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The v-Src SH3 Domain Binds Phosphatidylinositol 3′-Kinase

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Fibroblasts transformed by v-*src* or by related oncogenes encoding activated tyrosine kinases contain elevated levels of polyphosphoinositides with phosphate at the D-3 position of the inositol ring, as a result of the activation of phosphatidylinositol (PI) 3′-kinase. v-*src*-transformed cells also contain increased levels of PI 3′-kinase activity immunoprecipitable with anti-phosphotyrosine antibodies; furthermore, PI 3′-kinase can be detected in association with the v-Src tyrosine kinase. To identify regions of v-Src that can interact with PI 3′-kinase, the v-Src SH2 and SH3 domains were expressed in bacteria and incubated with lysates of normal chicken embryo fibroblasts. In vitro, the v-Src SH3 domain, but not the SH2 domain, bound PI 3′-kinase in lysates of uninfected chicken embryo fibroblasts. Substitutions of two highly conserved SH3 residues implicated in ligand binding abolished the ability of the v-Src SH3 domain to associate with PI 3′-kinase. Furthermore, the v-Src SH3 domain bound in vitro to the amino-terminal region of the p85α subunit of PI 3′-kinase. These results suggest that the v-Src SH3 domain may mediate an interaction between the v-Src tyrosine kinase and PI 3′-kinase, by direct binding to p85.

Phosphatidylinositol (PI) 3′-kinase was first described in cells transformed by polyomavirus middle-T antigen by virtue of its association with the transforming middle-T–c-Src complex (51). The enzyme has since been detected in association with receptor tyrosine kinases, to which it binds in a ligand-dependent manner (20, 22, 37, 39, 41, 48). The transforming v-Src tyrosine kinase and other activated cytoplasmic tyrosine kinases, such as v-Abl, v-Yes, and v-Fps, also associate with PI 3′-kinase (12, 14).

PI 3′-kinase phosphorylates PI or its derivatives [PI(4)P, PI(4,5)P_2] at the D-3 position of the inositol ring (50). Elevated levels of polyphosphoinositides with D-3 phosphate [PI(3,4)P_2 and PI(3,4,5)P_3)] have been observed in fibroblasts stimulated with platelet-derived growth factor and colony-stimulating factor type 1 (1, 16) and in cells transformed by v-*src* and *src*-related oncogenes (14, 47).

PI 3′-kinase consists of a heterodimer of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (4, 31). Sequencing of cDNAs encoding two distinct p85 isoforms (11, 35, 44) revealed the presence of an SH3 domain at the extreme N terminus and two SH2 domains, one in the middle of the molecule and the other at the extreme C terminus (11, 35, 44). A region of homology to the Rho/Rac GAP domain of BCR (breakpoint cluster region) is located between the SH3 domain and the N-terminal SH2 domain of p85. The p85 SH2 domains have been implicated in the interaction of PI 3′-kinase with the middle-T antigen–c-Src complex (52), with insulin receptor substrate 1 (2), and with members of the platelet-derived growth factor receptor subfamily of tyrosine kinases (19, 25, 30, 38) through binding to the conserved, tyrosine-phosphorylated motif [pY(M/V)XM] found in these molecules (3, 21, 23, 38, 45, 51). Tyrosine phosphorylation of PI 3′-kinase provides one potential means by which PI 3′-kinase might be activated. Recent evidence indicates that binding of phosphorylated peptides containing [pY(M/V)XM] motifs to PI 3′-kinase can stimulate its enzymatic activity, suggesting an alternative mode of activation (2).

The mechanism by which v-Src associates with or activates PI 3′-kinase is less clear, since v-Src lacks a [Y(M/V)XM] motif to which p85 SH2 domains might bind. In vitro, the amino-terminal half of v-Src, which contains the SH2 and SH3 domains, can bind PI 3′-kinase in lysates of uninfected cells. Binding is enhanced when lysates of v-*src*-transformed cells are used, presumably because of tyrosine phosphorylation of PI 3′-kinase (13). In vivo, binding of PI 3′-kinase to v-Src is reduced by deletions in the v-Src SH2 domain (13). Hence it has been proposed that v-Src associates with PI 3′-kinase through binding of tyrosine-phosphorylated p85 to the Src SH2 domain (3, 13). However, amino acid substitutions in the v-Src SH3 domain also affect PI 3′-kinase association (49), indicating a role for the SH3 domain in PI 3′-kinase binding.

To address these issues, we have studied the subcellular distribution of PI 3′-kinase and its p85 subunit in chicken embryo fibroblasts (CEF) following infection with Rous sarcoma virus, which contains the v-*src* oncogene. We have also examined the ability of different domains of v-Src to associate with PI 3′-kinase in vitro. The results of these experiments suggest that the v-Src SH3 domain can mediate binding to PI 3′-kinase prior to PI 3′-kinase tyrosine phosphorylation.

MATERIALS AND METHODS

Anti-p85 antibodies. Two antisera against p85 were raised in rabbits: one directed against a bacterial TrpE fusion protein (30) containing amino acids 312 to 722 of bovine p85α (35), and the other directed against a bacterial glutathione *S*-transferase (GST) fusion protein containing amino acids 2 to 83 of bovine p85α, corresponding to the SH3 domain. Both antisera specifically recognized an 85-kDa protein in lysates of either Rat-2 fibroblasts or CEF by Western immunoblotting. Immunoprecipitates from the same cell lysates

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with either anti-p85 antiserum contained PI 3′-kinase activity (data not shown). Affinity-purified anti-p85-SH3 antibodies were used for all experiments.

Synthetic peptides. The two synthetic peptides used in these studies have the following sequences: KPRP-PRPLPVA and NERQPAPALPPKG. They correspond to amino acids 88 to 98 and 299 to 310, respectively, of bovine p85α. To facilitate peptide synthesis, a glycine residue was added carboxy-terminal to lysine 310 in the latter peptide. Both peptides were over 90% pure as judged by high-pressure liquid chromatography analyses (data not shown).

Immunoblotting. Immunoblotting was carried out as previously described (38). For reprobing with a second antibody, following exposure of an ^{125}I-protein A-labeled nitrocellulose filter to X-ray film, the filter was briefly rinsed in TBST (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20). The rinsed filter was incubated in stripping buffer (5 M NaI, 1 mM sodium thiosulfate) at room temperature for at least 60 min with one buffer change. It was then rinsed several times with TBST and probed with a different primary antibody.

Infection of chicken embryo fibroblasts. CEFs were cultured and infected with RCAN v-*src* virus (9) essentially as described previously (10). Morphologically transformed cells were harvested by being gently scraped into phosphate-buffered saline (PBS) and collected by centrifugation. Cells were generally stored as pellets at −70°C until use.

Subcellular fractionation. Cells were resuspended at 2 × 10^7 cells per ml in cold hypotonic solution (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 10 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg each of leupeptin and aprotinin per ml). The cell suspension was incubated on ice for 15 min and then subjected to homogenization in a tight-fitting Dounce homogenizer (15 strokes). The homogenate was centrifuged twice at 900 × *g* for 5 min to remove unbroken cells and nuclei. The supernatant (postnuclear supernatant) was centrifuged at 100,000 × *g* for 40 min to separate the S100 (supernatant) and P100 (pellet) fractions.

Trichloroacetic acid precipitation of whole-cell suspensions and subcellular fractions. Samples were brought to 15% trichloroacetic acid and 1% Triton X-100. The mixtures were incubated on ice for 30 min. Precipitates were collected by centrifugation at 12,000 × *g* for 15 min and washed four times with cold acetone (stored at −20°C). The washed pellets were dried under vacuum and dissolved in sodium dodecyl sulfate (SDS) sample buffer.

Immunoprecipitation and PI kinase assays. Whole cells or subcellular fractions were lysed in PLC lysis buffer and immunoprecipitation was performed as described previously (38). Immunoprecipitates formed by using anti-p85 antibodies and complexes formed with immobilized GST or GST fusion proteins were assayed for associated PI kinase activity by the method of Fukui and Hanafusa (12) as described previously (37, 38).

In vitro transcription and translation of various forms of p85. Two restriction enzymes were used to digest pBSp85α, a p85α expression plasmid, for in vitro transcription and translation. Partial digestion with *Xho*I generates two linearized cDNAs encoding the full-length p85α and a truncated polypeptide ($p85_{1-338}$) of p85α. Digestion to completion with *Xho*I and *Sca*I generates cDNAs encoding the two truncated polypeptides ($p85_{1-338}$) and ($p85_{1-235}$), of p85α, respectively (see Fig. 4 for more detail). In vitro transcription with the linearized DNA as template was carried out as specified by the manufacturer. Usually, 2 μg of the resulting mRNA was used in a subsequent in vitro translation experiment in rabbit reticulocyte lysates supplemented with [^{35}S]methionine (total reaction, 50 μl).

Generation of v-Src SH3 mutants. Construction of cDNAs encoding bacterial GST fusion proteins containing wild-type v-Src SH2 (residues 148 to 251) and SH3 (residues 77 to 147) domains has been described elsewhere (28). Point mutations within the v-Src SH3 domain were generated by a two-step polymerase chain reaction with primers containing the mutations by the method of Vallette et al. (46) as described previously (29). The amplified cDNAs were subcloned into the bacterial expression vector pGEX-KT (15) as described previously (28).

In vitro binding experiments involving immobilized SH2 and SH3 domains. Bacterial cell lysates containing induced GST fusion proteins were incubated with glutathione-agarose beads. The beads were washed with PBS, and the amounts of bound proteins were determined by subjecting a small aliquot of beads to Bradford protein assays (Bio-Rad). Equal amounts (5 μg per sample) of each immobilized protein were incubated with cell lysates prepared in PLC lysis buffer (38) or with in vitro translation mixture diluted with PLC lysis buffer containing 1 mg of bovine serum albumin per ml. The complexes were washed three or four times with PLC lysis buffer. Bound proteins were either subjected to PI kinase assays or to SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography.

RESULTS

Subcellular distribution of PI 3′-kinase. Previous studies of the subcellular localization of PI 3′-kinase in v-*src*-transformed CEF have relied on either anti-phosphotyrosine antibodies or anti-v-Src antibodies. Anti-phosphotyrosine antibodies detect the tyrosine-phosphorylated form of PI 3′-kinase, and antibodies against v-Src immunoprecipitate PI 3′-kinase that associates with v-Src in these cells. By using the antibodies against recombinant p85 proteins (see Materials and Methods), we examined the subcellular distribution of total PI 3′-kinase in v-*src*-transformed CEF (CEF/v-*src*). Cells were hypotonically lysed and separated into S100 and P100 fractions. Immunoprecipitates were prepared from these subcellular fractions and assayed in vitro for PI kinase activity. Figure 1A shows that although a small amount of PI 3′-kinase was associated with the P100 fraction, most (~90%) was recovered from S100 fraction. The PI 3′-kinase p85 subunit had a very similar subcellular distribution. In contrast, v-Src was recovered exclusively from the particulate P100 fraction. The subcellular distribution of PI 3′-kinase and p85 in parental CEF was very similar to that in CEF/v-*src* (data not shown).

Tyrosine-phosphorylated p85 does not cofractionate with v-Src or the plasma membrane. Tyrosine phosphorylation of the p85 subunit of PI 3′-kinase has been reported to take place in v-*src*-transformed mouse fibroblasts (7). We have confirmed this observation in v-*src*-transformed CEF (Fig. 1B, lane 1), although only a relatively small percentage (less than 5%) of cellular p85 is phosphorylated on tyrosine as judged by quantitative immunoprecipitation with antibodies against phosphotyrosine (data not shown). In addition to p85, we detected two other phosphotyrosine-containing proteins (of 110 and 190 to 200 kDa) in anti-p85 immunoprecipitates from cell lysates of CEF/v-*src*. The 110-kDa protein may be the p110 catalytic subunit of PI 3′-kinase. The identity of the 190- to 200-kDa protein is unknown; it is not recognized by an antibody against the $p21^{ras}$ GTPase-acti-

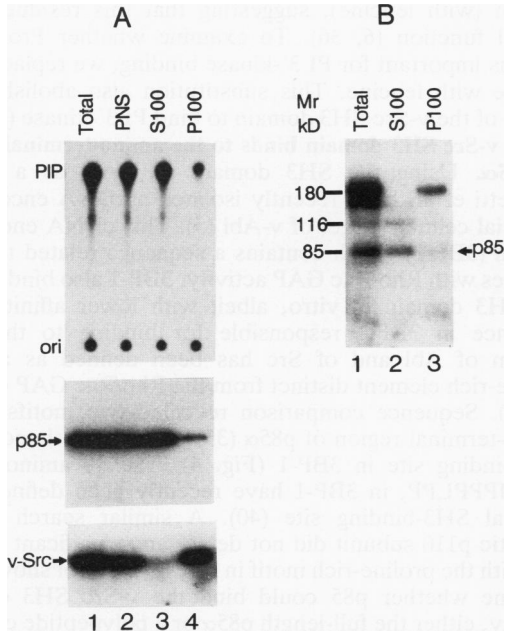

FIG. 1. Subcellular distribution of PI 3′-kinase in v-*src*-transformed CEF. (A) v-*src*-transformed CEF were lysed and fractionated as described in Materials and Methods. The initial lysate (total) (lane 1), the postnuclear supernatant (PNS) (lane 2), and the S100 (lane 3) and P100 (lane 4) fractions were immunoprecipitated with anti-p85 antibodies and subjected to PI kinase assays with one-third of each fraction (top panel of Fig. 1A). The remaining two-thirds of each fraction was precipitated with trichloroacetic acid, separated by SDS-PAGE, and immunoblotted with affinity-purified anti-p85 antibodies and ^{125}I-protein A (middle panel of Fig. 1A). The blot was then incubated in 5 M NaI–1 mM sodium thiosulfate to strip the anti-p85 antibodies and was reprobed with anti-v-Src antibody (Ab-1; Oncogene Science). A second antibody (rabbit anti-mouse immunoglobulin G) and ^{125}I-protein A were used for visualization (bottom panel of Fig. 1A). Abbreviations: ori, loading origin; PIP, phosphatidylinositol 3′-monophosphate. (B) Total lysate (lane 1) or S100 (lane 2) and P100 (lane 3) fractions of CEF/v-*src* were prepared and immunoprecipitated with affinity-purified anti-p85 antibodies. The anti-p85 immunoprecipitates were separated by SDS-PAGE and immunoblotted with affinity-purified polyclonal anti-phosphotyrosine antibodies followed by ^{125}I-protein A. Tyrosine-phosphorylated p85 is indicated. The mobilities of size markers are indicated.

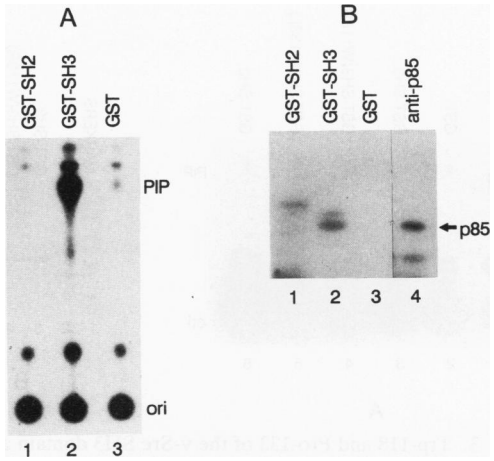

FIG. 2. v-Src SH3 domain binds p85 and PI 3′-kinase in vitro. GST fusion proteins containing the v-Src SH2 domain (lane 1), the v-Src SH3 domain (lane 2), or parental GST (lane 3) were immobilized on glutathione-agarose beads (5 μg of GST fusion protein per lane) and incubated with a lysate of 10^7 CEF. (A) One-third of the resulting complexes were then assayed for PI kinase activity. Designations are as in Fig. 1A. (B) The remaining two-thirds of the beads were boiled in SDS sample buffer, and the eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with affinity-purified anti-p85 antibodies. Antibody binding was detected with ^{125}I-protein A. An anti-p85 immunoprecipitate from CEF was included as a control (lane 4).

vating protein (GAP)-associated p190 protein (43) (data not shown). The band below the 180-kDa marker in lane 1, although specific for anti-p85 immunoprecipitates, was not consistently observed and may represent a breakdown product of the 190- to 200-kDa phosphotyrosine-containing protein.

Although the majority of PI 3′-kinase and of p85 was localized in the S100 fraction, we investigated the possibility that the tyrosine-phosphorylated form of p85 or of PI 3′-kinase is associated with the plasma membrane (P100). In fact, the majority of phosphotyrosine-containing p85 was found in the S100 fraction (Fig. 1B). Furthermore, anti-v-Src immunoprecipitates from the P100 fraction, although containing appreciable amounts of PI 3′-kinase activity and of p85, did not contain detectable amount of tyrosine-phosphorylated p85 (data not shown). Interestingly, the p85-associated 110-kDa and 190- to 200-kDa phosphotyrosine-containing proteins fractionated differently. The 110-kDa phosphoprotein was recovered primarily with phosphotyrosine-containing p85 in the S100 fraction. In contrast, the 190- to 200-kDa phosphoprotein was recovered exclusively from the P100 fraction, which contains p85 essentially free of phosphotyrosine.

The v-Src SH3 domain binds PI 3′-kinase from normal cells. Since tyrosine-phosphorylated p85 fractionates differently from v-Src, which is associated with the plasma membrane, we considered the possibility that the physical association between v-Src and PI 3′-kinase occurs prior to, or independently of, tyrosine phosphorylation of PI 3′-kinase.

To examine the element(s) in v-Src which might mediate its initial association with PI 3′-kinase, we tested the ability of the Src SH3-SH2 region to complex with PI 3′-kinase in lysates of uninfected CEF. A GST fusion protein containing the v-Src SH3-SH2 region proved capable of binding PI 3′-kinase when incubated with lysates of CEF (data not shown). To dissect the region of v-Src responsible for this interaction, we used GST fusion proteins containing either the SH3 domain or the SH2 domain of v-Src. Figure 2 shows that the GST-SH3 fusion protein was able to bind PI 3′-kinase in lysates of uninfected CEF, measured by PI kinase activity (Fig. 2A) or by immunoblotting of the associated proteins with anti-p85 antibodies (Fig. 2B). Neither the parental GST polypeptide nor a GST fusion protein containing the v-Src SH2 domain bound detectable levels of PI 3′-kinase or of p85 in cell lysates of normal CEF.

To investigate the specificity of this interaction, we introduced mutations into the coding region of the v-Src SH3 domain to form amino acid substitutions that are predicted to modify its ligand-binding activity. Recent resolution of the crystal structures of the SH3 domains of spectrin and c-Src has indicated that several hydrophobic residues, which are well conserved among different SH3 domains (26, 33), form a hydrophobic surface which probably binds SH3 ligands (32, 53). On the basis of these observations, Trp-118 was

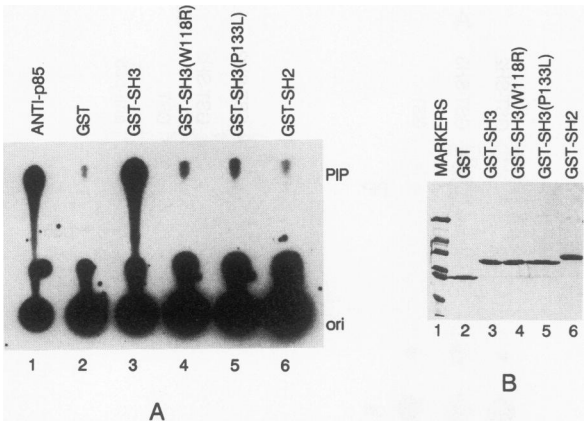

FIG. 3. Trp-118 and Pro-133 of the v-Src SH3 domain are essential for PI 3′-kinase binding. (A) Parental GST (lane 2) or GST fusion proteins containing the v-Src SH3 domain (lane 3), the W118R and P133L mutant v-Src SH3 domain (lanes 4 and 5, respectively), or the v-Src SH2 domain (lane 6) were immobilized on glutathione-agarose beads (5 μg of GST or GST fusion protein per lane) and incubated with a lysate from approximately 5×10^6 CEF. An anti-p85 immunoprecipitate from 2×10^6 CEF was included as a control (lane 1). The complexes were subjected to in vitro PI kinase assays. Designations are as in Fig. 1A. (B) Glutathione-agarose-immobilized parental GST (lane 2) or GST fusion proteins (lanes 3 to 6) (2 μg of GST or GST fusion protein per lane) was eluted with SDS sample buffer and analyzed by SDS-PAGE and Coomassie blue staining. Lane 1 contains protein molecular mass markers (from top: 67, 45, 36, 29, 24, and 20 kDa).

mutated to arginine, since this residue is one of the most highly conserved SH3 residues (33) and appears to participate in ligand binding (53). Figure 3 shows that the mutant v-Src SH3 domain [SH3(W118R)] was completely defective in binding to PI 3′-kinase from CEF. Another highly conserved SH3 residue is Pro-133. A severe defect in *Caenorhabditis elegans* vulval induction and sex myoblast migration is attributed to a substitution of the homologous proline in the amino-terminal SH3 domain of the Sem-5 protein (with leucine), suggesting that this residue has a critical function (6, 36). To examine whether Pro-133 of v-Src is important for PI 3′-kinase binding, we replaced this residue with leucine. This substitution also abolished the ability of the v-Src SH3 domain to bind PI 3′-kinase (Fig. 3).

The v-Src SH3 domain binds to the amino-terminal region of p85α. Using the SH3 domain of v-Abl as a probe, Cicchetti et al. have recently isolated a cDNA encoding a potential cellular target of v-Abl (5). This cDNA encodes a protein (3BP-1) which contains a sequence related to polypeptides with Rho/Rac GAP activity. 3BP-1 also binds to the Src SH3 domain in vitro, albeit with lower affinity. The sequence in 3BP-1 responsible for binding to the SH3 domain of Abl and of Src has been defined as a short proline-rich element distinct from the Rho/Rac GAP domain (5, 40). Sequence comparison revealed two motifs in the amino-terminal region of p85α (35) which are related to the SH3-binding site in 3BP-1 (Fig. 4). The 10 amino acids, APTMPPPLPP, in 3BP-1 have recently been defined as a minimal SH3-binding site (40). A similar search of the catalytic p110 subunit did not detect any significant homology with the proline-rich motif in 3BP-1 (data not shown). To examine whether p85 could bind the v-Src SH3 domain directly, either the full-length p85α or a polypeptide containing amino-terminal residues 1 to 338 ($p85_{1-338}$), including the potential SH3-binding sites described above, were expressed in vitro by transcription and translation in a rabbit reticulocyte lysate. Figure 5A shows that full-length p85 and $p85_{1-338}$, both of which contain the two potential SH3-binding motifs, were able to bind the v-Src SH3 domain in vitro. In contrast, another polypeptide ($p85_{1-235}$) containing p85α amino-terminal residues 1 to 235, which has only the more amino-terminal 3BP-1 homology, had a markedly reduced ability to bind the v-Src SH3 domain (Fig. 5B).

As expected, substitution of either Trp-118 (with arginine) or Pro-133 (with leucine) in the Src SH3 domain abrogated binding to both full-length p85 and the amino-terminal fragment of p85 ($p85_{1-338}$) translated in vitro (Fig. 6).

To further examine whether the two proline-rich motifs in p85α may be directly involved in binding to the v-Src SH3 domain, two synthetic peptides corresponding to these p85α

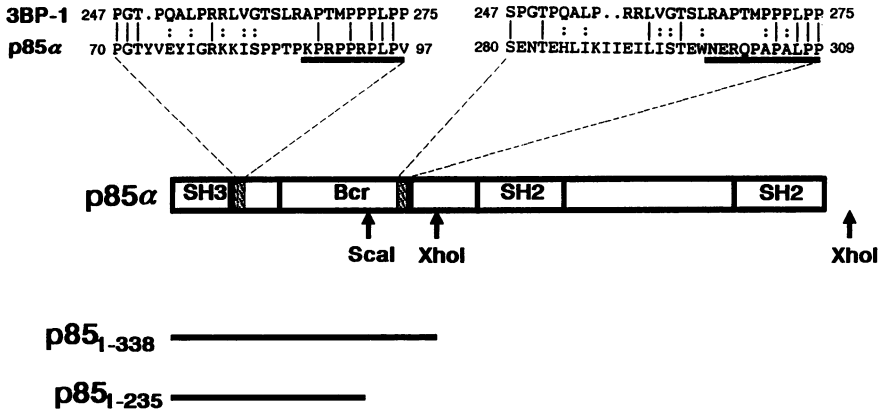

FIG. 4. Potential Src SH3-binding site in p85α. Sequence comparison between the SH3-binding site identified in 3BP-1 (amino acids 247 to 275) and two potential Src SH3-binding motifs in p85α (amino acids 70 to 97 and 280 to 309) is shown. Gaps (indicated as dots) were introduced into the 3BP-1 sequence to optimize the alignment. Sequence identity and similarity are indicated by a vertical line and two dots, respectively. The two underlined amino acid sequences of p85α were included in the two synthetic peptides used in competition experiments (see Fig. 7) (see Materials and Methods for more details). Other domains in the two molecules are indicated. Bcr is a sequence related to the BCR Rho/Rac GAP domain. Three restriction sites that were used to generate p85α cDNAs that give either full-length or two truncated forms of p85α in an in vitro transcription/translation system are indicated. The truncated forms of p85α are shown relative to intact p85.

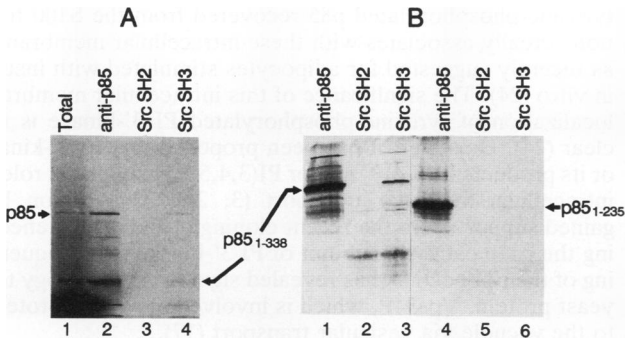

FIG. 5. The v-Src SH3 domain binds the N-terminal region of p85. (A) An aliquot of p85α plasmid was partially digested with *Xho*I, transcribed, and translated in a rabbit reticulocyte lysate, resulting in both full-length p85 and $p85_{1-338}$. Samples (2 μl) of the total translation products are shown in lane 1. Then 20 μl (lane 2) or 50 μl (lanes 3 and 4) of reticulocyte lysates containing the translation products was incubated with either 1 μg of affinity-purified anti-p85 antibody (lane 2) or 5 μg of immobilized GST-Src SH2 (lane 3) or GST-Src SH3 (lane 4). (B) The p85 cDNA was digested with *Xho*I (lanes 1 to 3) or *Sca*I (lanes 4 to 6) to completion. The resultant linearized cDNAs were transcribed and translated in vitro, yielding $p85_{1-338}$ (lanes 1 to 3) or $p85_{1-235}$ (lanes 4 to 6). Translation products were incubated with anti-p85 antibodies or GST fusion proteins, as in panel A. Immunoprecipitated or bound proteins were separated by SDS-PAGE followed by fluorography. The mobilities of the full-length (p85) and the two truncated ($p85_{1-338}$ and $p85_{1-235}$) polypeptides of p85α are indicated.

sequences were examined for their ability to compete for binding of PI 3′-kinase to v-Src SH3. Figure 7 shows that both peptides were able to inhibit binding of the v-Src SH3 domain to isolated p85α (Fig. 7A) or intact PI 3′-kinase (Fig. 7B). Half-maximal inhibition for SH3-p85 binding occurred at 250 and 100 μM for the two peptides, respectively (Fig. 7A). Interestingly, higher concentrations of the peptides were required to inhibit binding of the v-Src SH3 domain to PI 3′-kinase in lysates of CEF.

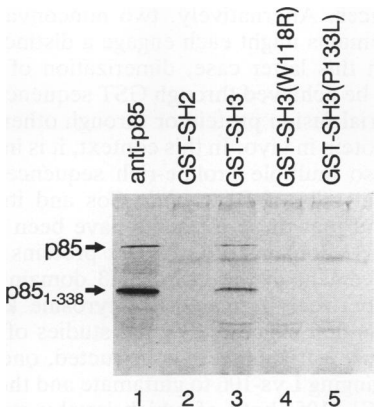

FIG. 6. Amino acid substitutions in the Src SH3 domain abolish binding to p85α. GST fusion proteins containing the v-Src SH2 domain, the v-Src SH3 domain, or the W118R and P133L mutant SH3 domains (lanes 2 to 5) (5 μg per lane) was immobilized on glutathione-agarose beads and incubated with 50 μl of in vitro translation product prepared as for Fig. 5A. Bound proteins were analyzed by SDS-PAGE and fluorography. Lane 1 is an anti-p85 immunoprecipitate from 20 μl of the same translation products. Designations are as in Fig. 5.

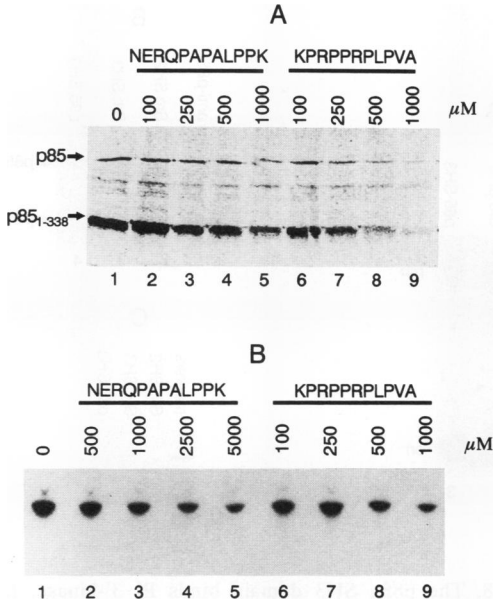

FIG. 7. Two synthetic peptides derived from p85α inhibit binding of the v-Src SH3 domain to p85α and PI 3′-kinase. (A) GST–v-Src SH3 fusion protein (5 μg) immobilized on glutathione-agarose beads was incubated with 50 μl of p85α in vitro translation products, prepared as described in the legend to Fig. 5A. Increasing concentrations (as indicated) of either one of the two synthetic peptides NERQPAPALPPK (lanes 2 to 5) and KPRPPRPLPVA (lanes 6 to 9) (see Fig. 4 and Materials and Methods for more detail) were included at the beginning of the incubation. Bound proteins were analyzed by SDS-PAGE. The figure is an image produced by scanning of the dried gel in a Molecular Dynamics PhosphorImager. The mobilities of both full-length (p85) and truncated ($p85_{1-338}$) p85α polypeptides are indicated. (B) GST-v-Src SH3 fusion protein (5 μg) immobilized on glutathione-agarose beads was incubated with a lysate from 3×10^6 CEF. Increasing concentrations (as indicated) of either one of the two synthetic peptides were included at the beginning of the incubation. Complexes were assayed for PI 3′-kinase activity.

p85α SH3 binds PI 3′-kinase. The p85α subunit of PI 3′-kinase contains an amino-terminal SH3 domain, whose function is not known. We tested the possibility that it also binds PI 3′-kinase. Figure 8A (lane 3) shows that the p85α SH3 domain indeed binds PI 3′-kinase, although with a much reduced efficiency compared with that of the v-Src SH3 domain. Similarly, the p85α SH3 domain also binds to the amino-terminal region of p85α (Fig. 8B), again with a similarly reduced efficiency in comparison with the binding of the v-Src SH3 domain.

DISCUSSION

The concentrations of the phospholipid products of PI 3′-kinase are elevated in fibroblasts transformed by v-*src* (14) and other oncogenes encoding cytoplasmic tyrosine kinases (14, 47). Fibroblasts expressing nonmyristylated variants of v-Src, which, unlike their wild-type counterpart, do not associate with the plasma membrane, fail to accumulate these phospholipids (14). The mechanism by which v-Src activates PI 3′-kinase and induces production of these phospholipids is unclear but is likely to involve a physical association between v-Src and PI 3′-kinase. Previous work has suggested that physical association of v-Src with PI 3′-kinase is mediated by the noncatalytic amino-terminal

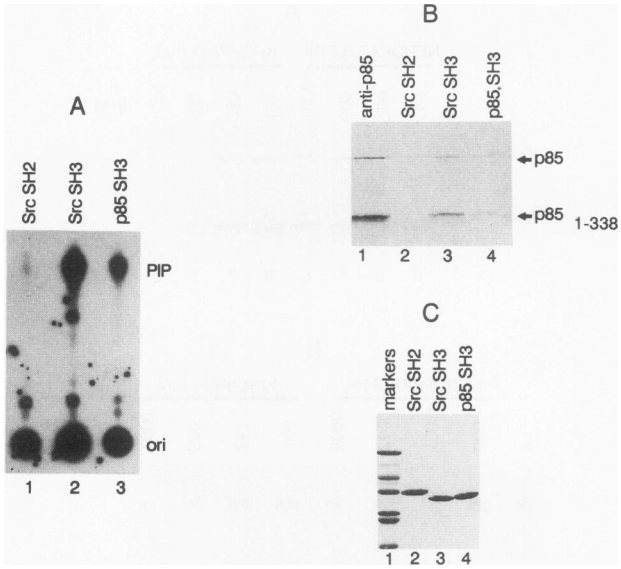

FIG. 8. The p85α SH3 domain binds PI 3′-kinase. (A) GST fusion proteins (5 μg) immobilized on glutathione-agarose beads were incubated with a cell lysate from 5×10^6 CEF. The complexes were washed and assayed for PI kinase activity. Designations are as in Fig. 1A. (B) GST fusion proteins (5 μg) immobilized on glutathione agarose beads (lanes 2 to 4) were incubated with 50 μl of in vitro translation reaction (prepared as described in the legend to Fig. 5A). The complexes were analyzed by SDS-PAGE and fluorography. Lane 1 is an anti-p85 immunoprecipitate from 30 μl of in vitro translation mixture. Designations are as in Fig. 5. (C) GST fusion proteins (2 μg) immobilized on glutathione-agarose beads (lanes 2 to 4) were eluted in SDS sample buffer and analyzed by SDS-PAGE followed by Coomassie staining. The molecular weight markers are as described in the legend to Fig. 3B.

region (13). Furthermore, mutations in both the v-Src SH2 and SH3 domains have been reported as affecting PI 3′-kinase association (13, 49). We have therefore investigated the ability of these distinct noncatalytic regions of v-Src to bind PI 3′-kinase and have assessed the role of tyrosine phosphorylation of PI 3′-kinase in its association with v-Src.

Tyrosine-phosphorylated p85 is not stably bound to v-Src. Tyrosine phosphorylation of an 85-kDa protein has been previously observed in v-*src*-transformed cells, and this has been attributed to phosphorylation of the p85 subunit of PI 3′-kinase (7, 8). Using antibodies raised against recombinant p85α, we have confirmed this prediction. However, the majority of the tyrosine-phosphorylated form of p85 is detected in the S100 fraction of v-*src*-transformed cells and is therefore physically separable from v-Src itself, which is recovered exclusively from the P100 fraction. This suggests that the association of v-Src and PI 3′-kinase does not necessarily require the tyrosine phosphorylation of p85. These results are consistent with an early study by Fukui and Hanafusa, who demonstrated that temperature-sensitive v-Src mutants remained capable of binding PI 3′-kinase at nonpermissive temperatures. Under these conditions, the mutant v-Src proteins exhibited very limited tyrosine kinase activity and phosphorylation of cellular proteins was restricted (12).

The subcellular fractionation procedure used in these studies is crude and does not separate some intracellular membranes, noticeably the low-density microsomes, from the cytoplasm fraction. Therefore, it is possible that the tyrosine-phosphorylated p85 recovered from the S100 fraction actually associates with these intracellular membranes, as recently suggested for adipocytes stimulated with insulin in vitro (24). The significance of this intracellular membrane localization of tyrosine-phosphorylated PI 3′-kinase is not clear (24). However, it has been proposed that PI 3′-kinase or its products PI(3,4)P_2 and/or PI(3,4,5)P_3 may play a role in intracellular vesicular transport (3, 24). This notion has gained support from the recent cloning of the cDNA encoding the p110 catalytic subunit of PI 3′-kinase (18). Sequencing of the p110 cDNA has revealed significant homology to a yeast protein, Vps34P, which is involved in sorting proteins to the vacuole via vesicular transport (17).

The v-Src SH3 domain associates with p85 and PI 3′-kinase. The observation that tyrosine-phosphorylated p85 is physically separable from v-Src in v-*src*-transformed CEF suggests that v-Src might interact with PI 3′-kinase lacking phosphotyrosine. To investigate the domain in v-Src that might mediate this proposed interaction, we performed in vitro binding experiments with bacterial fusion proteins containing different regions of v-Src. The results of these experiments showed that the v-Src SH3 domain could bind stably to PI 3′-kinase in CEF which contains no detectable phosphotyrosine. The specificity of this interaction is supported by the following observations. First, neither the parental GST protein nor a GST fusion protein containing the Src SH2 domain bound detectable amounts of PI 3′-kinase under the same conditions. Second, either one of two substitutions affecting highly conserved SH3 residues implicated in ligand binding abolished the ability of the v-Src SH3 domain to bind PI 3′-kinase. Third, the v-Src SH3 domain bound directly to a region of p85 that contains sequence motifs similar to the SH3-binding motif defined in 3BP-1 (5). In addition, two synthetic peptides corresponding to these proline-rich p85α motifs inhibited binding.

The presence of two proline-rich motifs in p85α potentially involved in binding to the v-Src SH3 domain (Fig. 7), together with the observation that deleting a region containing one of these motifs diminishes but does not eliminate SH3 binding, suggests that the v-Src SH3 domain may recognize both proline-rich elements on p85α. It is possible that one SH3 domain binds a single bipartite site containing both sequences. Alternatively, two nonconvalently-associated SH3 domains might each engage a distinct proline-rich sequence; in this latter case, dimerization of the SH3 domains might be achieved through GST sequences in the case of the bacterial fusion protein or through other components of the Src protein in vivo. In this context, it is interesting that there are also multiple proline-rich sequences in the carboxy-terminal tails of *Drosophila* Sos and its mammalian homologs and that these elements have been implicated in binding to SH3 domains of Grb2/Drk proteins (27, 34, 42).

The involvement of the v-Src SH3 domain in mediating association between activated Src tyrosine kinase and PI 3′-kinase was first suggested by the studies of Wages et al. (49). Two v-*src* variants were constructed, one with a point mutation changing Lys-106 to glutamate and the other with a deletion of Gly-105, both of which involve well-conserved SH3 residues. In contrast to CEF infected with the wild-type v-*src*, which are rounded and refractile, CEF infected with the mutant viruses were extremely elongated and fusiform. Correlating with this altered transforming ability, the mutant v-Src proteins had decreased levels of associated PI 3′-kinase activity compared with wild-type v-Src (49). Taken together, these results suggest that PI 3′-kinase is a physiological ligand for the v-Src SH3 domain.

What is the significance of the interaction between the v-Src SH3 domain and PI 3'-kinase? One interpretation of these results is that the v-Src SH3 domain mediates an initial interaction of PI 3'-kinase with v-Src. This interaction may result in tyrosine phosphorylation of PI 3'-kinase and subsequent dissociation of tyrosine-phosphorylated PI 3'-kinase from v-Src. Alternatively, the proposed SH3-mediated PI 3'-kinase binding to v-Src may have a function unrelated to tyrosine phosphorylation of PI 3'-kinase. Such an interaction could result in activation of PI 3'-kinase in vivo or translocation of PI 3'-kinase to the membrane by virtue of its association with v-Src.

The p85α SH3 domain also binds PI 3'-kinase and the amino-terminal region of p85α in vitro, although less efficiently than the v-Src SH3 domain does. This raises the possibility that the p85 SH3 domain participates in an intramolecular interaction with the SH3-binding site described above, which might regulate PI 3'-kinase activity or the function of the BCR-related domain of p85. Under these circumstances, binding of v-Src SH3 domain to the amino-terminal region of p85 might disrupt the proposed intramolecular interaction. This might affect PI 3'-kinase activity in vivo and/or its intracellular localization.

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