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The Interaction of Small Domains between the Subunits of Phosphatidylinositol 3-Kinase Determines Enzyme Activity

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Previous studies have suggested that the two subunits of phosphatidylinositol (PI) 3-kinase, p85 and p110, function as localizing and catalytic subunits, respectively. Using recombinant p85 and p110 molecules, we have reconstituted the specific interaction between the two subunits of mouse PI 3-kinase in cells and in vitro. We have previously shown that the region between the two Src homology 2 (SH2) domains of p85 is able to form a functional complex with the 110-kDa subunit in vivo. In this report, we identify the corresponding domain in p110 which directs the binding to p85. We demonstrate that the interactive domains in p85 and p110 are less than 103 and 124 amino acids, respectively, in size. We also show that the association of p85 and p110 mediated by these domains is critical for PI 3-kinase activity. Surprisingly, a complex between a 102-amino-acid segment of p85 and the full-length p110 molecule is catalytically active, whereas p110 alone has no activity. In addition to the catalytic domain in the carboxy-terminal region, 123 amino acids at the amino terminus of p110 were required for catalytic activity and were sufficient for the interaction with p85. These results indicate that the 85-kDa subunit, previously thought to have only a linking role in localizing the p110 catalytic subunit, is an important component of the catalytic complex.

Phosphatidylinositol (PI) 3-kinase has been implicated in the regulation of cell proliferation. Association of PI 3-kinase activity with Src-like or receptor tyrosine kinases correlates with the oncogenic or mitogenic responses induced by these protein kinases (3, 8, 10). The major known activity of PI 3-kinase is the phosphorylation of PI at the 3′ position of the inositol ring (1, 37). This reaction may be a step in the production of PI 3-phosphates which are candidate second-messenger molecules. The functions and downstream effectors of PI 3-phosphates have not yet been identified (6).

PI 3-kinase is a heterodimer consisting of an 85-kDa subunit (p85) and 110-kDa subunit (p110) (5, 26, 33). The genes encoding the subunits have been cloned from bovine (13, 28), human (33), and mouse (7; this report) tissues. It has been suggested that p110 is the catalytic subunit of PI 3-kinase (13). The 85-kDa subunit is thought to function as an adapter-like molecule that links the catalytic subunit to activated growth factor receptors. A number of functional domains have been identified in p85: a Src homology 3 (SH3) domain, a domain with homology to the breakpoint cluster region (bcr) and two SH2 domains which are separated by the inter-SH2 (iSH2) region (7, 28, 33). The SH2 domains of p85 direct the interaction of the PI 3-kinase complex with activated growth factor receptors and tyrosine kinase complexes (14, 19, 25, 29, 39, 40). We have shown previously that the p85 iSH2 domain mediates the interaction of p85 with the 110-kDa subunit in vivo. The iSH2-p110 complex is active in the absence of the p85 SH2 domains (20).

Recently, a homolog of the catalytic subunit of PI 3-kinase has been identified in the yeast *Saccharomyces cerevisiae*. Yeast PI 3-kinase is a heterodimer consisting of a catalytic subunit (Vps34p) and a serine/threonine kinase (Vps15p) (12, 35). Vps34p and Vps15p were initially discovered as components of the yeast vacuolar sorting machinery (12). *vps* mutants are defective in the sorting of several proteins destined for the yeast vacuole, the functional analog of the mammalian lysosome. Yeast strains carrying mutations which abolish Vsp15 and Vsp34 gene function display a severe growth defect and lack any detectable PI 3-kinase activity (12, 31, 35). Previously, additional molecules presumed to represent PI 3-kinase homologs were identified in *S. cerevisiae* after a screen for mutations conferring resistance to rapamycin (2, 22).

The regulation of PI 3-kinase activity following growth factor stimulation is poorly understood. PI 3-kinase associates with receptor proteins and other signaling molecules that contain a tyrosine-phosphorylated consensus sequence. In this way, it may be localized to membrane compartments where its substrate is located (15, 18). Phosphorylation may also modulate the enzymatic activity of PI 3-kinase (4, 17, 34). In thrombin-stimulated platelets, PI 3-kinase associates with the membrane skeleton and exhibits increased activity compared with that of the cytosolic fraction (41). The increase in PI 3-kinase activity in thrombin-stimulated platelets is dependent on the GTP-binding protein Rho, which participates in reorganization of the cytoskeleton (42). Whether analogous mechanisms regulate PI 3-kinase in other cell types is not known.

In this study, we demonstrate that p85 is more than just an adapter molecule which links the catalytic subunit of PI 3-kinase to activated growth factor receptors. The interaction of a 102-amino-acid segment of the p85 iSH2 domain with the catalytic 110-kDa subunit regulates PI 3-kinase activity. Furthermore, a small domain at the immediate amino terminus (N terminus) of p110 mediates the stimulatory effect of iSH2 and is required for PI 3-kinase activity. These findings suggest that the segment of p85 that interacts with p110 either is part of the catalytic region of PI 3-kinase or regulates the activity of the p110 catalytic subunit.

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MATERIALS AND METHODS

Cell culture. COS-7 cells were obtained from the American Type Culture Collection. BALB/c 3T3 cells, clone A31, were obtained from C. D. Scher, Children's Hospital of Philadelphia, Philadelphia, Pa. COS-7 and BALB/c 3T3 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).

Escherichia coli K-12 strain DH5α (Bethesda Research Laboratories) was used for plasmid propagation. *E. coli* TAP 90 (30) was used for plating λ cDNA libraries. *E. coli* B strain BL21(DE3)plysS (36) served as the host for bacterial expression vectors.

Antibodies. The rabbit anti-p110 antibodies R7481 and R7826 have been described previously (20). Murine monoclonal anti-p110 antibodies J1A and U3A were raised against a purified fragment of mouse p110 (spanning amino acids 575 to 1068) expressed in *E. coli*. Anti-p85 murine monoclonal antibody N7B was generated against the purified SH2-N fragment of p85 (19), which was overproduced in *E. coli*. Ascites fluid with the murine anti-influenza virus hemagglutinin 1 (HA1) monoclonal antibody 12CA5 (38) was kindly provided by Q. Hu (University of California, San Francisco). Purified murine monoclonal anti-Myc antibody 9E10 (9) was generously supplied by B. Eide (University of California, San Francisco).

Cloning of the mouse p110 cDNA. A cDNA fragment encoding the 5′ end of the catalytic subunit of PI 3-kinase was isolated from a λgt 11 random primed cDNA library prepared from mouse BALB/c brain (Clontech). A 500-bp DNA fragment derived from the 5′ end of the bovine p110 sequence obtained by PCR served as a probe. A positive clone with a cDNA insert of ~1 kb was plaque purified, and the insert was cloned via *Eco*RI ends into the polylinker of pBluescript II KS(+) [pKS(+)] DNA (Stratagene). The cDNA fragment, p110-5′, overlapped nucleotides 1 to 506 of the p110 coding region (A of the start codon is designated nucleotide 1) preceded by approximately 500 bp of 5′ untranslated sequence.

An oligo(dT)-primed cDNA library was generated from mouse embryo BALB/c 3T3 fibroblast poly(A)$^+$ RNA by the TimeSaver cDNA synthesis system (Pharmacia). Primary recombinants (10^7) were obtained after directional cloning of cDNA fragments (>500 bp) into λgt 11 arms (Promega) via *Eco*RI-*Not*I ends. Recombinants of the unamplified library (8×10^5) were screened in duplicate by using the 1-kb DNA fragment of clone p110-5′ as a probe. Hybridizing clones were plaque purified, and the cDNA inserts were ligated into compatible restriction sites in the polylinker of vector pKS(+). The largest cDNA clone obtained from this library (~4 kb; flanked by an *Eco*RI site at its 5′ end and a *Not*I site at its 3′ end) contained nucleotides 310 to 3204 of the mouse p110 coding region followed by a stop codon and approximately 1 kb of 3′ untranslated sequence. An *Eco*RI fragment, p110-m, containing nucleotides 310 to 1726 of the p110 cDNA was ligated into pKS(+) DNA. Fragment p110-3′ encoding the C terminus of p110 from nucleotide 1722 was cloned as an *Eco*RI-*Not*I fragment.

The full-length cDNA for mouse p110 was reconstituted by ligating three restriction fragments from the pKS(+) constructs described above: a *Sca*I-*Sph*I fragment containing the C-terminal half of the vector-encoded β-lactamase gene (*Sca*I), the origin of replication and p110-5′ sequence up to nucleotide 369 (*Sph*I); a *Sph*I-*Eco*RI fragment of p110-m spanning nucleotides 366 to 1726; and an *Eco*RI-*Sca*I fragment consisting of p110-3′ (from the *Eco*RI site at nucleotide 1722 of the p110 coding region) and vector sequences coding for the N terminus of the β-lactamase gene (truncated at *Sca*I). The sequence of the mouse p110 cDNA was determined by DNA sequence analysis.

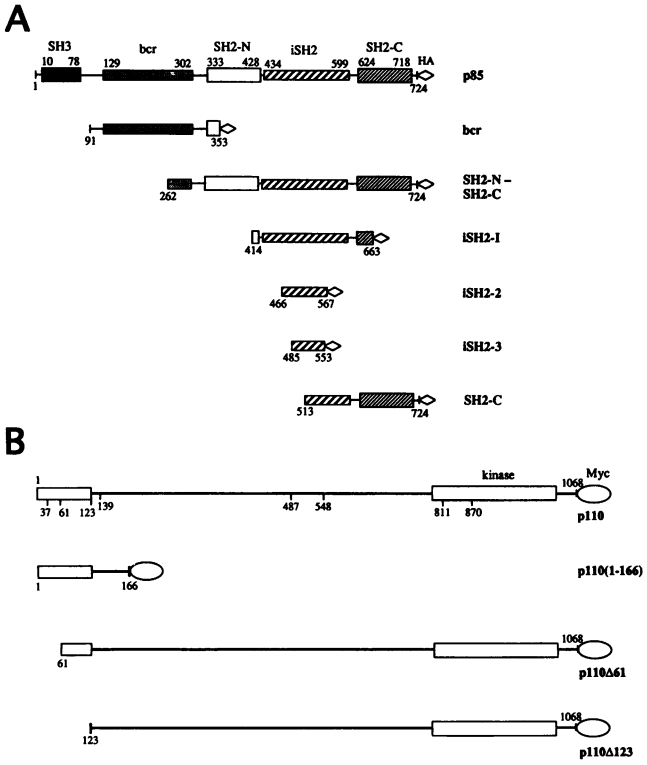


FIG. 1. (A) Schematic structure of full-length p85 and p85 fragments used in this study. All p85 molecules were tagged at the C terminus with the influenza virus HA epitope (diamond). The presumed functional domains (21, 29) are represented by the differently shaded boxes described above full-length p85. The first and last amino acids of each domain are numbered according to their relative positions within the p85 sequence (the initiator methionine is assigned number 1). The first and last amino acids of each recombinant p85 fragment are shown. Fragments iSH2-2 and SH2-C were also expressed as GST fusion proteins in *E. coli*. (B) Schematic structure of full-length p110 and fragments expressed by using reticulocyte lysates or in COS-7 cells. All p110 derivatives were modified at their C-terminal ends with the Myc tag (oval). The presumed consensus sequence for the lipid-kinase domain (13, 22) is depicted by the box labelled kinase. The domain responsible for binding p85 and required for enzymatic activity is shown as a smaller box at the p110 N terminus. The last amino acids (numbers) of various C-terminal truncations used in this study are indicated in the diagram of wild-type p110. p110(1-166) consists of amino acids 1 to 166 of p110. p110Δ61 and p110Δ123 are N-terminal truncations lacking the first 61 and 123 amino acids, respectively.

Plasmid constructions. Fragments of p85 or p110, which were expressed in bacterial or mammalian cells, are schematically shown in Fig. 1. The construction of mammalian expression vectors pCG-85 · HA, pCG–SH2-N–SH2-C · HA, pCG-iSH2-I · HA and pCG–SH2-C · HA was previously described in detail. The coding region for the 102-amino-acid iSH2-2 fragment of p85 was generated by PCR with mouse p85α cDNA (7) as the template with primer iSHV-s- (5′ CTT CTA GAA TGG CTC ATA TGT TAT ATG AGG AGT ACA CCC GT 3′), containing nucleotides 1396 to 1416 of the coding strand extended by *Xba*I and *Nde*I restriction sites, and primer iSHV-α- (5′ T CCC GGG CTT AAT ACT GTT CAT GCG

3′), containing nucleotides 1684 to 2001 of the noncoding strand extended by a *Sma*I-*Xma*I restriction site (A of the start codon is designated nucleotide 1, and nucleotides that are changed with respect to the wild-type sequence are underlined). The sequence was confirmed by DNA sequence analysis. For expression in mammalian cells, the iSH2-2 DNA fragment was ligated via *Xba*I-*Xma*I ends into a modified version of vector pCG carrying the 16-amino-acid HA epitope tag (20) (SYPYDVPDYASLGGPS [38]). pCG directs expression in mammalian cells from the human cytomegalovirus promoter/enhancer region; translation initiation is controlled by the 5′ untranslated region of the herpes simplex virus thymidine kinase gene (24). The coding region for the 69-amino-acid iSH2-3 fragment was isolated as a *Taq*I-*Pvu*II fragment, consisting of nucleotides 1452 to 1660 of the p85 cDNA, and fused in frame to the ATG in the polylinker region of vector pCG at the N terminus and to the HA epitope sequence at the C terminus.

Bacterial expression vectors coding for HA-tagged p85 molecules were generated by exchanging the KT3-tagged C-terminal fragments of previously described expression vectors (19) for the corresponding HA-tagged versions obtained from the pCG constructs described above: an *Acc*I-*Bam*HI fragment (nucleotides 1900 to 2172 extended by the HA tag sequence) was used to generate pHB-85 · HA and pHB–SH2-C · HA. In vector pHB40P, a pET derivative (36), protein expression is under control of the phage T7 ϕ10-promoter. The DNA fragment encoding a p85 domain homologous to the carboxy terminus (C terminus) of the bcr gene product was isolated as an *Nla*IV-*Ppu*MI DNA fragment (nucleotides 272 to 1061 of the p85 cDNA). The N-terminal end was fused in frame to the ATG of plasmid pHB40P; the C-terminal end was fused to the *Sma*I-*Xma*I site preceding the HA tag sequence described above. To obtain pHB-iSH2I · HA, the iSH2-I DNA fragment was isolated via *Ple*I-*Acc*I ends (nucleotides 1240 to 1989 of the p85 cDNA) and fused in frame to the ATG of the vector and the HA tag sequence at the C terminus. The following vectors directed the expression of p85 fragments as glutathione *S*-transferase (GST) fusion proteins. pHB-GST-iSHV · HA contains the HA-tagged iSH2-2 DNA fragment described above fused to the GST moiety (obtained by PCR from pGEX-3X [Pharmacia]) at the N-terminal end. pHB–GST–SH2-C · HA was derived from pHB–SH2-C · HA (see the description above).

The mouse p110 cDNA was cloned into the polylinker of vector pKS(+) (see the description above). To modify the C-terminal end of p110 with the 10-amino-acid Myc epitope (EQKLISEEDL [9]), a C-terminal fragment of the cDNA was amplified with primer p110-3′ *Hin*dIII (5′ CTG AGC AAG AAG CTT TGG 3′), consisting of nucleotides 3092 to 3109 of the coding strand overlapping a *Hin*dIII site, and primer p110 · Myc (5′ GGA TCC TCA GTT CAG GTC CTC CTC GGA AAT CAG CTT CTG CTC CCC GAG CTC GTT CAA AGC ATG CTG CTT GAT GGT GTG G 3′) containing nucleotides 3177 to 3204 of the noncoding strand (A of the start codon is designated nucleotide 1, and nucleotides that are changed with respect to the wild-type sequence are underlined). This extended the p110 C-terminal end by a sequence encoding amino acids DLG as a hinge region (overlapping a *Sac*I-*Ecl*136II site), which precedes the coding region for the Myc epitope, a stop codon, and a *Bam*HI restriction site. The wild-type C-terminal end was exchanged for the Myc-tagged sequence with *Hin*dIII and *Bam*HI. The N-terminal end of the p110 coding region was modified by PCR with primer p110-5′ *Bsm*I (5′ GGC CTG AGG AGG CAT TCT AAA G 3′), consisting of nucleotides 98 to 120 of the noncoding strand overlapping a *Bsm*I site, and primer p110-350 (5′ TCT AGA ATG GCT CAT ATG CCT CCA CGA CCA TCT TCG 3′) containing nucleotides 1 to 21 of the coding strand extended by *Xba*I and *Nde*I restriction sites. The wild-type N terminus was replaced by the newly generated N-terminal end via *Xba*I-*Bsm*I sites. The correct sequence of the p110 fragments modified by PCR was confirmed by DNA sequence analysis. p110Δ61, which lacks the N-terminal 61 amino acids of full-length p110, was constructed by fusing the p110 coding region, after blunting the *Alw*NI site at nucleotide 187, to an ATG of the modified p110 N-terminal region described above. p110Δ123 lacking the first 123 amino acids was generated in parallel by fusion of the blunted *Sph*I site at nucleotide 370 in frame to a start codon. For expression of full-length p110 and truncated p110 molecules tagged with the Myc epitope in COS-7 cells, the respective DNA fragments were cloned into mammalian expression vector pCG (see the description above) via *Xba*I-*Bam*HI ends. pCG-p110(1-166) · Myc was obtained by truncating the p110 cDNA at nucleotide 499 with *Acc*I and fusing it at the C terminus with the Myc epitope in a pCG derivative (20) at the *Sma*I-*Xma*I site.

Expression of recombinant p85 or p85 domains in bacteria. p85 and its truncated fragments were expressed in *E. coli* by using the T7 expression vectors described above (19). Cultures containing pHB-85 · HA, pHB-bcr · HA, or pHB–iSH2-I · HA were incubated for 2 h at 37°C after IPTG (isopropyl-β-D-thiogalactopyranoside) induction; cultures containing pHB–GST–iSH2-2 · HA or pHB–GST–SH2-C · HA were incubated for 4 h at 20°C after induction. Bacterial pellets were resuspended in bacterial lysis buffer {50 mM Tris-HCl [pH 7.5], 50% [vol/vol] glycerol, 5 mM EDTA, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*-*N*-*N*′-*N*′-tetraacetic acid], 10 μg of aprotinin per ml, 10 μg of pepstatin per ml, 10 μg of leupeptin per ml, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol} at 1/30 of the initial culture volume and subjected to one or more freezing-thawing cycles. Lysis was completed by sonicating the suspension five times for 5 s. After the addition of 0.5% (vol/vol) Triton X-100, the extracts were cleared by centrifugation for 15 min at 16,000 × *g* at 4°C. The concentration of soluble p85 fragments was assessed against protein standards visualized by Coomassie blue stain after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

In vitro transcription and translation of recombinant p110 derivatives. Plasmid pKS(+) DNA containing full-length or truncated versions of the p110 cDNA (Fig. 1B) was transcribed in vitro with T3 RNA polymerase, after linearizing the DNA with suitable restriction enzymes: *Bam*HI, cleaving downstream of the modified stop codon, for wild-type p110, p110Δ61, or p110Δ123; *Pst*I, cleaving at nucleotide 2612 of the coding region, to generate p110 truncated after amino acid 870; *Hpa*I (position 2435), *Asp*700 (position 1645), *Afl*III (position 1463), *Bst*BI (position 417), *Sph*I (position 369), *Alw*NI (position 183), and *Bsm*I (position 109) to generate C-terminally truncated p110 molecules consisting of 811, 548, 487, 139, 123, 61, and 37 amino acids, respectively. Typically, 10 μg of RNA was subjected to in vitro translation with reticulocyte lysates (Promega) in the presence of 125 μCi of [^{35}S]methionine (Amersham) in a 100-μl volume. The translation products were stored at −80°C, after the addition of 10% (vol/vol) glycerol.

Transient expression of recombinant p85 and p110 fragments in COS cells. COS-7 cells (60 to 70% confluent on a 10-cm-diameter plate) were transfected with the pCG-derived constructs described above by the DEAE-dextran method (11). Fifty-four hours after transfection, cells were lysed and the

recombinant p85 and p110 fragments were analyzed for complex formation and enzyme activity (see Results).

In vivo association of recombinant p85 and p110 molecules. Transiently transfected COS-7 cells were washed twice with cold phosphate-buffered saline. Cell lysates were prepared as previously described (27). In brief, cells were treated with mammalian cell lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 15% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mg of aprotinin per ml, 20 mM leupeptin, 2 mM benzamidine, and 1 mM sodium vanadate. Lysates were cleared by centrifugation at 14,000 × *g* for 5 min and incubated with monoclonal anti-Myc antibody 9E10 overnight at 4°C. Protein A-Sepharose beads (Sigma) were used to precipitate the immune complexes. The beads were washed twice with 50 mM Tris-HCl (pH 7.5)–0.5 M LiCl–0.5% (vol/vol) Triton X-100, twice with 50 mM Tris-HCl (pH 7.5)–0.5 M LiCl, and once with distilled water, all containing 0.1 mM sodium vanadate. The complexes were analyzed by immunoblotting with the indicated antibodies or by PI 3-kinase assay as described below.

In vitro association of recombinant p85 and p110 derivatives. Twenty microliters of various ^{35}S-labelled p110 derivatives synthesized in reticulocyte lysates were mixed with bacterial lysates containing the indicated amount of HA-tagged p85 molecules in 500 μl of mammalian cell lysis buffer and incubated for 2 h at 4°C. Anti-HA antibody 12CA5 was added for 1 h, and the immune complexes were precipitated with protein A-Sepharose beads. Control samples containing reticulocyte lysate-derived p110 were immunoprecipitated with anti-p110 antibodies. After serial washes (described above), the precipitates were separated by SDS-PAGE. The presence of full-length or truncated p110 molecules in the complexes was detected by fluorography.

Immunoblotting. Immunoprecipitates were boiled in Laemmli sample buffer (23), separated by SDS-PAGE, and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked in TBST buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% [vol/vol] Tween 20, 0.5% [wt/vol] sodium azide) containing 5% (wt/vol) 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-indolylphosphate (Promega). Alternatively, ^{125}I-labelled anti-mouse antibody (Amersham) was used after incubation with antibody 9E10 in order to quantitate Myc-tagged p110 bands by using a PhosphorImager (Molecular Dynamics).

Determination of PI 3-kinase activity in p110 complexes. The presence of PI 3-kinase activity in immunocomplexes 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, and 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 (37). 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 (16). The conversion of PI to PI 3-phosphate was determined by autoradiography and quantitated by using a PhosphorImager.

Nucleotide sequence accession number. The GenBank accession number for the mouse p110 cDNA sequence is U03279.

RESULTS

In vitro association of p85 fragments with p110. We have previously shown that the iSH2 region of p85 binds to the 110-kDa catalytic subunit of PI 3-kinase in vivo (20). We developed the following in vitro assay in order to map the region of p110 which binds to p85. Wild-type p85 and p85 fragments were expressed in *E. coli*. The p85 molecules were tagged with a 16-amino-acid epitope from influenza virus HA1, which is recognized by the monoclonal antibody 12CA5 (38). The concentrations of the p85 proteins were equalized after immunoblotting with anti-tag antibody 12CA5 (Fig. 2B). *E. coli* extracts containing full-length p85, iSH2-I (the iSH2 domain plus flanking regions) or iSH2-2 (a 102-amino-acid iSH2 fragment) (Fig. 1A) were mixed with [^{35}S]methionine-labelled mouse p110 synthesized by in vitro translation of p110 cDNA in rabbit reticulocyte lysate. p85 fragments were immunoprecipitated with anti-HA antibody 12CA5. ^{35}S-labelled p110 molecules coprecipitating with p85 fragments were detected by fluorography. As shown in Fig. 2A, wild-type p85, iSH2-I, and iSH2-2 were able to associate with p110 in vitro. No binding was observed in control precipitations in which p85 was omitted or in the presence of the SH2-C fragment of p85 (Fig. 2A). The SH2-C fragment includes half of the iSH2 domain and was previously found not to interact with p110 in vivo (20). Additional HA-tagged molecules such as the bcr homology region of p85 or the signaling protein Nck, which is also thought to be an adapter molecule, did not associate with p110 (Fig. 3 and data not shown).

A small region of the 110-kDa subunit of PI 3-kinase mediates the interaction with p85. Using the assay described above, we performed experiments to identify the domain in p110 that binds to p85. p110 fragments truncated at the C terminus were generated by in vitro transcription and translation of mutant cDNAs (Fig. 1B). Correct production of p110 derivatives was confirmed by immunoprecipitation of the mutant proteins from the corresponding reticulocyte lysates with anti-p110 antibodies (Fig. 3B). Association of ^{35}S-labelled p110 proteins with HA-tagged p85 was performed as described above. All the truncated proteins analyzed, including a molecule consisting of only the N-terminal 139 amino acids of p110, exhibited binding to p85 (Fig. 3A). The specificity of the interaction was confirmed by using *E. coli* lysate containing the HA-tagged bcr fragment of p85, which does not associate with p110 (20), as a control. These data suggested that the immediate N-terminal region of p110 binds to the 85-kDa subunit. We determined the minimal domain in p110 which could associate with p85 (Fig. 3C): mutant p110 molecules lacking 61 and 123 amino acids from the N terminus (Fig. 1B) no longer associated with p85. Fine mapping from the C-terminal end revealed that a fragment truncated at amino acid 123 was capable of binding to p85, while further C-terminal truncations failed to bind.

Interaction of the p85 iSH2 domain with p110 is required for PI 3-kinase activity. The experiments described above demonstrate that the subunits of PI 3-kinase interact via specific contacts between defined protein domains that are smaller than 130 amino acids. We investigated whether these domains are sufficient for binding in vivo. Both PI 3-kinase subunits and their smaller derivatives were transiently expressed by using the human cytomegalovirus promoter in COS-7 cells. The p85 molecules were tagged at the C terminus with the HA epitope and were detected with antibody 12CA5, while the p110 derivatives were tagged at the C-terminal end with a 10-amino-acid Myc epitope which is recognized by antibody 9E10 (9).

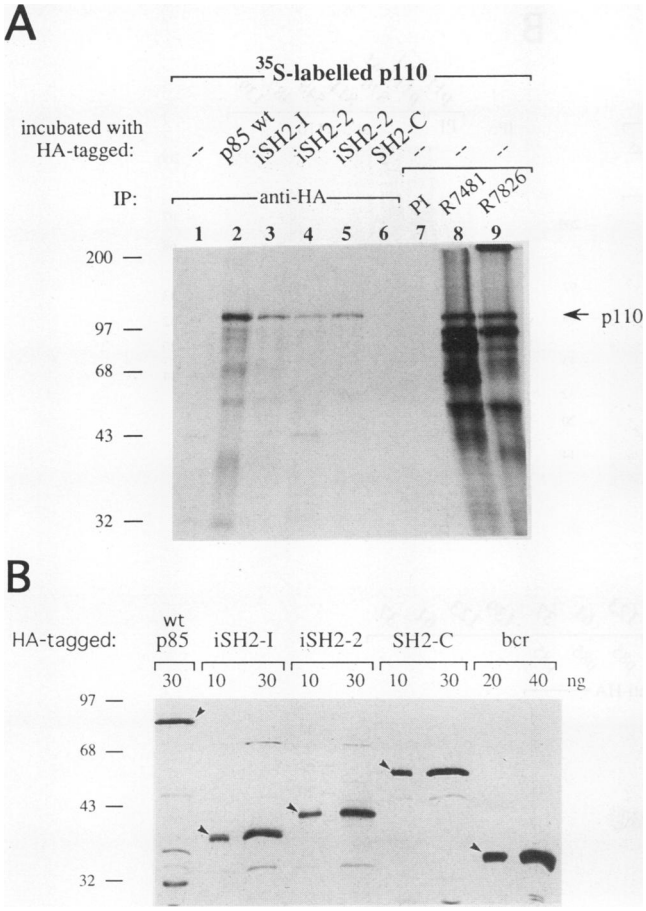


FIG. 2. In vitro association of p85 fragments with p110. (A) Reticulocyte lysates containing in vitro-translated, [^{35}S]methionine-labelled p110 were incubated with or without (− −) the indicated HA-tagged p85 molecules obtained by expression in *E. coli*: 50 ng of p85 (lane 2), 35 ng of iSH2-I (lane 3), 30 (lane 4) and 60 (lane 5) ng of iSH2-2, and 100 ng of SH2-C (lane 6). Protein complexes were precipitated with anti-HA antibody 12CA5, which recognizes the p85 fragments, and analyzed by SDS-PAGE (7% acrylamide) after stringent washes. Immunoprecipitations (IP) with anti-p110 antibodies R7481 and R7826 were performed in parallel to determine the mobility of full-length p110; parallel precipitations were done with preimmune serum (PI). ^{35}S-labelled p110 molecules coprecipitating with p85 fragments were detected by fluorography. The position of full-length p110 is indicated. The smaller species are truncated p110 molecules generated either by initiation from internal methionines during in vitro translation or by prematurely terminated translation. In the first case, molecules with N-terminal deletions that do not associate with p85 were produced; in the second case, C-terminally deleted p110 fragments that are still able to bind to p85 were produced. Molecular size markers are given in kilodaltons on the left. (B) Quantitation of bacterially expressed p85 molecules used in this study. Fragments iSH2-2 and SH2-C were expressed as GST fusion proteins. The protein concentrations were equalized by anti-HA immunoblot analysis of the respective bacterial lysates. The positions of recombinant p85 molecules are indicated by arrowheads. Minor species detected by the 12CA5 antibody are most likely proteolytic degradation products. wt, wild type.

Full-length p110 or a 166-amino-acid N-terminal fragment of p110 were coexpressed in COS cells with wild-type p85 or fragments of p85 overlapping the iSH2 region (SH2-N–SH2-C, iSH2-I, iSH2-2, and iSH2-3). p110 molecules were immunoprecipitated from cell lysates with the anti-Myc antibody, and then the precipitates were divided in half. For one half of the precipitates, p110 and coprecipitating p85 fragments were resolved by SDS-PAGE and visualized by immunoblotting (Fig. 4A). The other half of the precipitates was analyzed in parallel for the presence of PI 3-kinase activity (Fig. 4B). A p85 fragment as small as 102 amino acids (iSH2-2) overlapping the center of the iSH2 domain efficiently bound to p110 in vivo (Fig. 4A). Binding of a smaller, N- and C-terminally truncated version of this fragment (Fig. 1A, iSH2-3) was barely detectable, while SH2-C was not able to bind p110. As shown in Fig. 4A (lanes 9 to 11), this interaction is mediated by small domains within p110 and p85, as expected from the in vitro studies described above. The PI 3-kinase activity assay performed on aliquots of the same samples (Fig. 4B) demonstrated that wild-type p110 exhibits high-level enzyme activity only when bound to full-length p85 or iSH2-2 containing p85 fragments. Precipitates of p110 from cells coexpressing p85 fragments that are deficient in binding did not have PI 3-kinase activity above the level of p110 expressed alone. The fragment of p110 consisting of amino acids 1 to 166 was able to bind full-length p85 or iSH2-2 (Fig. 4A), but these complexes were enzymatically inactive (Fig. 4B).

To ensure comparable expression levels of the recombinant p110 and p85 molecules in the experiment described above, an aliquot of each lysate was analyzed by immunoblotting. The levels of protein expression were very similar in all samples, except for an increased amount of p110 when coexpressed with full-length p85 (Fig. 4C). Association of p85 might stabilize p110 by preventing its degradation. Fig. 4C also shows that after transient transfection recombinant mouse p110 was expressed at a much higher level than endogenous COS cell p110. The monoclonal anti-p110 antibodies used recognize COS and mouse p110 equally well (data not shown). This suggests that a large excess of recombinant p110 was produced. A fraction of the recombinant p110 may be activated by COS cell p85 (Fig. 4B, lane 2). To test this possibility, endogenous and/or recombinant p110 molecules were immunoprecipitated with anti-p110 or anti-Myc antibodies from COS cell lysates. The presence of p85 fragments in the complexes was visualized by immunoblotting with anti-p85 or anti-HA antibodies. COS cell p110 bound a slightly larger amount of endogenous p85 than recombinant p110, although the latter was present at a much higher level (Fig. 4D, lanes 1 and 2). This implies that the majority of recombinant p110 molecules are uncomplexed. This is further supported by the finding that the same amount of recombinant p110 in the presence of endogenous p85 was able to associate with a large amount of coexpressed iSH2-2 or p85 (lanes 3 and 4 and lanes 7 and 8). As shown in lanes 6 to 8 of Fig. 4D, anti-Myc-immunoprecipitated recombinant p110 was able to bind to recombinant p85 fragments and therefore was not saturated by endogenous p85. Because of the large amount of uncomplexed p110, only a slight increase of PI 3-kinase activity can be observed when p110 is expressed alone (Fig. 4B; also see Fig. 6A and B, lanes 9 to 11). The stimulation of PI 3-kinase activity observed when iSH2-2 fragments are coexpressed demonstrates that p85 is a limiting component for the formation of an active complex.

In order to obtain more quantitative information about the stimulatory effect of p85 coexpressed with full-length p110, the products of PI 3-kinase reactions of several experiments were quantitated and normalized for the amount of p110 in each reaction mixture (Fig. 4E). We observed more than a 40-fold increase in p110 enzyme activity in the presence of full-length p85 and more than a 20-fold increase in the presence of fragments containing the iSH2 region of p85. Therefore, the

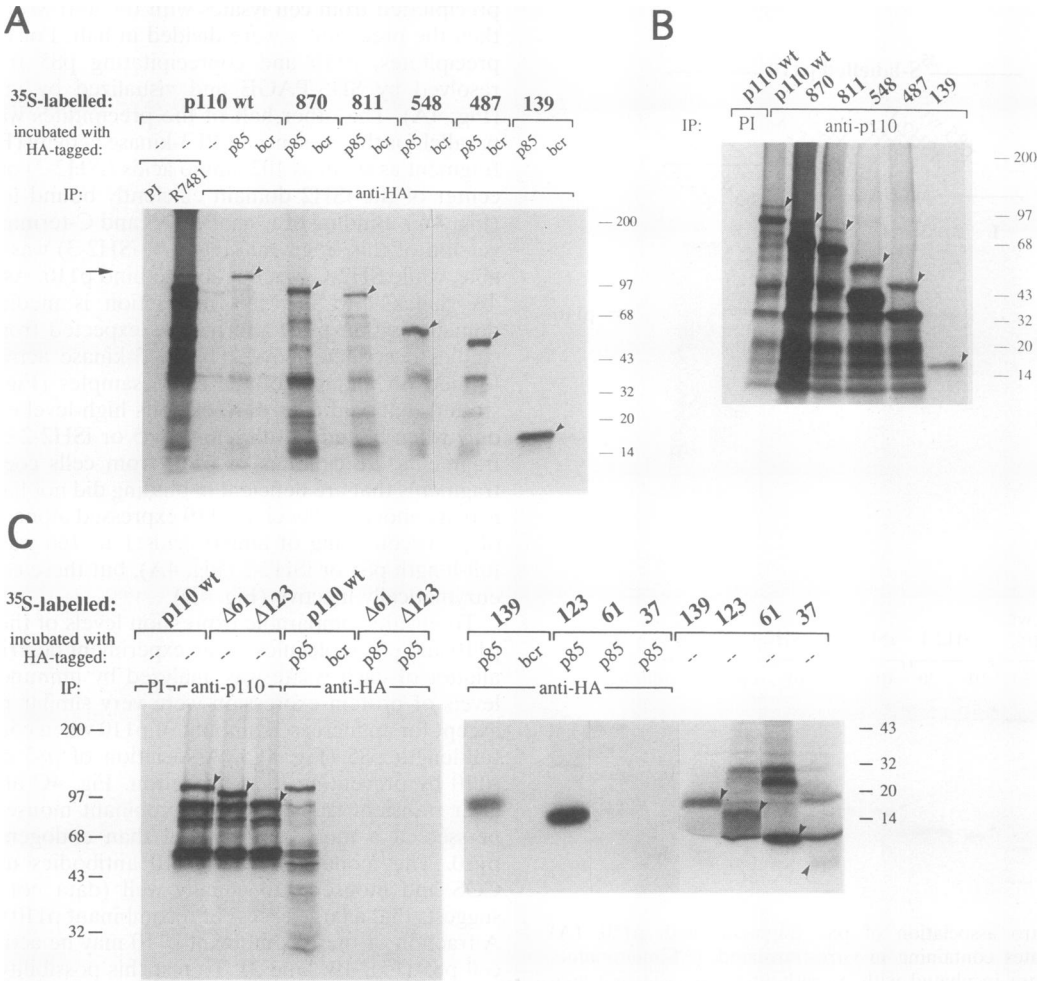
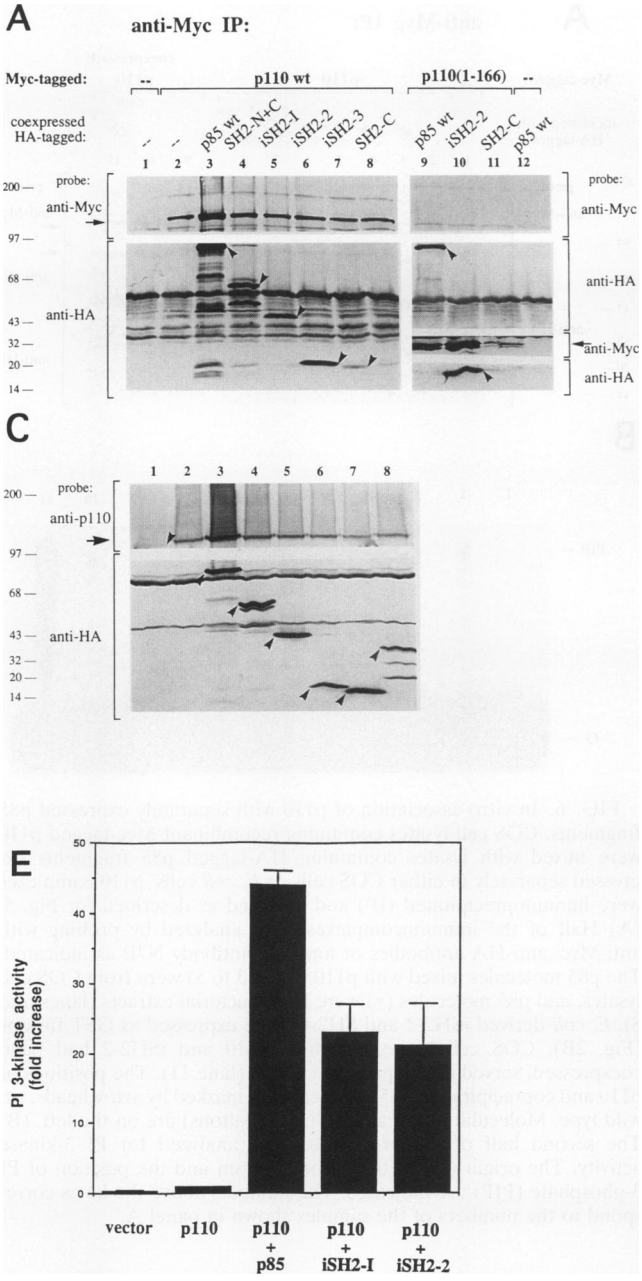


FIG. 3. Identification of the p85-interacting region in p110. (A) C-terminal deletions of the p110 cDNA generated by using suitable restriction enzymes (see Materials and Methods) and subjected to in vitro transcription and translation. The mutant p110 molecules (Fig. 1B) are designated by the number of their C-terminal amino acid. Reticulocyte lysates containing ^{35}S-labelled full-length p110 or various C-terminal truncations were incubated with or without (− −) 100 ng of HA-tagged p85 or 150 ng of bcr fragment and then precipitated with anti-HA antibody 12CA5. In control samples, p110 was immunoprecipitated (IP) with preimmune serum (PI) or anti-p110 antibody R7481. The protein complexes were analyzed in parallel on two gels by step-gradient SDS-PAGE (7 to 12% acrylamide). The presence of p110 molecules in the p85 complexes was detected by fluorography. The position of full-length p110 is indicated by the arrow on the left, and the positions of mutant p110 molecules are indicated by arrowheads. (B) Analysis of in vitro-translated p110 molecules. Reticulocyte lysates containing full-length p110 or its smaller derivatives were immunoprecipitated with anti-p110 antibody R7826. The immune complexes were analyzed as described for panel A. The mobility of each p110 molecule is marked (arrowheads). (C) Fine mapping of the p85-binding domain in p110. N-terminal truncations of p110 lacking 61 (Δ61) or 123 (Δ123) amino acids were translated in vitro and tested for their abilities to associate with p85 or the bcr fragment, as described above (left panel). Control samples of each construct were immunoprecipitated with preimmune serum or anti-p110 antibody R7481. C-terminally truncated p110 molecules consisting of 139, 123, 61, or 37 amino acids were also tested for interaction with p85 or the bcr fragment (right panel). Aliquots of lysates containing the C-terminal truncations were separated by step-gradient SDS-PAGE (8 to 14% acrylamide). The p110 fragments are shown by arrowheads. wt, wild-type. Molecular size markers (in kilodaltons) are shown beside the gels.

interaction between p110 and p85 is important for PI 3-kinase function and this interaction is mediated by small structural domains within p110 and p85.

The immediate N terminus of p110 is essential for its interaction with the 85-kDa subunit and enzyme activity. Our studies on the interaction between the two PI 3-kinase subunits indicated that a small domain at the N-terminal end of p110 mediates the interaction with p85. To investigate the role of the p110 N terminus in PI 3-kinase activity, we transiently expressed two mutant p110 molecules lacking either 61 (p110Δ61) or 123 (p110Δ123) amino acids with or without p85. In contrast to wild-type p110, which formed an enzymatically active complex with p85 or iSH2-2 (Fig. 5A and B), neither of the N-terminally truncated p110 molecules was able to bind or to be activated by p85. This demonstrates that the N-terminal region of p110 is essential for p85 binding and catalytic function.

Binding of p85 to p110 is necessary but not sufficient for PI 3-kinase activity. The series of experiments described above suggested a connection between the association of p85 with p110 and enzyme activity. We attempted to reconstitute PI 3-kinase activity by mixing the 110- and 85-kDa subunits. The two subunits were expressed separately in COS cells, and the cell lysates were mixed. The p110 complexes were immunopre-



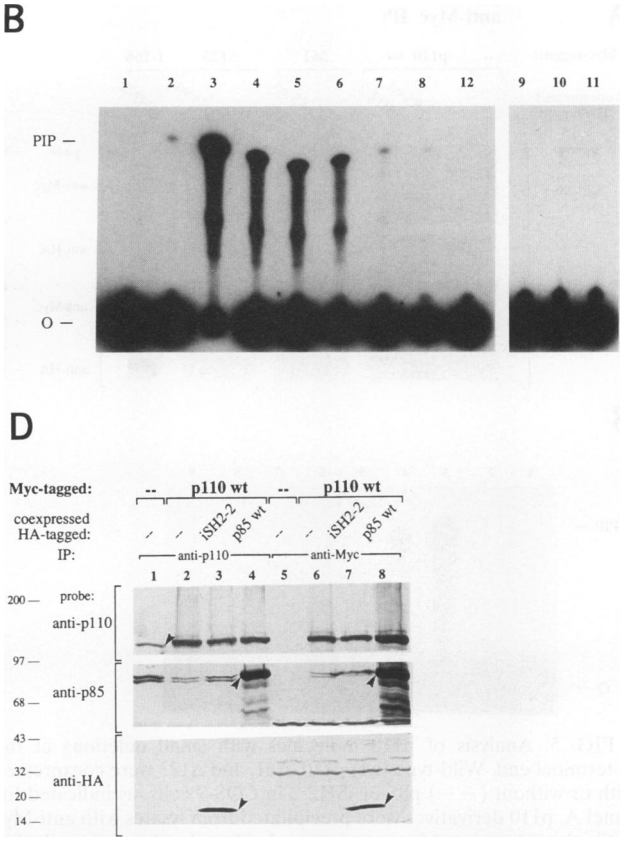


FIG. 4. In vivo association of p110 molecules with p85 fragments. Full-length p110 or its 166-amino-acid N-terminal fragment, p110(1-166), was expressed alone (− −) or coexpressed with the indicated HA-tagged p85 fragments in COS-7 cells. The N-terminal fragment of p110 used was slightly larger than the minimal binding fragment identified (Fig. 2), which enabled us to distinguish it from iSH2-2 by SDS-PAGE. Myc-tagged p110 molecules were immunoprecipitated (IP) from lysates with anti-Myc antibody 9E10, and half of the precipitates were resolved by SDS-PAGE (7 to 12% acrylamide). (A) The lanes shown are all part of one experiment analyzed in parallel on two gels. After transfer to nitrocellulose membrane, the presence of p110 molecules and p85 fragments in the immune complexes was monitored by using anti-Myc or anti-HA antibodies as probes. The positions of p110 and p110(1-166) are indicated by the arrows on the left and right of the diagram. The mobility of the associated p85 fragments is marked by arrowheads next to each lane. The immunoglobulin G heavy chain (50 kDa) and light chain (32 kDa) of the antibody used for the immunoprecipitations were also recognized by the alkaline phosphatase-conjugated anti-mouse antibody. (B) Active PI 3-kinase in the p110 complexes was assayed in parallel by thin-layer chromatography using the second half of the precipitates. The origin (O) of the chromatogram and the position of PI 3-phosphate (PIP) are indicated. The numbers above the lanes correspond to those of the samples shown in panel A. (C) Aliquots of the lysates used for panels A and B were examined for the expression levels of recombinant p110 and p85 molecules by probing with anti-p110 monoclonal antibodies J1A and U3A (upper panel) or anti-HA antibody 12CA5 (lower panel). The position of endogenous p110 is marked by an arrowhead next to lane 1. Recombinant p110 (indicated by the arrow on the left of the diagram) migrates above the endogenous p110 species because of the 12 additional amino acids of the Myc tag. The positions of the coexpressed p85 fragments are marked by arrowheads next to each lane. (D) The amount of endogenous COS cell p85 associated with endogenous and/or recombinant p110 was analyzed in the indicated samples after immunoprecipitation with anti-p110 antibody R7481 or anti-Myc antibody. The precipitates were immunoblotted with anti-p110 antibodies J1A and U3A, anti-p85 antibody N7B, or anti-HA antibody as shown. COS cell p85 is represented by two species. Lane 5 shows no endogenous p110 present in anti-Myc immune precipitates. (E) PI 3-phosphate production in immune complexes determined as shown for panel B was quantitated by using a PhosphorImager. The values for PI 3-phosphate were normalized for the amount of p110 in the associated complexes. The increase in PI 3-kinase activity is expressed relative to p110 expressed alone or coexpressed with non-interacting p85 fragments. Each bar represents the mean of at least three experiments ± the standard deviation. wt, wild type. Molecular size markers (in kilodaltons) are shown beside the gels.

cipitated with anti-Myc antibody, and the precipitates were assayed for p85 association and PI 3-kinase activity. A similar experiment was performed by mixing lysates of COS cells that express p110 with lysates of *E. coli* that express p85 or its derivatives. As shown in Fig. 6A, p85 and iSH2-2 expressed in COS or *E. coli* associated with p110 after mixing of the

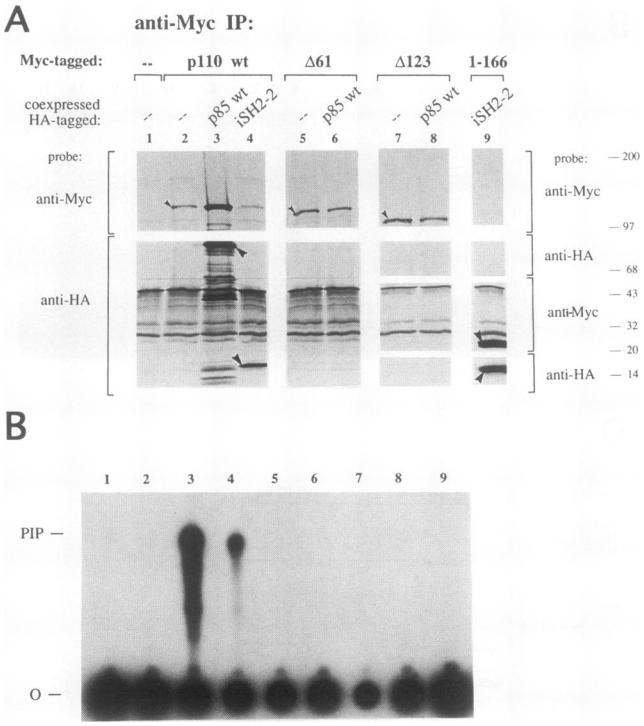


FIG. 5. Analysis of p110 molecules with small deletions at the N-terminal end. Wild-type (wt) p110, Δ61, and Δ123 were coexpressed with or without (− −) p85 or iSH2-2 in COS-7 cells, as indicated for panel A. p110 derivatives were precipitated from lysates with anti-Myc antibody and analyzed for association of p85 molecules as described in detail in the legend to Fig. 4. (A) Half of the precipitates were probed with a mixture of anti-HA and anti-Myc antibodies. Lane 9 serves as the control for the association of the N-terminal domain (amino acids 1 to 166) of p110 and iSH2-2 of p85. Molecular markers (in kilodaltons) are on the right. (B) The second half of the complexes was assayed in parallel for PI 3-kinase activity. The origin (O) of the chromatogram and the position of PI 3-phosphate (PIP) are indicated. The numbers above the lanes correspond to numbers of the samples shown in panel A.

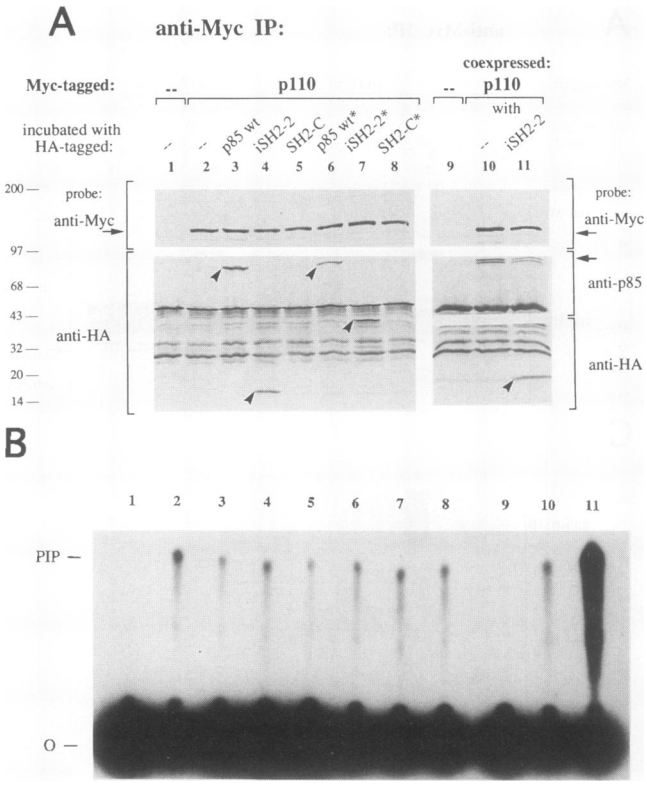


FIG. 6. In vitro association of p110 with separately expressed p85 fragments. COS cell lysates containing recombinant Myc-tagged p110 were mixed with lysates containing HA-tagged p85 fragments expressed separately in either COS cells or *E. coli* cells. p110 complexes were immunoprecipitated (IP) and analyzed as described for Fig. 5. (A) Half of the immunocomplexes were analyzed by probing with anti-Myc, anti-HA antibodies or anti-p85 antibody N7B as indicated. The p85 molecules mixed with p110 (lanes 3 to 5) were from COS cell lysates, and p85 molecules (*) were from bacterial extracts (lanes 6 to 8). *E. coli*-derived iSH2-2 and SH2-C were expressed as GST fusions (Fig. 2B). COS cell lysate, in which p110 and iSH2-2 had been coexpressed, served as the positive control (lane 11). The positions of p110 and coprecipitating p85 fragments are marked by arrowheads. wt, wild type. Molecular size markers (in kilodaltons) are on the left. (B) The second half of the precipitates was analyzed for PI 3-kinase activity. The origin (O) of the chromatogram and the position of PI 3-phosphate (PIP) are indicated. The numbers above the lanes correspond to the numbers of the samples shown in panel A.

extracts, while SH2-C did not associate. This result is consistent with those of the experiments described above. However, none of the p110-p85 complexes formed in vitro had more PI 3-kinase activity than p110 expressed alone (Fig. 6B). High-level enzymatic activity was only detected when p110 was coexpressed with iSH2-2, and the complex can form in vivo (Fig. 6B, lane 9). This suggests that the interaction between the subunits of PI 3-kinase is necessary but not sufficient for the formation of a functional complex.

DISCUSSION

We reconstituted the specific association between recombinant mouse p110 and p85α in vitro and in cells. We identified a 123-amino-acid domain that is sufficient to bind p85 in the N-terminal region of p110. We also demonstrated that a 102-amino-acid fragment of the iSH2 domain of p85 efficiently interacts with the 110-kDa subunit and is required for the formation of enzymatically active PI 3-kinase. Finally, we showed that binding of the iSH2 domain of p85 to the N-terminal region of p110 is necessary but not sufficient for the formation of a functional enzyme complex. These data, which are summarized in Fig. 7, indicate that p85 is more than just an adapter-like molecule and is required for the catalytic activity of PI 3-kinase.

Our results provide an explanation for the finding of Hiles et al. (13) that p110 did not exhibit PI 3-kinase activity in mammalian cells unless p85 was coexpressed. They also reported that p110 by itself had enzymatic activity when expressed in insect cells, which suggested that recombinant p110 from insect cells is somehow activated or p110 expressed in COS cells is specifically inhibited. Shibasaki et al. (32) described the purification of a catalytically active p110 species from bovine thymus in addition to the isolation of a functional p85-p110 complex. It cannot be ruled out that this p110 species is different from those described by Hiles et al. (13) and in our experiments. It is also possible that the PI 3-kinase activity that they observe is caused ectopically by protein kinases, phosphatases, or proteases during purification (13). The possible role of protein phosphorylation in the regulation of PI 3-kinase activity is discussed further below.

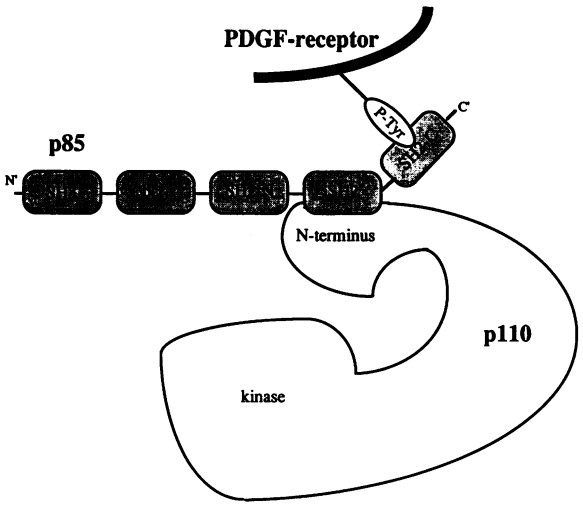


FIG. 7. Model for the formation of a functional complex between the subunits of PI 3-kinase. The high-affinity interaction between PI 3-kinase and the activated platelet-derived growth factor (PDGF) receptor is mediated via the C-terminal SH2 domain of the 85-kDa subunit (19). The iSH2 domain of p85 interacts with the N-terminal region of the catalytic 110-kDa subunit. This interaction is critical for enzymatic activity.

Prior comparison of bovine p110 with the yeast homolog Vps34p has suggested that the catalytic domain maps to the C-terminal half of the molecule (13). Mutations in the ATP-binding site of the proposed kinase domain of Vps34p abolished PI 3-kinase activity in yeast cells (31). Consistent with these data, the complex containing the N-terminal 166 amino acids of p110 and p85 or iSH2 is not functional (Fig. 4), because the kinase domain of p110 is missing. We demonstrate here that in addition to the catalytic center located in the C-terminal region of p110, a small domain at the N-terminal end is required for enzymatic activity. Small deletions from the N terminus of p110 destroy its ability to bind p85 and prevent PI 3-kinase activation (Fig. 5). Moreover, deleting the p85 binding domain did not render these p110 molecules constitutively active. This argues against the possibility that p85 promotes the activation of PI 3-kinase by sequestering an inhibitory domain in p110.

Stack et al. (35) reported that the catalytic activity of the yeast p110 homolog Vps34p is dependent on a phosphorylation event mediated by Vps15p. Vps15p was shown to be a serine/threonine protein kinase (12) that forms a complex with Vps34p in vivo (35). The mammalian 110-kDa subunit of PI 3-kinase is 33% identical and 55% similar to Vps34p over a stretch of 450 amino acids in the C terminus of each protein (13). The N-terminal halves of both molecules also contain some regions exhibiting low homology. Interestingly, the Vps34p molecule lacks the domain corresponding to the p110 N-terminal region, which interacts with p85. Therefore, Vsp34p probably does not associate with an analogous protein in *S. cerevisiae*. More recently, a 282-kDa *S. cerevisiae* molecule with homology to p110, TOR2/DRR2, was isolated. It remains to be shown whether it has PI 3-kinase activity (2, 22). The C-terminal half of TOR2 has significant homology to Vps34p and p110, with the highest degree of conservation around the catalytic center. The N-terminal half has no obvious homology to any known protein. In contrast to Vps34p, TOR2 possesses a region with low homology to the N-terminal p85-binding domain of p110. Therefore, it is possible that a yeast homolog of p85 exists.

At present, it is not known whether Vsp15p activates Vps34p in a manner analogous to the activation of mammalian PI 3-kinase activity by p85. The stimulatory effect of Vps15p is mediated by its kinase activity, since kinase-deficient Vps15p mutants associate with the catalytic Vps34 subunit, but the complex formed is inactive (35). Our data suggest that p85, which has no catalytic activity itself, stimulates PI 3-kinase activity by making specific contacts to p110 via a domain consisting of approximately 102 amino acids in the iSH2 region. The mechanism by which p85 bound to the N terminus of p110 activates the C-terminal catalytic center of p110 and consequently of PI 3-kinase remains to be elucidated. Our attempts to reconstitute a functional PI 3-kinase complex in a cell-free system have not been successful. Neither the p85-p110 complex formed after mixing p85 expressed in *E. coli* with in vitro-translated p110 nor the complex formed after mixing the two subunits, which had been separately expressed in COS cells or *E. coli*, exhibited PI 3-kinase activity (Fig. 2 and 6 and data not shown). We do not believe that this is due to p110 being completely denatured when expressed alone, since p110 is still able to form a specific complex with the iSH2 region of p85 (Fig. 2 and 6). There are several possible explanations for the observation that the formation of a p85-p110 complex in a cell-free system alone is not sufficient for PI 3-kinase activity. It is possible that an additional modification, such as phosphorylation, may be required. It was reported earlier that a serine/threonine kinase copurifies with PI 3-kinase and regulates its activity (4). In contrast to the situation for yeast, this protein kinase phosphorylates PI 3-kinase and inhibits its activity. Alternatively, the formation of an active PI 3-kinase complex may require the transient action of an additional factor after association of the subunits. This factor could recognize the interactive domains and lead to a stimulatory conformational change. Finally, p85 bound to the p110 N terminus may assist in the proper folding of the p110 catalytic domain in a co- or posttranslational manner, thus acting as a p110-specific chaperone. Additional experiments are required to distinguish between these and other possibilities.

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