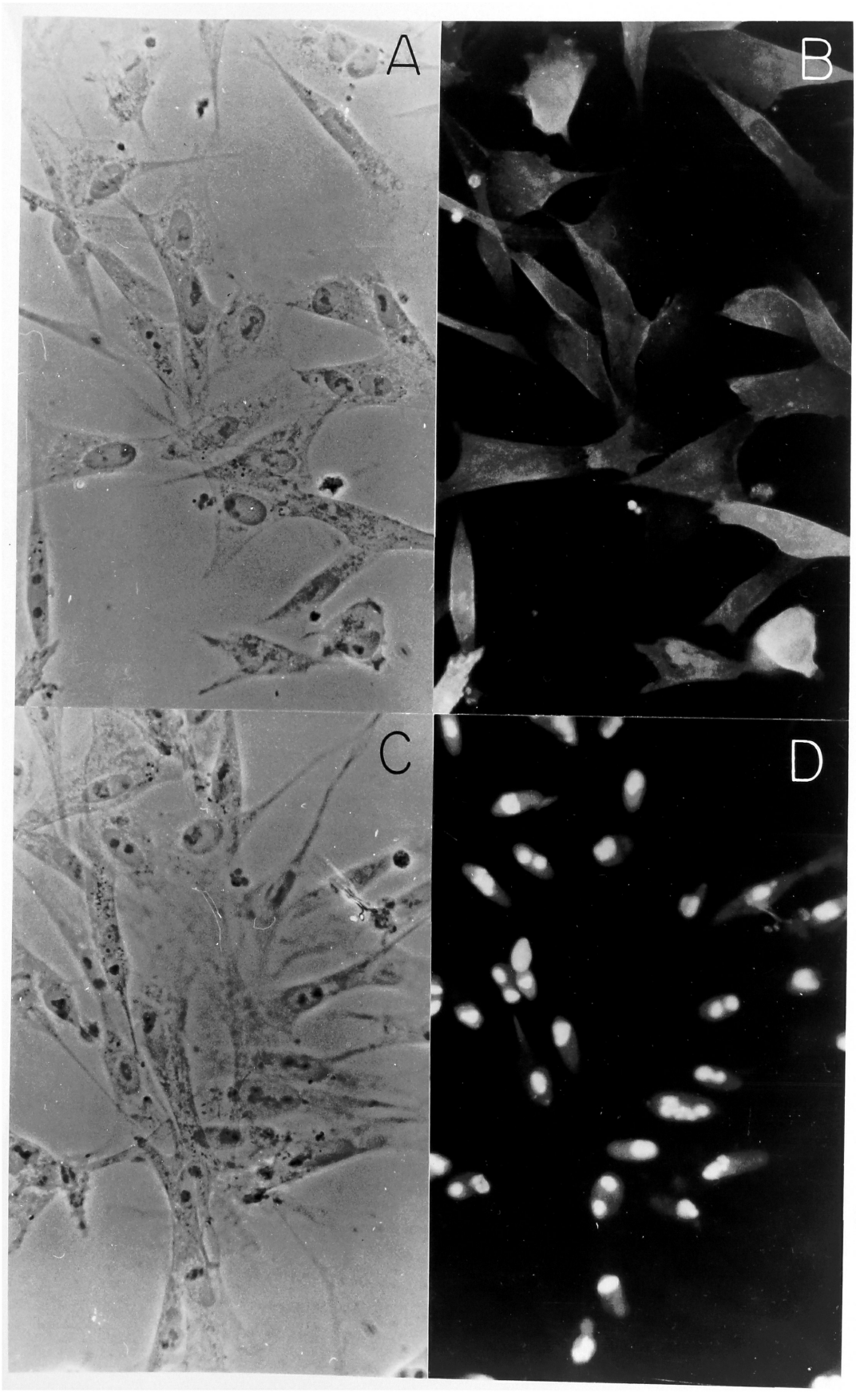
Fig. 38. Phosphoamino acid analysis of 34 kD phosphoprotein. The 34 kD phosphoprotein immunoprecipitated by the monoclonal antibody from normal and transformed cells was eluted from gels, hydrolyzed, and phosphoamino acids separated by two dimensional thin layer electrophoresis as described in Materials and Methods. (A) Analysis of 34 kD phosphoprotein from SR-A RSV infected CEFs; (B) Analysis of 34 kD phosphoprotein from uninfected CEFs. Marker phosphoamino acids were co-electrophoresed with the sample and visualized by ninhydrin staining. Radioactive phosphoamino acids were visualized by autoradiography.

primarily at a serine residue (Fig. 38). The phosphoamino acid analysis established that the 34 kD phosphoprotein immunoprecipitated by the 5E1 monoclonal antibody was phosphorylated at tyrosine in a transformation-specific manner.

Immunofluorescence Staining with 34 kD Monoclonals. Chick embryo fibroblasts were fixed, permeabilized, and stained with the two monoclonal antibodies to the 34 kD proteins. The antibody to the non-phosphorylated protein (12E3) did not stain the cytoplasm but bound specifically to the cell nucleus, particularly the nucleolus. Indirect immunofluorescence staining with 12E3 is shown in Fig. 39D. The cells are shown by phase contrast illumination in Fig. 39C. Staining of permeabilized CEFs by the monoclonal antibody to the 34 kD phosphoprotein is shown in Fig. 39B and phase contrast illumination of these cells is seen in Fig. 39A. The antibody to the 34 kD phosphoprotein stained diffusely, although in some cells a finely reticular, sometimes filamentous, network was detected at higher magnification (Fig. 40). In the absence of detergents to permeabilize the cell, no staining with either monoclonal antibody was observed, indicating that the antigens are not exposed at the cell surface.

Normal and SR-A RSV transformed CEFs were compared for staining with the monoclonal antibody to the 34 kD phosphoprotein (Fig. 41). The overall staining pattern was suggestive of membrane association since no nuclear shadow was visible and the fluorescence appeared in two distinct focal planes. In the transformed cells the staining was often more intense along the cell edges. This appeared to correlate with increased membrane ruffling and other morphological changes that occurred in the CEFs transformed by RSV.

Fig. 39. Indirect immunofluorescence staining of cultured cells with monoclonal antibodies to 34 kD proteins. (A and B), staining is with monoclonal 5E1, the antibody to the 34 kD phosphoprotein. (A), phase contrast illumination; (B), indirect immunofluorescence staining. (C and D) staining is with monoclonal 12E3, the antibody to the non-phosphorylated 34 kD protein. (C), phase contrast illumination; (D) indirect immunofluorescence staining.


A
B
C
D

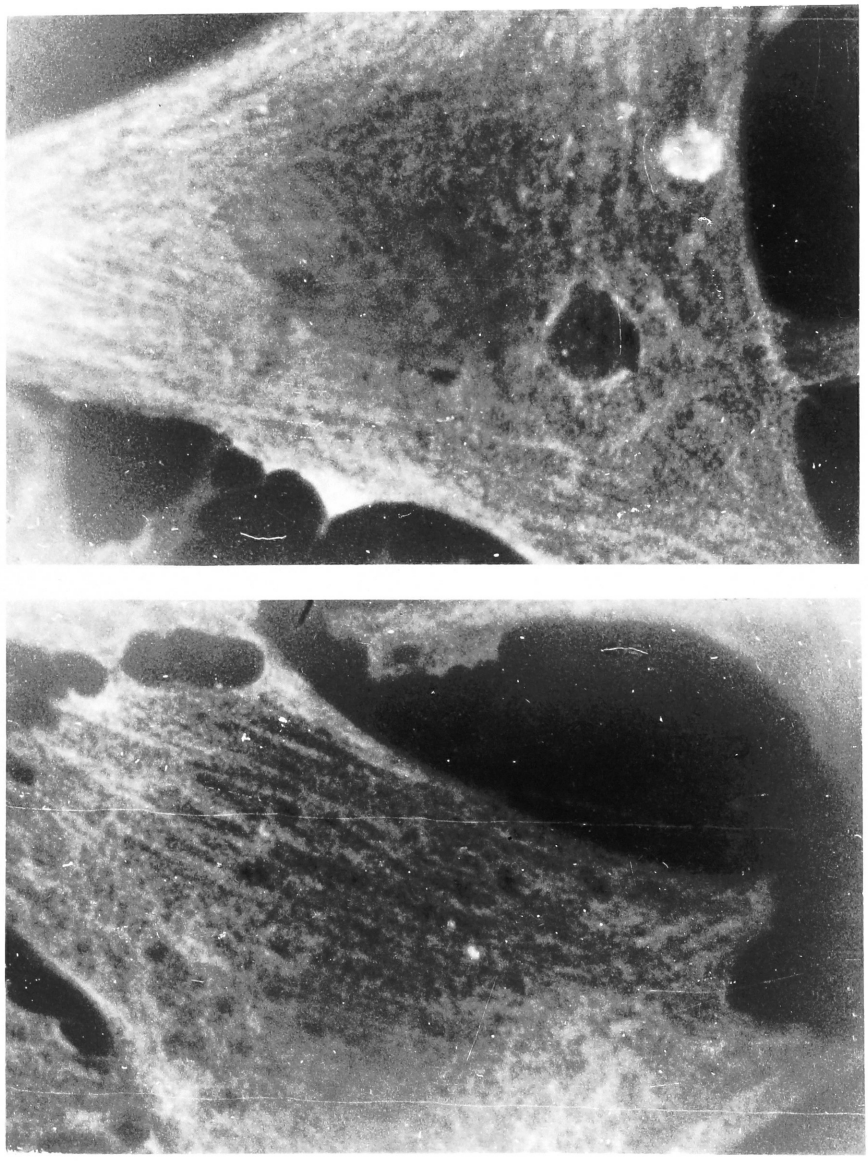


Fig. 40. Indirect immunofluorescence staining of cultured cells with the monoclonal antibody to the 34 kD phosphoprotein. Cells were fixed with formaldehyde and then permeabilized with methanol.

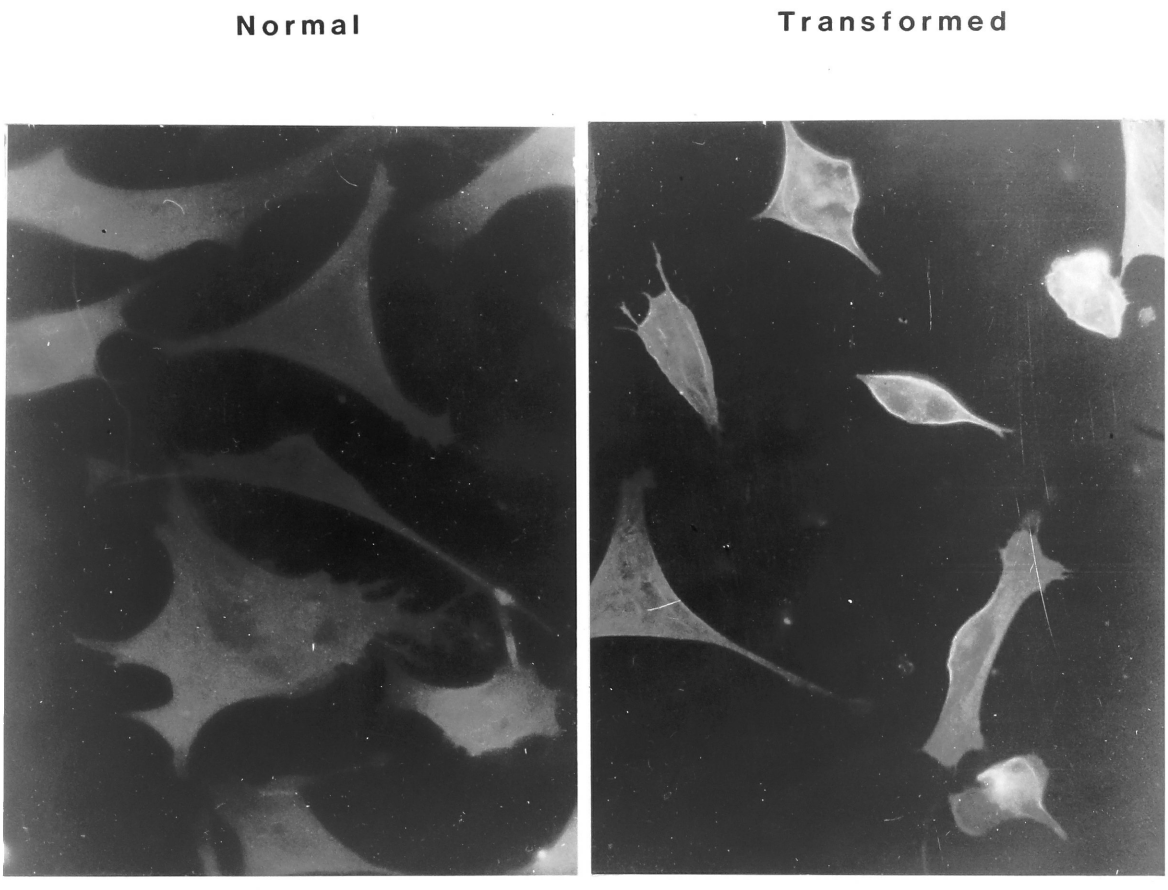


Fig. 41. Comparison of the immunofluorescence staining pattern of the monoclonal antibody to the 34 kD phosphoprotein in SR-A RSV infected and uninfected CEFs. Cells were fixed with formaldehyde and permeabilized with 0.1% Triton X100.

Purification and Characterization of Vinculin and the 34 kD Protein. In addition to the subcellular localization experiments the monoclonal antibodies also allowed the determination of the tissue distribution of vinculin and the 34 kD protein in the chick embryo. Significant amounts of both proteins were detected by immunofluorescence or immunoprecipitation in every tissue examined, including skeletal muscle, fibroblasts, gizzard, spleen, bursa, liver and brain.

In order to study functional differences between the $pp60^{src}$ substrates isolated from normal and transformed cells, rapid purification protocols were developed using monoclonal affinity columns. The monoclonal antibody to the 34 kD phosphoprotein was coupled to Sepharose and the 34 kD protein purified. Chicken gizzards, a convenient source of starting material, were homogenized in the presence of detergents and the extract (Fig. 42, lane 1) passed over a DEAE-cellulose column. Application of the unbound fraction to the monoclonal affinity column and elution at high pH gave an apparently homogeneous preparation of the 34 kD protein (Fig. 42, lane 2). Actin is present in large amounts in chick gizzard and binds nonspecifically to the affinity column if it is not removed by prior absorption to the DEAE-cellulose column. It was possible to purify the 34 kD protein from chick embryo fibroblasts, which contain signficantly less actin than do chicken gizzards, by a one-step protocol. Chick embryo fibroblasts were extracted in detergent, mixed with antibody coupled to Sepharose, washed extensively with detergent and high salt solutions, and the 34 kD protein was eluted at high pH. A homogeneous preparation of the 34 kD protein was obtained. Similar procedures were used to obtain highly purified 34 kD protein and

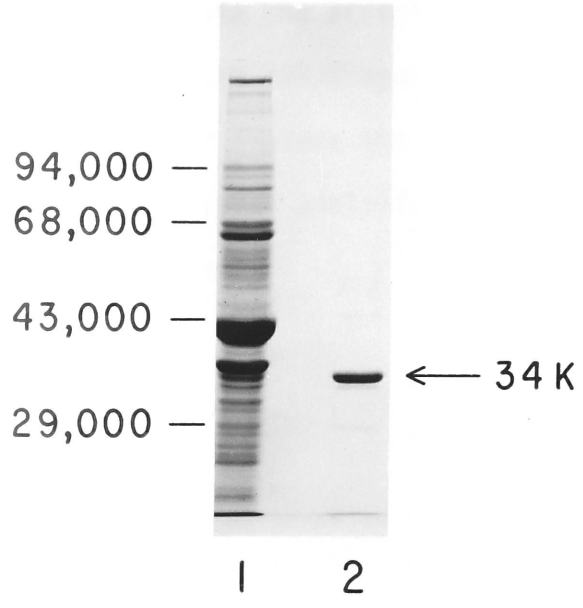


Fig. 42. SDS-polyacrylamide gel analysis of affinity purified 34 kD protein. Chicken gizzard whole cell homogenates, (lane 1); affinity purified 34 kD protein, (lane 2). The gel was stained with Coomassie brilliant blue to detect proteins.

vinculin from normal or transformed chick cells. The affinity purified 34 kD protein and vinculin are being used in structural and functional studies.

The function of the 34 kD protein is not known. Recently, however, Rübsamen et al. (1982) reported that a 34 kD phosphoprotein copurified with cytosolic malate dehydrogenase and suggested that the two proteins were identical. In preliminary experiments the affinity purified protein did not appear to be associated with malate dehydrogenase activity. In addition, the monoclonal antibody did not deplete the MDH activity from crude chick cell extracts. Future structural analysis of the two proteins is necessary to resolve this issue.

Conclusions

Monoclonal antibodies to two pp60^{src} substrates vinculin and the 34 kD phosphoprotein were prepared. The four antibodies raised against partially purified vinculin immunoprecipitated a 130,000 dalton phosphoprotein from both normal and transformed cells. By indirect immunofluorescence the antibodies stained chick embryo fibroblasts at sites of cell contact with the substratum. This immunoprecipitation and immunofluorescence staining data demonstrated that the four antibodies recognize vinculin.

Two monoclonal antibodies were identified which immunoprecipitated a 34 kD protein but only one of the antibodies recognized a 34 kD phosphoprotein. This 34 kD phosphoprotein was phosphorylated at a tyrosine in a transformation specific manner suggesting that it is probably the same protein originally identified by Radke and Martin (1979).

The tissue distribution and subcellular localization of the 34 kD proteins was examined using the monoclonal antibodies. The 34 kD phosphoprotein was localized, by indirect immunofluorescent staining, to the plasma membrane. The monoclonal antibody to the non-phosphorylated protein specifically stained the nucleoli.

Monoclonal antibodies coupled to Sepharose were used to affinity purify the pp60^{src} substrates. The availability of the highly purified substrates makes it possible to study the function of these proteins in normal and transformed cells.

VII. SUMMARY AND DISCUSSION

Transformation of cells by Rous sarcoma virus results from the activity of a single protein $pp60^{src}$. At the time this thesis work was started very little was known about the mode of action of this protein within the cell. The biochemical identification of $pp60^{src}$ in 1977 by Brugge and Erikson was followed by an intense effort in many laboratories aimed at understanding how this one protein effects the multiple complicated changes involved in cell transformation.

Much of the work done in other laboratories to characterize the structure and function of $pp60^{src}$ was summarized in Chapters I and II. As a result of this thesis work two procedures, described in Chapter III, were developed which allowed the purification of the src protein. In the first protocol the $pp60^{src}$-associated kinase activity was purified in detergent free solution but as a proteolytic cleavage product of $pp60^{src}$ with a molecular weight of 52,000 daltons. In the second scheme $pp60^{src}$ was fractionated, in the presence of detergents and protease inhibitors, as an intact protein.

The activity of the purified proteins was characterized *in vitro* (Chapter IV). Purification of $pp60^{src}$ resulted in the removal of phosphatases which made it difficult to study $pp60^{src}$ activity in solution. The partially purified $pp60^{src}$ was found to phosphorylate α-casein in solution specifically at tyrosine residue(s). The interaction of $pp60^{src}$ with cytoskeletal proteins was also examined and it was determined that the $pp60^{src}$ kinase phosphorylated a range of different cytoskeletal proteins *in vitro* at tyrosine residues. In addition the src kinase was

observed to phosphorylate a variety of other physiological, as well as, non-physiological substrates.

Further characterization of the partially purified src preparations, Chapter V, demonstrated that they contained a 50 kD protein that was phosphorylated in the presence of Ca^{2+}-calmodulin. The Ca^{2+}-calmodulin activated kD protein appeared to interact with $pp60^{src}$ in vitro by several criteria and is a potential src substrate. Ca^{2+}-calmodulin regulated control mechanisms were also implicated in the in vivo action of $pp60^{src}$. The calmodulin inhibitor chlorpromazine was found to inhibit the $pp60^{src}$ induced deregulation of DNA synthesis.

As a result of the experiments described in Chapters IV and V and experiments done in other laboratories detailed in the History and Background section several potential src substrates were identified. In an effort to further characterize these proteins monoclonal antibodies were generated against two of the proteins: the actin-associated protein vinculin and a 34 kD protein of unknown function (Chapter VI). The antibodies were used to purify and characterize these src targets from normal and transformed cells. Studies using antibodies and purified components have made it possible to begin to understand the complicated biochemical changes that occur during cellular transformation.

DISCUSSION

The original goal of this thesis work was to purify the src gene product, identify some of its targets, and to use this information to probe the mechanisms involved in the control of cell growth. Of particular interest was the possible interaction of $pp60^{src}$ with the cytoskeleton and how that might relate to the regulation of cell growth.

In order to purify $pp60^{src}$ it was first necessary to find a suitable source of starting material. RSV-transformed CEFs were useful for many studies of $pp60^{src}$ structure and function since they contain relatively high levels of $pp60^{src}$, but these cells cannot be passaged repeatedly in culture. It is therefore difficult to obtain the large quantities of cells necessary for purifying a protein such as $pp60^{src}$ which, even in transformed cells, is present as only 0.1% of the total cell protein (Collett et al., 1978; Karess et al., 1979). A variety of permanently transformed mammalian cell lines were screened for the presence of $pp60^{src}$ kinase using TBR serum. Of all the cells tested the rat tumor cell line RR1022 was found to contain the highest levels of $pp60^{src}$-associated kinase activity in TBR immunoprecipitates. Peptide analysis of the 60 kD protein immunoprecipitated from these cells with TBR serum established that the protein was $pp60^{src}$, and a scheme was developed for purifying the src protein from the RR1022 cells several thousand fold, in detergent free solution. The purification scheme provided the first available method for obtaining the $pp60^{src}$-associated kinase activity free of phosphatases and other inhibitors. The protocol proved to be particularly advantageous because $pp60^{src}$ was purified as a soluble

protein in the absence of detergents which often inhibit enzymatic assays and would certainly interfere with cellular microinjection experiments. The major disadvantage of the fractionation scheme was that $pp60^{src}$ was degraded during the purification and the resulting 52 kD proteolytic product could have different enzymatic properties and did have different solution properties from those of the intact enzyme.

Detergents were necessary to extract intact $pp60^{src}$ from cells in high yield, supporting the observations by others (Krueger et al., 1980 a,b; Courtneidge et al., 1980) using immunofluorescence microscopy and subcellular fractionation that $pp60^{src}$ interacts with cell membranes. The fact that the src protein is solubilized in detergent free solution when it is degraded to the 52 kD species suggests that the cleaved NH_2-terminal 8 kD fragment may be involved in anchoring this protein to the plasma membrane. Alternatively, cleavage of $pp60^{src}$ to $pp52^{src}$ could cause a conformational change in the protein which results in its release from membranes. These solution differences between $pp60^{src}$ and $pp52^{src}$ made it all the more likely that these two species might have different enzymatic properties so that it seemed worthwhile to attempt to purify $pp60^{src}$ as an intact species.

Erikson et al. (1979) reported the purification of $pp60^{src}$ in an undegraded form; however, the alternative protocol presented in Chapter III gave more reproducible results. $pp60^{src}$ was purified from RR1022 cells as an intact species in the presence of detergents and protease inhibitors. The $pp60^{src}$ was found to be quite stable to degradation even in crude cell extracts under the appropriate buffer conditions. In

some preparations, however, even after extensive purification pp60^{src} degraded to the 52 kD species. The mechanism involved in the degradation of pp60^{src} to the 52 kD protein is not known. Possibly the reaction is autocatalytic or else is due to a copurifying protease since the activity is still present in the more purified column fractions. Casein-Sepharose was a convenient last step in the purification procedure since it not only yielded a good fractionation of the src protein but was also useful for separating pp60^{src} from pp52^{src}. pp52^{src} did not bind as tightly to casein Sepharose as did pp60^{src}. This may reflect differences in the affinity of the two src protein species for substrates. Perhaps intact pp60^{src} will bind substrates more tightly than pp52^{src}.

The binding of pp60^{src} to the casein column is of particular interest because it suggests that pp60^{src} might complex with its physiological substrates as well. If this turns out to be true, it might be possible to design a highly specific affinity column using a src substrate coupled to Sepharose; this would allow the rapid purification of pp60^{src} in high yield.

After chromatography on four affinity supports, including casein Sepharose, the purified pp60^{src} was not homogeneous although it was the major component in the preparation. Some of the other proteins present in the preparation may be substrate proteins that bind to and copurify with pp60^{src}. The three major contaminants in the preparation are proteins with molecular weights of 34,000, 47,000 and 50,000 daltons. Cooper and Hunter (1981a) have reported that proteins with similar molecular weights are among the major proteins specifically phosphorylated at tyrosine residues in transformed cells and have suggested that

they may be pp60^{src} substrates *in vivo*. Purchio (1982) observed that similar proteins copurified with pp60^{src} under different conditions. The evidence that the proteins in the partially purified sample are *src* substrates is circumstantial, and more extensive characterization of these proteins is necessary to resolve this issue.

The partially purified preparations of pp52^{src} and pp60^{src} were used to study the activity of the *src* protein in solution. The purification of pp60^{src} successfully removed contaminating phosphatases that complicated the study of the *src* kinase activity in solution. This was demonstrated in an experiment which compared the ability of crude cell extracts and partially purified pp60^{src} to hydrolyze the phosphate from the ^{32}P labelled IgG heavy chain in TBR serum immunoprecipitates. The crude cell extracts contained significantly more phosphatase activity than the purified preparations. It was not determined whether the phosphatases were specific for phosphotyrosine. Recently several zinc sensitive phosphatases have been described that specifically remove the phosphate from phosphotyrosine residues (Gallis et al., 1981; Brautigan et al., 1981; Swarup et al., 1981). Dephosphorylation of ^{32}P labelled IgG heavy chain could be used as a specific assay for purifying the tyrosine phosphatases. The role of these phosphatases in the regulation of viral and normal cell pp60^{src} has not yet been adequately explored.

Removal of the phosphatases and other inhibitors made it possible to test directly for pp60^{src} kinase activity in solution. In solution, the partially purified pp52^{src} and pp60^{src} phosphorylated α-casein at a tyrosine residue(s). This suggests that the kinase activity detected in

TBR serum immunoprecipitates reflects an actual property of the pp60^{src} enzyme and that the lack of phosphorylation of substrates such as casein added exogenously to TBR serum immunoprecipitates is probably a consequence of pp60^{src} being bound up in the immune complex so that it is unavailable for reaction with substrates. The fact that the tyrosine kinase activity copurifies with pp60^{src} during four successive purification steps suggested further that the pp60^{src} kinase activity is an intrinsic property of pp60^{src}, although it does not rule out the possibility that the kinase activity could be due to a cellular kinase that binds to and copurifies with pp60^{src}. Recent experiments from other laboratories also support the view that the kinase activity is intrinsic to pp60^{src}. It was observed that pp60^{src} synthesized by cell-free translation, in the absence of most of the cellular kinases, was associated with a kinase activity (Sefton et al., 1979). Furthermore, pp60^{src} expressed in E. coli, as a result of the insertion of the src gene into the bacteria, was found to be associated with tyrosine kinase activity (Gilmer and Erikson, 1981; McGrath and Levinson, 1982).

Further characterization of the activity of partially purified pp52^{src} and pp60^{src} demonstrated that these proteins were both phosphorylated endogenously when incubated with [γ-^{32}P]ATP and magnesium. It is not clear whether this phosphorylation is due to autophosphorylation of pp60^{src} or is a result of the activity of another kinase in the enzyme preparation. Erikson et al. (1979) have also detected endogenous phosphorylation at a tyrosine residue in partially purified pp60^{src} preparations. Purchio (1982) recently exhaustively purified pp60^{src} by both immunoaffinity and classical column chromatography and reported that the pp60^{src} is

endogenously phosphorylated. Purchio suggested on the basis of his extensive purification that the endogenous phosphorylation is actually due to autophosphorylation. Levinson et al. (1980) have been unable to detect endogenous phosphorylation in their pp60^{src} preparations and have suggested that the endogenous phosphorylation of pp60^{src} is due to the presence of a contaminating kinase. The issue of endogenous versus autophosphorylation of pp60^{src} is unresolved. The mechanism by which pp60^{src} is phosphorylated is of considerable interest because it may be important in the regulation of pp60^{src} activity within the cell (reviewed by Erikson et al., 1980).

The state of phosphorylation of a protein can drastically affect its activity both in vivo and in vitro (reviewed by Krebs and Beavo, 1979). It would be useful to determine the state of phosphorylation of the partially purified pp60^{src} and to study how phosphorylation at tyrosine and serine affects its activity and substrate specificity in vitro. In order to do this it will be necessary to develop a method for phosphorylating and dephosphorylating pp60^{src} in vitro, and a procedure for separating the different phosphorylated forms of pp60^{src}.

Having established that partially purified pp60^{src} was associated with a casein kinase activity in solution, the next step was to define more physiological activities that might be associated with pp60^{src}. The original goal in purifying pp60^{src} was to investigate the interaction of this protein with the cytoskeleton. The purified src protein was tested in the microinjection assay, however, no disruption of the microfilaments was observed. In the initial injection experiments it was determined that the buffers used in the pp60^{src} purification scheme were

not compatible with cell microinjection. However, when the purified pp60^{src} was dialyzed into a buffer appropriate for microinjection the pp60^{src} kinase was inactivated, and the injected protein failed to induce dissolution of the microfilaments. Additional experiments are required to establish the appropriate buffer conditions so that active purified pp60^{src} can be tested in the microinjection assay. The state of phosphorylation of the partially purified pp60^{src} may also affect its activity in the microinjection assay. This issue also requires further investigation.

In other experiments it was not possible to detect any direct interaction of pp60^{src} with cytoskeletal proteins; however, the purified pp60^{src} was found to phosphorylate a variety of cytoskeletal proteins *in vitro* including tubulin, actin, vinculin, myosin and filamin. The phosphorylation of tubulin, actin, and vimentin by pp60^{src} *in vitro* has also been reported by Collett et al. (1980) and Levinson et al. (1980). After purification, pp60^{src} has a broad specificity as a kinase phosphorylating many cytoskeletal proteins, although tropomyosin and α-actinin were not phosphorylated under the conditions tested.

The *in vitro* phosphorylation of the cytoskeletal proteins is promising, although the results must be viewed cautiously because pp60^{src} was found to phosphorylate a range of physiological and non-physiological substrates in solution. Certainly it will be valuable to further study the specificity of pp60^{src} for the cytoskeletal proteins when it is possible to better regulate the activity of pp60^{src} itself by phosphorylation and dephosphorylation reactions *in vitro*. Currently, a worthwhile extension of the present studies would be to examine the kinetics of phosphorylation

of various cytoskeletal proteins by pp60^{src}. A comparison of V_{max} and K_m parameters for each of the pp60^{src} substrates would yield information concerning the relative affinity of pp60^{src} for the substrate and might suggest which substrates are relevant to pp60^{src}'s *in vivo* activity.

More importantly, the *in vitro* phosphorylation results must be correlated with pp60^{src}'s *in vivo* activity directly. Sefton et al. (1981b) have studied the phosphorylation of cytoskeletal proteins *in vivo* by labelling cells with [^{32}P]orthophosphate followed by immunoprecipitation of cell extracts with specific cytoskeletal antibodies. Vinculin was found to be phosphorylated at a tyrosine residue *in vivo* in a transformation specific manner. Other cytoskeleton proteins such as tubulin, actin, filamin, vimentin, and myosin were not significantly phosphorylated at tyrosine residues in normal or transformed cells. Nominally, these results suggest that vinculin is the primary cytoskeletal target of *src* action. However, the *in vivo* studies can not determine whether pp60^{src} interacts directly with vinculin or through an intermediate kinase(s). Also, the failure to detect significant amount of phosphotyrosine on other cytoskeleton proteins does not conclusively rule out these proteins as potential pp60^{src} substrates. The presence of phosphatases and kinases in the cell which may be activated when the cells are lysed make it difficult to interpret the *in vivo* results. For example, studies with substrates of the cAMP-dependent protein kinase (Ueda, T. and Greengard, 1977; Huttner et al., 1981) indicate that significant dephosphorylation of the substrate can occur upon cell lysis. *In vivo* experiments in which the cellular enzymes are inactivated at the time of extraction should give more conclusive results. The *in vivo* experiments

coupled with the *in vitro* experiments reported here and by others (Sefton et al., 1981b) suggest that vinculin may be a direct target of $pp60^{src}$. It has not yet been determined if vinculin is phosphorylated at the same site *in vivo* and *in vitro*. This experiment is important in evaluating the biochemical significance of the phosphorylation results.

The phosphorylation of microfilament-associated proteins such as vinculin and filamin by the *src* protein is of particular interest since these proteins are thought to be involved in anchoring the microfilaments to the plasma membrane. Phosphorylation of these proteins in the transformed cell might alter this anchorage resulting in disruption of the cytoskeleton. Similarly, phosphorylation of tubulin or a microtubule-associated protein might dramatically affect the state of the microtubules. The state of the microtubules has already been shown to be controlled by calcium levels and regulated by the cAMP-dependent protein kinase (Tash et al., 1981; Purich et al., 1981; Schliwa et al., 1981; Burke and DeLorenzo, 1981) and has been implicated in the regulation of cell growth. Presumably proteins that are involved in fundamental cellular processes such as the regulation of DNA synthesis will be regulated by several different kinase systems. A number of different proteins including glycogen synthase and phosphorylase kinase, which are involved in glycogen metabolism (Krebs and Beavo, 1979), and the neuron-specific protein 1, which may be involved in synaptic transmission (Huttner et al., 1981), have already been shown to be regulated by several different protein kinases. It would therefore not be surprising if the microtubules or microfilaments were regulated by the tyrosine kinases as well.

Additional *in vitro* pp60^{src} phosphorylation experiments allowed the identification of another potential *src* substrate. A 50,000 dalton protein present in the partially purified preparations of pp52^{src} and pp60^{src} appeared to be regulated by the Ca^{2+}-calmodulin system; its phosphorylation was stimulated by the addition of Ca^{2+}-calmodulin and was inhibited by chlorpromazine at concentrations known to inactivate calmodulin (Levin and Weiss, 1977; Weiss and Levin, 1978). It has not been established if the Ca^{2+}-calmodulin activated phosphorylation is an autophosphorylation event with the 50 kD protein acting as both the enzyme and substrate, or if the 50 kD protein is phosphorylated by another enzyme present in the preparation.

The Ca^{2+}-calmodulin stimulation of phosphorylation of the 50 kD protein was for the most part not dependent on the presence of pp60^{src}, since it was observed to occur in both normal cells and transformed cells to similar extents. Preliminary experiments indicate that the 50 kD protein from transformed cells is phosphorylated primarily at threonine and serine residues after Ca^{2+}-calmodulin activation. A lower level of phosphotyrosine was also detected by phosphoamino acid analysis. Possibly the 50 kD protein is a pp60^{src} substrate that is also regulated by or is itself a Ca^{2+}-calmodulin dependent protein kinase. It would be useful to compare the *in vivo* and *in vitro* sites of phosphorylation of the 50 kD proteins from both normal and transformed cells. Possibly phosphorylation at the tyrosine residue is specific to the 50 kD protein isolated from transformed cells.

Several lines of experimental evidence indicated that the Ca^{2+}-calmodulin activated 50 kD protein and pp60^{src} specifically interact.

Although further studies are needed to systematically define the solution properties necessary to stabilize the 50 kD protein-pp60^{src} interaction, preliminary experiments suggested that the interaction between the two proteins is promoted by the presence of salt.

A 50 kD and 90 kD protein were found to coprecipitate in TBR immunoprecipitates of transformed cell extracts (Brugge et al., 1981). These proteins, which were also found in normal cells, were observed to cosediment on glycerol gradients with a high molecular weight form of pp60^{src}. It has not yet been established if the 50 kD protein determined by Brugge et al. (1981) to interact with pp60^{src} is identical to the Ca^{2+}-calmodulin stimulated 50 kD protein reported here. Several lines of evidence suggest that the two proteins may be the same. Most convincing is the fact that both 50 kD proteins have been found to sediment with the high molecular weight form of pp60^{src}. In addition, experiments have indicated that the interaction of the two 50 kD proteins with pp60^{src} is promoted by the presence of salt.

The 50 kD protein described by Brugge and her collaborators was recently shown to be phosphorylated *in vivo* at serine and tyrosine residues in transformed cells but not at a threonine residue (Brugge and Darrow, 1982; Gilmore et al., 1982). The Ca^{2+}-calmodulin activated 50 kD protein is phosphorylated primarily at threonine and serine residues *in vitro* but its *in vivo* state of phosphorylation has not yet been determined. The *in vitro* calmodulin effects on phosphorylation must be correlated with an *in vivo* phosphorylation event, and further biochemical analysis is required to establish the identity of the two 50 kD proteins.

Binding of the 50 kD-pp60^{src} complex to the calmodulin-affinity column and elution with EGTA should provide a useful method for obtaining highly purified 50 kD protein and pp60^{src}. This would be worthwhile since, as yet nothing is known about the function or location of the Ca^{2+}-calmodulin activated 50 kD protein within the cell.

In an effort to see if the Ca^{2+}-calmodulin effects on the 50 kD protein were correlated with any of the pp60^{src} induced changes during cell transformation, the in vivo effects of the calmodulin inhibitor chlorpromazine were investigated. Chlorpromazine inhibited the pp60^{src} induced stimulation of DNA synthesis without affecting the activation of pp60^{src} kinase activity that occurs upon downshift of tsNY68-infected cells from the nonpermissive to permissive temperature. Although drug studies are difficult to interpret unambiguously, this result raises the possibility that calmodulin is involved in the sequence of events initiated by pp60^{src} and leading to an increased rate of DNA synthesis in the RSV-transformed cells. DNA synthesis in Chinese Hamster Ovary cells was recently found to be inhibited by several phenothiazines that inactivated calmodulin (Chafouleas et al., 1982). The inhibition of pp60^{src} induced DNA synthesis in tsNY68-infected cells is consistent with the observations of others (Gail et al., 1973; Dulbecco et al., 1975) which have established that cell proliferation is controlled at least in part by calcium. The data presented here may help to elucidate a biochemical pathway by which calcium controls DNA synthesis.

One intriguing, highly speculative biochemical model is that the Ca^{2+}-calmodulin activated phosphorylation of the 50 kD protein is required for the stimulation of DNA synthesis. In this model the activity of the

50 kD protein is further enhanced when it is phosphorylated at a tyrosine residue by $pp60^{src}$. The $pp60^{src}$-induced phosphorylation of the 50 kD protein is not sufficient by itself to activate the 50 kD protein and only enhances the protein's activity in the presence of Ca^{2+}-calmodulin. The drug chlorpromazine inhibits calmodulin resulting in the dephosphorylation of the 50 kD protein and a concomitant inhibition of DNA synthesis. The proposed model is consistent with the observations that the 50 kD protein is phosphorylated in a Ca^{2+}-calmodulin dependent manner and that it interacts with $pp60^{src}$. The model also considers that DNA synthesis is activated by $pp60^{src}$ but that the activation is abrogated by the addition of chlorpromazine, although this drug does not inhibit the $pp60^{src}$ kinase activity.

More work will be required to substantiate or disprove this model. The data currently do not provide direct biochemical evidence that the 50 kD protein is involved in controlling DNA synthesis. Furthermore, the in vivo experiments with chlorpromazine must be analyzed carefully. Ca^{2+}-calmodulin modulates many enzymatic systems within the cell (Cheung, 1980) and the effects of a drug such as chlorpromazine may be broad.

It would be worthwhile to investigate other aspects of the cell's response to chlorpromazine, as well as the effects of other calmodulin inhibitors on $pp60^{src}$-induced DNA synthesis. A more detailed analysis of the effects of chlorpromazine on the kinetics of DNA synthesis is required to determine where in the cell cycle this drug acts. One way in which it would be possible to study more directly the function of the 50 kD protein and its potential role in DNA synthesis regulation would be to purify this protein and prepare antibodies to it.

Three potential substrates of pp60^{src} have been identified which may mediate the effects of pp60^{src} in the transformed cell. Further characterization of the 50 kD Ca^{2+}-calmodulin activated protein, vinculin, and the 34 kD phosphoprotein (discussed in Chapters II and VI) is required in order to understand the function of these pp60^{src} substrates. It is not known if any of these substrates mediate the effect of pp60^{src} on DNA synthesis. An original goal of this work, which was to determine if cytoskeletal states are involved biochemically in the regulation of cell growth, remains to be achieved. Possibly pp60^{src} phosphorylation of vinculin or one of the other substrates causes a change in the cytoskeleton that influences the cell's growth state.

The effects of pp60^{src} and its substrates on the state of the cytoskeleton and on the regulation of DNA synthesis could be tested using the microinjection assay (McClain et al., 1978; Tijian et al., 1978). pp60^{src} substrates would be purified from normal and transformed cells, antibodies prepared to the substrates and both sets of reagents used in the microinjection experiment. After the microinjection of the src target, or the specific antibody to the target, cells would be monitored for disruption of the cytoskeleton and the stimulation of DNA synthesis. In this way it should be possible to determine if the phosphorylation of a given pp60^{src} target is directly responsible for these specific changes that occur during transformation. It should also be possible by this approach to determine if the disruption of the cytoskeleton is causally related to the deregulation of cell growth.

As a first step towards achieving this goal pp60^{src} was purified. Next, in an effort to better characterize the src substrates, monoclonal

antibodies were prepared against partially purified vinculin and 34 kD phosphoprotein. The monoclonal antibodies should be particularly useful for the microinjection experiments described above.

Preparation and characterization of the monoclonal antibodies to vinculin was relatively straight forward. Four monoclonal antibodies were generated that recognize vinculin. The antigenic specificity of the antibody has not yet been investigated. The different antibodies will be useful for perturbing different functions of vinculin, such as the interaction of vinculin with the cytoskeleton, the plasma membrane, or other proteins, during microinjection experiments and other in vitro assays of its activity.

Two monoclonal antibodies were obtained that immunoprecipitate a 34,000 dalton protein from cell extracts; however, only one of these antibodies recognized a protein specifically phosphorylated at a tyrosine residue in transformed cells. The other 34,000 dalton protein was not detectably phosphorylated. The subcellular localization of the two 34 kD proteins was strikingly different. The antibody to the 34 kD phosphoprotein stained the plasma membrane while the other antibody bound specifically to the nucleolus.

It has not yet been determined by peptide analysis if the two 34 kD proteins are structurally similar, differing perhaps only in their state of phosphorylation. Most likely the nuclear associated non-phosphorylated species is an unrelated protein that copurifies with the 34 kD phosphoprotein. Possibly this nuclear protein is a contaminant in the preparation and care should be taken in the preparation of polyspecific antibodies to the partially purified 34 kD protein(s). In preliminary experiments

it was determined, by two dimensional polyacrylamide gel electrophoresis, that the partially purified 34 kD protein preparation contains a basic 34 kD protein that is not separated from the 34 kD phosphoprotein and would not be detected during one dimensional SDS gel electrophoresis. Perhaps this basic protein will be recognized by the monoclonal antibody that binds to the nucleolus.

The monoclonal antibody to the 34 kD phosphoprotein was used to further study the subcellular localization of this protein. The overall staining pattern suggested that the 34 kD protein was associated with the plasma membrane. Cells were fixed and permeablized by a variety of different methods. Sometimes, rather than a diffuse membrane staining pattern, a fine reticular network was detected and it was difficult to rule out the possibility of a cytoskeletal interaction. Immunofluorescence staining experiments have obvious limitations. It is difficult to exclude the possibility of redistribution of the 34 kD protein as a result of permeabilization or fixation, and the immunofluorescent staining data were not sufficient to unambiguously localize the 34 kD protein to the plasma membrane. Cell fractionation techniques are currently being used to further examine the subcellular localization of this protein.

Localizing phosphoproteins within the cell is a difficult task. It is reasonable to expect that the state of phosphorylation of a protein may determine its subcellular localization. Phosphorylation of the 34 kD protein by pp60^{src} for example might cause a change in its ability to interact with membranes. If this occurs the 34 kD protein might be present in several subcellular fractions, further complicating the analysis of its location. Such is apparently the case for vinculin. In

normal cells it has been found both in focal adhesion plaques and in the cytoplasm, but vinculin's subcellular localization is altered in the transformed cell. It would be interesting to determine if the alteration of the location of vinculin is triggered directly by $pp60^{src}$ phosphorylation of a tyrosine residue.

In addition to aiding in the determination of the subcellular localization of the $pp60^{src}$ substrates the monoclonal antibodies were used to rapidly purify the 34 kD protein and vinculin from both normal and transformed cells. The availability of the purified src substrates as well as the highly specific antibodies to the substrates make it possible to begin studying the biochemical mechanisms by which the substrates effect cellular transformation.

The function of the src substrates in the normal cell is not yet well understood. Normal cellular vinculin has been shown (Wilkins and Lin, 1982) to interact with actin in vitro. Perhaps vinculin isolated from the transformed cell will interact with actin differently. The disruption of the microfilaments, in vivo, may be mediated via $pp60^{src}$ phosphorylation of vinculin. The other $pp60^{src}$ substrates are more of a mystery. Almost nothing is known about the function of the 34 kD phosphoprotein and the Ca^{2+}-calmodulin activated 50 kD protein. While significant progress has been made in the last few years towards understanding the molecular basis of $pp60^{src}$ action still very little is known about the way this protein causes the deregulation of cell growth. The biochemical studies presented in this thesis, however, provide a basis for future studies.

Analysis of the structure and function of $pp60^{src}$ resulted in the discovery of a new class of proteins, the tyrosine kinases, which have been implicated in the control of normal cell growth. Future studies aimed at understanding the function of $pp60^{src}$ targets should aid in further elucidating the biochemical mechanisms of cell growth regulation.

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