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The SH3 Domain of p56^{lck} Is Involved in Binding to Phosphatidylinositol 3′-Kinase from T Lymphocytes

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Many of the Src-like tyrosine kinases are thought to participate in multiprotein complexes that modulate transmembrane signalling through tyrosine phosphorylation. We have used in vitro binding studies employing bacterially expressed glutathione *S*-transferase–p56^{lck} fusion proteins and cell extracts to map regions on p56^{lck} that are involved in binding to phosphatidylinositol 3′-kinase (PI3K). Deletions within the SH3 domain of p56^{lck} abolished binding of PI3K activity from T-cell lysates, whereas deletion of the SH2 domain caused only a slight reduction in the level of PI3K activity bound to p56^{lck} sequences. The binding of PI3K from T-cell extracts to p56^{lck} was not blocked by antiphosphotyrosine antibodies, but p56^{lck}-bound PI3K activity was sensitive to phosphatase treatment. The SH3 domain of p56^{lck} also bound the majority of PI3K activity from uninfected chicken embryo fibroblasts. However, a drastically different binding specificity was observed with use of extracts of Rous sarcoma virus v-*src*-transformed cells, in which the majority of PI3K activity bound to the SH2 domain of p56^{lck} in a phosphotyrosine-dependent manner. These results suggest that are two modes of PI3K binding to p56^{lck}, and presumably to other Src-like tyrosine kinases. In one mode, PI3K from T cells or uninfected chicken embryo fibroblasts binds predominantly to the SH3 domain of p56^{lck}. In the other mode, involving PI3K from Rous sarcoma virus-transformed cells, binding is largely phosphotyrosine dependent and requires the SH2 domain of p56^{lck}.

The *lck* gene is a member of the *src* family of genes, which encode a series of eight closely related protein tyrosine kinases (for a review, see reference 23). The product of the *lck* gene is a 56-kDa protein (p56^{lck}) that is expressed predominantly in cells of the lymphoid lineage, primarily T lymphocytes and lymphoid tumor cell lines (28, 30, 37). Expression of p56^{lck} has also been observed in human carcinomas of the lung and colon (50).

Members of the Src family of tyrosine kinases are characterized by an NH_2-terminal unique region followed by three regions that contain different degrees of homology among all members of the family (23, 42). In p56^{lck}, Src homology region 3 (SH3) extends from amino acids 56 to 114. The SH2 domain includes amino acids 115 to 221 and the SH1, or catalytic, domain comprises most of the remainder of the C-terminal half of the protein. Recent evidence indicates that the SH2 region of Src-like kinases functions as a protein association domain that is important in the formation of multienzyme complexes that regulate signal transduction through tyrosine phosphorylation (5, 27).

Several proteins have been reported to form complexes with p56^{lck} in T lymphocytes. The NH_2-terminal unique region of p56^{lck} has been shown to associate with the C-terminal region of two T-cell transmembrane glycoproteins, CD4 and CD8 (3, 39, 40, 45, 48). The accessory role of CD4 and CD8 in T-cell signalling has led to the suggestion that p56^{lck} may participate in antigen-stimulated signal transduction events (18, 36). Other molecules observed to directly associate with p56^{lck} include the beta chain of the interleukin-2 (IL-2) receptor (20), phospholipase C-γ1 (PLC-γ1) (54), a 32-kDa protein with GTPase activity (43), and a 70-kDa protein similar to GTPase-activating protein-associated p62 (52). The beta chain of the IL-2 receptor, PLC-γ1, the 32 kDa GTPase protein, and p56^{lck}-associated p70 all become phosphorylated on tyrosine as a result of T-cell activation (20, 43, 52, 54). However, it has not been directly demonstrated that any of these proteins are in vivo substrates of p56^{lck}.

It has been suggested that the critical targets of tyrosine kinase oncogenes are likely to be enzymes that cause an amplification of the signal transduction cascade (5). Of the proteins found in association with p56^{lck} to date, both PLC-γ1 and the 32-kDa GTPase protein fit this description. The phosphatidylinositol (PI) pathway, in particular, provides a good example of growth factor-activated second-messenger amplification (reviewed in reference 57). Until recently, PI was thought to be phosphorylated only at positions D-4 and D-5 of the inositol ring. More recently, a novel PI kinase that phosphorylates inositol at the D-3 position has been identified (57). This enzyme, known as PI 3-kinase (PI3K), has subsequently been found to associate with several tyrosine kinase oncogenes and proto-oncogenes (2, 4, 7, 9, 14). Furthermore, a strong correlation between the mitogenic response and associated PI3K activity in receptor-type tyrosine kinases has been reported (9, 12, 13, 46). Similarly, PI3K association with transforming or activated forms of pp60$^{v\text{-}src}$ or pp60$^{c\text{-}src}$ and with polyomavirus middle-T antigen–c-Src complexes has suggested a possible correlation with cell transformation ability (4, 7, 10, 24, 56). The correlation between PI3K and receptor tyrosine kinases, as well as the link between PI3K and the transformation potential of pp60^{src}, is suggestive of a novel signal transduction pathway involving PI 3-phosphate (PI-3-P).

The molecular mechanisms responsible for the interaction between PI3K and tyrosine kinases have been most thoroughly characterized for the platelet-derived growth factor receptor (PDGFR). Active PI3K is a heterodimer composed of an 85-kDa regulatory subunit and a 110-kDa catalytic

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subunit (6, 21). Association between PI3K and the PDGFR is mediated by SH2 domains within the 85-kDa subunit of PI3K (22, 26, 31) and is dependent upon autophosphorylation of tyrosine residues within the kinase insert domain of the PDGFR (11, 22, 25, 31). Considerably less is known about the interaction between PI3K and nonreceptor tyrosine kinases. Nonreceptor tyrosine kinases do not contain sequences that share strong homology with the kinase insert domain of the PDGFR, nor do they require autophosphorylation for association with PI3K (15). Consequently, it appears that interactions between PI3K and nonreceptor tyrosine kinases differ considerably from those involving PDGF receptor-type kinases. However, it has been observed that $p60^{v\text{-}src}$-associated PI3K activity is reduced if the SH2 domain of $p60^{v\text{-}src}$ is deleted (15), indicating that the SH2 domain of Src family kinases is involved in this interaction. Another indication for the involvement of the SH2 domain of nonreceptor tyrosine kinases in PI3K interaction comes from the observation that two amino acid substitutions in the SH2 domain of $p60^{c\text{-}src}$ result in increased PI3K association as well as increased transformation potential (34). In addition, depletion of $p60^{v\text{-}src}$-associated PI3K activity following immunoprecipitation of lysates with antiphosphotyrosine antibodies suggests that PI3K requires tyrosine phosphorylation for activity and/or association with $p60^{v\text{-}src}$ (15). Interestingly, a point mutation in the SH3 domain of $p60^{v\text{-}src}$ also has been found to result in reduced association of PI3K activity (53), suggesting a role for the SH3 domain in the interaction of PI3K with Src-like nonreceptor tyrosine kinases.

In this report, we have utilized bacterially expressed glutathione *S*-transferase (GST)–$p56^{lck}$ fusion proteins to examine the binding interactions between PI3K and $p56^{lck}$. We have demonstrated that PI3K in lysates prepared from leukemic T lymphocytes or stimulated normal peripheral blood lymphocytes (PBLs) associated with $p56^{lck}$ in a manner that was primarily dependent on the SH3 region of $p56^{lck}$ and partially dependent on the SH2 region. In addition, this association of active PI3K from lymphocytes was dependent on PI3K phosphorylation. Similar results were obtained for PI3K from extracts of normal chicken embryo fibroblasts (CEFs). However, binding of PI3K isolated from Rous sarcoma virus (RSV)-transformed CEFs primarily involved the SH2 region of $p56^{lck}$ and was dependent on tyrosine phosphorylation of PI3K.

(A preliminary report of these findings was presented at the 1992 Meeting on Oncogenes, Frederick, Md.)

MATERIALS AND METHODS

Cell culture, T-cell activation, and immunoprecipitation. All human leukemic T-cell lines (Molt-4, Tib-195, Hut-78, Jurkat, and CCRM-CEF) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO). CEFs and RSV Schmitt Ruppin A (SRA) v-*src*-transformed CEFs were cultured in Dulbecco's modified Eagle medium supplemented with 5% calf serum and 10% tryptose phosphate broth. PBLs were isolated from buffy-layer blood samples obtained from the Canadian Red Cross, Calgary, Alberta, Canada. T cells were isolated from these samples as follows. Leukocytes were separated from erythrocytes by centrifugation at 1,500 × *g* for 10 min. The leukocyte layer was washed extensively in 10× phosphate-buffered saline (PBS) to remove platelets. Finally, T cells were separated from B cells, monocytes, and macrophages on a Percoll gradient as described previously (19). Following isolation, PBLs were cultured for 24 h in RPMI 1640–10% fetal calf serum supplemented with 5 μg of phytohemagglutinin L (PHA; Sigma) per ml. After induction with PHA, PBLs were expanded for 48 to 72 h in the presence of human recombinant IL-2 (20 U/ml; Sigma). PBLs were grown in the absence of IL-2 for 48 h prior to activation with either PHA or IL-2.

Fusion protein constructs. An *lck* clone was isolated from a cDNA library derived from the human leukemic T-cell line Molt-4. This *lck* cDNA was used to prepare deleted or mutated constructs that were expressed from the bacterial expression vector PGEX-2T or PGEX-3X (Pharmacia). The construct designated pG-wt encodes full-length wild-type $p56^{lck}$. In vitro mutagenesis was used to generate the pG-f505 construct, which contains a tyrosine-to-phenylalanine mutation at position 505. Constructs pG-c323, pG-c275, pG-c221, and pG-c117 were deleted by digestion with exonuclease III to codons 323, 275, 221, and 117, respectively. Each of these cDNA clones was isolated from Bluescript KS+ (Stratagene) as an *Nco*I-*Eco*RI fragment and ligated into *Sma*I-*Eco*RI-digested PGEX-2T. Construct pG-n89 was deleted from the 5′ end to codon 89 by digestion with exonuclease III. Construct pG-n57 was deleted from the 5′ end to codon 57 by digestion with the restriction enzyme *Nae*I. Both pG-n89 and pG-n57 were ligated into *Sma*I-*Eco*RI-digested PGEX-2T. Construct pG-n79 was deleted from the 5′ end to codon 79 by using a *Bgl*II restriction site a codon 79. This construct was ligated as a *Bgl*II-*Eco*RI fragment into *Sma*I-*Eco*RI-digested PGEX-3X. The construct designated pG-SH2 includes codons 108 to 221 and was generated from construct pG-221 by excising an *Nco*I-*Bgl*I fragment encompassing the first 107 codons. All deletions and mutations were verified by nucleotide sequence analysis prior to PGEX subcloning.

Expression of fusion protein was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG; Sigma). Harvesting and purification of the fusion proteins by affinity to glutathione agarose were carried out essentially as described by Smith and Johnson (41). Appropriate expression of all $p56^{lck}$-fusion protein constructs was verified by immunoblotting of purified proteins with anti-Lck sera. Fusion proteins bound to glutathione agarose were stored at −20°C as a 75% slurry in PBS containing 20% glycerol.

In vitro association, PI kinase assay, and high-pressure liquid chromatography (HPLC) analysis. All human T-cell lines and PBLs were treated identically with respect to preparation of lysates. Cells were collected by centrifugation, washed once with PBS, counted, and then lysed at 10^7/ml in ice-cold lysis buffer (0.15 M NaCl, 20 mM Tris-HCl [pH 8.0], 5 mM EDTA, 1% Nonidet P-40, 2% glycerol, 1.0 mM Na_3VO_4, 50 μg of leupeptin per ml, 7.5 mg of *p*-nitrophenylphosphate per ml) for 15 min on ice. Lysates were then cleared by centrifugation at 13,000 × *g* for 5 min. CEFs and SRA-infected CEFs were prepared in an identical manner except that they were lysed directly on 60-mm-diameter culture plates after reaching confluency. In vitro association with $p56^{lck}$-fusion protein was carried out as follows. Glutathione agarose-bound fusion protein (approximately 3 μg of fusion protein) was added to 300-μl aliquots of cell lysate and was left at 4°C for 2 h. Glutathione agarose was then collected by centrifugation and washed once with PBS–1% Nonidet P-40, once with 0.5 M LiCl–100 mM Tris-HCl (pH 7.4), once with PBS, and finally once with 10 mM Tris-HCl (pH 7.4)–100 mM NaCl–1 mM EDTA.

PI3K activity was measured by the method of Whitman et al. (56). Briefly, the washed glutathione agarose pellets were mixed with 50 μl of reaction buffer (20 mM Tris-HCl [pH

7.6], 100 mM NaCl, 10 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP [3,000 Ci/mmol; New England Nuclear], 0.2 mg of sonicated PI [Serdary Research Laboratories, London, Ontario, Canada] per ml, 0.01 mM ATP). Reaction mixtures were incubated for 10 min at 30°C, and reactions were stopped by the addition of 100 μl of 1 M HCl. Lipids were extracted with 300 μl of chloroform-methanol (1:1) and chromatographed on thin-layer chromatography (TLC) silica plates (G-25; Macherey and Nagel), using a developing solvent of chloroform–methanol–4.0 M ammonium (9:7:2). Incorporation of ^{32}P into PI was visualized by autoradiography and quantified by liquid scintillation counting of radioactive spots scraped from TLC plates. Relevant signals were identified by comparison with lipid standards (Sigma) stained with iodine vapors.

Spots identified as PI phosphate by autoradiography were scraped from TLC plates and prepared for HPLC analysis as described previously (2, 7, 57). Analysis of the deacylated PI kinase products (glycerophosphoinositol phosphates) was carried out on a Partisphere SAX (Whatman) HPLC column. Samples were loaded in water and eluted with a linear gradient of 0 to 0.109 M $(NH_4)_2HPO_4$ (pH 3.8) at 1.0 ml/min over 40 min, followed by 0.109 to 0.42 M over 10 min.

Immunoblotting, pretreatment with phosphatase, and pretreatment with antiphosphotyrosine antibodies. All samples were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose (Schleicher & Schuell) or polyvinylidene difluoride membranes (Immobilon; Millipore). Filters were blocked with Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 5% nonfat dry milk and 1 mM Na_3VO_4 (buffer A). Filters were incubated with affinity-purified rabbit antiphosphotyrosine antibody (our unpublished data) for 1.5 h at room temperature in TBS–0.1% Tween 20 containing 2.5% nonfat dry milk and 1 mM Na_3VO_4 (buffer B). Following incubation with the primary antibody, filters were washed extensively with TBS–0.1% Tween 20 and then incubated with ^{125}I-labelled donkey anti-rabbit antibody at a concentration of 1 ng/ml (10 μCi/μg; New England Nuclear) in buffer B, washed, and autoradiographed.

Pretreatment of cell lysates with alkaline phosphatase (Boehringer) was done for 15 min at room temperature, using a concentration of 20 U of phosphatase per ml of lysate. Pretreatment of cell lysates with monoclonal antiphosphotyrosine antibodies (Upstate Biotechnologies Inc.) was done for 30 min at 4°C, using an antibody concentration of 5 μg/ml of lysate. Control lysates were pretreated with monoclonal antibody (5 μg/ml) directed against α-tubulin (Sigma) for 30 min at 4°C.

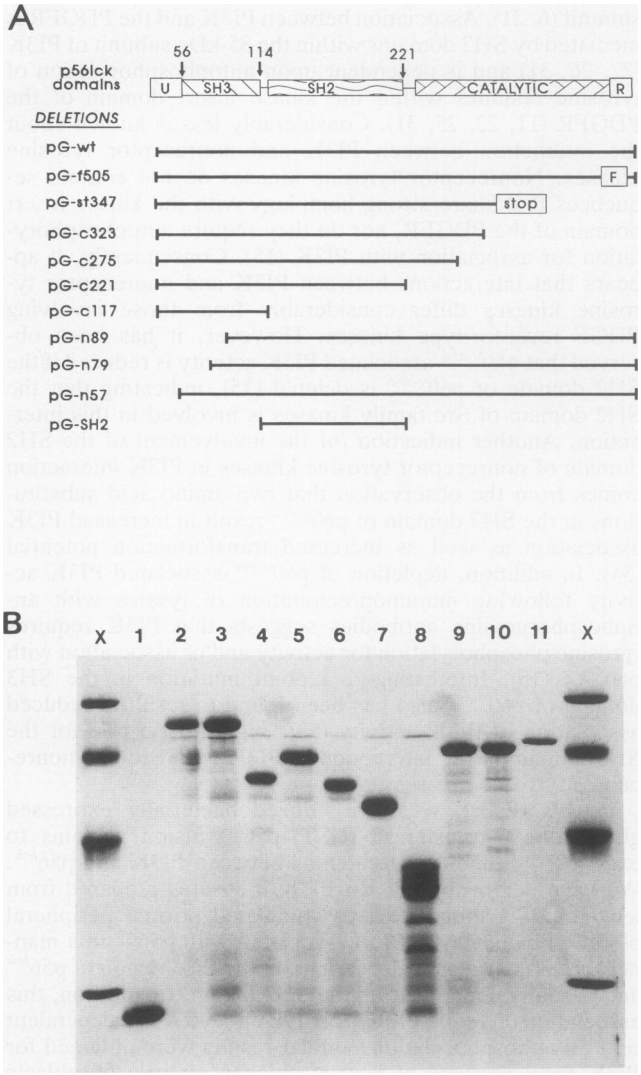

FIG. 1. (A) p56^{lck}-fusion protein constructs. The GST portion of the fusion protein (not shown) is 27 kDa and is continuous with the NH_2-terminal portion of the tyrosine kinase. (B) Coomassie blue staining of purified fusion proteins. Fusion proteins were purified by affinity to glutathione agarose and were resolved on a 10% polyacrylamide gel. Lanes: X, molecular weight markers (97, 66, 45, and 31 kDa); 1, GST; 2, pG-wt; 3, pG-f505; 4, pG-c323; 5, pG-st347; 6, pG-c275; 7, pG-c221; 8, pG-c117; 9, pG-n89; 10, pG-n79; 11, pG-n59.

RESULTS

PI3K activity is associated with p56^{lck}-fusion proteins in leukemic T-cell lysates and lysates of PBLs. Antibody cross-linking of the T-cell receptor by using anti-CD3 antibodies or cross-linking of the CD4 cell surface glycoprotein has been previously shown to result in phosphorylation and activation of p56^{lck} (1, 47, 49, 51). Simultaneous cross-linking of both the T-cell receptor and the CD4 glycoprotein has been shown to enhance some parameters of T-cell activation compared with cross-linking of either CD3 or CD4 alone (29, 33). Others have demonstrated an increase in p56^{lck}-associated PI3K activity in T cells following T-cell receptor activation (44). We have confirmed these results by using the human T-cell line Jurkat stimulated with an antibody cross-linking regimen employing anti-CD3 and anti-CD4 monoclonal antibodies (data not shown). Also, as previously demonstrated (44), this increase in PI kinase activity correlated with an increase in p56^{lck} kinase activity (data not shown).

To further characterize the structural requirements for association of PI3K with p56^{lck}, we used in vitro binding studies involving bacterially expressed forms of p56^{lck} and lysates of leukemic T cells and PBLs. Ten overlapping deletions of a human *lck* cDNA were inserted into a pGEX bacterial expression vector (Fig. 1A). The resulting GST-p56^{lck} fusion proteins were expressed to high levels by induction with IPTG and then purified from bacterial lysates by affinity to glutathione agarose. The full-length *lck* constructs, pG-wt and pG-f505, both encoded a fusion protein of approximately 85 kDa (Fig. 1B). The C-terminally truncated

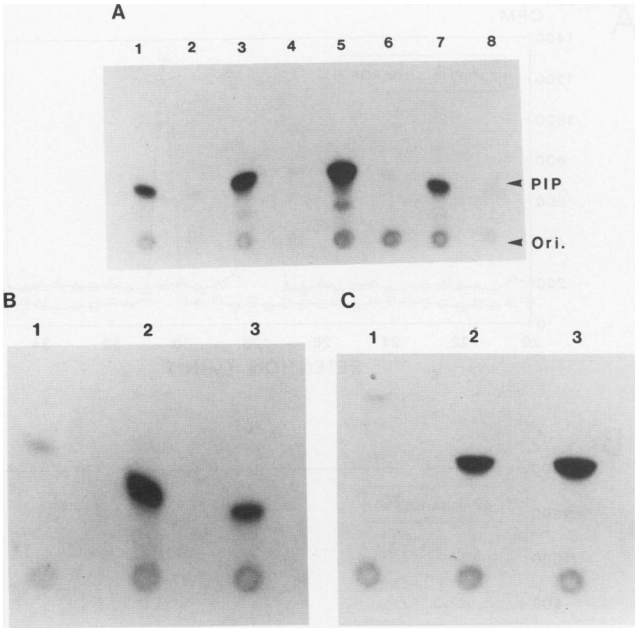

FIG. 2. Association of PI kinase activity with purified fusion proteins in vitro. (A) Purified p56^{lck}-fusion protein construct pG-st347 (3 μg) or GST immobilized on glutathione agarose beads was added to T-cell lysates (300 μl). Following incubation at 4°C, the p56^{lck}-fusion protein bound fraction was washed and assayed for PI activity. Lanes: 2, 4, 6, and 8, CCRF-CEM, Molt-4, Jurkat, and Tib-195 lysates, respectively, incubated with GST alone; 1, 3, 5, and 7, CCRF-CEM, Molt-4, Jurkat, and Tib-195 lysates, respectively, incubated with p56^{lck}-fusion protein construct pG-st347. Ori., origin. (B) Lysates of CEFs (lane 3) or SRA-infected CEFs (lane 2) were incubated with p56^{lck}-fusion protein pG-c347 (3 μg), and then the fusion protein bound fraction was isolated and assayed for PI kinase activity. Lane 1, GST incubated with SRA-infected CEFs and then assayed for PI3K activity. (C) Lysates of PBLs incubated with purified p56^{lck}-fusion protein pG-st347 (3 μg), washed, and assayed for PI kinase activity. Lanes: 1, quiescent PBLs; 2, PBLs following 5 min of PHA (5 μg/ml) treatment; 3, PBLs following 5 min of IL-2 (10 U/ml) treatment.

constructs pG-c347, pG-c323, pG-c275, and pG-c221 each encoded the SH3 and SH2 domains as well as progressively smaller segments of the catalytic domain. Construct pG-c117 encoded the p56^{lck} unique region as well as the SH3 domain, while construct pG-SH2 encoded the SH2 domain alone. Constructs pG-n89, pGn79, and pG-n57 contained deletions removing the unique domain and 34, 24 and 3 amino acids, respectively, from the N-terminal half of the SH3 domain. Each of the p56^{lck}-fusion proteins was recognized by polyclonal anti-p56^{lck} serum in immunoblots (data not shown). As a control, the bacterial expression vector without any inserted *lck* cDNA was transfected into *Escherichia coli*, and expression of the 27-kDa GST fragment of the fusion protein was induced with IPTG and purified from bacterial lysates by affinity to glutathione agarose.

Human leukemic T-cell lines and activated PBLs were lysed with nonionic detergents as described in Materials and Methods; lysates were incubated with immobilized p56^{lck}-fusion protein and then assayed for PI kinase activity. All of the leukemic T-cell lines showed a high level of PI kinase activity associated with the p56^{lck}-fusion protein pG-c347 (Fig. 2A, lanes 1, 3, 5, and 7). No PI kinase activity was observed to associate with the immobilized GST control following incubation with T-cell lysates (Fig. 2A, lanes 2, 4, 6, and 8). The highest level of PI kinase activity associating with p56^{lck} was detected in the Jurkat cell line. This cell line also exhibited the highest tyrosine kinase activity when kinase assays were performed with immunoprecipitates obtained with antisera to p56^{lck} from equivalent amounts of total cellular protein (data not shown). In contrast, quiescent PBLs exhibited very low p56^{lck} kinase activity (data not shown), and low levels of PI kinase activity were found to associate with the p56^{lck}-fusion proteins (Fig. 2C). Following stimulation with either IL-2 or PHA, there was a 5- to 10-fold increase in the level of PI kinase activity detectable by in vitro association with the p56^{lck}-fusion proteins. Although the difference was somewhat less pronounced, there was also a substantial increase in p56^{lck}-associated PI kinase activity in RSV SRA-transformed CEFs versus normal CEFs (Fig. 2B). Association of PI kinase activity with p56^{lck}-fusion protein could be reduced by 80% when competed for with a 100-fold excess of free fusion protein (Fig. 3, lane 2), and p56^{lck}-fusion protein alone had no associated PI kinase activity (Fig. 3, lane 4). These results indicate that PI kinase activity in human leukemic T-cell lysates specifically associated with p56^{lck} sequences in vitro.

The p56^{lck}-associated PI kinase was examined to determine whether this activity was characteristic of PI3K or PI4K. Inhibition of kinase activity by low levels of nonionic detergent is characteristic of PI3K, or type I PI kinase (55). This inhibition was observed for the PI kinase activity associated with the p56^{lck}-fusion proteins. As shown in Fig. 3 (lane 3), the addition of 0.5% Nonidet P-40 to the PI kinase reaction resulted in a 95% reduction in the amount of ^{32}P incorporated into the PI substrate. To confirm that the p56^{lck}-associated PI kinase was phosphorylating the inositol ring at the D-3 position (as opposed to the D-4 position phosphorylated by the type II kinase) (55), we used HPLC to resolve PI4P and the PI3P product of the p56^{lck}-associated PI kinase (Fig. 4A). The deacylated product of the p56^{lck}-associated PI3K eluted 3 min prior to the deacylated PI4P standard and coeluted with the deacylated product of the previously described v-*src*-associated PI3K, or type I PI kinase (14).

The SH3 domain of p56^{lck} is required for association of PI3K activity from T cells. Having observed PI3K activity associated with truncated p56^{lck}-fusion proteins representing the NH_2-terminal one-half of p56^{lck}, we wished to determine more precisely the p56^{lck} sequences required for this interaction. To address this question, we generated a series of overlapping C-terminal deletions through the SH2 domain of p56^{lck} as well as NH_2-terminal deletions into the SH3 domain (Fig. 1). The associated levels of PI3K activity from leukemic T-cell lysates were similar for intact and deleted p56^{lck} constructs with overlapping deletions of the catalytic domain (Fig. 5). Truncation of the catalytic domain in the pG-c347, pG-c323, pG-c275, and pG-c211 constructs, as well as the mutation of tyrosine 505 to phenylalanine in the Phe-505 construct, had no effect on the level of p56^{lck}-associated PI3K activity (Fig. 5, lanes 1 to 5). Deletion of the entire SH2 domain in construct pG-c117 resulted in a reduction of associated PI3K activity by approximately 25 to 30% (Fig. 5, lane 6). However, deletions of the first 89 (Fig. 5, lane 7) or 79 (Fig. 5, lane 8) amino acids of p56^{lck}, which remove 34 or 24 amino acids, respectively, of the SH3 domain of p56^{lck}, completely abolished association of PI3K activity. If the NH_2 terminus was deleted to amino acid 57 such that the SH3 domain was largely intact and the unique domain was removed, then the p56^{lck}-fusion protein retained the ability

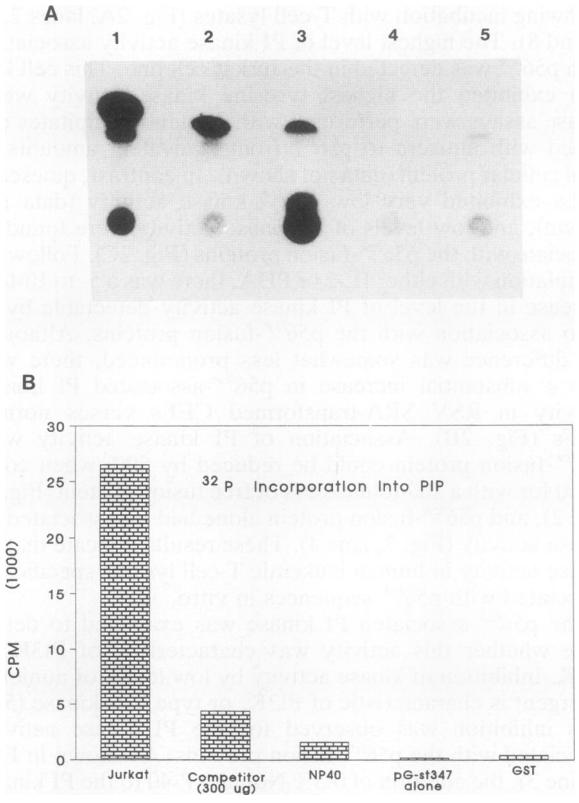

FIG. 3. (A) Association of PI kinase activity can be competed for with unbound fusion protein, and PI kinase activity is inhibited by the addition of nonionic detergent. Jurkat cell lysate (300 μl) was incubated with the glutathione agarose-immobilized p56^{lck}-fusion protein pG-st347 and then washed, and the fusion protein bound fraction was assayed for PI kinase activity as described in Materials and Methods, with the following modifications: lane 1, lysate was preincubated for 30 min with excipient buffer; lane 2, lysate was preincubated for 30 min with 300 μg of soluble p56^{lck}-fusion protein; lane 3, lysate was preincubated for 30 min with excipient buffer and the PI kinase assay was performed in the presence of 0.5% Nonidet P-40; lane 4, fusion protein was incubated in cell lysis buffer alone; lane 5, immobilized GST was incubated with Jurkat cell lysate. (B) Following autoradiography, spots were scraped from the TLC plate and ^{32}P incorporation was analyzed by fluorography, using a beta counter.

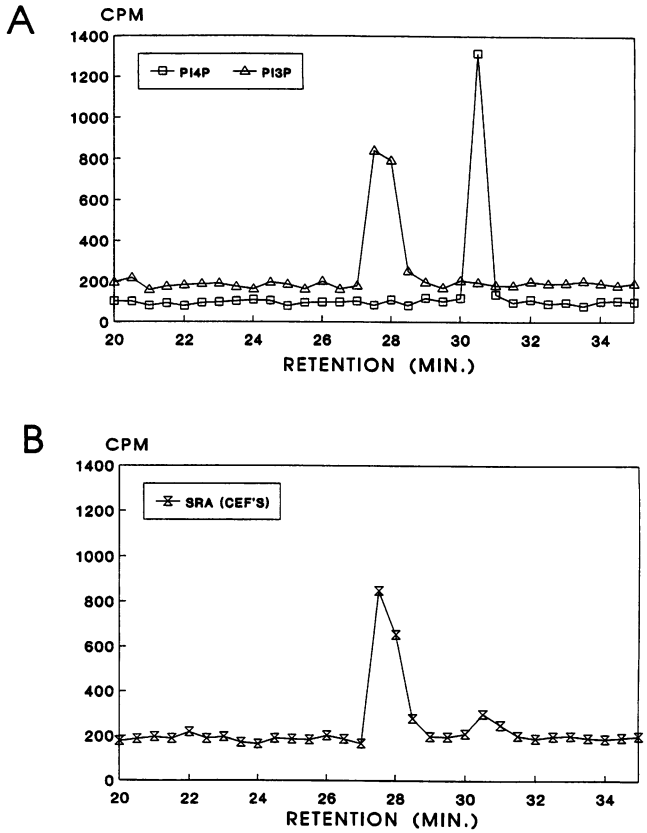

FIG. 4. HPLC analysis of PI kinase product. (A) PI kinase assays were performed on p56^{lck}-fusion protein bound fractions from Molt-4 cell lysates. Tritium-labelled PI4P and the ^{32}P-incorporating PI product of the p56^{lck}-associated activity were both deacylated with methylamine and resolved by anion-exchange chromatography. These products were then resolved by anion exchange, using HPLC. The deacylated product of p56^{lck}-associated PI kinase activity eluted 3 min prior to the deacylated PI4P standard. (B) As an external standard, the product of pp60$^{c\text{-}src}$-associated PI3K activity was chromatographed under the same conditions as for the product of the p56^{lck}-fusion protein-associated PI kinase.

to associate with PI3K activity (Fig. 5, lane 9). These results indicated that although both the SH2 and SH3 domains of p56^{lck} participated in PI3K association, the SH3 domain appeared to be required for interaction with a much larger proportion of PI3K activity in T-cell lysates than the SH2 domain was. They also indicated that the NH_2-terminal unique region was not required for PI3K association, although it may have the ability to augment PI3K binding somewhat.

Increased association of PI3K activity with p56^{lck}-SH2 peptides in SRA-transformed CEFs. The observation that association of PI3K activity with p56^{lck} in T cells was largely dependent on the SH3 domain prompted us to reexamine association of p56^{lck} to PI3K from RSV SRA-transformed CEFs. In vitro binding assays were performed by using either pG-c221, pG-c117, or pG-SH2 incubated with cell lysates from SRA-transformed CEFs, nontransformed CEFs, or the Molt-4 T-cell line (Fig. 6). All samples were assayed for PI3K activity, resolved by TLC, and autoradiographed. Spots corresponding to the PI-3-P signal on X-ray film were scraped from the TLC plates and quantified by liquid scintillation counting. For each cell type, the PI3K activity associated with either the SH2 or N-terminal plus

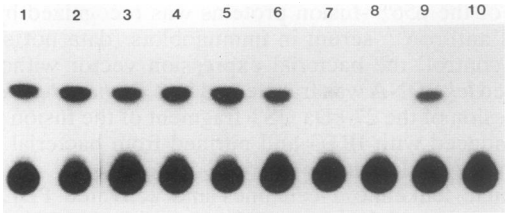

FIG. 5. Association of T-cell PI3K activity with deleted p56^{lck}-fusion protein constructs. Molt-4 cell lysates (300 μl) were incubated with p56^{lck}-fusion protein, and the fusion protein bound fraction was isolated and assayed for PI3K activity. Lanes 1 to 9 show PI3K activity associated with constructs pG-wt, pG-f505, pG-st347, pG-c275, pG-c221, pG-c117, pG-n89, pG-n79, and pG-n57, respectively. Lane 10, GST.

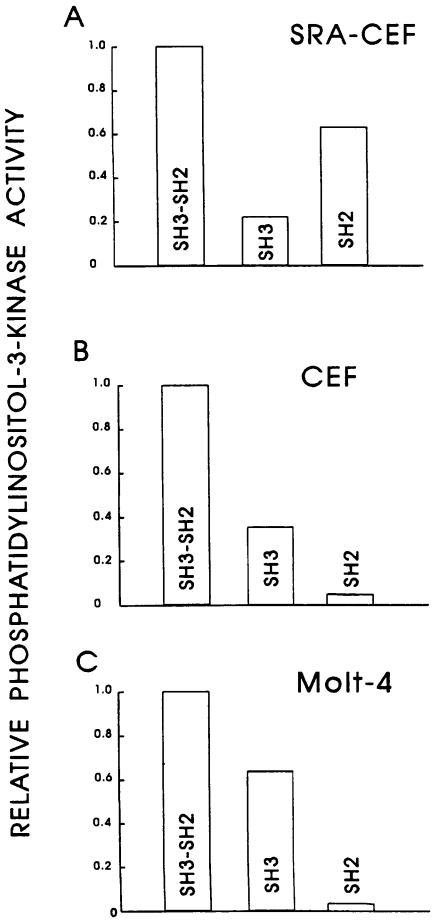

FIG. 6. Quantification of PI3K activity interacting with SH2-, SH3- or SH2-SH-3-containing peptides. PI3K assays were performed on p56^{lck}-fusion protein bound fractions, using construct pG-c221 (SH3-SH2), pG-c117 (SH3), or pG-SH2 (SH2); the products were separated by TLC, and ^{32}P incorporation was quantified by liquid scintillation counting. Values represent averages of at least two separate experiments. For each set of assays using lysates of SRA-infected CEFs (A), CEFs (B), or Molt-4 cells (C), a relative value of 1.0 was assigned to the total PI3K activity associated with the SH2-SH3-containing peptides.

SH3 domain independently was averaged from at least two separate experiments and compared with the total PI3K activity associated with the construct containing both an SH2 and an SH3 domain. Most of the PI3K activity from Molt-4 cells and CEFs associated preferentially with the N-terminal plus SH3 domain of p56^{lck} (Fig. 6B and C). In contrast, the PI3K activity from RSV SRA-transformed CEFs associated preferentially with the SH2 domain (Fig. 6A). The p56^{lck}-fusion protein constructs containing both an SH2 and an SH3 domain bound somewhat more PI3K activity than the total activity bound to separate SH2 and SH3 domains. This observation was made for all three cell lines tested and may suggest that SH2 and SH3 domains cooperate to form a binding pocket that might facilitate or stabilize PI3K binding (53).

The phosphorylation dependence of PI3K association to either SH2- or SH3-containing p56^{lck}-fusion proteins was also assessed. Treatment of cell lysates with alkaline phosphatase in the absence of phosphatase inhibitors reduced the overall phosphotyrosine content of cell lysates by greater than 90%, as assessed by antiphosphotyrosine immunoblotting (Fig. 7A, lanes 3 and 4). Presumably, this treatment also reduced phosphothreonine and phosphoserine content. Preincubation with alkaline phosphatase also dramatically reduced the PI3K activity bound to both SH3- and SH2-containing p56^{lck}-fusion proteins (Fig. 7B, first six lanes). This effect was due to dephosphorylation, as further confirmed by a control experiment in which phosphatase inhibitors prevented the alkaline phosphatase-induced reduction of bound PI3K activity (Fig. 7C). The simplest explanation for these results is that phosphorylation of PI3K was required for binding to the SH2 and SH3 domains. To clarify this observation, we attempted to block the association between PI3K and p56^{lck} by preincubating cell lysates with an antiphosphotyrosine monoclonal antibody. Lysates from SRA-transformed CEFs and Molt-4 cells were preincubated with either antiphosphotyrosine antibodies (Fig. 8A, lanes 1 to 4) or control antibodies directed against α-tubulin (Fig. 8A, lanes 5 to 8). Pretreated lysates were then assayed for p56^{lck}-associated PI3K activity as described above. Pretreatment with antiphosphotyrosine antibodies abolished the SH2-associated PI3K activity in lysates of SRA-transformed CEFs (Fig. 8A, lane 1) but had no effect on SH3-associated PI3K activity in SRA-transformed CEFs or Molt-4 cells (Fig. 8A, lanes 2 and 4; Fig. 8B, columns 2 and 4). Pretreatment with antiphosphotyrosine antibodies also reduced the SH2- and SH3-associated PI3K activity in Molt-4 lysates by approximately 25% (Fig. 8A, lane 3), suggesting that a minor component of T-cell PI3K activity interacted with the SH2 domain of p56^{lck}. This result is consistent with the finding (Fig. 5) that deleting the SH2 domain of p56^{lck} resulted in a reduction of associated PI3K activity of approximately 30%. In addition, we have found that phosphotyrosine reduced 20 to 30% by the PI3K activity associated with p56^{lck}-fusion proteins containing both an SH2 and an SH3 domain (data not shown). These results suggest that PI3K can exist in two forms: one form that binds primarily to the SH3 domain of p56^{lck} and is independent of tyrosine phosphorylation, and an additional tyrosine-phosphorylated form which binds primarily to the SH2 domain of p56^{lck}.

DISCUSSION

In T lymphocytes, PI3K activity has been observed to coimmunoprecipitate with two nonreceptor protein tyrosine kinases of the Src family. IL-2 stimulation of T lymphocytes resulted in an increase in PI3K activity associated with p59^{fyn} (2), and antibody-mediated T-cell receptor activation led to increased PI3K activity associated with p56^{lck} (44). We have also observed an increase in PI3K activity in p56^{lck} immunoprecipitates following cross-linking with anti-CD3 and anti-CD4 monoclonal antibodies (data not shown).

In this study, we wished to analyze the nature of the interaction between PI3K and nonreceptor tyrosine kinases. To address this issue, we used bacterially expressed p56^{lck} to develop an in vitro assay designed to directly examine the PI3K binding properties of p56^{lck}. As p56^{lck} has been observed to associate with PI3K in a stimulation-dependent manner (our observations and reference 44), the use of p56^{lck}-fusion proteins in this study is probably also relevant to the characterization of PI3K interactions with other Src family tyrosine kinases.

We have observed that PI3K activity from lysates of T lymphocytes specifically associated with bacterially expressed p56^{lck}-fusion proteins. Association of PI3K activity

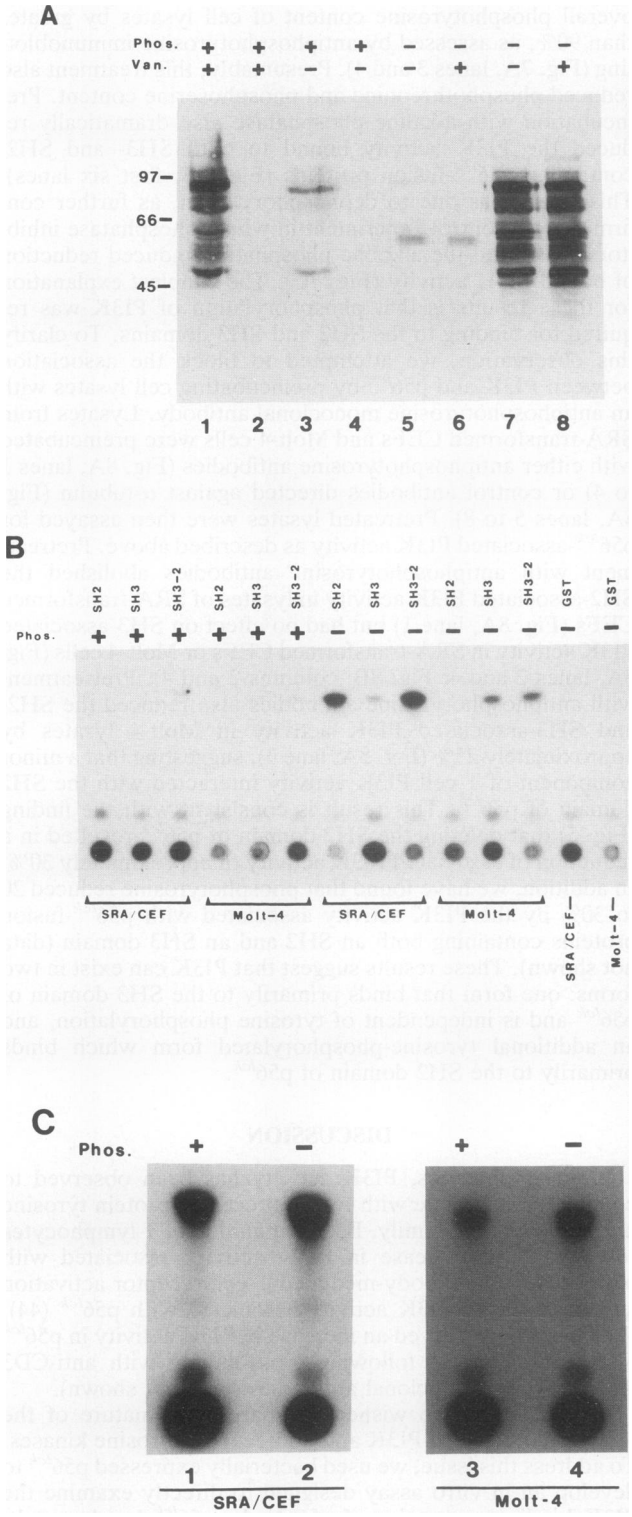

FIG. 7. Dephosphorylation of cell lysates abolishes p56^{lck}-associated PI3K activity. (A) Antiphosphotyrosine immunoblot of lysates from SRA-infected CEFs (lanes 1, 3, 7, and 8) or Molt-4 cells (lanes 2, 4, 5, and 6) lysed in the presence (+) or absence (−) of 1 mM Na_3VO_4 (Van.) and either treated (+) or untreated (−) with 20 U of alkaline phosphatase (Phos.) per ml for 15 min at room temperature (B) PI3K assays performed on the p56^{lck}-fusion protein bound fractions from lysates of Molt-4 cells or SRA-infected CEFs (SRA/CEF). Lysates either contained 1 mM Na_3VO_4 and were not treated with alkaline phosphatase (−) or contained no Na_3VO_4 and were treated with alkaline phosphatase (20 U/ml) for 15 min at room temperature (+). (C) PI3K assays were performed on the p56^{lck}-fusion protein bound fractions of control lysates from SRA-infected CEFs (lanes 1 and 2) or Molt-4 cells (lanes 3 and 4) containing the phosphatase inhibitors Na_3VO_4 (1.0 mM) and *p*-nitrophenylphosphate (7.5 mg/ml) that were either treated with alkaline phosphatase (20 U/ml) for 15 min at room temperature (+) or were incubated for 15 min at room temperature without the addition of alkaline phosphatase (−).

from T lymphocytes with p56^{lck} was not substantially affected by deletion of the entire p56^{lck} SH2 domain but was abolished by small deletions in the NH_2-terminal half of the SH3 domain. These and other results suggested that the *lck* SH3 domain was required for binding of PI3K from T lymphocytes. This observation was initially somewhat surprising to us, as previously published reports suggested that PI3K from RSV SRA-transformed cells bound primarily to the SH2 domain of pp60$^{v\text{-}src}$ (16, 17). However, while our work was in progress, we became aware of a report indicating that a point mutation affecting the SH3 domain of a "partial"-transformation mutant of pp60$^{v\text{-}src}$ substantially reduced the amount of pp60$^{v\text{-}src}$-associated PI3K in immunoprecipitates from mutant-transformed cells (53). Our results indicate that PI3K from T lymphocytes and uninfected CEFs associated predominantly with the SH3 domain of p56^{lck}. However, in agreement with previously published reports (16, 17), PI3K from RSV-transformed cells associated primarily with the SH2 domain. This difference in PI3K binding specificity was apparently caused by tyrosine phosphorylation on the PI3K species in RSV-transformed cells, as the SH2-directed binding could be abolished by preincubation of cell lysates with monoclonal antiphosphotyrosine antibodies.

Less than 20% of the PI3K activity associated with the SH2- and SH3-containing p56^{lck} construct was blocked by preincubation of T-cell lysates with antiphosphotyrosine antibodies (Fig. 8A, lanes 3 and 7). However, the level of T-cell PI3K activity associated with the p56^{lck} construct containing only an SH3 domain was not affected by preincubation with antiphosphotyrosine antibodies. These results indicated that the majority of PI3K activity in T cells associated with the SH3 domain of p56^{lck} and that this interaction was independent of tyrosine phosphorylation of PI3K. In contrast, approximately 70% of the PI3K activity from SRA-transformed CEFs that associated with GST-p56^{lck} constructs required a p56^{lck} SH2 domain for binding (Fig. 6A), and this binding was entirely dependent upon an accessible phosphotyrosine residue on PI3K (Fig. 8A, lanes 1 and 5; Fig. 8B, columns 1 and 5). The SH3 dependence of the p56^{lck} interactions with PI3K from CEFs and T cells and the SH2 dependence observed with PI3K from transformed CEFs suggests that a fundamental difference in the binding properties of PI3K may result from increased tyrosine phosphorylation of PI3K in v-*src*-transformed cells. Similar findings regarding binding of PI3K from uninfected cells and RSV-transformed cells to SH3 and SH2 domains have been observed by Liu and Pawson (29a). These observations are also consistent with previous suggestions that cellular transformation by *src* family tyrosine kinases results in a modification of PI3K that enhances its association with pp60$^{v\text{-}src}$ and that this modification is likely to involve tyrosine phosphorylation (15).

The PI3K from T lymphocytes and RSV-transformed cells

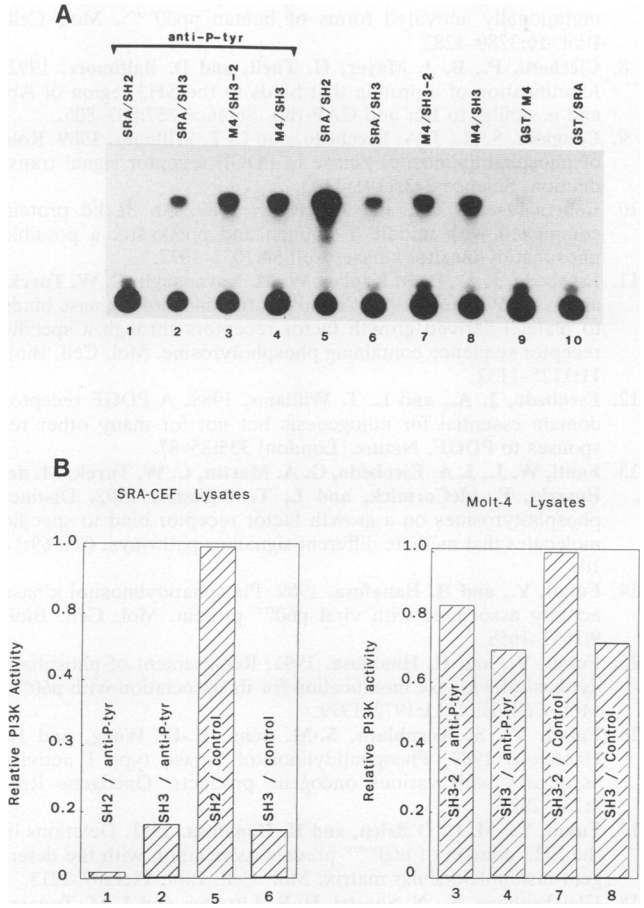

FIG. 8. Pretreatment of lysates with antiphosphotyrosine monoclonal antibodies abolishes p56^{lck}-associated PI3K activity in lysates of SRA-infected CEFs but not in Molt-4 cell lysates. (A) Molt-4 (M4) or SRA-infected CEF (SRA) cell lysates were either preincubated with monoclonal antiphosphotyrosine (anti-P-tyr) antibodies (5 μg/ml) for 30 min at 4°C (lanes 1 to 4) or preincubated with monoclonal antitubulin antibodies (5 μg/ml) for 30 min at 4°C (lanes 5 to 8). Lysates were then incubated with p56^{lck}-fusion protein pG-c221 (SH3-2), pG-c117 (SH3), or pG-SH2 (SH2), and the fusion protein bound fraction was isolated and assayed for PI3K activity. (B) PI-3-P spots from panel A were scraped from TLC plates, and ^{32}P incorporation was quantified by liquid scintillation counting. A relative PI3K activity of 1.0 was assigned to columns 5 and 7, corresponding to the SH2 bound fraction from SRA-CEF lysates and the SH3-2 bound fraction from Molt-4 lysates, respectively.

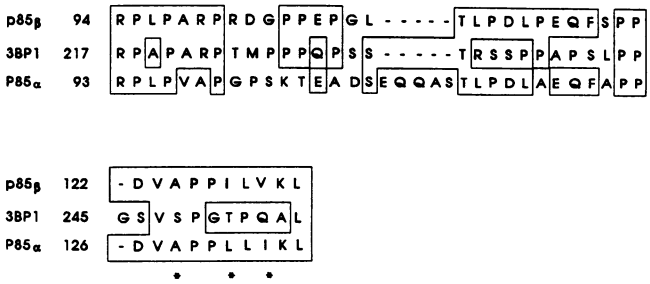

FIG. 9. Comparison of the SH3 binding sequence of 3BP1 to the amino acid sequences of p85α and p85β. Amino acids for the p85 and 3BP1 proteins are numbered as in references 35 and 8, respectively. Boxing shows sequence identity. In p85β, 16 of the 38 amino acids shown are identical to the 3BP1 sequence. In p85α, 12 of the 43 amino acids shown are identical to the 3BP1 sequence. Amino acid similarities are indicated by asterisks.

demonstrated a similarity in that p56^{lck} did not associate with PI3K from lysates prepared without phosphatase inhibitors (data not shown) or in lysates pretreated with phosphatase (Fig. 7). However, treatment of cell lysates with alkaline phosphatase did not allow a distinction to be made between dephosphorylation of serine, threonine, or tyrosine residues. Consequently, it is possible that the loss of SH3-bound PI3K activity following phosphatase treatment of T-cell lysates reflects a requirement for serine or threonine phosphorylation of PI3K in order to bind p56^{lck}. It is also possible that the binding of PI3K species to the p56^{lck} SH3 domain was dependent upon a phosphotyrosine residue in PI3K that was not recognized by our monoclonal antiphosphotyrosine antibody. Alternatively, phosphorylation of serine, threonine, or tyrosine may affect the catalytic activity of p56^{lck}-bound PI3K.

The nature of the non-phosphotyrosine-dependent association between PI3K and the SH3 domain of p56^{lck} remains to be determined. However, examination of the primary structure of the PI3K subunits suggests one possibility regarding this interaction. The primary structure has been determined for two forms of the 85-kDa subunit of PI3K, p85α and p85β (35). Both proteins contain an NH_2-terminal SH3 domain followed by two widely spaced SH2 domains. Between the SH3 domain and the NH_2-terminal SH2 domain there is a region of sequence homology to the Bcr protein (32). Interestingly, a protein (3βP1) that demonstrates binding to the SH3 domain of Abl (8) contains a region of sequence similarity to both the C-terminal region of Bcr and the Bcr-like sequences found within p85α and p85β. We have identified a sequence within the Bcr-like region of p85β that shares 42% amino acid identity with a region of 3BP1 that overlaps the SH3 binding domain (Fig. 9). The corresponding region of p85α contains a five-amino-acid insert and has a somewhat lower amino acid identity of 28% with this region of 3BP1. Although somewhat speculative at present, it is possible that these Bcr-like regions of p85β and p85α may represent sites of interaction with the SH3 domains of p56^{lck} and other Src-type kinases. We are currently in the process or examining interactions between SH3 domains and the Bcr-like sequences in p85.

Our results suggest that different forms of PI3K exist in the cells tested here. One form may preferentially bind to the SH3 domain of p56^{lck}, and another form which is phosphorylated on tyrosine may preferentially bind to the SH2 domain. In addition, it was noted that an increased level of PI3K activity from all cell lines was found to associate with SH3- and SH2-containing fusion proteins compared with the total PI3K activity binding to either SH2 or SH3 alone (Fig. 6). This observation may indicate that the SH2 and SH3 domains interact to form a PI3K binding pocket that is somewhat more efficient at binding PI3K than are the SH3 and SH2 regions by themselves. Such a possibility has been previously suggested by Wages et al. (53), who observed reduced PI3K activity associated with an RSV mutant having a point mutation causing a lysine-to-glutamic acid change at amino acid 106 in the SH3 region of pp60$^{v\text{-}src}$; interestingly, the corresponding SH3 region of p56^{lck} lies within the SH3 region that was deleted in the constructs used here.

It has been suggested that differential expression or assembly of the various PI3K subunits may confer binding specificity between receptor tyrosine kinases (22, 35). Spec-

ificity of PI3K for binding sites within certain receptor tyrosine kinases is supported by the derivation of a consensus sequence for sites of interaction with p85α (5) and the demonstration of sequence discrimination by both SH2 domains of p85α (22, 26, 38). In addition, the difference in binding specificity between isolated p85 and p85 complexed with p110 has aroused speculation that the p110 subunit of PI3K modulates the binding specificity of the p85 subunit (35). Similarly, alternative expression or assembly of subunits may allow for binding specificity to nonreceptor tyrosine kinases. Analysis of these possibilities awaits the availability of specific antisera directed against the known isomers of the 85- and 110-kDa subunits of PI3K.

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ADDENDUM IN PROOF

After the manuscript was submitted, binding of PI3K to SH3 domains of other Src family members has been reported by others: K. V. S. Prasad, O. Janssen, R. Kapeller, M. Raab, L. C. Cantley, and C. Rudd, Proc. Natl. Acad. Sci. USA **90:**7366–7370, 1993; X. Liu, L. E. M. Marengere, C. A. Koch, and T. Pawson, Mol. Cell. Biol. **13:**5225–5232, 1993; and C. M. Pleiman, M. R. Clark, L. K. T. Gauen, S. Winitz, K. M. Coggeshall, G. L. Johnson, A. S. Shaw, and J. C. Cambier, Mol. Cell. Biol. **13:**5877–5887, 1993.

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