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Structural and functional domains of the Rous sarcoma virus transforming protein ($pp60^{src}$)

(protein kinase/phosphoprotein/membrane protein/viral oncogenesis)

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ABSTRACT **The transforming protein ($pp60^{src}$) of Rous sarcoma virus (RSV) is a phosphoprotein with the enzymatic ability to phosphorylate tyrosine in protein substrates. Previous work has indicated that the bulk of $pp60^{src}$ may be attached to the plasma membrane of infected cells. In an effort to better understand the mechanism by which $pp60^{src}$ induces the neoplastic phenotype, we have characterized further the attachment of $pp60^{src}$ to the plasma membrane, and we have identified separate molecular domains that are responsible for the attachment to membranes and for the protein kinase activity. Our results indicate that $pp60^{src}$ may be an integral membrane protein that is nevertheless synthesized on soluble polyribosomes. Subsequent to its synthesis, the protein attaches to plasma membrane without concomitant cleavage of a signal polypeptide. The amino-terminal quarter (or some portion thereof) of $pp60^{src}$ anchors the protein to the plasma membrane by forces that can be disrupted only with detergents. By contrast, protein kinase activity is located in the carboxyl-terminal half of the molecule. It appears that $pp60^{src}$ is designed on the one hand for tethering to the plasma membrane and on the other hand for enzymatic activity beyond the confines of the membrane. The fact that $pp60^{src}$ is but one of at least four different viral transforming proteins located on the plasma membrane implies that neoplastic transformation may commonly originate in events that occur at the periphery of the cell.**

The oncogene (*src*) of Rous sarcoma virus (RSV) encodes a 60,000-dalton phosphoprotein ($pp60^{src}$) that apparently possesses the enzymatic activity of a protein kinase (1–5). Both crude (6) and purified (4, 7) preparations of $pp60^{src}$ phosphorylate tyrosine residues in protein substrates, and cells transformed by RSV contain more phosphotyrosine than do untransformed cells (6, 8); it has therefore been proposed that phosphorylation of tyrosine in particular cellular proteins may represent the mechanism by which *src* induces neoplastic transformation. Pursuit of this hypothesis will require further characterization of the enzymatic activity of $pp60^{src}$, localization of the protein within the transformed cell, and identification of the cellular proteins on which the viral protein acts.

Several lines of evidence have indicated that at least a substantial portion of $pp60^{src}$ is associated with the plasma membrane: immunofluorescence has revealed concentrations of $pp60^{src}$ underlying cell–cell junctions as well as in adhesion plaques (9); the results of immunoelectron microscopy suggest that $pp60^{src}$ is located at the cytoplasmic aspect of the plasma membrane (10); and the bulk of $pp60^{src}$ fractionates with plasma membranes (but not other membrane fractions) in biochemical procedures (11, 12). We therefore undertook to characterize the nature of the association of $pp60^{src}$ with the plasma membrane and to determine how this association might constrain the enzymatic activity of the protein. Our strategy exploited the use of limited hydrolysis by proteolytic enzymes, as described previously for the study of proteins in the plasma membrane of the erythrocyte (for example, see ref. 13). We identified the portions of $pp60^{src}$ that were either released from or retained on the plasma membrane after controlled attack by protease, and we located the protein kinase activity on individual products of the digestion. In addition, we used pulse–chase labeling to follow the fate of $pp60^{src}$ subsequent to its synthesis. We conclude that $pp60^{src}$ may be an integral membrane protein whose attachment to the plasma membrane occurs subsequent to, rather than coincident with, translation and is not accompanied by cleavage of a signal polypeptide. The protein is anchored to the membrane by some portion of its amino-terminal domain, whereas the protein kinase activity is carried by the carboxyl-terminal half of the molecule.

MATERIALS AND METHODS

General Procedures. We have described our procedures for the propagation and isotopic labeling of cultured cells, the preparation of antisera from rabbits bearing tumors induced by the Schmidt–Ruppin (SR) strain of RSV, the immunoprecipitation of virus-specific proteins, the assay of the protein kinase associated with $pp60^{src}$, and the fractionation of proteins by electrophoresis in polyacrylamide gels (2, 14). NRK cells (rat kidney fibroblasts) and NRK cells transformed by SR-RSV (SR-NRK) were provided by L. Turek.

Subcellular Fractionation. Cells were broken and fractionated as described (12). Briefly, monolayers of cells were harvested by scraping into phosphate-buffered saline (140 mM NaCl/1.18 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4/0.7 mM $CaCl_2$/0.5 mM $MgCl_2$, pH 7.4) and collected by centrifugation. The cells were resuspended in 5 mM KCl/1 mM $MgCl_2$/20 mM Hepes, pH 7.2, and allowed to swell at 4°C. After breakage of the cells in a Dounce homogenizer, nuclei were removed by centrifugation at 1000 × *g*. The supernatant was centrifuged at high speed to yield a crude membrane pellet and a soluble fraction (see figure legends for the centrifugal forces used in each experiment).

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Abbreviations: RSV, Rous sarcoma virus; SR-RSV, the Schmidt–Ruppin strain of RSV; *src*, the gene responsible for oncogenesis by RSV; $pp60^{src}$, a phosphoprotein encoded in *src*; $Pr76^{gag}$, a polyprotein precursor of RSV structural proteins; NRK cells, normal rat kidney fibroblasts; SR-NRK cells, NRK cells transformed by the SR-RSV; TosPheCH$_2$Cl, 1-tosylamido-2-phenylethyl chloromethyl ketone; Pipes, 1,4-piperazinediethanesulfonic acid.

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Pulse–Chase Labeling. Four 100-mm dishes of SR-NRK cells were incubated at 37°C in methionine-free medium for 1 hr. Five hundred microcuries of [^{35}S]methionine (1160 Ci/mmol; 500 μCi/ml; 1 Ci = 3.7 × 10^{10} becquerels) was then added to each dish, and incubation at 37°C was continued for a further 5 min. The medium was then removed and the plates were washed twice with 5 ml of complete medium. One of these dishes was then placed on ice, while the remaining three were incubated with 5 ml of complete medium for 5, 10, and 15 min. In each case, the pulse was ended by removing the medium and placing the plate on ice. Cells were scraped from each of these dishes into phosphate-buffered saline, harvested by centrifugation, and broken and fractionated as described above. Each subcellular fraction was assayed for pp60^{src} by immunoprecipitation (14).

Membrane Extractions. Membrane pellets were prepared as described, and resuspended in 0.5 ml of the appropriate buffer (see legend to Table 1) by passage three times through a 26-gauge needle. These suspensions were then incubated on ice for 2 hr and repelleted at 50,000 × *g* for 1 hr. Both pellet and supernatant were harvested and analyzed for pp60^{src} and *src* protein kinase activity.

Proteolytic Digestion of pp60^{src}. A crude membrane pellet was prepared from approximately 10^7 cells as described, resuspended by Dounce homogenization in 1 ml of 0.15 M KCl/0.1 mM EDTA/50 mM (1,4-piperazinediethanesulfonic acid (Pipes), pH 6.8, and incubated with trypsin [treated with 1-tosylamido-2-phenyl ethyl chloromethyl ketone (TosPheCH_2Cl)] at 10 μg/ml for 15 min at room temperature. Digestion was terminated by the addition of phenylmethylsulfonyl fluoride (to 0.3 mM) and beef pancreas trypsin inhibitor (to 0.1 mg/ml). Control experiments showed that no proteolytic digestion occurred in the presence of these inhibitors (data not shown). The samples were then pelleted at 50,000 × *g* for 30 min, and the supernatants and pellets were separately analyzed for pp60^{src} as described above.

Gel Filtration of Proteolytic Fragments. SR-NRK cells (1 × 10^7) labeled for 3 hr with [^{35}S]methionine were lysed with 0.6 ml of 1% Nonidet P-40/1 mM EDTA/0.2 M NaCl/50 mM Tris·HCl, pH 7.5. The lysate was clarified by centrifugation (10,000 × *g* for 10 min at 4°C) and divided into halves. Tos-PheCH_2Cl-treated trypsin was added to one half (final concentration 170 μg/ml). Both halves were then incubated for 10 min at room temperature. Phenylmethylsulfonyl fluoride was then added to 2.5 mM; bovine serum albumin (68,000 daltons), ovalbumin (42,000 daltons), and cytochrome *c* (17,000 daltons) were added as markers to 5 mg/ml each and the lysate was applied to a 20 × 0.6 cm column containing Sephadex G-100 (superfine) equilibrated with 0.2 M KCl/0.2% Nonidet P-40/0.1 mM EDTA/10% (vol/vol) ethylene glycol/25 mM Hepes, pH 7.2. Thirty-six fractions were collected and selected fractions were analyzed for elution of size markers, for pp60^{src}-related fragments by immunoprecipitation, and for *src*-specific kinase.

RESULTS

pp60^{src} May Be an Integral Membrane Protein. We have reported previously that the bulk of pp60^{src} in infected cells is firmly bound to vesicles of plasma membrane (12); by contrast, a degradation product of pp60^{src} (pp52^{src}) is less readily retained in membranous fractions (4, 12, 15). We pursued these findings as follows. Membrane vesicles were prepared and incubated in various buffers designed to remove peripheral and nonspecifically adherent proteins (16); the amount of pp60^{src} released from the vesicles was then evaluated by immunoprecipitation. It has been reported that EDTA will strip peripheral proteins from the membrane (16). Incubation in such a buffer removes no more than 10% of pp60^{src} and pp52^{src} from the membrane (Table 1), although the same treatment does remove substantial amounts of other proteins (data not shown). Similarly, incubating the membranes in high concentrations of salt (designed to remove nonspecifically adherent and peripheral proteins) (16) does not remove a significant amount of pp60^{src}, although it does remove approximately 40% of pp52^{src} (Table 1) as well as other proteins (data not shown). Table 1 also shows that whereas none of the *src* kinase activity is solubilized by EDTA, 25% of the activity is solubilized by high salt concentrations. This finding presumably reflects the fact that pp52^{src}, while not being so firmly bound to the membrane, retains kinase activity (4).

pp60^{src} Does Not Have a Cleavable Signal Sequence. Although pp60^{src} is firmly bound to the plasma membrane, the protein is apparently synthesized on soluble polyribosomes (17). We therefore used pulse–chase labeling in order to explore the means by which pp60^{src} becomes associated with the membrane. Cells were labeled with [^{35}S]methionine for 5 min and then chased with complete medium for 0, 5, 10, and 15 min (Fig. 1 *A*, *B*, *C*, and *D*, respectively). pp60^{src} first appeared in the soluble fraction (*A*) but rapidly became associated with the membranous elements of the cell (*B*). Thus, within 10 min of synthesis (*C*), greater than 80% of the protein was in the membrane fraction. After a 15-min chase (*D*), essentially all of pp60^{src} was membrane associated. Fig. 1 also shows that pp60^{src} did not appear to be made in precursor form. If the protein were to have a cleavable signal sequence, one would expect it to be cleaved upon insertion of the protein into the membrane. That this is not the case is shown in Fig. 1*B*. Both the soluble and membrane-bound forms of the protein had the same mobility in sodium dodecyl sulfate gels. We conclude, therefore, that although pp60^{src} is synthesized on soluble polyribosomes and thereafter makes its way to the plasma membrane, it does not appear to be guided to the membrane by a cleavable signal sequence.

Protein Domain that Anchors pp60^{src} to the Plasma Membrane. The association of pp60^{src} with the plasma membrane appears to take the form of direct interaction with lipid, because procedures that are known to remove peripheral proteins from the membrane do not disrupt the association. In an effort to

Table 1. Extraction of pp60^{src} and pp52^{src} from membrane vesicles

Buffer	Fraction	% of total recovery		
		Kinase activity	pp60^{src}	pp52^{src}
20 mM Pipes, pH 6.8/5 mM KCl	Pellet	95	89	91
	Supernatant	5	11	9
20 mM Pipes, pH 6.8/5 mM KCl/10 mM EDTA	Pellet	94	90	90
	Supernatant	6	10	10
20 mM Pipes, pH 6.8/500 mM KCl	Pellet	77	88	63
	Supernatant	13	12	37

SR-NRK cells were labeled with [^{35}S]methionine, and membranes were prepared by centrifugation at 50,000 × *g* and resuspended in the indicated buffers by Dounce homogenization. After incubation for 2 hr at 4°C, the samples were recentrifuged at 50,000 × *g*, and both pellets and supernatants were analyzed for the presence of pp60^{src}, pp52^{src}, and *src* protein kinase activity.

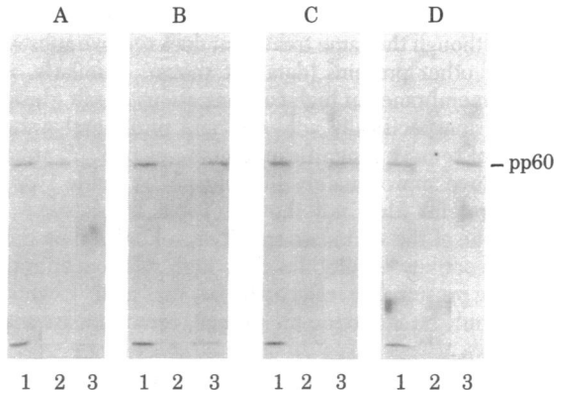

FIG. 1. Distribution of pp60^{src} among subcellular fractions at various times after its synthesis. SR-NRK cells were labeled with [^{35}S]methionine for 5 min and then chased with unlabeled medium for 0 min (*A*), 5 min (*B*), 10 min (*C*), or 15 min (*D*). Cells were harvested and fractionated into soluble and membrane components by centrifugation at 100,000 × *g*. Each fraction was adjusted to 1% Nonidet P-40. The distribution of pp60^{src} was monitored by immunoprecipitation, followed by electrophoresis on 10% polyacrylamide gels. Lanes 1, lysate of total cells; lanes 2, soluble fraction; lanes 3, membrane vesicles.

identify the portion of pp60^{src} that interacts with the plasma membrane, we resorted to the use of limited hydrolysis with proteases. Membrane suspensions were treated with trypsin; solubilized material was then analyzed by immunoprecipitation for polypeptides derived from pp60^{src}. No pp60^{src} was released from membrane vesicles in the absence of protease treatment, although the protein was efficiently extracted from sedimentable material by disruption with detergent (data not shown; see also ref. 12). However, limited treatment of membrane vesicles with trypsin released a protein of 47,000 daltons (p47) that could be specifically precipitated with antiserum directed against pp60^{src}.

In order to determine which portion of pp60^{src} was represented in p47, we analyzed the protein by partial hydrolysis with the V8 protease of *Staphylococcus aureus*, according to the procedure of Cleveland *et al.* (18). Limited cleavage of pp60^{src} with the V8 protease divides the protein into two fragments—a larger fragment denoted A that represents the amino-terminal portion of the molecule, and a smaller carboxyl-terminal fragment denoted B (19). Fig. 2*B* demonstrates that p47 retains the full B fragment of pp60^{src}. The A fragment is missing, however, and is replaced by a shorter fragment approximately 13,000 daltons lighter. It therefore appears that p47 is derived from pp60^{src} by the loss of 13,000 daltons from the amino terminus of the molecule. This conclusion is supported by the demonstration that limited hydrolysis with chymotrypsin generates a 31,000-dalton fragment from the amino terminus of pp60^{src} that remains bound to the membrane (data not shown). We conclude that pp60^{src} is bound to the plasma membrane by an amino-terminal domain that is no larger than 13,000 daltons.

Localization of the Catalytic Site of pp60^{src}. Having located the molecular domain that binds pp60^{src} to the plasma membrane, we next sought to localize the protein kinase activity within the molecule. We obtained pp60^{src} for these analyses by treating infected cells with nonionic detergent. Extracts prepared in this manner contained pp60^{src} and the large precursor of viral structural proteins, Pr76^{gag}, both of which were precipitated by tumor antisera (Fig. 3*A*, lane b). Controlled trypsin digestion generated immunoprecipitable fragments of 52,000, 50,000, and 30,000 daltons (Fig. 3*B*, lane b). None of these proteins were precipitated by normal rabbit serum (Fig. 3*B*, lane a). Untreated and trypsin-treated extracts were applied to columns of Sephadex G-100. Fig. 3*A*, lanes c, illustrates that in the untreated homogenate, both Pr76^{gag} and pp60^{src} eluted in the void volume as expected. The fragments resulting from trypsin digestions were effectively separated from one another and eluted in the order expected. We mapped these fragments to regions within pp60^{src} by the use of V8 protease, as described above. Fig. 2*C* illustrates that the 52,000-, 50,000-, and 30,000-dalton species all retained the B fragment of pp60^{src}, and in each case the A fragment was replaced by a smaller species. Thus, all the fragments generated by trypsin digestion represent sequences from the carboxyl-terminal portion of pp60^{src}.

Fractions from the gel filtrations were also analyzed for *src* protein kinase activity. As expected, the column loaded with the undigested lysate exhibited a peak of *src* kinase activity coincident with the elution of intact pp60^{src} (Fig. 3*A*). However, the column loaded with the trypsin-digested homogenate exhibited two peaks of *src* kinase activity: a minor peak corresponded to the elution positions of the 50,000- and 52,000-dalton proteins, the major peak to the elution position of p30. It thus appears that the fragments of pp60^{src} are able to carry out the phosphorylation of immunoglobulin. We can therefore assign the catalytic site of pp60^{src} to the carboxyl-terminal half of the molecule.

DISCUSSION

How Does pp60^{src} Associate with the Plasma Membrane? Previous studies have revealed that pp60^{src} in RSV-transformed cells is associated with the plasma membrane. Membrane proteins have been classified into two major categories: integral proteins, which directly interact with the lipid bilayer of the membrane, and peripheral proteins, whose association with the membrane is mediated through other interactions. Our present studies indicate that pp60^{src} may interact directly with the lipid bilayer of the membrane, because only agents capable of disrupting this bilayer release pp60^{src} from the membrane. Agents capable of removing a variety of peripheral membrane proteins fail to release significant quantities of pp60^{src}.

Two general views of the process by which proteins are inserted into the membrane bilayer have been advanced. The first postulates the existence within the membrane of specific

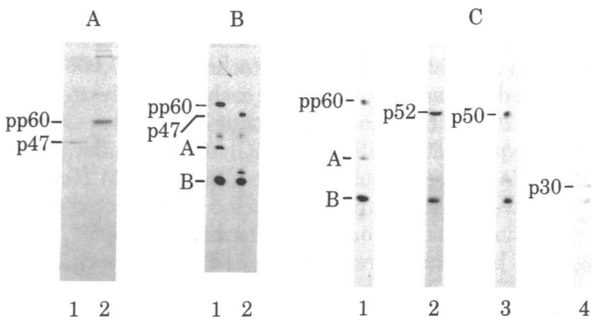

FIG. 2. Analysis of proteins by limited proteolysis. (*A*) Membranes from SR-NRK cells labeled with [^{35}S]methionine were prepared by centrifugation at 50,000 × *g*. After limited trypsin digestion, the sample was centrifuged at 50,000 × *g*, and peptides related to pp60^{src} were detected in the released fraction (lane 1) and sedimentable fraction (lane 2) by immunoprecipitation. (*B*) [^{35}S]Methionine-labeled pp60^{src} (lane 1) and p47 (lane 2) from *A* were subjected to partial proteolysis with *S. aureus* V8 protease and mapped according to the procedure of Cleveland *et al.* (18) on 12% polyacrylamide gels. (*C*) [^{35}S]Methionine-labeled pp60^{src} (lane 1), p52 (lane 2), p50 (lane 3), and p30 (lane 4) generated by limited trypsin digestion of cellular lysates (see Fig. 3*B*) were subjected to partial proteolysis as described in *B*.

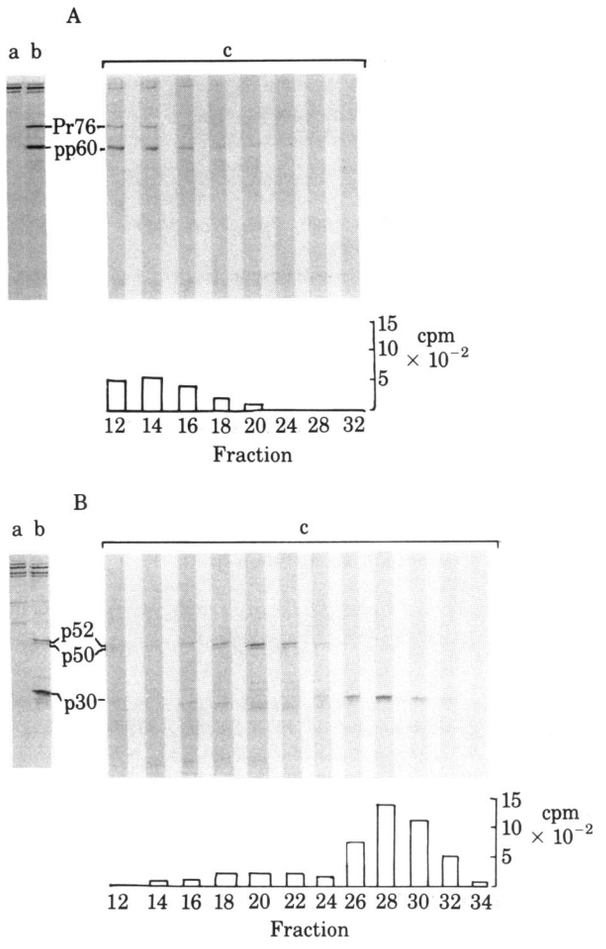

FIG. 3. Gel filtration of pp60^{src} before and after partial proteolysis. (*A*) Proteins immunoprecipitated from clarified lysates of [^{35}S]methionine-labeled SR-NRK cells with control (lane a) or immune (lane b) serum. Lanes c represent the results of gel filtration in Sephadex G-100. Column fractions were analyzed for pp60^{src} and other viral proteins by immunoprecipitation, and for *src* kinase activity with immune serum as substrate. The gel lanes display the results of immunoprecipitations, the bar graph represents kinase activity. Proteins added as internal size markers eluted in fraction 16 (bovine serum albumin, 68,000 daltons), fraction 23 (ovalbumin, 43,000 daltons), and fraction 32 (cytochrome *c*, 12,000 daltons). (*B*) Same as *A*, except the clarified extract was treated with trypsin before gel filtration.

protein assemblies that facilitate the transfer of the growing polypeptide into (or through) the bilayer (20, 21). A cleavable signal sequence directs the growing polypeptide to the rough endoplasmic reticulum. The second postulate emphasizes self-assembly, assuming protein insertion to be a spontaneous process (22). We have explored the means by which pp60^{src} associates with the plasma membrane and have obtained evidence on two major issues. First, virtually all of pp60^{src} may be found in a soluble fraction of the cell immediately after synthesis (Fig. 1). This observation indicates to us that the perceived association of pp60^{src} with the plasma membrane is not a reflection of adventitious binding of the protein to the membrane after breakage of the cell. Second, pp60^{src} associates with the plasma membrane without concomitant reduction in size. (We do not at present know whether the protein proceeds directly to the plasma membrane, or whether it first enters other membranous fractions of the cell and then subsequently localizes to the plasma membrane.) We conclude that the protein may be synthesized without a cleavable signal sequence and may insert into the membrane by a spontaneous process.

Functional Domains of pp60^{src}. We have used controlled proteolysis to release fragments of pp60^{src} from plasma membrane vesicles. Cleavage at a site *ca.* 13,000 daltons from the amino terminus of pp60^{src} releases the remaining 47,000 daltons of the protein from its association with the plasma membrane. Peptide maps of the released fragment indicate that it derives from the carboxy-terminal portion of pp60^{src}. We conclude that a molecular domain at the amino terminus of pp60^{src} anchors the protein to the plasma membrane and that the remainder of the molecule is exposed to the cytoplasm (10). To date, efforts to detect any portion of pp60^{src} on the external surface of the plasma membrane have failed. For example, we have treated intact RSV-infected cells with sufficient concentrations of several proteases (bromelain, trypsin, chymotrypsin, and Pronase) to degrade 80% of iodinated cell surface proteins without effect on the integrity of pp60^{src}; similar treatments completely destroyed solubilized pp60^{src} (unpublished data).

In order to localize protein kinase activity to a specific domain within pp60^{src}, we subjected the intact protein to partial cleavage with trypsin and resolved the various digestion products by gel filtration. The kinase activity was detected on several products of the digestion, each of which contained at least the carboxy-terminal half of pp60^{src}. In fact, the specific activity of one such digestion product (p30) was considerably higher than that of either pp52 or pp60^{src}. We cannot explain these differences in specific activity with any certainty. The enzymatic reactions were performed with immune complexes in which the antibody molecules served as acceptors for phosphate. Perhaps steric hindrance by the amino-terminal domain of pp60^{src} impedes access of the catalytic site to the acceptor site on the immunoglobulin molecules. We have yet to measure the enzymatic activities of the tryptic fragments of pp60^{src} on other substrates. This localization of the enzymatically active domain to the carboxyl-terminal half of the molecule extends a previous observation that a 45,000-dalton protein encoded in *src*, lacking amino-terminal sequences, possessed protein kinase activity (23).

Fig. 4 illustrates our present view of the molecular topography of pp60^{src}. It appears that the protein is designed on the one hand for tethering to the plasma membrane, and on the other hand, for enzymatic activity beyond the confines of the membrane. The nature of the interaction between pp60^{src} and the membrane has not been fully elucidated. Our data indicate that the protein is firmly bound to membrane vesicles and in

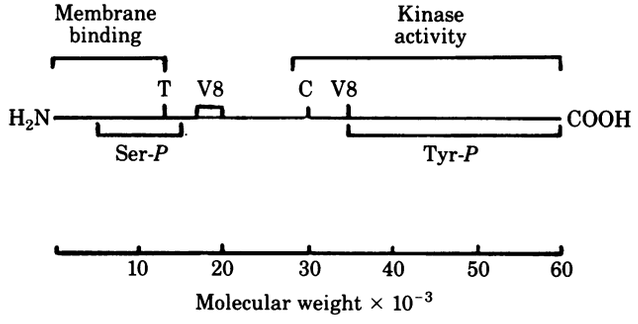

FIG. 4. Functional domains of pp60^{src}. The diagram represents the approximate locations within pp60^{src} of: phosphorylated serine (Ser-*P*); phosphorylated tyrosine (Tyr-*P*); a preferred site for cleavage by trypsin (T); a preferred site for cleavage by chymotrypsin (C); preferred sites for cleavage by the V8 protease (V8); the molecular domain that binds pp60^{src} to the plasma membrane; and the molecular domain that carries protein kinase activity. The sites for trypsin, chymotrypsin, and V8 protease were located by reference to the deduced amino acid sequence of pp60^{src} (24).

this regard obeys the operational criteria for an integral membrane protein. But we are still lacking direct evidence that $pp60^{src}$ is imbedded in the lipid bilayer rather than being bound by strong hydrophobic and ionic forces to another protein on the cytoplasmic face of the plasma membrane. The domain of $pp60^{src}$ that binds the protein to the membrane contains a serine residue that is phosphorylated by cyclic nucleotide-dependent protein kinase (19). We presently have no indication as to what role this phosphorylation might play in the localization or enzymatic activity of $pp60^{src}$. The carboxy-terminal half of $pp60^{src}$ carries protein kinase activity and contains a tyrosine residue that is phosphorylated (6), perhaps by the *src* kinase itself (3). It is conceivable that this phosphotyrosine lies within the active site of the enzyme.

Membrane Proteins and Neoplastic Transformation by Tumor Viruses. At least four tumor viruses encode transforming proteins that have been localized to the plasma membrane: RSV (refs. 9–12 and the present paper); the Harvey strain of murine sarcoma virus (25); the Abelson murine leukemia virus (26); and polyoma virus (27). We presently have little reason to believe that all of these viruses transform cells by precisely the same mechanism, but the subcellular localization of the transforming proteins recalls previous proposals that neoplastic transformation might originate with events at the periphery of the cell (28) and directs the search for "targets" of the transforming protein to the plasma membrane and its associated structures.

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