Identification of Two SH3-binding Motifs in the Regulatory Subunit of Phosphatidylinositol 3-Kinase*

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Src homology 3 (SH3) domains have been recently shown to bind to proline-rich sequences contained in 3BP1, 3BP2, and SOS. In a recent study we demonstrated that phosphatidylinositol 3-kinase (PI 3-kinase) associates with the Fyn SH3 domain. Here we show that p85, the regulatory subunit of PI 3-kinase, binds directly to the SH3 domains of Abl, Lck, Fyn, and p85 itself. An examination of p85 amino acid sequence revealed two proline-rich sequences in its N-terminal region similar to those present in 3BP1, 3BP2, and SOS. To test whether these sequences mediate the association of p85 with SH3 domains two peptides with amino acid composition corresponding to the p85c proline-rich sequences were synthesized and used in competition assays. Both peptides worked equally well in inhibiting the binding of PI 3-kinase activity and p85c to Fyn SH3 domain, whereas a control peptide had no effect. These results indicate that, as in 3BP1 and SOS, the proline-rich sequences in p85 mediate its interaction with SH3 domains. These results also suggest that the SH3 domain of p85 may "self-associate" with the proline-rich motifs of the same subunit as part of the PI 3-kinase regulatory mechanism.

Protein-protein interactions have been recently implicated as a major mechanism by which intracellular signaling pathways communicate with each other (1, 2). In recent years two elements have been identified that play major roles in regulating protein-protein interactions: the Src homology 2 and 3 domains (SH2 and SH3 domains) (3). They are present in a wide variety of proteins, some that contain intrinsic enzymatic activity as in the members of the Src family of tyrosine kinases, phospholipase Cγ and GTPase-activating protein (GAP),1 or in adaptor proteins such as the regulatory subunit of PI 3-kinase (p85), c-Crk, Nck, and the Sem-5/Grb2 molecules (reviewed in Ref. 3). Although the adaptor proteins do not contain intrinsic enzymatic activity, their targets do. The SH2 domains confer association with tyrosine-phosphorylated proteins via sequence-specific recognition of the phosphotyrosine moiety (1, 4-7).

The function of SH3 domains has been less well characterized to date. These domains have been implicated in regulating cytoskeletal functions, because they have been identified in several proteins associated with the cytoskeleton in both yeast and higher eukaryotes (myosin, spectrin, BEM 1, ABP1, FUS 1, and in a ras guanine nucleotide exchange factor, CDC25) (3, 8). It has also been suggested that SH3 domains may play a negative regulatory function in the context of cytosolic protein-tyrosine kinases: mutations and deletion of the SH3 elements in Abl and Src lead to the activation of their transformation capacity (9-12). Cicchetti et al. (13) have cloned a protein by its ability to associate with the Ahb SH3 domain. This protein, named 3BP1, contains sequence homology to rho-GAP, the C-terminal region of Bcr and n-chimerin, all proteins that contain intrinsic GTPase activity (18), indicating that 3BP1 is a GAP. Furthermore, the SH3 domains of Grb2/SEM-5/Drk have been found to associate with SOS, a guanine nucleotide exchange factor for ras (14-17). The interactions between Abl SH3 and 3BP1 have been mapped to a proline-rich 10 amino acid sequence (APTMPPPLPPVPP) in the C-terminal portion of the molecule (19). Amino acid substitutions have indicated that the proline residues are critical in the association of this sequence with the Abl SH3 domain. Four similar sequences have been identified in the C-terminal domain of SOS in the region that has been shown to associate with the SH3 domain of Grb2 (14, 15, 17).

We have recently shown that p59*-c (T) and p56*-c associate with PI 3-kinase activity via their SH3 domains (20, 27). PI 3-kinase phosphorylates the D-3 position of the inositol ring of phosphatidylinositol and has been shown to be activated by a wide range of growth factors and other cellular activators (1). PI 3-kinase is a heterodimer complex composed of an 85-kDa protein (regulatory subunit) and a 110-kDa protein (catalytic subunit) (21-25). The 85-kDa subunit has two SH2 domains, one SH3 domain and a domain with homology to BCR and various rho and rac GAPS. In this work, we show that PI 3-kinase interacts with the SH3 domains of Abl, Lck, Fyn, and p85 via its regulatory subunit (p85). Examination of the p85 amino acid sequence revealed two proline-rich sequences homologous to 3BP1 and SOS, suggesting that these may be the contact sites for the SH3 domains. Two synthetic peptides containing these sequences were able to block the association of PI 3-kinase and p85c to a GST-Fyn SH3 fusion protein, indicating that these proline-rich sequences in p85 are indeed SH3-binding motifs. These results suggest that SH3-containing proteins may be involved in linking tyrosine kinases not only to ras
signaling pathways but to other signal transduction elements as well.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Tissue culture media was purchased from Life Technologies, Inc. Acrylaldehyde and Bis-acrylaldehyde were obtained from National Diagnostics (Manville, NJ). Phosphotyrosinylidinitol, glutathione-Sepharose 4B beads, (γ-32P)ATP, and silica gel plates were purchased from Avanti (Alabaster, AL), Sigma, DuPont NEN, and E. Merck (Germany), respectively. The HPLC system used is from Hewlett-Packard (Germany), and the amion exchange Partisep SAX column from Whatman (Clifton, NJ). Anti-p85 polyclonal antibodies used in this study were raised against a GST fusion protein containing the N-terminal SH2 domain of rat p85α. Antibodies against the C-terminal SH2 domain of p85α were obtained from Transduction (Lexington, KY).

**Cell Culture**—The T lymphoblastoid cell line HPB-ALL was cultured in RPMI 1640, 10% heat-inactivated fetal calf serum containing 2 mM L-glutamine at 37 °C and 5% CO2. Spodoptera frugiperda (SF9) cells were cultured as described elsewhere (26). SF9 cells were plated in 60-mm plates and infected with baculovirus-p85α (multiplicity of infection = 10). The cells were harvested 40–60 h post-infection, washed twice with 120 mM-buffered saline, and sonicated on the plates or as cell pellets at −70 °C and used as a source of p85α.

**Preparation of Cell Lysates**—HPB-ALL cells were pelleted and washed twice with ice-cold phosphate-buffered saline. The cell pellets were incubated in ice-cold lysis buffer (157 mM NaCl, 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin) for 30 min at 4 °C with constant rocking. The lysates were cleared by centrifugation at 15,000 rpm for 10 min followed by passage through 0.2-µm filters. Protein concentration was then determined (Bio-Rad micro-assay), and the lysates were used at a concentration of 2 mg/ml/sample. Bovine serum albumin was added to the lysates at a final concentration of 2 mg/ml. 3 × 106 SF9 cells over-expressing p85α (SF9/p85α) were lysed in 500 µl of lysis buffer (same as above) and subjected to the same clearing procedure. Bovine serum albumin was also added at a final concentration of 2 mg/ml/µl aliquot used per sample.

**Glutathione S-Transferase Fusion Proteins**—Escherichia coli cells transformed with PGEX-2T vector containing the SH3 domain of Abl (residues 84–132) (13) were kindly provided by Dr. Richard Van Eten (Center for Blood Research, Harvard Medical School). DNA sequences corresponding to the SH3 domains of Fyn (residues 82–148), Lck (residues 62–126), and p85 (residues 1–80) were amplified by polymerase chain reaction from vectors containing full-length Fyn, Lck, and p85 cDNA, using specific primers and subcloned into PGEX-2T vectors at the BamHI and EcoRI sites (20, 27). The transformed bacteria (JM109 or DH5α) were grown, induced with isopropyl-1-thio-β-D-galactopyranoside (0.5 mM), lysed as described (26), and the GST-SH3 fusion proteins affinity-purified with glutathione-Sepharose beads. The beads were kept in aliquots as a 50% slurry in NETN (20 mM Hepes (pH 7), 40 mM NaCl, 1 mM EDTA, 100 µM vanadate) at −70 °C until further use.

**Synthetic Peptides**—Peptides corresponding to the sequences SP60-PKRPPRPLP (residues 82–96 of p85α) and ERQPAPALPPKPPK (residues 300–314 of p85α) were synthesized on a MILLIGEN EXCELL using a polyurethane support (PAL) which gives an amide on the proteins affinity-purified with glutathione-Sepharose beads. The peptides were stored as substrate (a 50% slurry in NETN (20 mM Hepes (pH 7), 40 µM ATP, 10 mM MgCl2, and 100 µM vanadate). The reaction was carried out for 10 min at 37 °C and the lipid kinase procedure to verify that the amounts of GST-Fyn SH3 fusion protein was the same in all samples. The final peptide concentration in the final 200-µl volume of the assays is indicated in the figures. These conditions were determined by time course studies that revealed that preincubating the GST fusion protein beads with the peptides for longer periods of time or at room temperature did not increase the amount of inhibition of baculovirus p85α or HPB-ALL-derived PI 3-kinase binding to GST-Fyn SH3 fusion proteins.

**RESULTS**

**Association of PI 3-Kinase with SH3 Domains**—GST fusion proteins containing the SH3 domains of p85 (regulatory subunit of PI 3-kinase), Abl, Lck, and Fyn were bound to glutathione-Sepharose beads and incubated with HPB-ALL cell lysates. Lipid kinase activity was assayed on the precipitates, and the reaction products were analyzed by TLC and HPLC (see "Experimental Procedures"). Fig. 1A depicts a TLC analysis of one of these experiments which shows that GST-SH3 fusion protein precipitated comparable amounts of PI kinase activity, whereas GST alone (lane 1) did not precipitate detectable amounts of PI kinase activity. Although the same amounts of GST-SH3 fusion proteins were used (10 µg), GST-S6SH3 was the most efficient in precipitating PI kinase activity followed in order of efficiency by GST-Abl SH3, GST-Fyn SH3, and GST-Lck SH3. Further analysis of the reaction product by HPLC of the deacylated lipid demonstrated one single peak that co-migrated with glycerol-phosphorylinositol 3-phosphate (p1-3-P) (Fig. 1, B and C), confirming the identity of the SH3 precipitable lipid kinase activity as PI 3-kinase.

The Regulatory Subunit of PI 3-Kinase (p85) Mediates the Direct Association of PI 3-Kinase with SH3 Domains—It was next of interest to determine whether the association of PI 3-kinase with the SH3 domains was direct or mediated by an adaptor molecule. We first examined the available amino acid sequences of p85 and p110 (the catalytic subunit of PI 3-kinase) for motifs homologous to the proline-rich sequences identified in β1,3, and 2 and 5 which are homologous to the proline-rich sequences in p85α as depicted in Fig. 2. The first sequence is located just downstream of the SH3 domain (residues 82–96 in p85α), and the second sequence is just upstream of the N-terminal SH2 domain (residues 300–314 in p85α). These proline-rich sequences flank the region of p85α that is homologous
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A

**FIG. 1.** GST-SHS fusion proteins precipitate PI 3-kinase activity from HPB-ALL cell lysates. A, GST (lane 1), GST-85SH3 (lane 2), GST-Abl SH3 (lane 3), GST-Lck SH3 (lane 4), and GST-Fyn SH3 (lane 5) fusion proteins (10 μg each) immobilized on glutathione-Sepharose beads were incubated with HPB-ALL cell lysates for 15 min at 4 °C with constant rocking. The beads were then extensively washed and assayed for precipitable PI kinase activity by the addition of PI and [γ-32P]ATP. The lipids were chloroform-extracted and separated by TLC. An autoradiograph is shown. The position of PIP standard and origin are indicated. B, the PIP produced in the above reactions were extracted, deacylated, and submitted to HPLC analysis. The figure shows the reaction product generated by the lipid kinase reaction on GST-85SH3 precipitate. The migration positions of gPI-3-P and gPI-4-P are indicated. GST (○), GST-85SH3 (○), and [3H]gPI-4-P standard (solid line). No lipid is detected in the GST precipitate, whereas one single peak that co-migrates with gPI-3-P standard is detected on the GST-85SH3 precipitate, indicating that the 85SH3 domain associates with PI 3-kinase. All the other reactions produced similar products (data not shown). C, for PI-3-P standard we used the lipid produced on a lipid kinase reaction performed on anti-p85 immunoprecipitate. α-p85 (○) and [3H]gPI-4-P (solid line) indicate the retention times of gPI-3-P and gPI-4-P, respectively.
sequences within the BCR domain, which is just downstream of P1, and the N-terminal part of the molecule and is followed by the first proline-rich sequences. The SH3 domain is located in the sequence of P1 and P2 are shown and the residues that are identified in 3BP1, 3BP2, and SOS are shown, as well as the sequences homologous to 3BP1, 3BP2, and SOS that have been implicated in direct binding to the SH3 domains of p85a and Grb2, respectively (13, 15, 19) (Fig. 2). To investigate whether these sequences can bind to SH3 domains two peptides with amino acid composition corresponding to residues 82–96 (SPTPKPRPPLP) and 300–314 (ERPAPALPPKKP) of p85a (see Fig. 2) were synthesized (see "Experimental Procedures"). The control peptide sequence contained residues 1195–1207 (YWAPAPHLKPK). These peptides were used in competition studies as a positive control. No p85a was detected in the GST lane (lane 2), indicating that p85a interacts directly with the SH3 moiety of the GST fusion proteins.

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Fig. 3. The GST-SH3 fusion proteins associate with baculovirus-p85a. Immobilized anti-p85 antibody (lane 1) or glutathione-Sepharose beads containing GST (lane 2), GST-85SH3 (lane 3), GST-Abl SH3 (lane 4), GST-Lck SH3 (lane 5), and GST-Fyn SH3 (lane 6) were incubated with SF9/p85a lysates for 15 min at 4 °C with constant rocking. The beads were washed twice with buffer containing 1% Nonidet P-40 and 2 x loading buffer was added. The samples were boiled for 5 min, separated on a 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. An anti-p85 immunoblot is shown. The position of p85a is indicated. p85a was detected in the precipitates of GST-85SH3, GST-Abl SH3, GST-Lck SH3, and GST-Fyn SH3 (lanes 3–6). It was also detected in the anti-p85 immunoprecipitate (lane 1, positive control). No p85a was detected in the GST lane (lane 2), indicating that p85a interacts directly with the SH3 domain of the GST fusion proteins.

Fig. 2. Schematic representation of p85a, the proline-rich sequences within and homologous to 3BP1, 3BP2, and SOS. p85a is represented schematically showing the relative positions of the different domains in a linear fashion. The SH3 domain is located in the N-terminal part of the molecule and is followed by the first proline-rich sequence (P1). The second proline-rich sequence (P2) is located between the BCR domain, which is just downstream of P1, and the N-terminal SH2 domain. The C-terminal SH2 domain is also shown. The amino acid sequences of P1 and P2 are shown and the residues that are underlined make up the sequence of the synthetic peptides used in this study (P82–P96 and P890–P914, the numbers represent the residue positions in the p85a amino acid sequence). The proline-rich sequences identified in 3BP1, 3BP2, and SOS are shown, as well as the sequences in p85a. The residues in boldface in 3BP1 indicate the critical residues involved in the interaction with the Abl SH3 domain (see text). The analogous residues are also indicated in boldface in the other sequences.

The C-terminal region of BCR. The lower part of Fig. 2 depicts the p85a of the 3BP1, 3BP2, and SOS. Mutation analysis of the 3BP1 proline-rich sequence has pointed out the critical residues involved in the interaction with the Abl SH3 domain (19). Mutation of residues Ala1, Pro5, Pro7, and Pro8 abolish binding to Abl SH3, whereas changing Pro9 decreases dramatically the binding affinity (residues in boldface). The critical proline residues (Pro2, Pro5, and Pro8) are present in both p85a sequences, and they are indicated in boldface. The presence of these proline-rich sequences suggested that p85a may be sufficient to provide the direct association of PI 3-kinase with SH3 domains.

To study SH3/p85 association we opted for a baculovirus expression system where human p85a is overexpressed in a background of insect proteins (see "Experimental Procedures"). In this system the possibility that another protein may be mediating the binding of p85 to the SH3 domains is virtually eliminated allowing us to determine whether p85 binds directly to the SH3 domains. GST-SH3 fusion proteins on beads were assayed for PI kinase activity, whereas the GST-Fyn SH3 precipitates from HPB-ALL cells were assayed for PI kinase activity (Fig. 3). Both peptides were equally capable of inhibiting binding of p85a (Fig. 4A) and PI 3-kinase activity (Fig. 4B) to GST-Fyn SH3. The 50% maximal inhibition for both peptides occurred at a concentration between 200 and 400 μM peptide. In addition, an 85-kDa protein was the most abundant protein observed in GST-Fyn SH3 precipitates from SF9/p85a cells by Coomassie Blue staining and was the only one that disappeared when GST-Fyn SH3 beads were preincubated with proline-rich peptides, indicating that this interaction was not mediated by another protein endogenous to SF9 cells (data not shown). The control peptide had no effect on the binding of p85a to the Fyn SH3 domain; however, in the PI kinase assay it somewhat increased the total precipitable activity. Two other highly charged but unrelated peptides also caused a similar slight increase in precipitable PI 3-kinase activity when added at millimolar concentration (not shown), suggesting that this is a nonspecific effect.

2 The same effect was observed on immunoprecipitable PI 3-kinase activity when protein A-Sepharose beads were incubated with multiple control peptides, including polylysine, prior to immunoprecipitation.
A GST-fynSH3

P82-96  P300-314  P control

GST  0 µM  10 µM  100 µM  1000 µM

0 1 2 3 4 5 6 7 8 9 10 11 12

B

P control

P82-96

P300-314

GST-fynSH3

1 10 100 1000

Peptide Concentration (µM)

Fig. 4. The synthetic peptides P82-96 and P300-314 block the binding of baculovirus-p85α and PI 3-kinase to GST-Fyn SH3. A, immobilized GST-Fyn SH3 fusion proteins were incubated with 10, 100, or 1000 µM of peptides P82-96 (lanes 4–6), P300–314 (lanes 7–9), and P control (lanes 10–12) or no peptide (lane 3) for 5 min at 4 °C with constant rotation. 100 µl of SP9/p85α cell lysates were added per sample, and the beads were incubated for another 10 min at 4 °C with constant rocking. An anti-p85 immunoprecipitation (lane 1, positive control) and a GST precipitation (lane 2, negative control) were carried out in parallel. The beads were washed and processed as described (see "Experimental Procedures"). An anti-p85 immunoblot is shown. The position of p85 is indicated. The immunoblot was quantitated with a Bio-Rad imaging system. Normalizing to the amount of p85 detected in lane 3 (GST-Fyn SH3, no peptide) as 100%, lane 4 (10 µM P82-96) is also 100%, lane 5 (100 µM P82-96) is 77%, lane 6 (1000 µM P82-96) is 20%, lane 7 (10 µM P300–314) is 91%, lane 8 (100 µM P300–314) is 65%, and lane 9 (1000 µM P300–314) is 17%. The amounts of p85α detected in lanes 10–12 corresponding to the control peptide remained constant at about 95%, even at the highest concentration of peptide used. No p85α was detected in the GST lane (lane 2). B, immobilized GST-Fyn SH3 fusion proteins were incubated for 5 min at 4 °C with constant rocking with different concentrations of peptides P82-96, P300–314, and P control or no peptide, as indicated in the figure. 100-µl HPB-ALL cell lysates were added to individual samples, and they were further incubated for 10 min at 4 °C with rocking. A GST precipitation was carried out in parallel. The beads were washed and subjected to lipid kinase assay. Lipids were extracted and analyzed as described. Following separation by TLC, individual PIP spots were quantitated by a Bio-Rad imaging system. The data are expressed as percent of control, i.e. 100% corresponds to the amount of PI-3-P detected in the GST-Fyn SH3 precipitates in the absence of peptide. The data presented here are the average of three distinct experiments. GST (■), GST-Fyn SH3 + P82–96 (○), GST-Fyn SH3 + P300–314 (□), GST-Fyn SH3 + P control (▲).

investigated if the proline-rich peptides had any effect on the PI 3-kinase activity of purified enzyme. The peptides (P82-96, P300–314, and P control) were incubated for 5 min at room temperature with purified PI 3-kinase prior to lipid kinase assay. The peptides at concentrations ranging from 1 to 1000 µM had no apparent effect on the activity of purified PI 3-kinase (data not shown).

However, these peptides had no effect in the activity of soluble PI 3-kinase. These data suggest that the peptides do not specifically affect the soluble enzymatic activity but that they rather preserve the enzymatic activity of PI 3-kinase during precipitation, probably by blocking hydrophobic sites on the beads that cause inactivation.

DISCUSSION

In this report we investigate the mechanism of association of PI 3-kinase with SH3 domains. We have shown previously that PI 3-kinase associates with the SH3 domains of the Src-like kinases p56lck (27) and p55γ (T) (20). In this study, we provide evidence that the 85-kDa subunit of PI 3-kinase mediates the association with the SH3 domains of Lck, Fyn, Abl, and p85 itself, suggesting that PI 3-kinase may be a target of several SH3-containing proteins. These results indicate that PI 3-kinase may use two modes for its interaction with upstream elements: one that is dependent on tyrosine phosphorylation.
and is mediated by its SH2 domains as in the case of its association to activated receptor-tyrosine kinases and one that is phosphorylation-independent, in which p85 associates with the SH3 domains of upstream molecules. Both interactions may occur in parallel in the case of activated cytosolic-tyrosine kinases that contain SH2 and SH3 domains.

Examination of the p85 amino acid sequence revealed the presence of two proline-rich motifs highly homologous to the ones identified in 3BP1, 3BP2, and SOS. These sequences have been implicated in mediating the interaction of 3BP1 and 3BP2 to the Ab1 SH3 domain and SOS to the SH3 domains of Grb2/SEM-5/Drk (13-17, 19). In this work, we show that the p85 proline-rich sequences are indeed SH3-binding motifs. Both sequences (P62-96 and P300-314, synthetic peptides) were able to inhibit binding of PI 3-kinase activity and p85α to a GST-Fyn SH3 fusion protein, whereas an unrelated sequence had no effect. The peptide concentrations required for 50% inhibition of SH3 binding to p85 (~200 μM) were quite high compared with concentration of phosphopeptides that inhibit SH2 domain interactions (~10 μM). However, they were similar to the concentrations of dynamin peptides that bind to the SH3 domain of Drk (30-34). The affinity of the GST-SH3 to retain association with bacular-upregulated p85 despite relative harsh washing conditions suggests that the affinity of p85 for SH3 domains is much higher than is the affinity of the individual proline-rich peptides. This could be explained by the ability of multiple SH3 domains on the same bead to simultaneously bind a single p85 protein via interaction with both proline-rich domains. The actual affinity of the proline-rich peptides for immobilized SH3 domains are probably underestimated in the competition experiment (Fig. 4) because of the entropic advantage of binding the bidentate sites in p85 over the individual peptides. Preliminary experiments using full-length p85 to block binding of lymphocyte-derived PI 3-kinase to the Ab1 SH3 domain and SOS to the SH3 domains of Grb2/SEM-5/Drk (13), all of which have been shown to contain GTPase activity, were quite high with concentrations of dynamin peptides that bind to the SH3 domain of Drk (30-34). The SH3 domain of p85 binds to a single p85 protein via interaction with both SH3 domains and SOS, respectively, and has revealed the mechanism by which tyrsoine kinases may communicate with the ras signaling pathway (13-17, 19). In this work, we show that the p85 SH3 domain associates to regulate interactions with other proteins.

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