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Mutations in the SH3 Domain of the *src* Oncogene Which Decrease Association of Phosphatidylinositol 3′-Kinase Activity with pp60$^{v\text{-}src}$ and Alter Cellular Morphology

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To analyze the signaling pathways utilized in malignant transformation by pp60$^{v\text{-}src}$, we have isolated and characterized *src* mutants which possess normal levels of protein tyrosine kinase activity but which cause only a partially transformed phenotype. Our hypothesis is that such mutants are partially defective for transformation because they are defective in their ability to activate specific components of the cellular signaling machinery while still activating others. In this communication, we report on the molecular and biochemical characterization of one such mutant, CU12 (D. D. Anderson, R. P. Beckmann, E. H. Harms, K. Nakamura, and M. J. Weber, J. Virol. 37:455–458, 1981). Cells infected with this mutant are capable of anchorage-independent growth, but rather than exhibiting the rounded and refractile morphology characteristic of wild-type-infected cells, they display an extremely elongated, fusiform morphology. The morphological properties of this mutant *src* could be accounted for entirely by a single mutation in the SH3 domain (lysine 106 to glutamate). Other mutations were constructed in this region by in vitro mutagenesis, both in a v-*src* and in an activated c-*src* background, and several of them also induced a fusiform morphology. All of the mutations inducing fusiform morphology also resulted in decreased association of pp60^{src} with phosphatidylinositol 3′-kinase activity. In addition, association of pp60^{src} with some tyrosine-phosphorylated proteins was altered. We propose that the SH3 domain participates (along with the SH2 domain) in the interaction of pp60^{src} with cellular signaling proteins, and we speculate that the association with phosphatidylinositol 3′-kinase plays an important role in the regulation of cellular morphology.

pp60$^{v\text{-}src}$, the product of the *src* oncogene, is a nonreceptor protein tyrosine kinase whose enzymatic activity is essential to its ability to transform cells (reviewed in reference 52). Despite considerable effort over the past 10 years, knowledge is fragmentary concerning the signaling pathways utilized in cell transformation by pp60$^{v\text{-}src}$. It is clear that the tyrosine kinase activity and membrane association of pp60$^{v\text{-}src}$ are both necessary for transformation, but neither is sufficient. In no case has phosphorylation of a specific cellular substrate protein been proven to be essential for transformation.

One approach to identifying potential functionally significant substrates for pp60$^{v\text{-}src}$ utilizes *src* mutants which are completely or partially defective in transforming ability (2, 3, 14, 37–39, 57, 58, 67, 68). In this approach, tyrosine phosphorylation of individual proteins is correlated with specific manifestations of transformation, with the expectation that proteins whose phosphorylation is associated with transformation may be causally involved in oncogenesis. The receptor for insulinlike growth factor I (46, 47) and a 120-kDa protein (46) were identified as candidate functional targets for pp60$^{v\text{-}src}$ in this way, and similarly the tyrosine phosphorylations of calpactin (14, 50) and vinculin (4, 43, 44) were shown not to be necessary for transformation.

The use of *src* mutants to analyze transformation also has helped elucidate functional domains of pp60$^{v\text{-}src}$ which are important for substrate recognition and transformation (52). The amino terminus of pp60$^{v\text{-}src}$ begins with a myristylation signal sequence, and myristylation is necessary for appropriate membrane association of pp60$^{v\text{-}src}$ and for transformation (40, 41). Following the myristylation signal sequence is a unique domain of approximately 70 amino acids, whose function is unknown. The SH3 domain extends from residues 85 to 137 of pp60^{src}, and similar SH3 domains are found in other nonreceptor tyrosine kinases as well as in the v-*crk* oncogene, *fus-1* of *Saccharomyces cerevisiae* and two neutrophil oxidases (48). Little is known about the function of the SH3 domain, although mutations in this domain can activate the c-*src* proto-oncogene, implying that this domain participates in the negative regulation of the pp60$^{c\text{-}src}$ kinase activity (35, 36, 54). Residues 137 to 241 encompass the SH2 domain. There is recent evidence that this domain participates in multiprotein complex formation that is involved in intracellular signaling by tyrosine phosphorylation (11, 45). The carboxy-terminal half of pp60$^{v\text{-}src}$ has the tyrosine kinase domain.

Considerable current interest has been focused on phosphatidylinositol (PI) 3′-kinase as a functionally significant target for pp60$^{v\text{-}src}$ and other tyrosine kinases. This enzymatic activity was first reported to associate with polyomavirus middle T/pp60$^{c\text{-}src}$ complexes (70). It has subsequently been found associated with other tyrosine kinases, including pp60$^{v\text{-}src}$, products of the *fyn* and *yes* oncogenes, and receptor tyrosine kinases such as receptors for epidermal growth factor, platelet-derived growth factor, colony-stimulating factor 1, and insulin (5–8, 11, 13, 20, 21, 24–27, 31, 32, 59, 63, 64). Association with so many proteins involved in growth control suggests PI 3′-kinase activity may play a role in growth signaling.

PI 3′-kinase can phosphorylate inositol on the D-3 hydroxyl position, thereby producing PI 3-phosphate (PI-3-P), PI 3,4-bisphosphate (PI-4,5-P_2), and PI 3,4,5-triphosphate (5, 6, 11, 17). These products of PI 3′-kinase are distinct from those of the classic PI turnover pathway because they do not

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appear to be substrates of phospholipase C-γ (60). PI 3′-kinase activity has been purified (12), and the kinase activity appears to reside in a heterodimer consisting of 85- and 110-kDa proteins. The 85-kDa subunit has been cloned and sequenced and found to contain one SH3 domain and two SH2 domains (22, 51, 61). By itself, the 85-kDa subunit does not have PI 3′-kinase activity.

In this report, we show that immunoprecipitates of the $pp60^{v\text{-}src}$ encoded by a partially transforming *src* mutant, CU12 (2), display decreased association with PI 3′-kinase activity. CU12-infected cells assume a strikingly needlelike, fusiform morphology, and both the morphological properties and the decreased association with PI 3′-kinase can be accounted for by a single point mutation at residue 106 in the SH3 domain of *src*. Other SH3 mutations which caused a fusiform morphology also reduced association with PI 3′-kinase, raising the possibility that the association with PI 3′-kinase is important for full morphological transformation. Immunoprecipitates of these mutant $pp60^{src}$ proteins differed from those of the wild type also with respect to some of the tyrosine-phosphorylated proteins which were associated with $pp60^{src}$. These results show that the SH3 domain is important for association of $pp60^{src}$ with cellular proteins such as PI 3′-kinase, although they do not determine whether SH3 directly participates in these interactions. Our results also suggest that these protein-protein associations are necessary for $pp60^{src}$ to induce a fully transformed morphology.

MATERIALS AND METHODS

Vector construction and mutagenesis. DNA from cells infected with CU12 was digested with *Eco*RI, and DNA in the 3-kb size range (corresponding to the size of the R1 fragment of *src* from Rous sarcoma virus) was cloned into λgt10. Colonies were screened with a nick-translated v-*src* probe, and the insert from positive colonies was recloned into a Rous sarcoma virus expression vector, using an approach similar to that described by Cross et al. (15, 16). After ensuring the cloned CU12 induced morphological features and growth-stimulatory properties identical to those induced by the partially transforming mutant virus, the v-*src* gene was cloned into M13mp18 for sequencing and further manipulation. The mutant v-*src* was sequenced with the Sequenase kit from United States Biochemical Co. (Cleveland, Ohio). Oligonucleotides for sequencing were generously supplied by the laboratory of J. T. Parsons or were synthesized in our core facility.

To determine which of the point mutations found in CU12 was responsible for its partially transforming phenotype, we took advantage of the *Mlu*I site which bisects the *src* gene and constructed chimeras consisting of the 5′ half of CU12 with the 3′ half of wild-type virus, or the converse. Plasmids were transfected onto chicken embryo fibroblasts (CEFs), and morphological properties and growth in soft agar were assessed.

Mutagenesis was performed with the Promega (Madison, Wis.) Altered Sites kit and appropriate 25-mer oligonucleotides. The presence of the desired mutation was confirmed by sequencing.

Plasmids containing c-*src* G2A/Y527F and *dl*92/Y527F clones were gifts from the laboratory of J. T. Parsons.

Cell culture. CEFs were prepared from gs^- (group-specific antigen-negative) embryos from Spafas Co. (Norwich, Conn.) and were maintained in Dulbecco's modified Eagle's medium supplemented with 0.2% heat-inactivated chicken serum and 4% fetal calf serum. Penicillin G was added to 50 U/ml, and streptomycin was added to 50 μg/ml. The cells were grown at 37°C with 7.5% CO_2.

Kinase assays. The PI 3′-kinase assay was similar to that described by Whitman et al. (69, 70). Anti-$pp60^{src}$ immunoprecipitations for PI 3′-kinase assays and autokinase assays were performed essentially the same way. Immunoprecipitations were generally from cell lysates containing 800 μg of protein, using 4 μl of EC10 monoclonal antibody to $pp60^{src}$ (53). This procedure was followed by a 1-h incubation with protein A-Sepharose or fixed *Staphylococcus aureus* (Immunoprecipitin; Bethesda Research Laboratories, Gaithersburg, Md.). Lysates and washes were similar for both lipid and protein kinase assays except that after the last wash the protein A-Sepharose or Immunoprecipitin was resuspended in 50 μl of 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 7.2)–10 mM $MnCl_2$ for the protein kinase assay. Then 5 μCi of [γ-^{32}P]ATP was then added, and the tubes were placed at 37°C for 2 min. The reaction was stopped by adding 50 μl of 2× electrophoresis sample buffer, boiled for 2 min, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

Decreased PI 3′-kinase associated with an SH3 mutant of $pp60^{v\text{-}src}$. A preliminary screen of the partially transforming Rous sarcoma virus mutants reported by Anderson et al. (2) showed that immunoprecipitates of $pp60^{v\text{-}src}$ from cells infected with the CU12 *src* mutant, which had an extreme fusiform morphology, had reduced PI 3′-kinase activity, whereas the other partially transforming mutants showed associated PI 3′-kinase activity comparable to that of wild-type infected cells (60a). These results indicated a correlation between the partially transforming properties of CU12 and PI 3′-kinase.

To further analyze the molecular basis of the defect in CU12, the mutant *src* DNA was sequenced and found to contain two mutations: E at amino acid 106 (in the SH3 domain) had been mutated to K (E106K), and V at position 461 (in the kinase domain) had been mutated to M (V461M). By taking advantage of the *Mlu*I site near the midpoint of the *src* gene, we were able to form chimeras between wild-type and mutant *src* DNAs which each contained only one of the mutations found in CU12. We found that the fusiform morphology of CU12 could be accounted for entirely by the E106K mutation (Fig. 1) and that the V461M mutation by itself was without effect (28; data not shown). Therefore, the E106K mutant was used in subsequent experiments.

We next established the linearity of the PI 3′-kinase assay as a function of amount of lysate. Assays were performed on three different amounts of lysate from uninfected CEFs, cells infected with wild-type virus, and cells infected with the E106K mutant. Over the three different amounts of lysate used, the amount of PI-3-P produced was less in the E106K immunoprecipitates than in the wild-type immunoprecipitates (Fig. 2A). The E106K immunoprecipitates showed only slightly more PI-3-P produced than did the uninfected CEF negative control. Western immunoblot analysis revealed that comparable amounts of $pp60^{v\text{-}src}$ were present in lysates from wild-type- and mutant-infected cells (Fig. 2B). The wild-type and mutant $pp60^{v\text{-}src}$ immunoprecipitates also displayed comparable amounts of protein kinase activity (Fig. 2C). Previous results have shown that the overall amount and pattern of tyrosine phosphorylation in CU12-infected cells did not differ significantly from results

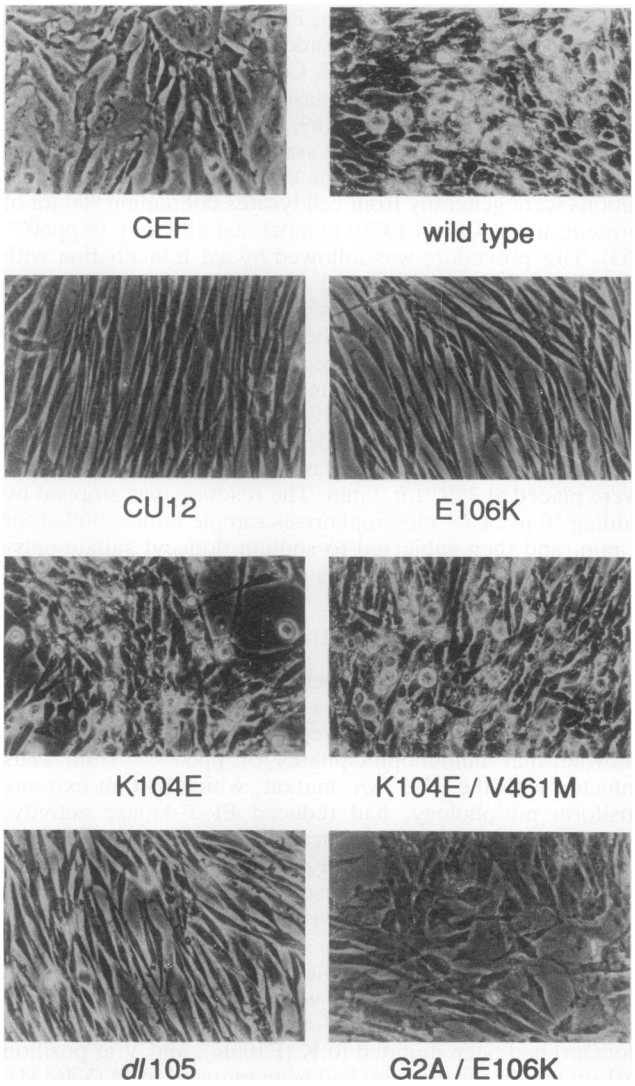

FIG. 1. Morphology of cells expressing SH3 mutants of *src*. Wild-type, Schmidt-Ruppin strain subgroup A. CU12, partially transforming mutant containing E to K at amino acid 106 and V to M at amino acid 461. Other mutants are as designated.

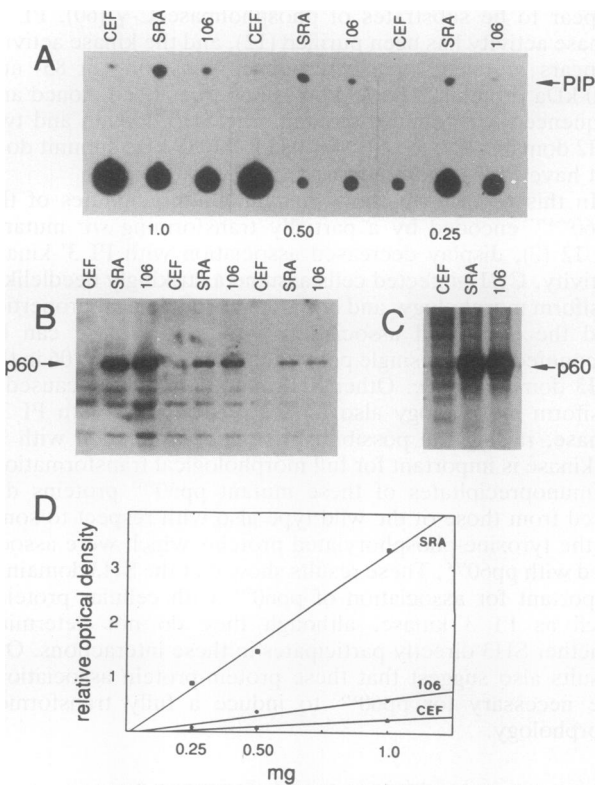

FIG. 2. Decreased PI 3′-kinase activity associated with E106K mutant pp60$^{v\text{-}src}$. (A) PIP produced in immunoprecipitates of pp60$^{v\text{-}src}$, indicated by the arrow. Radioactivity at the origin is variable isotope carried over in the $CHCl_3$-methanol extractions. (B and C) Western blots and autokinase activities, respectively, of pp60$^{v\text{-}src}$ in the immunoprecipitates shown in panel A. (D) Quantitation of PIP production shown in panel A, determined by densitometric scanning with a BioImage Visage. The ordinate is relative arbitrary optical density units. SRA, wild-type *src* (Schmidt-Ruppin strain subgroup A); 106, E106K *src* mutant.

for wild-type infected cells, as detected by antiphosphotyrosine immunoblotting of whole cell lysates which had been separated by SDS-PAGE (46). Therefore, under conditions controlling for the amount and activity of pp60^{src}, E106K anti-pp60$^{v\text{-}src}$ immunoprecipitates have less PI 3′-kinase activity associated with them than do wild-type anti-pp60$^{v\text{-}src}$ immunoprecipitates. Quantitation of these data is shown in Fig. 2D.

Assays comparing PI 3′-kinase activity associated with the wild type with that associated with E106K were repeated nine times. The results were quantitated by densitometry of the PI-3-P spots and/or by scraping the thin-layer chromatography plates and counting the silica in a liquid scintillation counter. When the amount of PI-3-P was normalized to the amount of pp60^{src} present in the lysate as determined by immunoblotting, a significant difference ($P < 0.05$) was found between the activity associated with the wild type and that associated with E106K. On average, five times more PI 3′-kinase activity was associated with wild-type pp60$^{v\text{-}src}$ than with E106K.

Nonmyristylated 106 mutants of pp60^{src}. The amino acid change that occurs in E106K, a lysine substituting for glutamate, could affect the association of pp60$^{v\text{-}src}$ with PI 3′-kinase in two different ways. E106K pp60$^{v\text{-}src}$ could have an altered intracellular distribution and therefore might not be able to bind PI 3′-kinase because the *src* protein was in an inaccessible location. Alternatively, the 106 mutation could affect the PI 3′-kinase binding site of pp60$^{v\text{-}src}$ and thus reduce association between the proteins. To distinguish between these two possibilities, we took advantage of the fact that myristylation of pp60^{src} is necessary for its membrane association (40, 41). We made a nonmyristylated version of E106K, which we designate G2A/E106K, by replacing with alanine the amino-terminal glycine to which myristic acid is normally attached.

Mutants of pp60$^{v\text{-}src}$ which are nonmyristylated but are otherwise wild type are nontransforming (40, 41). Nonmyristylated pp60^{src} associates with PI 3′-kinase, indicating that while PI 3′-kinase association may be necessary for transformation, it is not sufficient (24, 31). We reasoned that if decreased association of PI 3′-kinase with the E106K mutant was a consequence of the mutant pp60 being sequestered in

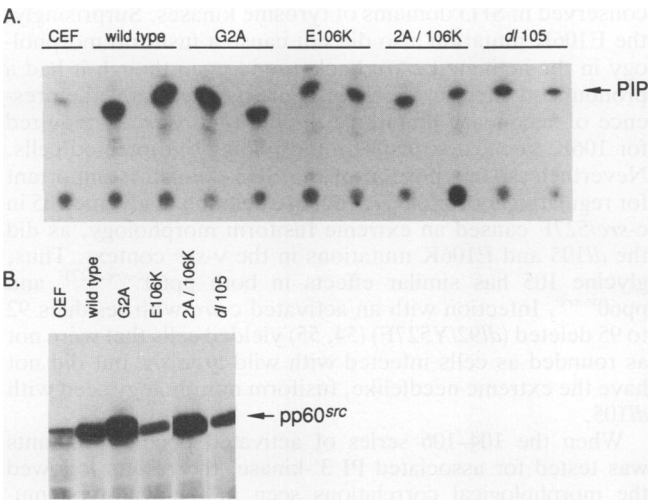

FIG. 3. PI 3′-kinase activity in immunoprecipitates of pp60$^{v\text{-}src}$ SH3 mutants. (A) PI 3′-kinase assay. Duplicate samples are shown (except for CEF); (B) anti-pp60^{src} Western blot for this experiment. Note that the elevated levels of pp60$^{v\text{-}src}$ in cells infected with nonmyristylated mutants are commonly observed but that associated PI 3′-kinase levels are nevertheless lower in cells infected with the 2A/106K mutant.

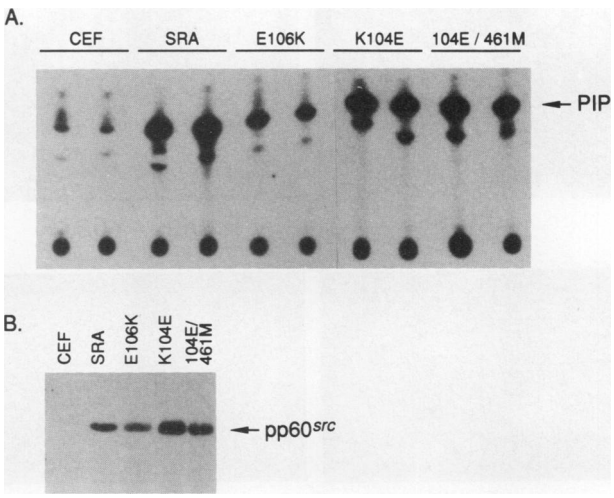

FIG. 4. PI 3′-kinase activity in immunoprecipitates of pp60$^{v\text{-}src}$ SH3 mutants. (A) PI 3′-kinase activity; (B) anti-pp60^{src} Western blot for this experiment. SRA, Schmidt-Ruppin strain subgroup A.

an inaccessible membrane location, then freeing the mutant pp60 from the membrane by blocking its myristylation would allow it to bind PI 3′-kinase (which is also cytosolic) as much as does G2A pp60$^{v\text{-}src}$. There thus should be equal amounts of PI 3′-kinase activity in the nonmyristylated wild type and in the double mutant. If, on the other hand, the E106K mutation affects more directly the binding site of PI 3′-kinase, there would be less PI 3′-kinase activity associated with G2A/E106K pp60$^{v\text{-}src}$ immunoprecipitates than in G2A pp60$^{v\text{-}src}$ immunoprecipitates.

Our results indicate that the E106K mutation affects the binding of pp60$^{v\text{-}src}$ to PI 3′-kinase even when the pp60$^{v\text{-}src}$ is nonmyristylated and cytosolic. When cells are infected by G2A/E106K, they have a nontransformed phenotype (Fig. 1) similar to that of G2A pp60$^{v\text{-}src}$ (data not shown). In three separate experiments with assays performed in duplicate, PI 3′-kinase activity associated with immunoprecipitates of G2A/E106K pp60$^{v\text{-}src}$ was seen to be less than that associated with G2A pp60$^{v\text{-}src}$ (Fig. 3). Therefore, we conclude that residue 106 in the SH3 domain of pp60$^{v\text{-}src}$ plays a role in PI 3′-kinase association by altering the binding site for PI 3′-kinase rather than by altering the location of pp60$^{v\text{-}src}$ in the cell.

Mutation of glycine 105 decreases PI 3′-kinase association with pp60$^{v\text{-}src}$. Much of the SH3 domain is strongly conserved among nonreceptor tyrosine kinases and in a wide variety of other proteins as diverse as phospholipase C-γ and myosin 1B. The residues near glutamate 106 of pp60^{src} are among the most strongly conserved. While some deletions of the SH3 domain have been made (35, 36, 54), few mutations have been reported in this conserved area of the SH3 domain near residue 106.

To investigate this region further, we made mutants of wild-type pp60$^{v\text{-}src}$ by deleting the glycine at position 105 and by altering the charge of the residue at position 104 by changing the lysine to a glutamate. We reasoned that the glycine might be important in its potential role as a helix breaker and that a deletion would alter the structure of this part of the SH3 domain. We made the charge change at residue 104 to investigate the possibility that PI 3′-kinase binds to pp60^{src} by alternating charged residues, that is to say, by having a lysine at 104 and then an oppositely charged glutamate at residue 106. We also made a 104E/461M mutant reminiscent of CU12 (2) to determine whether the carboxy-terminal half of pp60^{src} could affect this SH3 mutation. We designated the glycine deletion mutant *dl*105 and the 104 mutants K104E and K104E/V461M, respectively.

When we transfected CEFs with K104E, K104E/V461M, and *dl*105, we found that both K104E and K104E/V461M produced a rounded morphology indistinguishable from the wild-type morphology (Fig. 1). In contrast, deletion of amino acid 105 caused an extreme fusiform morphology indistinguishable from that of E106K (Fig. 1). The association of PI 3′-kinase activity with pp60$^{v\text{-}src}$ immunoprecipitates correlated with the morphological changes: both K104E and K104E/V461M displayed associated PI 3′-kinase activity comparable to wild-type levels (Fig. 4), while the value for immunoprecipitate of *dl*105 was very similar to the low level of E106K when normalized to the amount of pp60^{src} protein (Fig. 3).

PI 3′-kinase association with activated (Y527F) c-*src* mutants. Activation of pp60$^{c\text{-}src}$ results in increased association with PI 3′-kinase (13), and we therefore wished to determine the effects of SH3 mutations on the association with activated forms of the proto-oncogene. To do this, we made mutations in c-*src* and the activated c-*src*/Y527F (in which Y527, which is the site of negative regulatory phosphorylation, is replaced by F) similar to those described above in v-*src*. That is, we altered the charge at residue 104 by replacing lysine with glutamate, deleted glycine 105, or replaced glutamate 106 with a lysine, making the activated c-*src* equivalent of E106K.

When plasmids with the 104, 105, and 106 mutations in a normal c-*src* background were transfected onto CEFs, no morphological changes were noted (data not shown). Thus, these mutations do not activate c-*src* with respect to morphological transformation. However, when cells were transfected with plasmids carrying these c-*src* mutations in combination with the activating Y527F mutation, a variety of morphologies were seen (Fig. 5). Cells transfected with

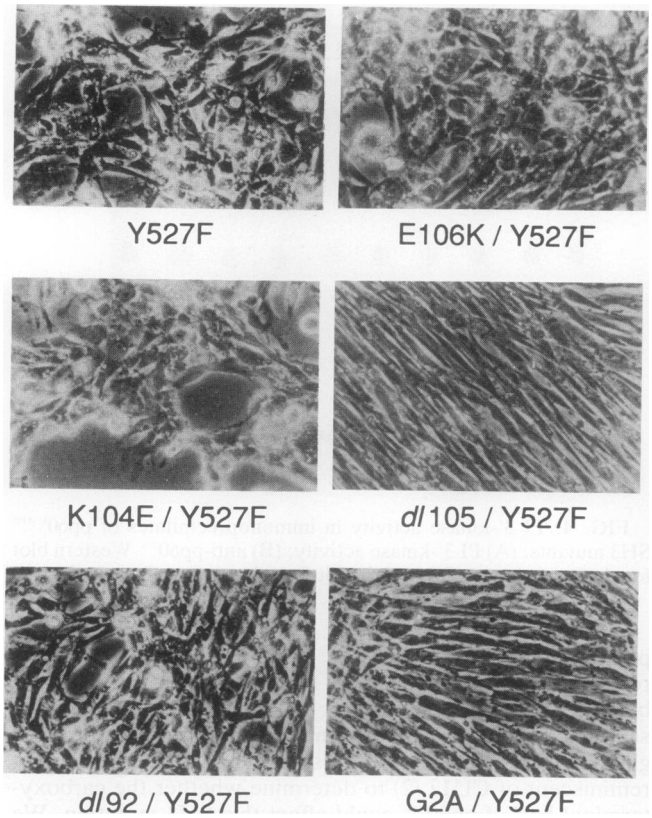

FIG. 5. Morphology of cells expressing SH3 mutants of activated c-*src*.

K104E/Y527F looked as completely transformed as those which had received Y527F. Thus, the charge mutation at position 104 had no detectable effects on c-*src*/Y527F, c-*src*, or v-*src* despite the fact that K at this position is highly conserved in SH3 domains of tyrosine kinases. Surprisingly, the E106K mutation also did not cause a fusiform morphology in the activated c-*src* background even though it had a pronounced effect in the v-*src* context. Therefore, the presence of secondary mutations in wild-type v-*src* is required for 106K to cause a fusiform morphology in infected cells. Nevertheless, this portion of the SH3 domain is important for regulating morphology, because deletion of glycine 105 in c-*src*/527F caused an extreme fusiform morphology, as did the *dl*105 and E106K mutations in the v-*src* context. Thus, glycine 105 has similar effects in both $pp60^{c\text{-}src/527F}$ and $pp60^{v\text{-}src}$. Infection with an activated c-*src* with residues 92 to 95 deleted (*dl*92/Y527F) (54, 55) yielded cells that were not as rounded as cells infected with wild-type *src* but did not have the extreme needlelike, fusiform morphology seen with *dl*105.

When the 104–106 series of activated $pp60^{c\text{-}src}$ mutants was tested for associated PI 3′-kinase, the results followed the morphological correlations seen in the wild-type mutants: fusiform cells had lowered PI 3′-kinase activity associated with $pp60^{src}$. Thus, we found equivalent association of PI 3′-kinase activity with $pp60^{c\text{-}src/527F}$ and in the fully transforming mutants $pp60^{c\text{-}src106K/527F}$ (Fig. 6) and $pp60^{c\text{-}src104E/527F}$ (data not shown), whereas deletion of glycine 105 resulted in reduced association of PI 3′-kinase (Fig. 6), even when the lower level of $pp60^{src}$ was accounted for. The *dl*92/Y527F mutant displayed little if any reduction in p60-associated PI 3′-kinase activity. Interestingly, the unmyristylated version of this mutant (G2A/*dl*92/Y527F) showed very little associated PI 3′-kinase activity; perhaps membrane association can compensate for some of the structural defects in this mutant p60.

Association of p130 and p110 with SH3 mutants of $pp60^{src}$. Because part of the PI 3′-kinase enzyme complex is approximately 110 kDa (12), we examined $pp60^{src}$ immunoprecipitates of these SH3 mutants to determine whether the presence of the 110-kDa phosphotyrosine protein studied by Parsons and associates (42, 55) correlated with $pp60^{src}$-associated PI 3′-kinase activity. We also tested for the

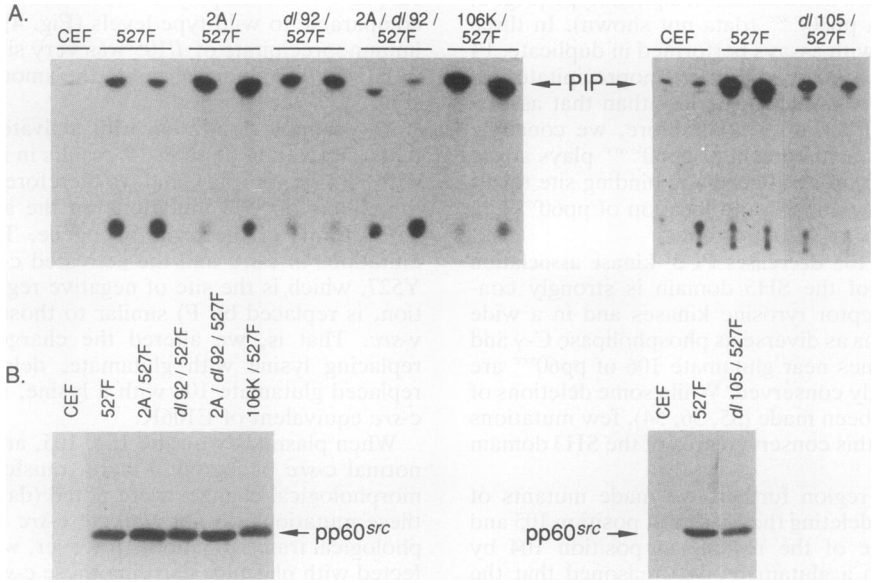

FIG. 6. PI 3′-kinase activity in immunoprecipitates of activated $pp60^{c\text{-}src}$ SH3 mutants. (A) PI 3′-kinase activity; (B) anti-$pp60^{src}$ Western blot for this experiment.

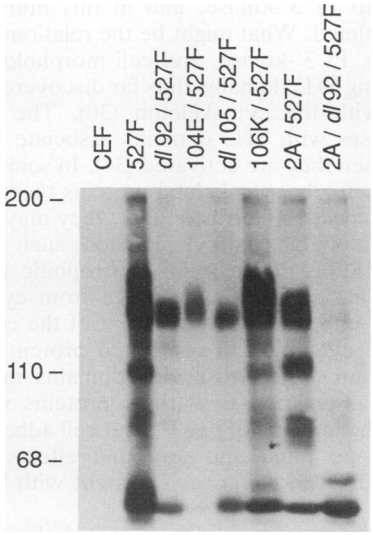

FIG. 7. Tyrosine-phosphorylated cellular proteins associated with SH3 mutants of activated pp60$^{c\text{-}src}$. pp60 was immunoprecipitated from infected cells, the immunoprecipitate was separated by SDS-PAGE and was then probed with antiphosphotyrosine antiserum. Sizes are shown in kilodaltons at the left.

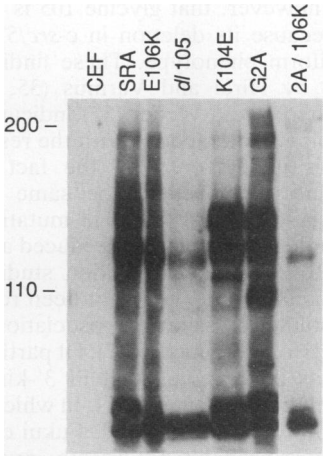

FIG. 8. Tyrosine-phosphorylated cellular proteins associated with SH3 mutants of Schmidt-Ruppin strain subgroup A (SRA) pp60$^{v\text{-}src}$. Conditions were as for Fig. 7. Sizes are shown in kilodaltons at the left.

presence of a 130-kDa tyrosine-phosphorylated protein which associates with pp60$^{v\text{-}src}$. Immunoprecipitates of pp60^{src} were separated by SDS-PAGE and then immunoblotted with antiphosphotyrosine antibodies to detect associated p110 and p130 that are phosphorylated on tyrosine.

As reported previously (42, 55), immunoprecipitates of pp60$^{c\text{-}src/527F}$ contained associated tyrosine-phosphorylated proteins of 110 and 130 kDa (Fig. 7), whereas the SH3 mutant *dl*92/Y527F associated with p130 but not p110. This pattern also was observed for the transforming K104E/Y527F and the fusiform *dl*105/Y527F. However, the transforming E106K/Y527F mutant appeared to associate with p110, albeit to a somewhat reduced degree. Because *dl*92/Y527F and K104E/Y527F have PI 3′-kinase activity associated with their immunoprecipitates to a degree comparable to that of pp60$^{c\text{-}src/527F}$ yet have little to no associated tyrosine-phosphorylated pp110, these results would imply, but not prove, that the p110 seen here is not the 110-kDa subunit of PI 3′-kinase.

Analysis of tyrosine-phosphorylated proteins which coimmunoprecipitate with the wild-type Schmidt-Ruppin strain of pp60$^{v\text{-}src}$ used by us has previously been less complete than for the Prague strain pp60$^{v\text{-}src}$ or pp60$^{c\text{-}src/527F}$. Interestingly, the pattern of tyrosine-phosphorylated p60-associated proteins differs between the two strains. We found little association of p110 with Schmidt-Ruppin pp60$^{v\text{-}src}$ (Fig. 8), even though cells infected with this strain are fully transformed and the pp60$^{v\text{-}src}$ associates with PI 3′-kinase activity. The one exception is that the unmyristylated mutant displayed association with p110. Similar results were obtained when the immunoprecipitates were blotted with antibody against p110 (42) (data not shown). However, this strain of pp60$^{v\text{-}src}$ displayed association with tyrosine-phosphorylated proteins of 130 and 145 kDa. The SH3 mutants which induced a fusiform morphology displayed reduced association with these tyrosine-phosphorylated proteins, most dramatically in the case of the *dl*105 mutant. The fact that a different pattern of coimmunoprecipitating proteins is seen with Schmidt-Ruppin strain *src* compared with Prague strain *src* and c-*src* derivatives may be a consequence of the three additional SH3 domain mutations found in the Schmidt-Ruppin strain (52).

DISCUSSION

Work presented in this report demonstrates that mutations in the SH3 domain can result in reduced amounts of active PI 3′-kinase in immunoprecipitates of pp60^{src}. These mutations also cause an altered, fusiform cell morphology. Although this connection between lowered PI 3′-kinase activity and altered morphology is correlational, these findings suggest a potential function for the SH3 domain and raise the possibility that PI 3′-kinase activity plays a role in morphological transformation by pp60^{src}.

It is important to emphasize that while these experiments show that mutations in the SH3 domain of pp60^{src} can alter the PI 3′-kinase activity associated with pp60^{src}, the experiments do not distinguish between effects on the binding of the PI 3′-kinase protein to pp60^{src} and effects on the activation of the PI 3′-kinase enzyme.

The mutation discovered to be responsible for the fusiform morphology of cells infected with CU12, glutamate 106 to lysine, is in a particularly conserved region of the SH3 domain. Despite the conserved nature of these residues, Hirai and Varmus found that alteration of nearby residue 109 had little effect on transforming ability (35, 36). Similarly, we found that changing adjacent residues could have dramatically different effects. In wild-type pp60$^{v\text{-}src}$, residue 104 can undergo a charge change with no effect on phenotype, while deletion of glycine 105 or a charge change at residue 106 causes a fusiform morphology. Mayer et al. report that a derivative of mutant PA101 which has a substitution mutation at residue 105, along with other amino-terminal changes, has a fusiform morphology (49). Our results suggest that the change at position 105 may be sufficient for the fusiform morphology caused by PA101.

Although the SH3 sequences in c-*src* and v-*src* are very similar, the 106 mutation has no obvious effect when placed in the c-*src*/527F background. This indicates that some of the other mutations in v-*src* interact with the SH3 domain. It

would appear, however, that glycine 105 is a critical structural residue because its deletion in c-*src*/527F or in v-*src* causes the fusiform phenotype. These findings along with those reported by Hirai and Varmus (35, 36) and those discussed in Parsons and Weber (52) indicate that the SH3 domain has complex interactions with the rest of the pp60^{src} protein. This is underscored by the fact that changing residues 105 and 106 leads to the same pattern for PI 3′-kinase association: all of the SH3 mutations inducing a fusiform morphology also caused a reduced association with PI 3′-kinase. PI 3′-kinase association studies on mutants made by Hirai and Varmus have not been reported.

Fukui et al. studied PI 3′-kinase association with pp60$^{v\text{-}src}$ by using a variety of mutants (24–27). Of particular relevance to our work was their finding that PI 3′-kinase associates with the SH3 deletion mutant NY311, in which residues 15 to 149 of the v-*src* protein are deleted. Fukui et al. concluded that this region was probably not directly involved in binding PI 3′-kinase. Conversely, small deletions in the SH2 domain were found to reduce PI 3′-kinase association, and such SH2 mutants induced a fusiform morphology (15). Similarly, Liu et al. (48a) have found that a *src* SH2 domain expressed as a bacterial fusion protein can bind to PI 3′-kinase but that the equivalent construct containing only an SH3 domain cannot. Thus, it seems likely that the SH2 domain is directly involved in binding to PI 3′-kinase and that the SH3 domain is neither necessary nor sufficient for such binding.

How can these reports be reconciled with our finding that a point mutation or a single amino acid deletion within the SH3 domain nonetheless reduces association with PI 3′-kinase activity? We interpret our results as pointing to an interaction between the SH3 domain and the other regions of pp60$^{v\text{-}src}$ (in particular SH2) which are involved in binding of PI 3′-kinase. Mutations in the SH3 domain which result in decreased PI 3′-kinase association could cause intramolecular interactions which block the accessibility of PI 3′-kinase to the remainder of this binding site, whereas mutations which do not decrease PI 3′-kinase association could leave the binding site accessible.

These results and the complex nature of the mutations reported by Hirai and Varmus (35, 36) and Wang and Parsons (66) suggest the SH3 domain has interactions both with other parts of the pp60^{src} protein and also with proteins that associate with pp60^{src}. Depending on the location and type of mutation, lesions in the SH3 domain appear to either activate or inhibit tyrosine kinase activity (52). Results in this report also show that different changes in the SH3 domain have diverse effects on proteins that coimmunoprecipitate with pp60^{src}. The SH3 mutants described here show varying associations with the 110- and 130-kDa phosphotyrosine proteins described by Reynolds et al. (42, 55). In an activated c-*src* background, K104E, *dl*105, and E106K all associate with p130; K104E and *dl*105 do not associate with a p110, while E106K, which is phenotypically indistinguishable from K104E, does associate with p110. Moreover K104E has PI 3′-kinase activity associated with it to the same degree as does the activated c-*src*/Y527F without additional mutations.

Cell morphology, SH3 domains and PI 3′-kinase: a speculation. Work presented in this report provides the first instance in which association of PI 3′-kinase activity with an oncogene product correlates with a specific parameter of transformation, namely, cellular morphology: all of the mutants examined by us and by Fukui et al. (24–27) which displayed reduced association with PI 3′-kinase induced a fusiform morphology. Only one fusiform mutant, NY311 (25), bound to PI 3′-kinase, and in this mutant the SH3 region was deleted. What might be the relationship between SH3 domains, PI 3′-kinase, and cell morphology? All proteins containing SH3 domains thus far discovered have some connection with the cytoskeleton (30). The nonreceptor tyrosine kinases with SH3 domains associate with the cytoskeleton when they are activated (34). In some cases, SH3 proteins are actually cytoskeletal proteins themselves, such as myosin or spectrin; in other cases, they may be enzymes that actively modulate the cytoskeleton, such as *S. cerevisiae* ABP1 (18). In the case of neutrophilic p47 and p67 oxidases, upon activation they move from cytosol to the cytoskeleton, in a manner reminiscent of the conversion of pp60$^{c\text{-}src}$ to a cytoskeleton-associated protein upon mutational activation (48). Thus, SH3 domains may serve to regulate the association of various proteins with the cytoskeleton. The location of pp60$^{v\text{-}src}$ at cell adhesion plaques (10, 56) and its rapid and profound effects on cellular morphology (1, 9, 57, 65) are consistent with this speculation.

PI 3′-kinase may be a component of this cytoskeletal signaling complex. Recently it has been shown that an intermediate in the classic PI cycle, PI-4,5-P_2, can regulate the actin-binding protein profilin (29, 30, 48). Profilin is a low-molecular-weight protein which can function as an actin-sequestering agent (23, 33, 62). Releasing profilin from actin would allow actin monomers to polymerize into filaments. Thus, a connection between PI metabolism and cytoskeletal control has been established. However, there are some problems with the idea that PI-4,5-P_2 is the agent regulating profilin. Conditions associated with morphological changes and actin polymerization can be accompanied by a decrease in PI-4,5-P_2 levels (19, 23). However, activation of PI 3′-kinase and/or increased PI-3-P metabolites have been described during agonist-induced actin polymerization and surface ruffling (3, 19). Therefore, it is quite possible that the physiological regulator of profilin is PI-3-P or one of its metabolites.

Thus, we speculate that SH3 domains serve to regulate the formation of a ternary complex between pp60$^{v\text{-}src}$, the cytoskeleton, and PI 3′-kinase and that the products of PI 3′-kinase regulate local actin polymerization by inhibiting profilin. The availability of molecular and chemical reagents to study PI 3′-kinase and its products will make it possible to test this speculation.

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