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The *src* Protein Contains Multiple Domains for Specific Attachment to Membranes

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The proteins encoded by the oncogene v-*src* and its cellular counterpart c-*src* (designated generically here as pp60^{src}) are tightly associated with both plasma membranes and intracellular membranes. This association is due in part to the amino-terminal myristylation of pp60^{src}, but several lines of evidence suggest that amino-terminal portions of the protein itself are also involved. We now report that pp60^{src} contains at least three domains which, in conjunction with myristylation, are capable of mediating attachment to membranes and determining subcellular localization. We identified these domains by fusing various portions of pp60^{src} to pyruvate kinase, which is normally a cytoplasmic protein. Amino acids 1 to 14 of pp60^{src} are sufficient to mediate both myristylation and the attachment of pyruvate kinase to cytoplasmic granules. In contrast, amino acids 38 to 111 mediate association with the plasma membrane and perinuclear membranes, whereas amino acids 204 to 259 mediate association primarily with perinuclear membranes. We conclude that pp60^{src} contains independent domains that target the protein to distinctive subcellular locations and thus may facilitate diverse biological functions of the protein.

The products of the retroviral oncogene v-*src* and its cellular counterpart c-*src* (designated generically here as pp60^{src}) are protein tyrosine kinases that are associated with membranes. Association of pp60^{src} with membranes is essential for transformation by v-*src* (19). In various cell types, the viral and cellular *src* proteins are associated with plasma membranes (7, 8, 21, 44), with perinuclear membranes (36), with secretory organelles in both chromaffin cells and platelets (30, 33), and with growth cones in developing neurons (27, 41). Little is known about how pp60^{src} is specifically targeted to these subcellular locations.

The amino terminus of pp60^{src} is covalently coupled to a 14-carbon fatty acid, myristic acid (4, 39). The myristyl moiety promotes the association of pp60^{src} with membranes (2, 11). On the other hand, not all myristylated *src* proteins are associated with membranes (3, 11), and some nonmyristylated *src* proteins are membrane associated, albeit weakly (12, 20). These results suggest that pp60^{src} contains amino acid sequences which, in conjunction with amino-terminal myristylation, mediate the association of the protein with membranes.

Previous work has implicated the amino-terminal 10 kilodaltons (kDa) of pp60^{src} in attachment to membranes (22, 35). Here we report work that dissects the amino-terminal domain into several regions that independently target and attach the protein to membranes in distinctive subcellular locations.

MATERIALS AND METHODS

Materials. The following were gifts: monoclonal anti-pp60^{src} antibody 327 (MAb327), from J. Brugge (25); rabbit antibodies specific for the chicken M1 pyruvate kinase (PK), from B. Roberts; simian virus 40 expression vectors containing the chicken M1 PK cDNA (RL142PK10X, RL18PK8X, and RL18PK12X, which differ by the reading frame at a polylinker sequence), from B. Roberts (16); and a plasmid containing the chicken fibroblast c-*src* cDNA, p5H, from H. Hanafusa. The E3 *src* gene is a deletion mutant which lacks the sequence encoding amino acids 8 to 37 of pp60^{src}, as previously described (17). Reagents were obtained as follows: protease inhibitors, paraformaldehyde, bovine serum albumin (fraction V), and detergents from Sigma Chemical Co.; fluorescein-conjugated goat anti-mouse immunoglobulin G, goat anti-rabbit immunoglobulin G, and normal goat serum from the Jackson Immunochemicals Co.; protein A conjugated to Sepharose CL-4B from Pharmacia Fine Chemicals; rhodamine-conjugated phalloidin from Molecular Probes Inc.; L-[^{35}S]methionine from ICN Radiochemicals; [^{3}H]myristic acid, 22.4 Ci/mmol (50 mCi/ml in dimethyl sulfoxide) from Du Pont, NEN Research Products; restriction enzymes, *Escherichia coli* DNA polymerase I, and T4 DNA ligase from New England BioLabs, Inc.; human serum fibronectin from Collaborative Research Inc.; and Gold Seal glass cover slips (18 by 18 mm; thickness, 1.5 mm) from VWR Scientific.

Metabolic labeling, immunoprecipitation, and subcellular fractionation. COS7 cells were labeled with L-[^{35}S]methionine for 18 h in Dulbecco modified Eagle medium containing 10% the normal concentration of L-methionine, 10% dialyzed fetal calf serum, and 200 μCi of L-[^{35}S]methionine per ml. COS7 cells were labeled with [^{3}H]myristic acid for 18 h in Dulbecco modified Eagle medium with 10% fetal calf serum, 1% dimethyl sulfoxide, and 500 μCi of [^{3}H]myristic acid per ml (5). Labeled cell extracts were prepared with lysis buffer (20 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 50 μg of soybean trypsin inhibitor per ml, 50 μg of aprotinin per ml, 20 μg of leupeptin per ml, and 1 mg of fraction V bovine serum albumin per ml). Immunoprecipitations with polyclonal rabbit sera and monoclonal antibodies were performed as described previously (21). Crude cell extracts prepared by Dounce homogenization of hypotonically swelled cells were fractionated into cytosol and membrane fractions by differential centrifugation, as previously described (21). Subcellular fractionations

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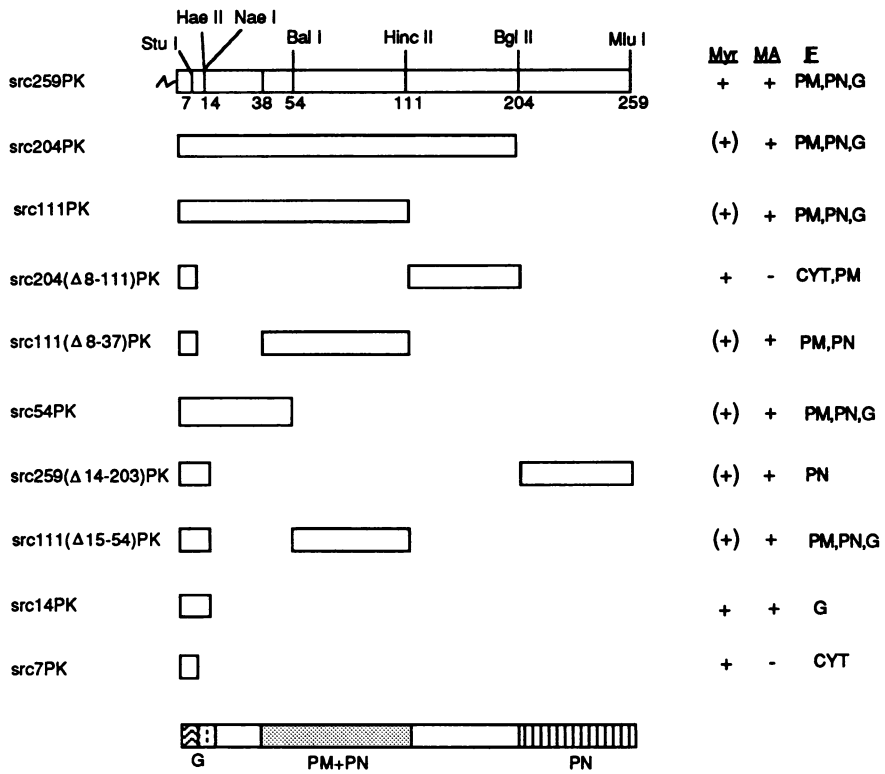

FIG. 1. Summary of the membrane association of *src*-PK proteins. The sequences derived from pp60^{src} in all of the *src*-PK proteins are depicted. The restriction sites used to create internal deletions of *src* sequences and to fuse the *src* sequences to the coding region of PK are indicated. Residue numbers correspond to the sequence of pp60^{src} encoded by the Prague C strain of Rous sarcoma virus (39). Myristylation (Myr) of hybrid proteins, when analyzed, is indicated by +. When *src*-PK proteins associated with membranes, myristylation was assumed [and denoted (+)] but not tested. Membrane association (MA) of hybrid proteins, as determined by biochemical fractionations, is indicated by +. The intracellular distribution of hybrid proteins was determined by immunofluorescence (IF). Association of hybrid proteins with plasma membranes (PM), with the cytoplasm (CYT), with perinuclear membranes (PN), or with cytoplasmic granules (G) is indicated. At the bottom of the figure the N-terminal 259 amino acids of pp60^{src} are divided into functional domains. The first 7 amino acids of pp60^{src} ( ) act as a recognition sequence for myristylation. Amino acids 1 to 14 cause association with cytoplasmic granules (G). Amino acids 38 to 111 ( ), together with the myristylation signal, cause association with both the plasma membrane and perinuclear membranes (PM+PN). Amino acids 204 to 259 ( ), together with the first 14 amino acids, causes association solely with perinuclear membranes (PN). The myristyl moiety is indicated by the crooked line in the diagram of *src*259PK (top of figure).

were quantitated by scanning autoradiograms with a Bio-Rad densitometer.

Construction of recombinant *src*-PK genes. The N-terminal amino acid sequence of the hybrid *src*-PK proteins is derived from pp60^{src} (B77 strain of Rous sarcoma virus), whereas the C-terminal amino acids are derived from PK. The *src*-PK proteins are designated by the C-terminal boundary of the domain of pp60^{src} that is fused to PK, and in cases in which this domain contains internal deletions, the amino acids that are lost are indicated in parentheses. Residue numbers reported correspond to the sequence of the Prague C strain of Rous sarcoma virus (40). The amino acids derived from the chicken M1 PK (residues 120 to 529) are the same in all cases (26). The nucleotide sequence encoding PK was derived from plasmid RL142PK10X, RL18PK8X, or RL18PK12X as required to maintain an open reading frame. The *src*7PK plasmid (previously known as R7-PK) was described previously (17). Sequences encoding various N-terminal amino acids of pp60^{src} were fused to the coding region of PK by cutting both genes with restriction endonucleases, repairing the ends with *E. coli* DNA polymerase I, and fusing the resulting blunt ends. Internal deletions of *src* sequences were constructed by cutting the *src* gene with restriction endonucleases and fusing the repaired ends. The restriction sites used in these constructions are indicated in Fig. 1. The *Stu*I site at codon 7 is a restriction polymorphism which was created by converting lysine 7 to arginine (17). A simian virus 40 vector expressing the chicken fibroblast pp60^{src}, pSV5H, was constructed by replacing the PK cDNA of RL142PK10X with the c-*src* cDNA derived from p5H. Further details about the construction of these genes will be provided upon request.

Transfection of recombinant *src*-PK genes. COS7 cells were exposed for 18 h to calcium phosphate precipitates containing 20 μg of recombinant *src*-PK DNA and then subjected to a 2-min shock with Tris-buffered saline (0.8% NaCl, 0.1% glucose, 0.038% KCl, 0.2% Tris hydrochloride, 0.06% Tris base, 4.5 mg of phenol red per liter) containing 25% dimethyl sulfoxide (15). Transfected COS7 cells were labeled for 18 h with either L-[^{35}S]methionine or [^{3}H]myristic acid starting 36 h after the transfection had begun.

Immunofluorescence. Glass cover slips were coated with human serum fibronectin (100 μg/ml in Dulbecco phosphate-buffered saline [PBS]) for 1 h at 37°C in a humidified chamber. Transfected COS7 cells were seeded onto glass cover slips 24 h after the transfection had begun. After 8 to 12 h, the cover slips were washed with PBS, cells were fixed with 3.5% paraformaldehyde in PBS for 20 minutes and

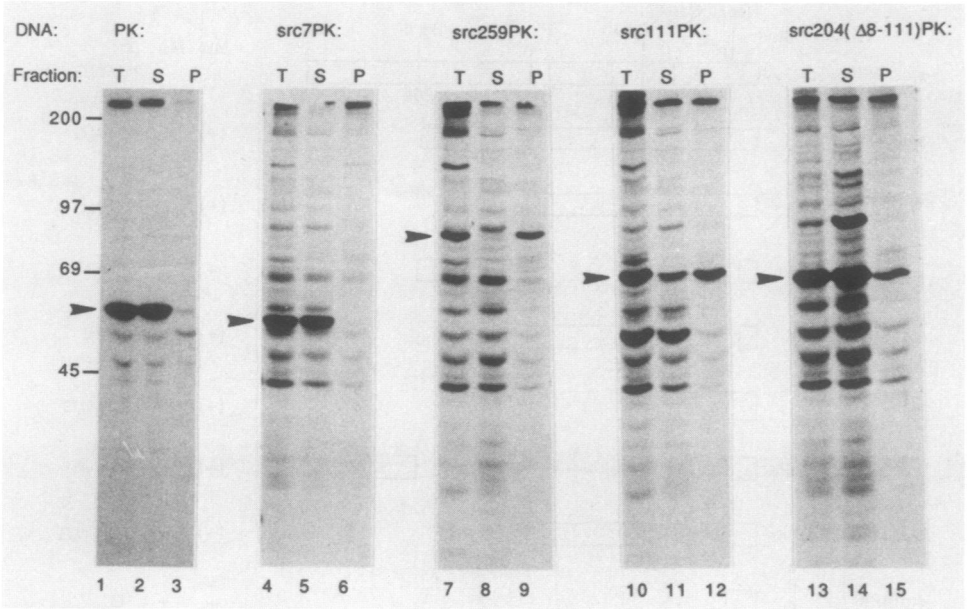

FIG. 2. The first 111 amino acids of pp60^{src} contain a membrane-anchoring domain. COS7 cells were transfected with plasmids encoding native PK (lanes 1 to 3), *src*7PK (lanes 4 to 6), *src*259PK (lanes 7 to 9), *src*111PK (lanes 10 to 12), or *src*204(Δ8–111)PK (lanes 13 to 15) as described in Materials and Methods. Transfected cells were labeled with L-[^{35}S]methionine for 12 h. Crude extracts (T; lanes 1, 4, 7, 10, and 13) were fractionated into cytosol (S; lanes 2, 5, 8, 11, and 14) and a membrane pellet (P; lanes 3, 6, 9, 12, and 15) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The positions of 200-, 97-, 69-, and 45-kDa molecular mass markers are indicated. The *src*-PK proteins are indicated by the arrowheads.

permeabilized with 0.1% Triton X-100 in PBS, and cover slips were coated with 10% normal goat serum in PBS to block nonspecific binding sites. Primary antibodies used were monoclonal anti-pp60^{src} 327 (10 μg/ml in PBS) and polyclonal rabbit anti-PK (1/200 dilution in PBS). Fluorescein-conjugated antibodies were used at a dilution of 1/200 in PBS. Rhodamine-conjugated phalloidin was used as recommended by the manufacturer. Stained cover slips were washed several times with PBS and then with absolute ethanol and were embedded in glycerol containing 2% propyl gallate, an antibleaching reagent. Microscopy was done with either a Zeiss Photomicroscope III or an inverted Olympus fluorescence microscope. Cells were photographed with Kodak Tri-X film at ASA 200. In general, 1 to 5% of the transfected COS7 cells gave a fluorescent signal following this procedure; this signal presumably corresponds to the fraction of cells that took up the DNA. The surrounding nonfluorescent cells acted as an internal control for the specificity of the antibodies used.

RESULTS

To test the role of N-terminal amino acid sequences of pp60^{src} in association with membranes and in subcellular localization, we constructed a set of genes encoding hybrid *src*-PK proteins (Fig. 1). Since myristylation is normally required for membrane association, we included the myristylation signal in all of these hybrid genes. The association of the hybrid proteins with membranes was analyzed first by a crude biochemical fractionation and subsequently by immunofluorescence.

Myristylation is not sufficient for membrane anchorage. We have previously shown that the first 7 amino acids of pp60^{src} act as a sufficient recognition sequence for myristylation (17). Fusing sequences encoding the first 7 amino acids of pp60^{src} to the coding region of PK creates a hybrid gene, *src*7PK, which encodes a myristylated protein (see Fig. 6). If myristylation is a sufficient cause of membrane association, the *src*7PK protein should attach to membranes.

Cells expressing either the native PK protein or the *src*7PK protein were fractionated into soluble and membrane fractions, and these fractions were assayed for PK proteins (Fig. 2). Virtually all of the native PK protein (Fig. 2, lanes 1 to 3) and the *src*7PK protein (Fig. 2, lanes 4 to 6) was recovered in the soluble fraction (Fig. 2, lane 2 and 5, respectively), indicating that a second domain of pp60^{src} is required in conjunction with myristylation to cause membrane association.

The gels illustrated in Fig. 2 contained variable amounts of proteins that appeared ancillary to the specific products of the transfected DNAs (marked by arrowheads). We do not know the identities of these ancillary proteins, but they were present in all specimens, including cells that had not been transfected. We therefore attribute them to nonspecific immunoprecipitation.

Anchoring PK to membranes with a domain from pp60^{src}. Since previous reports had implicated N-terminal sequences in pp60$^{v\text{-}src}$ as membrane-anchoring domains (9, 12, 19, 22, 34), we fused sequences encoding the first 259 amino acids of the protein to the coding sequences of PK, creating a hybrid gene called *src*259PK. Cells expressing the *src*259PK protein were fractionated into cytosol and membrane fractions, and these fractions were assayed for the *src*259PK protein (Fig. 2, lanes 7 to 9). Most (ca. 90%) of the *src*259PK protein was recovered in the membrane fraction (lane 9). This result demonstrates that sequences in the N-terminal half of pp60^{src}, together with myristylation, are sufficient to mediate association with membranes.

The *src*259PK protein and pp60^{src} associate with membranes in a similar manner. Before further characterizing the membrane-anchoring domains made up of the N-terminal

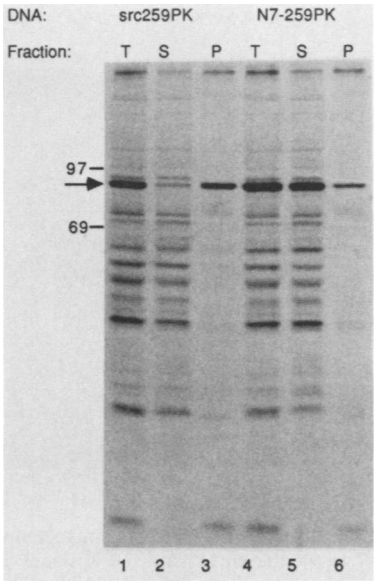

FIG. 3. Influence of myristylation of subcellular localization. COS7 cells were transfected with plasmids encoding either the *src*259PK protein (lanes 1 to 3) or the N7-259PK protein (lanes 4 to 6). Transfected cells were labeled with L-[^{35}S]methionine for 12 h. Crude extracts (T; lanes 1 and 4) were fractionated into cytosol (S; lanes 2 and 5) and a membrane pellet (P; lanes 3 and 6) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The *src*259PK protein is indicated by the arrow. The positions of 97- and 69-kDa molecular size markers are indicated.

259 amino acids of pp60^{src}, we tested whether the *src*259PK and pp60^{src} proteins associate with membranes in a similar manner. We first asked whether myristylation is required for the association of *src*259PK with membranes. Replacement of lysine 7 with an asparagine largely abolishes myristylation of pp60^{src} (17). Therefore, we converted lysine 7 to asparagine in *src*259PK and evaluated the subcellular distribution of the mutant protein (Fig. 3, lanes 4 to 6). The protein (designated N7-259PK) was found predominantly (ca. 87%) in the soluble fraction of cells (Fig. 3, lane 5), implying that the membrane association of *src*259PK, like that of pp60^{src} itself, requires N-terminal myristylation.

A second hallmark of the association of pp60^{src} with membranes is that, like other peripheral membrane proteins, pp60^{src} can be removed from membranes by alkaline extractions (Fig. 4, lanes 4 and 5). Similarly, the *src*259PK protein is extracted from membranes following extraction with base (Fig. 4, lanes 9 and 10). On the other hand, neither pp60^{src} (Fig. 4, lanes 2 and 3) nor *src*259PK (Fig. 4, lanes 6 to 8) is efficiently extracted from membranes by 0.3 M NaCl. We conclude that by several criteria, *src*259PK and pp60^{src} appear to associate with membranes in a similar manner.

The first 111 amino acids of the *src* protein are sufficient for membrane association. Previous work indicated that the N-terminal 10 kDa of pp60^{src} is required to anchor the protein to membranes (22, 34). To better define the responsible amino acid sequences, we fused the coding region of PK to sequences encoding each of the following domains: the first 204 amino acids of pp60^{src} (*src*204PK); the first 111 amino acids of pp60^{src} (*src*111PK); and the first 204 amino acids of pp60^{src}, from which residues 8 to 111 were deleted [*src*204(Δ8–111)PK]. Cells expressing these proteins were fractionated as described above (Fig. 2).

Although 75% of the *src*204(Δ8–111)PK protein was recovered in the cytosol (Fig. 2, lanes 13 to 15), most of the *src*204PK protein (not shown in Fig. 2, but see Fig. 4, lanes 11 to 15) and ca. 65% of the *src*111PK protein (Fig. 2, lanes 10 to 12) were recovered with the membrane pellet (Fig. 2, lanes 10 to 12). The proteins encoded by both *src*204PK (Fig. 4, lanes 11 to 15) and *src*111PK (Fig. 4, lanes 16 to 20) associated with membranes in a manner similar to pp60^{src},

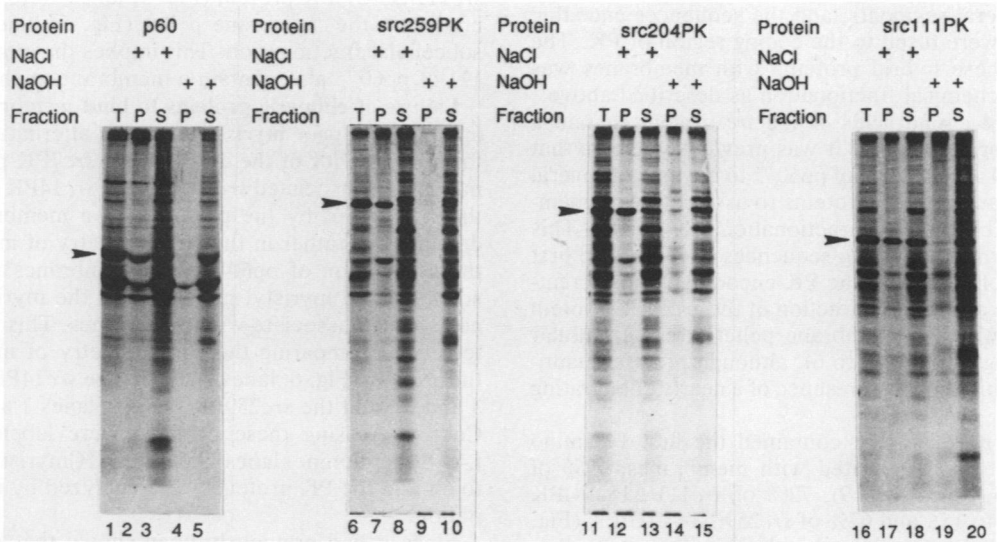

FIG. 4. The *src*-PK proteins and pp60^{src} associate with membranes in a similar manner. B31 cells (lanes 1 to 5) or COS7 cells transfected with plasmids encoding *src*259PK (lanes 6 to 10), *src*204PK (lanes 11 to 15), or *src*111PK (lanes 16 to 20) proteins were labeled with L-[^{35}S]methionine for 12 h. Crude extracts (T; lanes 1, 6, 11, and 16) were adjusted to either 0.3 M NaCl (lanes 2, 3, 7, 8, 12, 13, 17, and 18) or 0.1 M NaOH (lanes 4, 5, 9, 10, 14, 15, 19, and 20) and then fractionated into cytosol (S; lanes 3, 5, 8, 10, 13, 15, 18, and 20) and a membrane pellet (P; lanes 2, 4, 7, 9, 12, 14, 17, and 19) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with either MAb327 antibody (lanes 1 to 5) or a polyclonal rabbit anti-PK antibody (lanes 6 to 20). The positions of pp60^{src} and the *src*-PK proteins are indicated by the arrowheads.

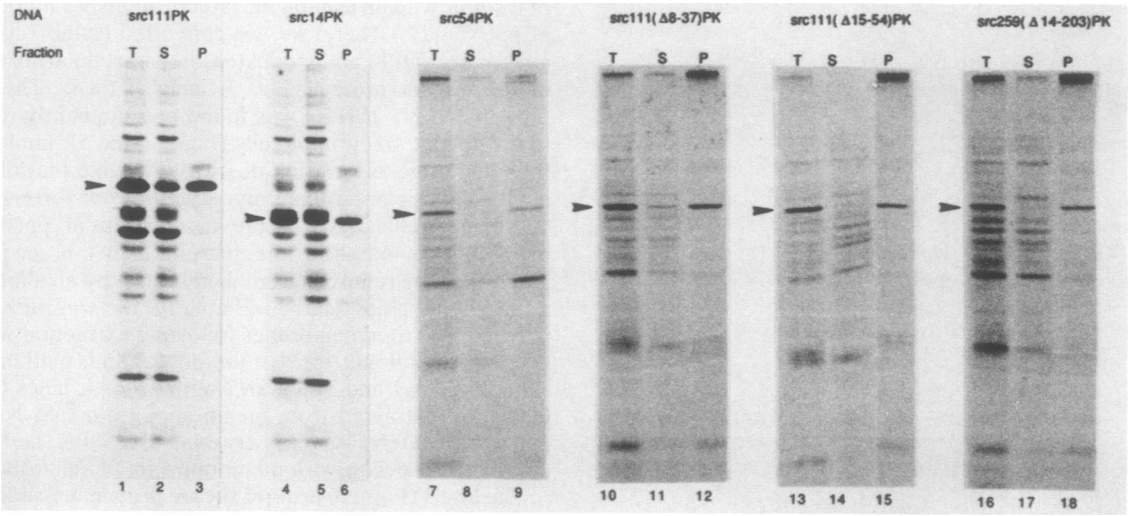

FIG. 5. The first 111 amino acids of pp60^{src} contain at least two membrane-anchoring domains. COS7 cells were transfected with plasmids encoding the *src*111PK (lanes 1 to 3), the *src*14PK (lanes 4 to 6), the *src*54PK (lanes 7 to 9), the *src*111(Δ8–37)PK (lanes 10 to 12), the *src*111(Δ15–54)PK (lanes 13 to 15), or the *src*259(Δ14–203)PK (lanes 16 to 18) proteins. Transfected cells were labeled with L-[^{35}S]methionine for 12 h. Crude extracts (T; lanes 1, 4, 7, 10, 13, and 16) were fractionated into cytosol (S; lanes 2, 5, 8, 11, 14, and 17) and a membrane pellet (P; lanes 3, 6, 9, 12, 15, and 18) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The *src*-PK proteins are indicated by the arrowheads. Note that the *src*14PK protein migrates slightly farther than a background protein, which prevented quantitation of the membrane association of *src*14PK.

because they were extracted only partially from membranes by 0.3 M NaCl, but were extracted quantitatively by base.

We conclude that the first 111 amino acids of pp60^{src} are sufficient to cause association with membranes and that the association is similar to that found with native pp60^{src}. By contrast, amino acids 111 to 204, together with N-terminal myristylation, permit only limited association with membranes.

The first 111 amino acids of the *src* protein contain at least two independent membrane-anchoring domains. The domain represented by the first 111 amino acids of pp60^{src} was divided into several segments, and the sequences encoding these segments were fused to the coding region of PK. The association of these hybrid proteins with membranes was analyzed by biochemical fractionation as described above.

(i) The first 14 amino acids of the *src* protein contain a membrane-anchoring domain. It was previously shown that fusing the first 14 amino acids of pp60^{src} to any one of several other proteins causes these proteins to associate with membranes in crude biochemical fractionations (1, 31, 32). This result was confirmed by fusing sequences encoding the first 14 amino acids of pp60^{src} to the PK-encoding region, creating *src*14PK. An appreciable fraction of the *src*14PK protein was recovered with the membrane pellet after subcellular fractionation (Fig. 5, lanes 4 to 6), although precise quantitation was prohibited by the presence of a nearly comigrating protein.

Other hybrid proteins that contained the first 14 amino acids of pp60^{src} also associated with membranes: 72% of *src*54PK (Fig. 5, lanes 7 to 9), 72% of *src*111(Δ15–54)PK (Fig. 5, lanes 13 to 15), and 67% of *src*259(Δ14–203)PK (Fig. 5, lanes 16 to 18) were recovered with the membrane pellet. These results leave open the possibility that amino acids 15 to 54, 55 to 111, and 204 to 259 also contain membrane-anchoring domains, a possibility that will be confirmed below by immunofluorescence (see Fig. 7).

(ii) Amino acids 38 to 111 of the *src* protein contain a membrane-anchoring domain. Two previous observations indicated that portions of pp60^{src} outside the first 14 amino acids contribute to membrane attachment. First, removal of more than 8 kDa from the amino terminus of pp60^{src} failed to detach the protein from membranes (22). Second, the protein encoded by the deletion mutant E3 *src*, which lacks amino acids 8 to 37, attaches to membranes (17).

We sought and identified a second membrane-anchoring domain by fusing the sequence encoding the first 81 amino acids of the E3 *src* protein to the coding region of PK, creating the *src*111(Δ8–37)PK gene. Most (66%) of the *src*111(Δ8–37)PK protein (Fig. 5, lanes 10 to 12) was recovered with the membrane pellet (Fig. 5, lane 12) following subcellular fractionation. This implies that amino acids 38 to 111 of pp60^{src} also contain a membrane-anchoring domain.

Failure of chimeric proteins to bind membranes cannot be attributed to poor myristylation. An alternative explanation for the behavior of the cytoplasmic *src*7PK protein and the membrane-associated *src*259PK and *src*14PK proteins is that they differ not by the presence of a membrane-anchoring domain, but rather in the stoichiometry of myristylation. If the association of pp60^{src} with membranes were mediated solely by the myristyl moiety, only the myristylated molecules would associate with membranes. This possibility was tested by comparing the stoichiometry of myristylation of the *src*7PK (Fig. 6, lanes 2 and 5), the *src*14PK (Fig. 6, lanes 3 and 6), and the *src*259PK (Fig. 6, lanes 1 and 4) proteins. Cells expressing these proteins were labeled with either L-[^{35}S]methionine (lanes 1 to 3) or [^{3}H]myristic acid (lanes 4 to 6), and the PK proteins were analyzed by immunoprecipitation.

Since it had previously been shown that 84% of pp60^{src} molecules are myristylated (4), we arbitrarily used the same value for the stoichiometry of myristylation of *src*259PK. In comparison, approximately 60% of the *src*14PK protein and 17% of the *src*7PK protein were myristylated (estimates derived from Fig. 6). Thus, if myristylation were sufficient to cause membrane association, 17% of the *src*7PK protein

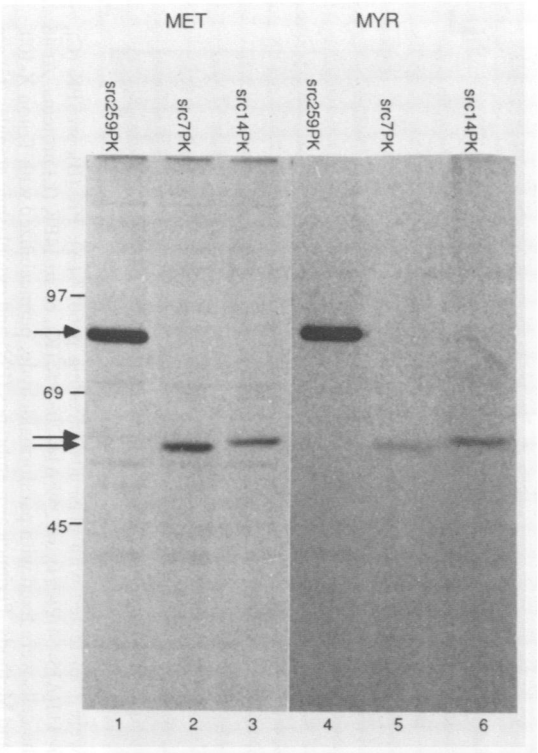

FIG. 6. The stoichiometries of myristylation of the *src*259PK, the *src*7PK, and the *src*14PK proteins are similar. COS7 cells were transfected with plasmids encoding the *src*259PK (lanes 1 and 4), the *src*7PK (lanes 2 and 5), or the *src*14PK (lanes 3 and 6) proteins. Transfected cells were labeled with either L-[^{35}S]methionine (lanes 1 to 3) or [^{3}H]myristic acid (lanes 4 to 6) for 12 h. Labeled cells were solubilized with lysis buffer, and PK proteins were immunoprecipitated with a polyclonal anti-PK antibody. The positions of 97-, 69-, and 45-kDa molecular mass markers are indicated. The PK proteins are indicated by the arrows. Stoichiometries mentioned in Results were calculated as follows, by using data from densitometry:

$$\frac{(\text{myr-x}/\text{myr-}src259)}{(^{35}\text{S-x}/^{35}\text{S-}src259)}.$$

would be expected to be membrane associated, whereas only 3% was observed.

We also analyzed the membrane association of myristylated proteins. If the only relevant difference between the cytoplasmic *src*7PK and *src*204(Δ8–111)PK proteins, on the one hand, and the membrane-associated *src*259PK and *src*14PK proteins, on the other, were the proportion of the molecules that are myristylated, all of the myristylated molecules would be membrane associated. Cells expressing these proteins were labeled with [^{3}H]myristic acid and subsequently divided into membrane and cytosol fractions. Virtually all of the myristate-labeled *src*7PK protein was recovered in the cytoplasmic fraction, whereas most of the myristate-labeled *src*259PK protein and the *src*14PK protein was recovered with the membrane pellet (data not shown). Similarly, much of the myristate-labeled *src*204(Δ8–111)PK protein was cytoplasmic. We conclude that the inability of the *src*7PK and *src*204(Δ8–111)PK proteins to associate with membranes does not reflect inefficient myristylation.

Localization of *src*-PK proteins by immunofluorescence. The intracellular distribution of pp60^{src} was previously analyzed by immunofluorescence and electron microscopy (30, 36, 37, 41, 44). The results indicated that in different types of cells, pp60^{src} may be associated with a variety of subcellular compartments, including plasma membranes (typically at sites of focal adhesion), perinuclear membranes, and secretory granules. Therefore, we used immunofluorescence to characterize the intracellular distribution of *src*-PK proteins.

(i) pp60^{src} associates with plasma membranes, perinuclear membranes, and cytoplasmic granules in transfected COS7 cells. The distribution of pp60^{src} in COS7 cells following transfection with a plasmid containing the c-*src* gene was determined by immunofluorescence. The intracellular distribution of pp60^{src} in COS7 cells appeared to be a composite of three subcellular locations: plasma membranes, perinuclear membranes, and cytoplasmic granules (Fig. 7A).

(ii) The PK protein, the *src*7PK protein, and the *src*204(Δ8–111)PK protein are cytoplasmic. The intracellular distribution of the PK and hybrid proteins was analyzed by staining fixed cells with a polyclonal anti-PK antibody. The PK and *src*7PK proteins had a distribution representing the cytoplasm (Fig. 7B and C): the margins of cells expressing these proteins were poorly visualized after staining with anti-PK antibody, and the intensity of the staining was proportional to the thickness of the cell (i.e., to the volume of underlying cytoplasm). The *src*204(Δ8–111)PK protein appeared to be equally distributed between the plasma membrane and the cytoplasm (Fig. 7G). These results confirm our interpretation of the biochemical fractionations described above.

(iii) Most of the membrane-associated *src*-PK proteins have an intracellular distribution similar to pp60^{src}. The *src*259PK protein (Fig. 7D), the *src*204PK protein (Fig. 7E), the *src*111PK protein (Fig. 7F), the *src*111(Δ8–37)PK protein (Fig. 7H), the *src*54PK protein (Fig. 7I), and the *src*111(Δ15–54)PK protein (Fig. 7J) had a distribution similar to that of pp60^{src} itself. Often cells expressing *src*-PK proteins that associate with the plasma membrane appeared to have a filamentous component to the fluorescent signal (for example, see Fig. 7E). The filamentous structures also contained actin, as demonstrated by staining with rhodamine-conjugated phalloidin, an actin-specific stain (data not shown). We do not know the nature of these filamentous structures, but they conceivably could correspond to sites of adhesion.

(iv) The *src*259(Δ14–203)PK protein associates specifically with perinuclear membranes. Interestingly, the *src*259(Δ14–203)PK protein associated primarily with perinuclear membranes (Fig. 7K). Since the distribution of the *src*259(Δ14–203)PK and the *src*14PK (see below) proteins were different, we conclude that amino acids 204 to 259 contain a domain that preferentially targets proteins to perinuclear membranes.

(v) The *src*14PK protein associates with cytoplasmic granules. Cells expressing the *src*14PK protein had a punctate pattern of cytoplasmic fluorescence after staining with anti-PK antibody (Fig. 7L). These fluorescent spots had an apparent diameter of 0.2 μm and were uniformly distributed throughout the cytoplasm. We suspect that this distribution corresponds to a membranous organelle, rather than a large proteinaceous complex, because the *src*14PK protein does not sediment rapidly after dissolution of membranes with 1% Triton X-100 (data not shown). (The photographic reproduction of Fig. 7L is not adequate to exclude a perinuclear location for some of the *src*14PK protein. However, other exposures of the same field and examination of numerous other cells sustain our conclusion that *src*14PK is located principally in cytoplasmic granules, rather than in either of the other two locations reported here.)

The *src*14PK is the only hybrid protein that associates principally with cytoplasmic granules. Although most of the

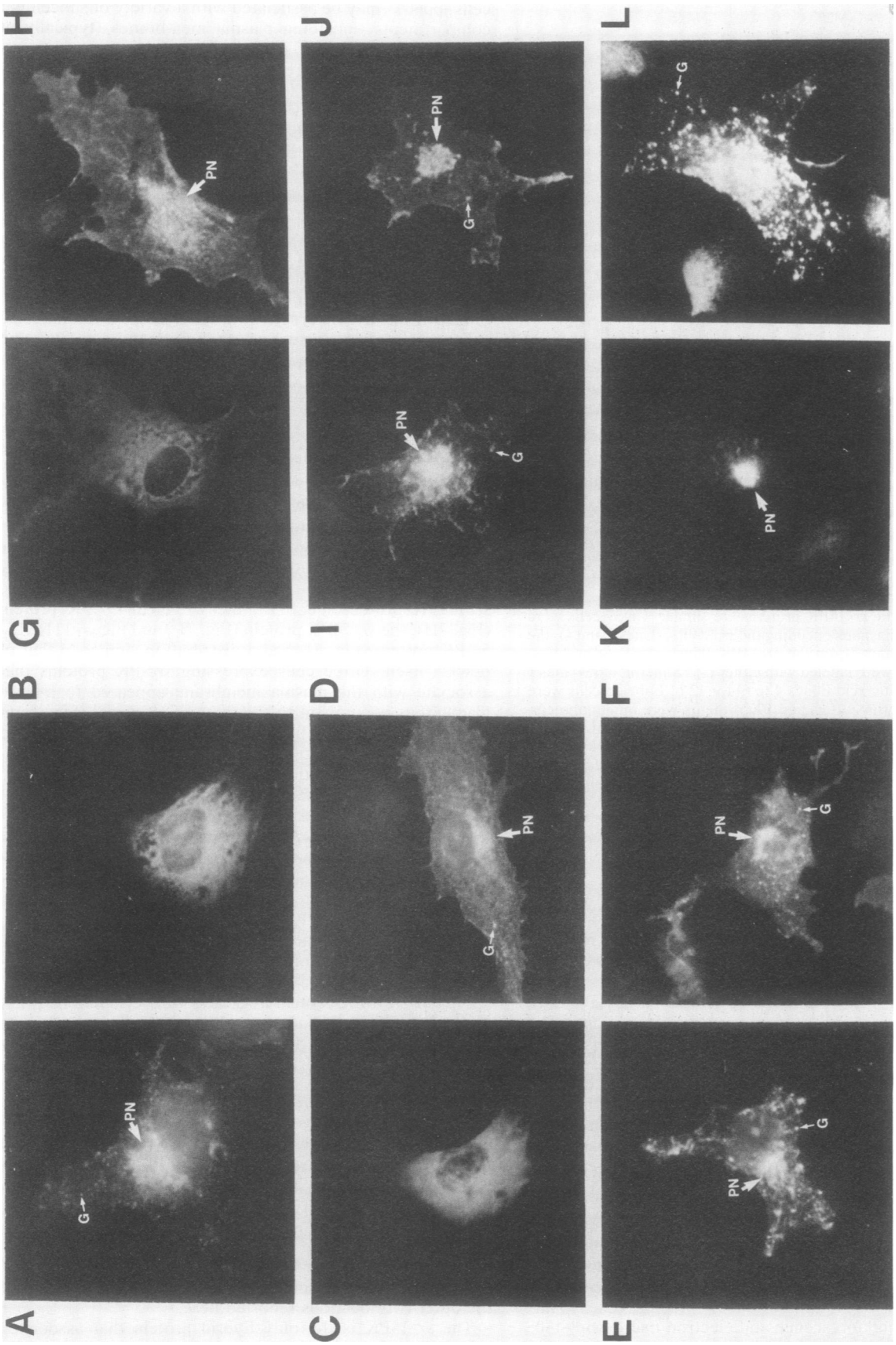

FIG. 7. Membrane-anchoring domains target proteins to specific subcellular locations. COS7 cell were transfected with plasmids encoding pp60^{src} (A), PK (B), *src*7PK (C), *src*259PK (D), *src*204PK (E), *src*111PK (F), *src*204(Δ8–111)PK (G), *src*111(Δ8–37)PK (H), *src*54PK (I), *src*111(Δ15–54)PK (J), *src*259(Δ14–203)PK (K), or *src*14PK (L) proteins. At 48 h after exposure to these DNAs, the cells were seeded onto glass cover slips. After an additional 24 h, the cells were fixed with paraformaldehyde, permeabilized with Triton X-100, and stained with either anti-pp60^{src} MAb327 or a polyclonal anti-PK antibody, as described in Materials and Methods. Perinuclear membranes (PN) and cytoplasmic granules (G) are indicated.

src-PK proteins that contain amino acids 1 to 14 were also found in cytoplasmic granules, proteins that contain additional portions of pp60^{src} associated with other membranes as well. For example, both the *src*54PK protein (Fig. 7I) and the *src*111(Δ15–54)PK protein (Fig. 7J) associated with the plasma membrane and perinuclear membranes, in addition to cytoplasmic granules. Because the distribution of these proteins differs from *src*14PK, amino acids 15 to 54 and 55 to 111 must contain additional sorting information. These results imply that multiple domains of pp60^{src} can affect the intracellular distribution of a protein.

DISCUSSION

Several lines of evidence suggest that the association of pp60^{src} with membranes is mediated in part by interaction between pp60^{src} and one or more membrane proteins. First, pp60^{src} is found in diverse subcellular locations that vary from one type of cell to another. These locations include the plasma membrane in fibroblasts (8, 21, 44), particularly focal contacts (37); perinuclear membranes in fibroblasts (36); cytoplasmic granules in platelets (33) and chromaffin cells (30); and growth comes in neurons (27, 41). These specificities suggest that pp60^{src} interacts with other proteins in the various locations. Second, reconstitution of purified pp60^{src} into lipid vesicles in vitro requires the addition of membrane proteins (34), and the binding of pp60^{src} to membranes utilizes at least one protein receptor that is both specific and saturable (35). Third, the protein tyrosine kinase encoded by *lck* associates with two transmembrane glycoproteins, the CD4 and CD8 antigens of T lymphocytes (38, 42), and the catalytic activity of *lck* can be modulated by cross-linking the CD4 antigen (43). Since the *lck* protein is closely related to pp60^{src}, it seems likely that the latter also interacts specifically with membrane proteins and that the interaction may help govern the activity of pp60^{src}.

If the attachment of pp60^{src} to membranes resembles that of the *lck* protein, the following predictions should be borne out: (i) pp60^{src} should behave like a peripheral membrane protein in schemes for solubilization; (ii) myristylation should not be sufficient for the association of pp60^{src} with membranes; (iii) specific domains of pp60^{src} should mediate its association with membranes; and (iv) these membrane-anchoring domains will target pp60^{src} to specific subcellular locations. The work reported here fulfills each of these predictions.

We acknowledge one caveat. Our work was performed with chimeric proteins formed between portions of pp60^{src} and the cytosolic protein PK. We cannot presently refute the possibility that the conformation (or other features) of these chimeras has influenced the outcome of our experiments in ways that are artifactual. It is reassuring, however, that the chimera *src*259PK (which contains all of the membrane-targeting domains that we have defined within pp60^{src}) distributes within cells in a manner identical to that of pp60^{src} itself. In addition, the conclusions reached here are in accord with previous observations on the roles of myristylation and other domains of pp60^{src} in the attachment to membranes (for examples, see references 22, 34, and 35).

The *src* protein is a peripheral membrane protein. Previous reports have demonstrated that the association of pp60^{src} with membranes cannot be disrupted by chelating agents or by extreme concentrations of salt, whereas nonionic detergents completely extract pp60^{src} from membrane vesicles (21, 22). These results led to the provisional conclusion that pp60^{src} is an integral membrane protein. In contrast, we have shown that pp60^{src} is a peripheral membrane protein, because it can be extracted from membrane vesicles with alkali. Similarly, hybrid *src*-PK proteins that are associated with membranes are also extracted with alkali. Perhaps extraction from membranes with alkali will be a general feature of myristylated proteins associated with membranes.

The role of myristylation in the membrane association of pp60^{src}. Myristylation is neither necessary nor sufficient for the association of pp60^{src} with membranes. Several forms of pp60^{src} are myristylated yet cytoplasmic (3, 11, 29). Conversely, nonmyristylated forms of pp60^{src} have been described which still associate with membranes (12, 20, 22). Our results provide further support for the notion that the myristyl moiety is not sufficient for the association of pp60^{src} with membranes: the myristylated proteins *src*7PK and *src*204(Δ8–111)PK are cytoplasmic when analyzed by either biochemical fractionation or immunofluorescence (see Fig. 1 for a summary).

What, then, is the role of myristylation in the attachment of pp60^{src} to membranes? First, the modification may increase the affinity of the protein for membranes: forms of pp60^{src} that lack myristylation but are nevertheless found on membranes can be solubilized by ionic strength alone. Second, myristylation may initiate the binding of pp60^{src} to membranes: typically, nonmyristylated forms of pp60^{src} never attach to membranes. The full association of pp60^{src} with membranes requires one or more portions of the protein itself, however, and once the association is established, it may be independent of myristylation: cleavage of membrane-bound pp60^{src} with protease removes 8 kDa from the amino terminus, yet fails to disrupt the association of the remainder of the protein with the membranes (22).

Membrane-anchoring domains target molecules to distinct subcellular locations. Previous reports have demonstrated that pp60^{src} is found in plasma membranes, perinuclear membranes, and cytoplasmic granules. The results reported here are unusual in that pp60^{src} is found in all of these locations in a single type of cell, a circumstance that we cannot presently explain but that might be due either to the use of COS7 cells or to the exceptional abundance of pp60^{src} produced in the cells (10- to 20-fold more than found in cells transformed by Rous sarcoma virus [data not shown]). Whatever its cause, this circumstance allowed us to demonstrate that distinctive domains of pp60^{src} are required for targeting the protein to each subcellular location.

Amino acids 1 to 14 of pp60^{src} cause proteins to associate primarily with cytoplasmic granules in COS7 cells. Perhaps these amino acids specifically target pp60^{src} to granules in chromaffin cells and platelets as well. Amino acids 38 to 111 cause proteins to associate with both plasma membranes and perinuclear membranes. We suspect that these amino acids make up multiple membrane-anchoring domains, because in conjunction with amino acids 1 to 14, either amino acids 15 to 54 or amino acids 55 to 111 are sufficient to cause association with plasma membranes, perinuclear membranes, and cytoplasmic granules. Amino acids 204 to 259, together with amino acids 1 to 14, cause a protein to associate primarily with perinuclear membranes. We conclude that the subcellular localization of pp60^{src} is mediated by multiple domains within the protein.

Is the subcellular distribution of pp60^{src} controlled by phosphorylation? How is it that in most types of cells, pp60^{src} is localized to one subcellular compartment and not to others? It appears possible that the activity of membrane-anchoring domains is regulated by phosphorylation. For example, we have shown that amino acids 1 to 14 of pp60^{src}

target proteins specifically to the membranes of cytoplasmic granules. Within and close to this domain are serine residues that are known to be phosphorylated by cellular kinases: serine 12 by kinase C (13, 14) and serine 17 by the cyclic AMP-dependent protein kinase (6, 10, 18). Perhaps phosphorylation of these sites helps regulate the subcellular location of pp60^{src}. There is precedent for this speculation in the finding that phosphorylation of the epidermal growth factor receptor by kinase C causes internalization of receptor molecules (24).

The viral and cellular *src* proteins differ in their amino acid sequence, including regions used here in chimeras. Since the chimeras contained sequences derived only from viral *src*, the conclusions that we have reached regarding the targeting of *src* protein to membranous compartments apply rigorously only to the viral version of the protein. Alternative splicing in neurons results in the synthesis of a novel form of pp60^{src} (pp60^{+}) (23, 28). Perhaps the localization of pp60^{+} in neurons to growth cones is in part due to these structural alterations.

Where within the cell is the transforming activity of pp60^{src} effected? Transformation by the viral version of pp60^{src} requires that the protein be associated with membranes (see reference 19 for a review), presumably because the crucial substrates of pp60^{src} are also membrane associated. Since pp60^{src} is capable of associating with various membranes, it is not clear whether transformation results from the activity of pp60^{src} at a single or at multiple subcellular locations.

Surprisingly, many of the mutant *src* proteins with deletions that begin at codon 15 retain transforming activity. None of these proteins has been analyzed by immunofluorescence. Our results indicate that these transforming *src* proteins would associate primarily with perinuclear membranes and cytoplasmic granules.

No mutant allele of *src* has been described in which all of the membrane-anchoring domains have been deleted. In most of the available mutants, the encoded proteins retain the first 14 amino acids of pp60^{src}; among these mutant proteins, only the product of the NY18-3 allele is not associated with membranes (11). The NY18-3 *src* gene lacks only the sequences that normally encode amino acids 169 to 264, yet it encodes a myristylated protein that is located in the cytoplasm. Our results do not explain these properties. Perhaps the conformation of this protein precludes association with membranes.

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