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A Region of the 85-Kilodalton (kDa) Subunit of Phosphatidylinositol 3-Kinase Binds the 110-kDa Catalytic Subunit In Vivo

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Phosphatidylinositol (PI) 3-kinase is a heterodimer consisting of an 85-kDa subunit (p85) and 110-kDa subunit (p110). The 85-kDa noncatalytic subunit, which contains two Src homology 2 (SH2) domains, one SH3 domain, and a domain homologous to the carboxy terminus of the breakpoint cluster region gene product, is known to mediate the association of the PI 3-kinase complex with activated growth factor receptors. We previously demonstrated that the C-terminal SH2 domain of p85 is responsible for the interaction of PI 3-kinase with phosphorylated platelet-derived growth factor receptor. To define the region in p85 that directs the complex formation with the PI 3-kinase catalytic subunit, a series of truncated p85 mutants was analyzed for association with p110 in vivo. We found that a fragment of p85 containing the region between the two SH2 domains was sufficient to promote the interaction with p110 in vivo. The complex between the fragment of p85 and p110 had PI 3-kinase activity that was comparable in magnitude to the activity of p110 associated with full-length p85. The binding with p110 was abolished when this domain in p85 was disrupted. These results identify a novel structural and functional element that is responsible for localizing the catalytic subunit of PI 3-kinase.

Phosphatidylinositol (PI) 3-kinase was discovered as an activity that phosphorylates PI at the D-3′ position of the inositol ring (1, 33). It was proposed that 3′-phosphorylated inositides might represent a novel class of second messenger molecules, the functions and downstream effectors of which remain to be elucidated. Further interest in PI 3-kinase was gained by experiments demonstrating the important regulatory role of this activity in cell growth and transformation. Studies on mutants of polyomavirus middle-T antigen, $pp60^{v\text{-}src}$, and $pp130^{gag\text{-}abl}$ showed a strong correlation between oncogene-associated PI 3-kinase activity and cell transformation (2). Platelet-derived growth factor receptor mutants that are unable to bind PI 3-kinase are also unable to induce a mitogenic response after growth factor stimulation (6, 9, 12).

Purified PI 3-kinase was shown to be a heterodimer consisting of an 85-kDa (p85) and a 110-kDa (p110) subunit (4, 25, 30). The amino acid sequence of p85, which was cloned from different sources, revealed that this subunit contains several functional domains (8, 27, 28, 31) (Fig. 1): a Src homology 3 (SH3) domain, a domain with homology to the breakpoint cluster region (*bcr*), and two SH2 domains, which are separated by a region encompassing 200 amino acids with a predicted α-helical structure named here the inter-SH2 (iSH2) region. p85 is thought to function as an adaptor molecule, since it mediates the interaction of PI 3-kinase with activated receptor tyrosine kinases and the middle-T antigen–$pp60^{c\text{-}src}$ complex but does not have PI 3-kinase activity itself (7, 8, 27). It is well established that the SH2 domains of p85 direct the interaction of PI 3-kinase and tyrosine kinase complexes (16, 19, 24, 29, 35, 36). The cDNA for the 110-kDa subunit has recently been cloned, and the protein was shown to have PI 3-kinase activity after expression in insect and mammalian cell systems (15).

The regulation of PI 3-kinase activation after growth factor stimulation is not yet understood. The growth factor-stimulated translocation of the enzyme to the plasma membrane, where it binds to tyrosine-phosphorylated proteins and where its substrate is located, is thought to be important in the action of PI 3-kinase. Alternatively, a change in the phosphorylation state of the subunits after association with activated receptor molecules may induce a conformational change and thereby modulate PI 3-kinase activity (3, 18, 32).

In this study, we demonstrate that coexpression of a small fragment of previously unknown function within p85, which encompasses the regions between the two SH2 domains, is sufficient to direct the association with p110 in vivo. We also show that the protein complex formed between the iSH2 region of p85 and the 110-kDa subunit has PI 3-kinase catalytic activity even though the other domains of p85 are not present.

MATERIALS AND METHODS

Cell culture. COS-7 and CHO-K1 cells were obtained from the American Type Culture Collection. COS-7 cells were cultured at 37°C in Dulbecco's modified Eagle medium containing 10% bovine calf serum, penicillin (50 μg/ml), and streptomycin (50 μg/ml). CHO cells were grown in Ham's F12 medium supplemented with 10% calf serum, penicillin (50 μg/ml), and streptomycin (50 μg/ml). Hygromycin-resistant cell transfectants were propagated in the same medium containing hygromycin B (200 μg/ml; Calbiochem). Cotransfection of CHO cells with expression vectors containing p85 fragments and pL1-3hyg was carried out by using Lipofectin (Bethesda Research Laboratories) according to the manufacturer's instructions. Cell lines stably expressing p85 fragments were established by selecting for resistance against

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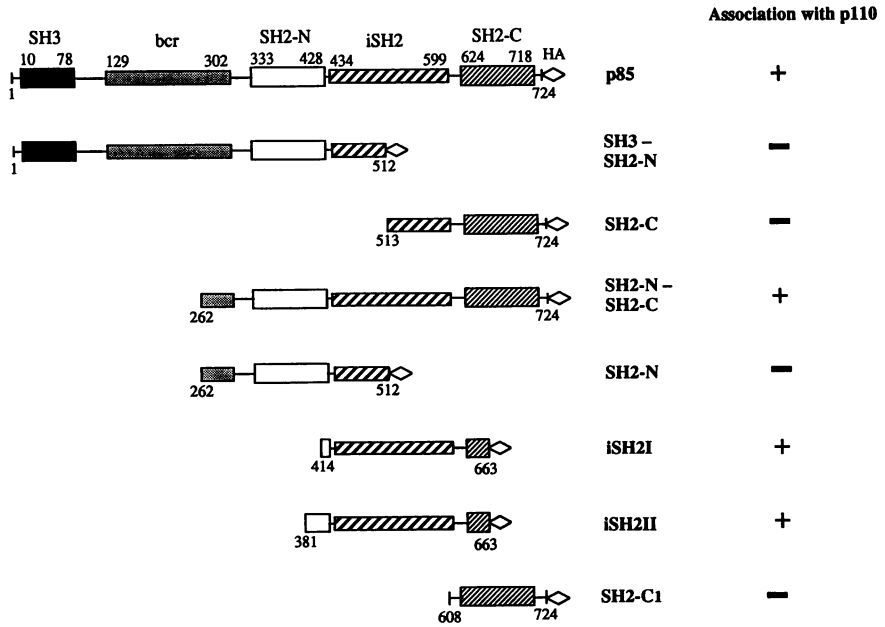

FIG. 1. Schematic structure of full-length p85 and p85 fragments expressed in COS and CHO cells. The presumed functional domains SH3, *bcr*, SH2-N, iSH2, and SH2-C are represented by black, shaded, white, and hatched boxes as indicated above the diagram for the full-length p85 molecule. The first and the last amino acids of each domain are numbered according to their relative positions within the wild-type p85 sequence (the initiator methionine is assigned position 1). The borders of the respective domains were numbered according to references 21 and 28. The first and last amino acids of each recombinant p85 fragment are indicated. The influenza virus HA epitope, which was fused to the C-terminal end of each construct, is indicated by a diamond. The ability to associate with p110–PI 3-kinase activity is shown at the right of each fragment (+, binding to p110; –, no binding to p110).

hygromycin B. COS-7 cells (60 to 70% confluent on a 10-cm-diameter plate) were transiently transfected by the DEAE-dextran method (13). At 72 h after transfection, the cells were lysed. The lysates containing recombinant full-length p85 and fragments of p85 were tested for the ability to associate with PI 3-kinase activity (see below). *Escherichia coli* K-12 strain DH5α (Bethesda Research Laboratories) was used for plasmid propagation.

Antibodies. The rabbit anti-p110 antibodies were directed against synthetic peptides derived from amino acids 127 to 145 (R7826), 513 to 532 (R7481), and 715 to 743 (R7484) of the mouse p110 gene (10). Murine anti-p85 monoclonal antibody FIA was raised against the purified SH2-C_1 fragment (spanning amino acids 608 to 724 of p85) expressed in *E. coli*. The anti-p85 rabbit polyclonal antibody R1176 was raised against the purified N-terminal fragment containing amino acids 1 to 282 of p85 and used at a 1:2,000 dilution for immunoprecipitations. Ascites fluid with the murine anti-influenza virus hemagglutinin 1 (HA1) monoclonal antibody 12CA5 (34) was prepared and used at dilutions of 1:2,500 for immunoprecipitations and 1:3,000 for immunoblotting. The murine monoclonal anti-Myc antibody 9E10 (11) was purified from tissue culture supernatant.

Plasmid constructions. Mammalian expression vectors containing p85 fragments to be expressed in COS or CHO cells are shown schematically in Fig. 1. The tagged C-terminal SH2 domain of p85 (SH2-C) was generated by using mouse p85α cDNA (8) as the template with primer tk · SH2-C (5′ CT TCT AGA ATG GCT CAT ATG AAA CGC GAA GGC AAC GAG AAA GAA 3′), containing nucleotides 1537 to 1560 of the coding strand extended by *Xba*I and *Nde*I restriction sites, and primer SH2-C · HA (5′ TAT GGA TCC TCA GGA AGG TCC TCC CAG GCT GGC ATA GTC AGG CAC GTC ATA AGG ATA GCT TCC CCC GGG TCG CCT CTG TTG TGC ATA TAC TGG GTA 3′). Alternatively, primer SH2-C · mycN (5′ TAT GGA TCC TCA GTT CAG GTC CTC CTC GGA AAT CAG CTT CTG CTC TCC CCC GGG TCG CCT CTG TTG TGC ATA TAC TGG GTA 3′) was used to modify the 3′ end of the SH2-C sequence (A of the start codon is nucleotide 1; nucleotides that are changed with respect to the wild-type sequence are underlined). This generated fragments in which the wild-type p85 coding region was extended by sequences encoding amino acids PGG as a hinge region (overlapping a *Sma*I site), which precedes the 16-amino-acid HA epitope (SYPYDVPDYASL GGPS [34]) or the 10-amino-acid Myc epitope (EQKLISE EDL [11]), followed by a stop codon and a *Bam*HI site. The DNA ends were repaired with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase. The DNA fragments were ligated into the *Sma*I site of pBluescript II KS+ (pKS) DNA (Stratagene), in which the *Acc*I polylinker site had been deleted. The sequences were confirmed by DNA sequence analysis.

For expression of p85 and various p85 fragments tagged with the HA or Myc epitope in COS-7 or CHO cells, the respective DNA fragments were cloned into the mammalian expression vector pCG via *Xba*I-*Bam*HI ends. pCG is a derivative of pEVRF (23) with a modified polylinker and contains the human cytomegalovirus enhancer/promoter region and the translation initiation region of the herpes simplex virus thymidine kinase (*tk*) gene. This generated translational fusions of the tagged SH2-C constructs with the first three amino acids of the herpes simplex virus *tk* gene. The following expression vectors were prepared by combining restriction fragments of the pKS and pCG constructs described above with restriction fragments of the p85 cDNA

or previously reported constructs (18, 19): pCG-85 · HA and pCG-85 · mycN, containing the N-terminal portion of p85 on an *Nde*I-*Acc*I fragment (nucleotides 1 to 1989 of the p85 coding region) and an *Acc*I-*Bam*HI DNA fragment with the remainder of SH2-C (nucleotides 1990 to 2172) and the respective tag sequences; pCG-SH2-N–SH2-C · HA, containing the two SH2 domains of p85 in their wild-type configuration on a *Bsm*I-*Bam*HI DNA fragment (nucleotides 784 to 2172); pCG-SH3–SH2-N, covering 70% of the p85 N-terminal sequence (nucleotides 1 to 1536) with the SH3 domain, the *bcr* homology region, and the N-terminal SH2 domain (SH2-N) on an *Nde*I-*Dra*I fragment; pCG-iSH2 · HA I and II, expressing the iSH2 region on a *Ple*I-*Acc*I fragment (nucleotides 1240 to 1989) or an *Asn*I-*Acc*I fragment (nucleotides 1141 to 1989); and pCG-SH2-C · HA, encoding the C-terminal SH2 domain on a *Dra*I-*Bam*HI fragment (nucleotides 1537 to 2172). pCG-SH2-C_1 · HA, coding for SH2-C on an *Nde*I-*Bam*HI fragment (nucleotides 1822 to 2172), was obtained from a polymerase chain reaction fragment by using primer SH2-C_1 (5′ GAA GAT CAT ATG AGT CTG GTA GAA GAT GAT GAG 3′), introducing a start codon and an *Nde*I restriction site at the 5′ end, and a primer overlapping a vector sequence downstream of the *Bam*HI site of pKS-SH2-C · HA.

Vector pL1-3hyg was used for cotransfections in CHO cells and confers hygromycin B resistance. It carries the *E. coli* gene for hygromycin phosphotransferase under control of a modified *tk* enhancer-promoter region and the *tk* polyadenylation signal (14) on a pKS origin.

In vivo association of p85 and p110. COS-7 or CHO transfectants were washed twice with cold phosphate-buffered saline. Cell lysates were prepared as previously described (26). In brief, cells were treated with lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 20 μM leupeptin, and 1 mM sodium vanadate. Lysates were cleared by centrifugation at 14,000 × *g* for 10 min and incubated with monoclonal anti-HA antibody 12CA5 overnight at 4°C. Protein A-Sepharose beads (Sigma) were used to precipitate the immune complexes. The beads were washed twice with wash buffer 1 (50 mM Tris-HCl [pH 7.5], 0.5 M LiCl, 0.5% Triton X-100), twice with wash buffer 2 (50 mM Tris-HCl [pH 7.5], 0.5 M LiCl), and once with distilled water. Proteins associated with the p85 fragments were analyzed by immunoblotting with the appropriate antibodies or by PI 3-kinase assays as described below.

Immunoblotting. p85 immunoprecipitates were boiled in Laemmli sample buffer (22), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked in TBST buffer (10 mM Tris-HCl [pH 7.5], 0.9% NaCl, 0.05% Tween 20, 0.2% sodium azide) containing 3% dried milk. The respective antibodies were added in TBST at appropriate dilutions. Bound antibody was detected with anti-mouse or anti-rabbit antibody conjugated to alkaline phosphatase (Promega) in TBST, washed, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega).

Determination of PI 3-kinase activity in p85 immune complexes. Lysates were immunoprecipitated with antibody 12CA5 or 9E10. p85 immune complexes were precipitated with protein A-Sepharose beads. After a series of sequential washes (see above), the presence of PI 3-kinase activity in the complexes was determined by incubating the beads with 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4)–30 mM $MgCl_2$–50 μM ATP–200 μM adenosine–0.2 mg of sonicated PI per ml–10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol) for 20 min at 25°C. Adenosine was added to inhibit any contaminating PI 4-kinase activity (33). Reactions were stopped by adding 100 μl of 1 M HCl, and the phospholipids were extracted with 200 μl of a 1:1 mixture of chloroform and methanol. The reaction products were separated by thin-layer chromatography as previously described (17). The conversion of PI to PI 3-phosphate was determined by autoradiography and quantitated by using a PhosphorImager (Molecular Dynamics).

Metabolic labeling of COS-7 cells. Transiently transfected COS-7 cells were metabolically labeled by incubating them for 6 h at 37°C in methionine-free Dulbecco's modified Eagle medium with 0.5 mCi of [^{35}S]methionine per 10-cm-diameter plate. Cell extracts were incubated with antibody 12CA5 as described above. The precipitates were separated by SDS-PAGE and analyzed by fluorography.

RESULTS

Identification of the region in p85 that mediates association with PI 3-kinase activity in COS-7 cells. To identify the region in p85 responsible for the interaction with the 110-kDa catalytic subunit of PI 3-kinase, we expressed various truncations of p85 tagged at the C terminus with a 16-amino-acid epitope of influenza virus HA1, which is recognized by monoclonal antibody 12CA5 (34). Vectors expressing the respective p85 constructs under control of the human cytomegalovirus promoter were transiently transfected into COS-7 cells (Fig. 1).

COS cell lysates containing recombinant full-length p85, a fragment spanning the SH3 domain together with the *bcr* homology region and the N-terminal SH2 domain (SH3–SH2-N), a fragment overlapping both SH2 domains of p85 (SH2-N–SH2-C), two fragments each encompassing the region between the SH2 domains with different sizes of flanking regions (iSH2I and iSH2II), the C-terminal SH2 domain with a 111-N-terminal-amino-acid flanking region (SH2-C), and a fragment with the C-terminal SH2 domain and 17-amino-acid flanking region (SH2-C_1) (Fig. 1) were immunoprecipitated with antibody 12CA5. COS cell lysate containing p85 tagged with a 13-amino-acid Myc-derived epitope was immunoprecipitated with monoclonal antibody 9E10 (11) as an additional control. The immune complexes were immobilized on protein A-Sepharose beads and washed extensively. The presence of PI 3-kinase activity was assayed by measuring PI 3-phosphate production in the immune complexes (see Materials and Methods). The relative amounts of p85 and mutant p85 proteins in the complexes were similar in all samples (Fig. 2B). As shown in Fig. 2A, only fragments which contain the iSH2 region (full-length p85, SH2-N–SH2-C, iSH2I, and iSH2II) precipitated PI 3-kinase activity. No or very little PI 3-kinase activity was associated with fragments containing either half of the iSH2 region (SH3–SH2-N or SH2-C), even when those half-sites were attached to a longer adjacent portion of the p85 molecule.

To obtain more quantitative information, the products of the PI 3-kinase reaction were analyzed by using a PhosphorImager. The amounts of PI 3-kinase activity associated with the various p85 fragments (Fig. 2C) suggested that the region between the SH2 domains is important for p110 binding.

Despite the presence of PI 3-kinase activity in a complex with p85 fragments, it was not possible to detect p110 protein by this transient assay, either by metabolic labeling of COS

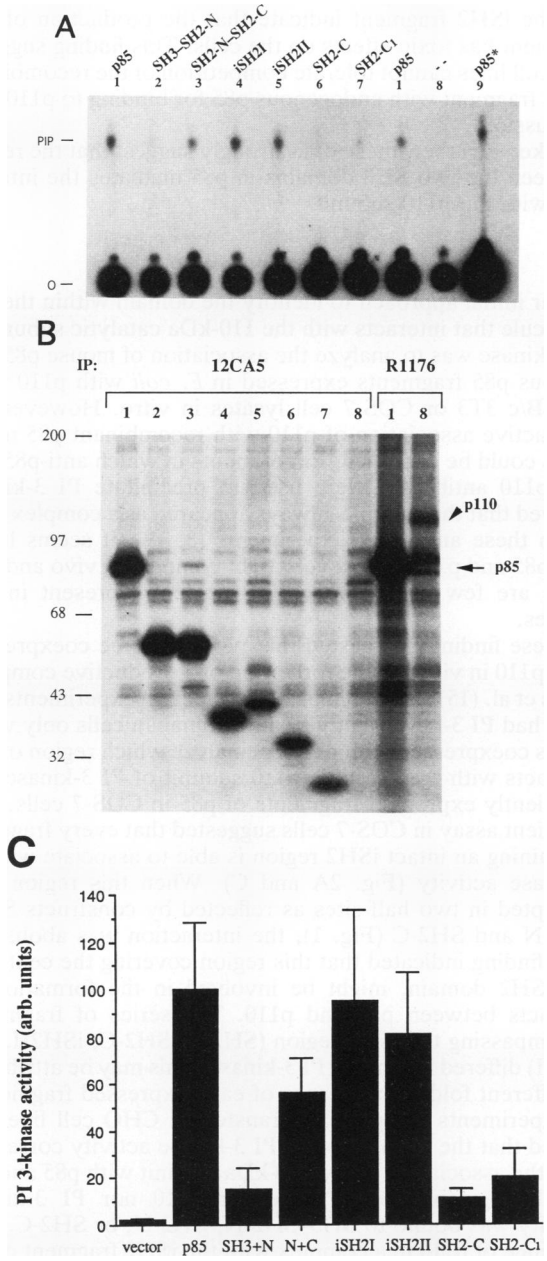

FIG. 2. (A) Association of transiently expressed p85 fragments with PI 3-kinase in COS-7 cells. COS-7 cells were transiently transfected with expression vectors containing full-length p85, p85 fragments, or vector DNA alone as shown above each lane. Cell lysates containing HA-tagged fragments and control lysate were immunoprecipitated with antibody 12CA5 (lanes 1 to 8); lysate with Myc-tagged p85 (p85*) and control lysate were immunoprecipitated with antibody 9E10 (lanes 8 and 9). After serial washes, the immune complexes were analyzed for the presence of PI 3-kinase activity by thin-layer chromatography as described in Materials and Methods. The origin (O) of the chromatogram and the position of PI 3-phosphate (PIP) are shown. (B) Levels of p85 and p85 fragments expressed in COS cells. Transiently transfected COS cells were metabolically labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with anti-tag antibody 12CA5. After extensive washes, the immune complexes were separated by 7 to 12% step gradient SDS-PAGE. Immunoprecipitations with anti-p85 antibody R1176 were performed to determine the positions of recombinant p85 and COS cell-derived p85 and p110. Numbers above the lanes correspond to numbers of the samples shown in panel A. The positions of p110 and p85 molecules are indicated on the right. Molecular size markers are indicated in kilodaltons on the left. (C) Relative amounts of PI 3-kinase activity associated with transiently expressed p85 and p85 fragments. PI 3-phosphate production determined by the assay described for panel A was quantitated with a PhosphorImager. Each bar represents the mean of at least three data points ± standard deviation for each p85 fragment. The relative amount of bound PI 3-kinase is given in arbitrary (arb.) units. SH3+N and N+C represent fragments SH3–SH2-N and SH2-N–SH2-C, respectively.

cells with [^{35}S]methionine (Fig. 2B) or by immunoblotting with an anti-p110 antibody (not shown). Most likely, only a very small fraction of the excess recombinant p85 molecules generated after transient transfection could associate with endogenous p110 within each COS cell; therefore, the association of PI 3-kinase with p85 was undetectable by any method except the very sensitive PI 3-kinase activity assay. To circumvent the problem of detecting p110 in complexes derived from transiently transfected cells, we studied this issue in cell lines stably expressing recombinant p85 fragments.

The 110-kDa subunit of PI 3-kinase is associated with the iSH2 region of p85 in vivo. Expression vectors coding for recombinant p85, SH3–SH2-N, SH2-N, SH2-C, and iSH2 with the HA1 tag were stably transfected into CHO cells. Several cell lines expressing comparable amounts of the different p85 fragments were selected. To analyze the association of recombinant p85 and its smaller derivatives with endogenous p110 in CHO cells, cell lysates were immunoprecipitated with antibody 12CA5. After several stringent washes of the beads, the presence of p110 in the immune complexes was monitored by immunoblotting with a mixture of three polyclonal anti-p110 antibodies. As shown in Fig. 3A, full-length p85 as well as iSH2I coprecipitated p110. No full-length p85 was detected in the immune complexes with iSH2I, indicating that the iSH2I fragment did not bind to p85 from lysate. Therefore, the association of p110 and iSH2I could not be attributed to coprecipitation of p85 and iSH2. In CHO lysates containing SH3–SH2-N, SH2-N, and SH2-C and in untransfected cell lysate, no p110 band was detected.

The iSH2 region of p85 can form a functional complex with PI 3-kinase in vivo. To determine whether the association with p110 shown in Fig. 3A corresponds to the presence of active PI 3-kinase, lysates from CHO cells stably expressing p85, SH3–SH2-N, SH2-N, SH2-C, or iSH2I were immunoprecipitated with antibody 12CA5 and the precipitates were analyzed by a PI 3-kinase activity assay. Full-length p85 as well as the iSH2 fragment coprecipitated PI 3-kinase activity from CHO cell lysates (Fig. 3B). The amount of PI 3-phosphate generated correlated with the relative amounts of p110 associated with p85 or iSH2I fragments in the immune complexes (Fig. 3A). No PI-3 kinase activity above the background level was observed in immunoprecipitates from cell lysates containing SH3–SH2-N, SH2-N, or SH2-C fragments, which correlated with the lack of p110 association with these fragments. A quantitative evaluation of several experiments obtained from at least two independently isolated cell lines of each construct is depicted in Fig. 3C. The only fragment capable of forming a functional complex with PI 3-kinase comparable to that of full-length p85 is the fragment containing the complete iSH2 region of p85. The amount of p110–PI 3-kinase activity associated with iSH2I was approximately 30% of that of full-length p85. Data obtained from approximately 10 different cell lines express-

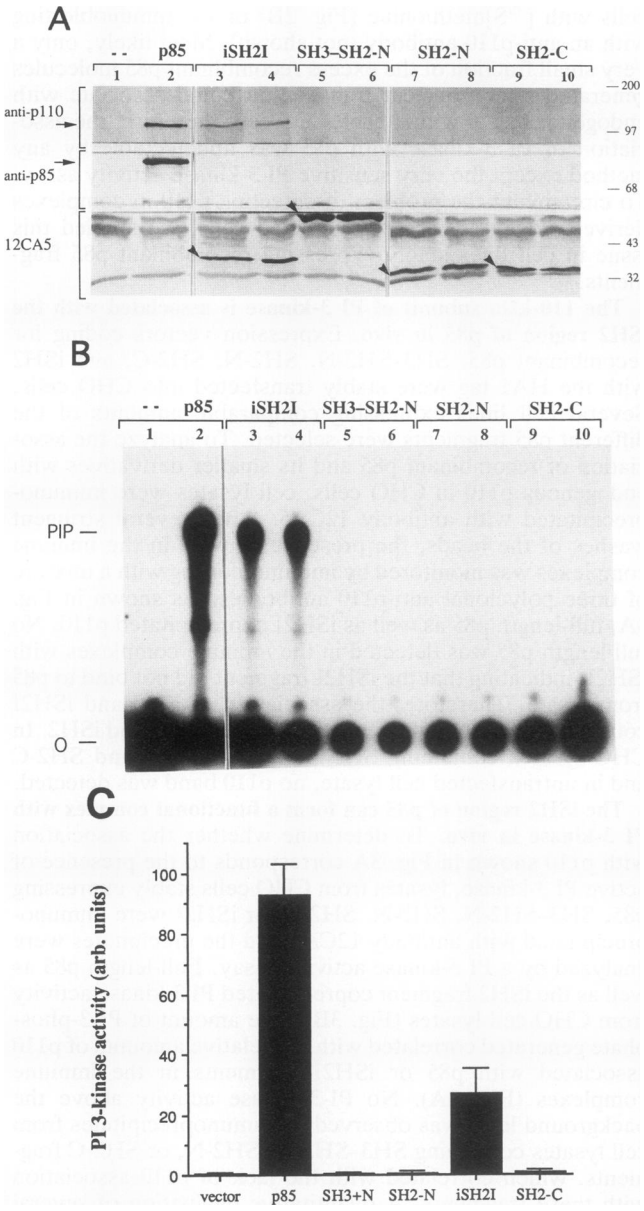

FIG. 3. In vivo association of full-length p85 and p85 fragments with p110. Lysates of CHO cell lines stably expressing full-length p85 or different p85 fragments were immunoprecipitated with antibody 12CA5. After extensive washes, the immune complexes were analyzed in parallel for the presence of the 110-kDa subunit of PI 3-kinase (A) and for the presence of PI 3-kinase activity (B). Lanes: 1, untransfected cell lysate; 2, lysate from a cell line expressing full-length p85, which served as a positive control; 3 and 4, lysates from CHO lines expressing the iSH2 fragment, lysates containing SH3–SH2-N (lanes 5 and 6), SH2-N (lanes 7 and 8) and SH2-C (lanes 9 and 10) from two independent cell lines each. The lanes shown are all part of one experiment analyzed in parallel on two gels. In panel A, the immune complexes were separated by 7 to 12% step gradient SDS-PAGE and transferred to nitrocellulose membranes. p110 molecules coprecipitating with full-length p85 or with p85 fragments were visualized by immunoblot analysis using a mixture of anti-p110 antibodies. The positions of associated p110 from CHO cells and of recombinant p85 are indicated by arrows on the left. The positions of the recombinant iSH2I, SH3–SH2-N, SH2-N, and SH2-C fragments are marked by arrowheads in the panel labeled 12CA5. The immunoglobulin G heavy-chain (50- and 54-kDa) and light-chain (34-kDa) bands of antibody 12CA5 used for the immunoprecipitations were also recognized by the alkaline phosphatase-conjugated anti-mouse antibody. Fragment SH3–SH2-N comigrates with the upper band of the immunoglobulin G heavy chain but can be distinguished by its darker staining. Molecular size markers on the right are indicated in kilodaltons. In panel B, the immune complexes were analyzed for PI 3-kinase activity. The origin (O) of the chromatogram and the position of PI 3-phosphate (PIP) are indicated. (C) Relative amounts of PI 3-kinase activity associated with p85 and p85 fragments stably expressed in CHO cells. PI 3-phosphate production of immune complexes analyzed as shown in panel B was quantitated by using a Phosphor-Imager. Each bar represents the mean of at least four data points ± standard deviation obtained from two or more cell lines expressing the respective p85 fragments. The relative amounts of bound PI 3-kinase are given in arbitrary (arb.) units. SH3+N stands for fragment SH3–SH2-N.

ing the iSH2 fragment indicate that the production of this fragment has toxic effects on the cells. This finding suggests that cell lines cannot tolerate competition of the recombinant iSH2 fragment with endogenous p85 for binding to p110 (see Discussion).

Taken together, these data strongly suggest that the region between the two SH2 domains in p85 mediates the interaction with the p110 subunit.

DISCUSSION

Our initial approach to identify the domain within the p85 molecule that interacts with the 110-kDa catalytic subunit of PI 3-kinase was to analyze the association of mouse p85 and various p85 fragments expressed in *E. coli* with p110 from BALB/c 3T3 or COS-7 cell lysates in vitro. However, no productive association of p110 with recombinant p85 molecules could be observed. Experiments in which anti-p85 and anti-p110 antibodies were used to precipitate PI 3-kinase showed that the subunits always appeared as a complex (10). From these and other experiments (8, 19), it seems likely that p85 and p110 form a very tight complex in vivo and that there are few if any free p110 molecules present in cell lysates.

These findings suggested that p85 had to be coexpressed with p110 in vivo for the formation of a productive complex. Hiles et al. (15) showed in p110 transfection experiments that p110 had PI 3-kinase activity in mammalian cells only when it was coexpressed with p85. To analyze which region of p85 interacts with the catalytic p110 subunit of PI 3-kinase, we transiently expressed fragments of p85 in COS-7 cells. The transient assay in COS-7 cells suggested that every fragment containing an intact iSH2 region is able to associate with PI 3-kinase activity (Fig. 2A and C). When this region was disrupted in two half-sites as reflected by constructs SH3–SH2-N and SH2-C (Fig. 1), the interaction was abolished. This finding indicated that this region covering the center of the iSH2 domain, might be involved in the formation of contacts between p85 and p110. The series of fragments encompassing the iSH2 region (SH2-N–SH2-C, iSH2II, and iSH2I) differed in binding PI 3-kinase. This may be attributed to different folding properties of each expressed fragment.

Experiments using stably transfected CHO cell lines revealed that the association of PI 3-kinase activity correlates with the association of the 110-kDa subunit with p85 and the iSH2I fragment (Fig. 3). Neither p110 nor PI 3-kinase activity was bound to SH3–SH2-N, SH2-N, or SH2-C. The presence of p110 in a complex with the iSH2 fragment could not be attributed to dimerization of iSH2 with p85, which in turn bound to p110, since no p85 was detected in immuno-

precipitations of iSH2 probed with an anti-p85 antibody (Fig. 3A). The iSH2 fragment also did not coprecipitate p110 by binding to activated receptor molecules that interact with PI 3-kinase (not shown). Receptor association is unlikely to occur, since iSH2 does not contain a functional SH2 domain. Therefore, the iSH2 region by itself must represent a structural domain within the p85 molecule which mediates specific protein-protein contacts to p110. The association between the iSH2 region of p85 and p110 is independent of the presence of SH2 domains and is thought to represent a high-affinity interaction, because the complex can be disrupted only by using denaturing conditions.

The association of p85 or the isolated iSH2 domain with the catalytic p110 subunit of PI 3-kinase was not affected by stimulation of the cells with platelet-derived growth factor (20). This result further supports our finding that p85 and p110 always form a tight complex. The observation that the iSH2 region by itself is capable of forming a functional complex with the p110 subunit (Fig. 2A and 3B) suggests that neither the SH2 domains nor the SH3 domain of p85 are required for the catalytic activity of p110. This observation also suggests that p85 might play a role solely as an adaptor molecule that links the catalytic subunit of PI 3-kinase bound to the iSH2 region to activated growth factor receptor molecules.

The stably expressed iSH2I fragment bound comparable amounts of p110–PI 3-kinase; however, it did not reach quite the same efficiency as did full-length p85 (Fig. 3C). Irrespective of its expression level, the iSH2I fragment associated on average with 30% of the number of PI 3-kinase molecules relative to p85. Analyzing a number of independently isolated cell lines expressing the iSH2 fragment, we observed that the respective cell lines could be propagated for only a limited number of passages, which indicates a possible toxic effect of the fragment. We believe that the iSH2I fragment might act as a dominant negative p85 mutant that competes with endogenous p85 for the association with p110. Association of the catalytic PI 3-kinase subunit with iSH2I instead of p85 renders it unresponsive to regulatory signals of activated growth factor receptors. Since PI 3-kinase is thought to play an important role in the regulation of cell growth, viable cells might not be able to tolerate a dramatic change in their PI 3-kinase level.

In this report, we describe a novel protein-protein interaction between the iSH2 domain of the p85 subunit and the catalytic p110 subunit of PI 3-kinase. Panayotou et al. (28) predicted that the iSH2 region of p85 adopts an α-helical structure. This independent structural unit is suggested to occupy most of the iSH2 region space. Our finding that the binding to p110 was completely abrogated when the iSH2 region was disrupted at amino acids 512 to 513 (Fig. 2A; Fig. 3A and B) representing the center of the presumed functional domain would therefore agree with those data. The minimal region of the iSH2 domain necessary for the interaction with p110 remains to be determined.

Our data disagree with results obtained by Cooper and Kashishian, who suggest that both SH2 domains of p85 individually bind to p110 after transient transfection, as judged from PI 3-kinase activity assays (5). At present we have no explanation for their findings. The experiments described here clearly demonstrate that the p85 SH2 domains do not interact with the 110-kDa subunit of PI 3-kinase (Fig. 2A; Fig. 3A and B). Furthermore, in our study both SH2 domains were expressed as significantly larger fragments containing flanking regions and were found to represent functional SH2 domains in vivo (20). Our study, which is based on a stable expression system, suggests that the iSH2 region of p85 is responsible for the interaction with p110 because of (i) the finding that exclusively iSH2 binds the 110-kDa protein and (ii) the presence of PI 3-kinase activity in the same complex in vivo.

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REFERENCES

1. **Auger, K. R., L. A. Serunian, S. P. Scoltoff, P. Libby, and L. C. Cantley.** 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell **57**:167–175.
2. **Cantley, L., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64**:281–302.
3. **Carpenter, C. L., K. R. Auger, B. C. Duckworth, W.-M. Hou, B. Schaffhausen, and L. C. Cantley.** 1993. A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. Mol. Cell. Biol. **13**:1657–1665.
4. **Carpenter, C. L., B. C. Duckworth, K. R. Auger, B. Cohen, B. S. Schaffhausen, and L. C. Cantley.** 1990. Purification and characterization of phosphoinositide 3-kinase from rat liver. J. Biol. Chem. **265**:19704–19711.
5. **Cooper, J. A., and A. Kashishian.** 1993. In vivo binding properties of SH2 domains from GTPase-activating protein and phosphatidylinositol 3-kinase. Mol. Cell. Biol. **13**:1737–1745.
6. **Coughlin, S. R., J. A. Escobedo, and L. T. Williams.** 1989. Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science **243**:1191–1194.
7. **Escobedo, J. A., D. R. Kaplan, W. M. Kavanaugh, C. W. Turck, and L. T. Williams.** 1991. A phosphatidylinositol-3 kinase binds to platelet-derived growth factor receptors through a specific receptor sequence containing phosphotyrosine. Mol. Cell. Biol. **11**:1125–1132.
8. **Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams.** 1991. cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β-receptor. Cell **65**:75–82.
9. **Escobedo, J. A., and L. T. Williams.** 1988. A PDGF receptor domain essential for mitogenesis but not for many other responses to PDGF. Nature (London) **335**:85–87.
10. **Escobedo, J. A., and L. T. Williams.** Unpublished data.
11. **Evan, G., G. Lewis, G. Ramsay, and J. M. Bishop.** 1985. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. Mol. Cell. Biol. **5**:3610–3616.
12. **Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. del Rosario, F. McCormick, and L. T. Williams.** 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell **69**: 413–423.
13. **Gorman, C.** 1985. High efficiency gene transfer into mammalian cells, p. 143–190. *In* D. M. Glover (ed.), DNA cloning, a practical approach, vol. II. IRL Press, Oxford.
14. **Groger, R. K., D. M. Morrow, and M. L. Tykocinski.** 1989. Directional antisense and sense cDNA cloning using Epstein-Barr virus episomal expression vectors. Gene **81**:285–294.
15. **Hiles, I. D., M. Otsu, S. Volinia, M. J. Fry, I. Gout, R. Dhand, G. Panayotou, F. Ruiz-Larrea, A. Thompson, N. F. Totty, J. J. Hsuan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. Cell **70**:419–429.
16. **Hu, P., B. Margolis, E. Y. Skolnik, R. Lammers, A. Ullrich, and J. Schlessinger.** 1992. Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-

derived growth factor receptors. Mol. Cell. Biol. **12**:981–990.
17. **Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M. White, L. Cantley, and T. M. Roberts.** 1986. Phosphatidylinositol metabolism and polyoma-mediated transformation. Proc. Natl. Acad. Sci. USA **83**:362–364.
18. **Kavanaugh, W. M., A. Klippel, J. A. Escobedo, and L. T. Williams.** 1992. Modification of the 85-kilodalton subunit of phosphatidylinositol-3 kinase in platelet-derived growth factor-stimulated cells. Mol. Cell. Biol. **12**:3415–3424.
19. **Klippel, A., J. A. Escobedo, W. J. Fantl, and L. T. Williams.** 1992. The C-terminal SH2 domain of p85 accounts for the high affinity and specificity of the binding of phosphatidylinositol 3-kinase to phosphorylated platelet-derived growth factor β receptor. Mol. Cell. Biol. **12**:1451–1459.
20. **Klippel, A., J. A. Escobedo, and L. T. Williams.** Unpublished data.
21. **Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson.** 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science **252**:668–674.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
23. **Matthias, P., M. M. Mueller, E. Schreiber, S. Rusconi, and W. Schaffner.** 1989. Eukaryotic expression vectors for the analysis of mutant proteins. Nucleic Acids Res. **17**:6418.
24. **McGlade, C. J., C. Ellis, M. Reedijk, D. Anderson, G. Mbamalu, A. D. Reith, G. Panayotou, P. End, A. Bernstein, A. Kaslauskas, M. D. Waterfield, and T. Pawson.** 1992. SH2 domains of the p85α subunit of phosphatidylinositol 3-kinase regulate binding to growth factor receptors. Mol. Cell. Biol. **12**:991–997.
25. **Morgan, S. J., A. D. Smith, and P. J. Parker.** 1990. Purification and characterization of bovine brain type I phosphatidylinositol kinase. Eur. J. Biochem. **191**:761–767.
26. **Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of the serine/threonine kinase activity of *raf*-1 through tyrosine phosphorylation by the PDGF β-receptor. Cell **58**:649–657.
27. **Otsu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. Morgan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1991. Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60$^{c\text{-}src}$ complexes, and PI3 kinase. Cell **65**:91–104.
28. **Panayotou, G., B. Bax, I. Gout, M. Ferderwisch, B. Wroblowski, R. Dhand, M. J. Fry, T. L. Blundell, A. Wollmer, and M. D. Waterfield.** 1992. Interaction of the p85 subunit of PI 3-kinase and its N-terminal SH2 domain with a PDGF receptor phosphorylation site: structural features and analysis of conformational changes. EMBO J. **11**:4261–4272.
29. **Reedijk, M., X. Liu, P. van der Geer, K. Letwin, M. D. Waterfield, T. Hunter, and T. Pawson.** 1992. Tyr 721 regulates specific binding of the CSF-1 receptor insert to PI 3′-kinase SH2 domains: a model for SH2-mediated receptor-target interactions. EMBO J. **11**:1365–1372.
30. **Shibasaki, F., Y. Homma, and T. Takenawa.** 1991. Two types of phosphatidylinositol 3-kinase from bovine thymus. J. Biol. Chem. **266**:8108–8114.
31. **Skolnik, E. Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger.** 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell **65**:83–90.
32. **Soltoff, S. P., S. L. Rabin, L. C. Cantley, and D. R. Kaplan.** 1992. Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with *trk* tyrosine kinase. J. Biol. Chem. **267**:14472–14477.
33. **Whitman, M., C. P. Downes, M. Keeler, T. Keller, and L. Cantley.** 1988. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol 3-phosphate. Nature (London) **332**:644–646.
34. **Wilson, I. A., H. L. Niman, R. A. Hougten, A. R. Cherenson, M. L. Conolly, and R. A. Lerner.** 1984. The structure of an antigenic determinant in a protein. Cell **37**:767–778.
35. **Yoakim, M., W. Hou, Y. Liu, C. L. Carpenter, R. Kapeller, and B. S. Schaffhausen.** 1992. Interactions of polyomavirus middle T with the SH2 domains of the pp85 subunit of phosphatidylinositol-3-kinase. J. Virol. **66**:5485–5491.
36. **Yonezawa, K., H. Ueda, K. Hara, K. Nishida, A. Ando, A. Chavanieu, H. Matsuba, K. Shii, K. Yokono, Y. Fukui, B. Calas, F. Grigorescu, R. Dhand, I. Gout, M. Otsu, M. D. Waterfield, and M. Kasuga.** Insulin-dependent formation of a complex containing an 85-kDa subunit of phosphatidylinositol 3-kinase and tyrosine-phosphorylated insulin receptor substrate 1. 1992. J. Biol. Chem. **267**:25958–25966.