MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7677–7688
0270-7306/93/127677-12$02.00/0

Vol. 13, No. 12

Cloning of a Novel, Ubiquitously Expressed Human Phosphatidylinositol 3-Kinase and Identification of Its Binding Site on p85

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Received 14 July 1993/Returned for modification 13 August 1993/Accepted 24 September 1993

Phosphatidylinositol 3-kinase (PI 3-kinase) has been implicated as a participant in signaling pathways regulating cell growth by virtue of its activation in response to various mitogenic stimuli. Here we describe the cloning of a novel and ubiquitously expressed human PI 3-kinase. The 4.8-kb cDNA encodes a putative translation product of 1,070 amino acids which is 42% identical to bovine PI 3-kinase and 28% identical to Vps34, a *Saccharomyces cerevisiae* PI 3-kinase involved in vacuolar protein sorting. Human PI 3-kinase is also similar to Tor2, a yeast protein required for cell cycle progression. Northern (RNA) analysis demonstrated expression of human PI 3-kinase in all tissues and cell lines tested. Protein synthesized from an epitope-tagged cDNA had intrinsic PI 3-kinase activity and associated with the adaptor 85-kDa subunit of PI 3-kinase (p85) in intact cells, as did endogenous human PI 3-kinase. Coprecipitation assays showed that a 187-amino-acid domain between the two *src* homology 2 domains of p85 mediates interaction with PI 3-kinase in vitro and in intact cells. These results demonstrate the existence of different PI 3-kinase isoforms and define a family of genes encoding distinct PI 3-kinase catalytic subunits that can associate with p85.

Phosphatidylinositol 3-kinase (PI 3-kinase), an activity that phosphorylates PI at the 3′-OH of the inositol ring (63), was first identified as an activity that associates with a variety of viral oncoproteins (36, 54, 65). Studies with various mutants of polyomavirus middle T antigen demonstrated a correlation between association with PI 3-kinase and the ability to transform cells (15, 26, 58, 65). The finding that PI 3-kinase also associates with activated growth factor receptor tyrosine kinases (14, 25, 61) implicated this activity as a potential participant in mitogenic signaling.

PI 3-kinase phosphorylates PI, PI-4-P, and PI-4,5-P_2 in vitro (8, 38). Measurements of polyphosphoinositides in virally transformed cells and in cells treated with growth factors demonstrate elevations in levels of PI-3,4-P_2 and PI-3,4,5-P_3 (2, 9, 13, 20, 24, 34, 43, 44, 46, 52, 58, 60, 61). Cells expressing nontransforming mutants of viral oncoproteins do not exhibit elevated levels of PI-3,4-P_2 or PI-3,4,5-P_3 (34, 46, 58, 60), corroborating previous correlations between PI 3-kinase activation and the transformed phenotype. However, the observation that PI 3-kinase products are synthesized in response to nonmitogenic stimuli (31, 55, 56) suggests that PI 3-kinase is involved in physiological processes unrelated to mitogenesis. Since the products of PI 3-kinase are poor substrates for phospholipase C (35, 47), it has been suggested that they may constitute a second messenger pathway distinct from that involving the hydrolysis of PI-4,5-P_2 to generate diacylglycerol and inositol trisphosphate (4).

The association of PI 3-kinase activity with polyomavirus middle T antigen and antiphosphotyrosine immunoprecipitates from platelet-derived growth factor (PDGF)-treated fibroblasts correlated with the presence of an 85-kDa phosphoprotein (15, 25), suggesting that this protein might be a component of PI 3-kinase. This was confirmed by the demonstration that purified PI 3-kinase consists of 85- and 110-kDa subunits (8, 17, 38, 48).

Cloning of cDNAs encoding the 85-kDa subunit of PI 3-kinase (p85 [17, 40, 50]) demonstrated that the conceptual cDNA translation product contained two Src homology 2 (SH2) domains, one Src homology 3 (SH3) domain, and a region of homology to the breakpoint cluster region (*bcr*) gene translocated in chronic myelogenous leukemia. Overexpression of p85 blocks association of PI 3-kinase with activated PDGF receptors (17, 23), and p85 binds to activated growth factor receptor tyrosine kinases and other tyrosine phosphoproteins through its SH2 domains (3, 23, 29, 33, 37, 39, 42). p85 has no intrinsic PI 3-kinase activity (17, 40); these observations taken together suggest that p85 functions as a regulatory subunit of PI 3-kinase that couples the catalytic 110-kDa subunit (p110) to activated growth factor receptors.

In many cases, endogenous p85 is not phosphorylated on tyrosine residues in response to growth factor treatment (3, 23), although tyrosine-phosphorylated p85 is detectable in some cell lines (27, 52). Recent studies have demonstrated that binding of tyrosine phosphopeptides to p85 activates PI 3-kinase in the absence of tyrosine phosphorylation (3, 6). This binding elicits a conformational change in p85 detectable by circular dichroism (41, 49). These findings are consistent with a model of PI 3-kinase activation in which binding of tyrosine phosphoproteins to p85 causes a conformational change that is transferred to the catalytic p110 subunit. However, since the magnitude of activation by tyrosine phosphopeptides in vitro is less than the increase in levels of PI 3-kinase products observed upon growth factor treatment in vivo (3, 44), it is likely that translocation of PI 3-kinase to the membrane, where PI 3-kinase substrates are in a relatively high concentration, also contributes to a rise in levels of 3′-phosphoinositides.

Subsequent cloning of a bovine p110 cDNA demonstrated that p110 possesses PI 3-kinase activity (22). Bovine p110

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has significant homology with two *Saccharomyces cerevisiae* proteins: Vps34, which is a PI 3-kinase required for proper targeting of vacuolar proteins (21, 45), and Tor2, which is a protein mediating rapamycin resistance of certain yeast strains (32). Tor2 is required for vegetative growth (32), whereas Vps34 is not (21). This is consistent with potential roles of mammalian PI 3-kinase in mitogenic and nonmitogenic signaling pathways (although Tor2 has not been shown to be a PI 3-kinase). p85α mRNA is widely expressed (50), consistent with the ubiquitous presence of PI 3-kinase in cell lines; however, the tissue distribution of bovine p110 mRNA has not been described.

In order to better understand the functions and regulation of mammalian PI 3-kinase, we sought to isolate cDNAs encoding the 110-kDa catalytic subunit. In this paper, we report the cloning of a novel and ubiquitously expressed p110 isoform, p110β, and the identification of its binding site on p85.

MATERIALS AND METHODS

Cloning and sequencing of p110β. To generate an appropriate probe for identifying cDNAs related to bovine p110, primers that corresponded to two 7-amino-acid stretches conserved between bovine p110 and yeast Vps34 were designed: GDDLRQD, corresponding to residues 804 to 810 and 626 to 632 of p110 and Vps34, respectively, and SCAGYCV, corresponding to residues 900 to 906 (p110) and 716 to 722 (Vps34). Inosine was incorporated into the primers at nucleotide residues not conserved between p110 and Vps34. The primers were tailed with *Bam*HI and *Eco*RI restriction sites to facilitate subcloning. Polymerase chain reaction (PCR) was performed with cDNA synthesized from Jurkat total RNA as a template. The annealing temperature was set at 37°C for 5 cycles, followed by 55°C for 30 cycles. The 333-nucleotide PCR product was subcloned into Bluescript (Stratagene), and the plasmid was sequenced directly by the dideoxy chain termination method with Sequenase (United States Biochemical). This DNA fragment was labeled by random priming in the presence of [α-^{32}P]dCTP and used to screen a λZAP (Stratagene) cDNA library (*Eco*RI to *Xho*I, 5′ to 3′) constructed from 293 human embryonic kidney cell mRNA. To obtain a full-length clone, the library was rescreened with a PCR product corresponding to nucleotides 1184 to 1533 of the p110β cDNA.

Phage plaques were screened at a density of approximately 40,000 plaques per 15-cm petri plate. Nitrocellulose filters were prehybridized at 42°C in 50% formamide–5× SSC (20× SSC = 3 M NaCl plus 0.3 M sodium citrate) –20 mM Tris (pH 7.6)–1× Denhardt's solution (50× Denhardt's solution = 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin)–0.1% sodium dodecyl sulfate (SDS)–100 μg of salmon sperm DNA per ml for 4 h. Denatured random-primed probe was added at ~10^6 counts/ml, and hybridization was performed at 42°C for 18 h. The filters were washed once at room temperature in 2× SSC–0.1% SDS for 15 min and then three times in 0.2× SSC–0.1% SDS for 15 min each time. Filters were exposed wet overnight at −70°C.

Positive clones were plaque purified, and the cDNA inserts were rescued from the phage by coinfection with Exassist helper phage (Stratagene). The resultant excised Bluescript plasmids containing cDNA inserts were purified on Qiagen midicolumns and sequenced directly. Both strands of the open reading frame of p110β were sequenced in their entirety.

A full-length cDNA encoding p110β was assembled from clones 7-1 and 9-1 in two steps. Initially, a 3.2-kb *Bbs*I-*Xho*I fragment from clone 7-1 and a 0.95-kb *Bam*HI-*Bbs*I fragment from clone 9-1 were ligated to *Bam*HI-*Xho*I-cut Bluescript vector to generate clone 6. Ligation of a 3.95-kb *Afl*III-*Xho*I fragment from clone 6 and a 0.85-kb *Eco*RI-*Afl*III fragment from clone 9-1 to *Eco*RI-*Xho*I-cut Bluescript vector generated pBSp110β, which contains a full-length cDNA insert.

Epitope tagging and transient expression assays. The full-length p110β cDNA was tagged with a 9-amino-acid epitope (YPYDVPDYA [19]) derived from influenza virus hemagglutinin. A PCR product generated with *Bst*XI-tailed oligonucleotide primers and a template containing a 114-bp cassette encoding this epitope in triplicate (57) was subcloned into a unique *Bst*XI site at nucleotide 93 of the p110β cDNA to create pBSp110Tag. The tagged cDNA encodes p110β containing a 38-amino-acid insertion 30 amino acids carboxy terminal to the first potential initiator methionine residue.

To generate an epitope-tagged cDNA encoding the p85 inter-SH2 domain, a PCR product synthesized with a *Kpn*I-tailed N-terminal primer, a *Kpn*I-*Hin*dIII-tailed C-terminal primer (*Kpn*I site 5′ to *Hin*dIII site), and a p85α cDNA template (50) was subcloned into *Kpn*I-cut pBSp110Tag. Ligation in the proper orientation results in a cDNA encoding amino acids 427 to 613 of human p85 fused to the epitope tag and the first 45 amino acids of p110β. Since this construct encodes the first 45 amino acids of p110β in addition to the p85 inter-SH2 domain, we cannot rule out the possibility that these residues may play a role in associating with full-length p110β in vivo. However, in vitro experiments with the glutathione *S*-transferase (GST)–inter-SH2 fusion protein suggest that this is unlikely.

A 4.9-kb *Eco*RI-*Xho*I fragment encompassing the entire tagged p110β cDNA and a 1.1-kb *Eco*RI-*Hin*dIII fragment encoding the tagged p85 inter-SH2 domain were subcloned into a cytomegalovirus enhancer-based expression vector (23) to generate p110Tag and pINTTag, respectively. 293 human embryonic kidney cells were transfected as described previously (10). Briefly, cells were plated at a density of ~10^6 cells per 10-cm fibronectin-coated dish in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After overnight incubation at 37°C in the presence of 5% CO_2, cells were transferred to a 3% CO_2 incubator for 1 h prior to transfection with 10 μg of plasmid DNA in 1 ml of 1× BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid]-buffered saline (2× BES-buffered saline = 50 mM BES [pH 6.95], 280 mM NaCl, and 1.5 mM Na_2HPO_4) and 0.125 M $CaCl_2$ per 10-cm dish. Transfected cells were incubated 12 to 16 h in the presence of 3% CO_2, washed with phosphate-buffered saline (PBS), and incubated overnight in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in the presence of 5% CO_2 prior to lysis.

Antibodies. Rabbit polyclonal anti-p85 antibodies for immunoprecipitation and immunoblotting are directed against a human p85α peptide (amino acids 500 to 518) and a GST fusion protein containing amino acids 265 to 523 from human p85α, respectively. Mouse monoclonal antibody 12CA5 (Babco) reactive to the influenza virus hemagglutinin epitope YPYDVPDYA (19) was used to detect epitope-tagged proteins. Rabbit polyclonal anti-p110β antibodies for immunoprecipitation and immunoblotting are directed against a carboxy-terminal peptide (amino acids 1056 to 1070) and a GST fusion protein containing amino acids 586 to 665 from p110β, respectively.

Cell lysis, immunoprecipitation, and immunoblotting. Cells were washed twice with ice-cold PBS prior to lysis with 400

μl of lysis buffer {50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM tetrasodium PP_i, and 100 mM sodium fluoride} per 10-cm dish. Lysates were incubated for 5 min on ice and centrifuged for 15 min at 4°C and 16,000 × *g*.

Equal amounts of total protein as measured by Bio-Rad protein assay were immunoprecipitated with antibodies prebound to protein A-Sepharose beads (Zymed) in the presence of 20 mM HEPES, pH 7.5. Protein A-Sepharose-antibody complexes were incubated with cell lysates for 2 h at 4°C prior to immunoblot analysis or PI 3-kinase assay.

For immunoblot analysis, immunoprecipitates were washed three times with ice-cold lysis buffer, boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer for 5 min, subjected to SDS–8% PAGE, transferred to nitrocellulose (MSI), and blotted as previously described (67). Antibodies were detected with ^{125}I-protein A (ICN) or by enhanced chemiluminescence (Amersham).

PI 3-kinase assay. Immunoprecipitates were assayed for PI 3-kinase activity as described previously (2). Briefly, immunoprecipitates were washed three times with PBS containing 1% Nonidet P-40 (NP-40), twice with 0.5 M LiCl–0.1 M Tris (pH 7.6), twice with TNE (10 mM Tris [pH 7.6], 100 mM NaCl, 1 mM EDTA), and twice with 20 mM HEPES (pH 7.5)–50 mM NaCl–5 mM EDTA–0.03% NP-40–30 mM tetrasodium PP_i–200 μM sodium orthovanadate–10 μg of aprotinin per ml–1 mM phenylmethylsulfonyl fluoride. A total of 50 μl of 1× kinase buffer (20 mM Tris [pH 7.6], 75 mM NaCl, 10 mM $MgCl_2$, 200 μg of PI [Avanti] per ml sonicated in 20 mM HEPES [pH 7.5], 20 μM ATP, 200 μM adenosine, and 10 μCi of [γ-^{32}P]ATP [6,000 Ci/mmol; New England Nuclear] per sample) was added to each immunoprecipitate, and the samples were shaken at room temperature for 20 min. A total of 100 μl of 1 N HCl was added to stop the reaction. Lipids were extracted with 200 μl of 1:1 (vol/vol) chloroform-methanol. The organic phase was dried, resuspended in 10 μl of 1:1 (vol/vol) chloroform-methanol containing a PI 4-phosphate standard, and spotted on a silica gel 60 thin-layer chromatography plate (Merck). Chromatography was performed in chloroform–methanol–4.0 M NH_4OH (9:7:2 [vol/vol/vol]), and labeled PI 3-phosphate was visualized by autoradiography. Assays of substrate specificity were performed as described above, except that a sonicated 1:1 mixture of substrate-phosphatidylserine (200 μg/ml) was used in the reaction buffer.

Northern (RNA) analysis. Poly(A)$^+$ RNA was extracted from tissues and cells with SDS and proteinase K and directly purified by oligo(dT)-cellulose chromatography as described previously (62). Two micrograms of mRNA was electrophoresed on a 1% formaldehyde–agarose gel and transferred to Nytran (Schleicher and Schuell) overnight in 10× SSC. The blot was probed with a random-primed [α-^{32}P]dCTP-labeled *Kpn*I-*Kpn*I restriction fragment corresponding to nucleotides 139 to 4285 of the p110β cDNA. Hybridization was performed in 0.5 M sodium phosphate (pH 7.2)–7% SDS–1 mM EDTA–100 μg of salmon sperm DNA per ml at 62°C for 18 h. The blot was washed in 40 mM sodium phosphate (pH 7.2)–1% SDS–1 mM EDTA once for 10 min at room temperature, twice for 10 min at 62°C, and once for 10 min at room temperature prior to autoradiography.

Generation of GST-p85 fusion proteins. The construction of plasmids expressing GST-N-SH2, GST-C-SH2, and GST-N+C-SH2 has been described previously (23). GST-SH3-bcr and GST–inter-SH2 were generated by PCR with oligonucleotides flanking the appropriate domains of p85α and tailed with appropriate restriction sites. PCR reaction products were subcloned into pGEX2T (Pharmacia), and recombinant clones were screened by SDS-PAGE of *Escherichia coli* protein lysates. Fusion proteins were purified by glutathione-agarose affinity chromatography (Sigma) as previously described (51), except that 10 mM dithiothreitol was included in the lysis buffer and in all subsequent purification steps. The GST-SH3-bcr and GST–inter-SH2 fusion proteins express amino acid residues 5 to 321 and 427 to 613 of human p85α, respectively.

In vitro binding assays. Equal amounts of lysate from 293 cells transfected with an epitope-tagged p110β cDNA were incubated with approximately 5 μg of various immobilized GST fusion proteins for 2 h at 4°C. Bound proteins were washed three times with ice-cold lysis buffer, boiled in SDS-PAGE sample buffer for 5 min, separated by SDS–10% PAGE, and immunoblotted with anti-Tag antibodies as described earlier.

RESULTS

Cloning of a novel PI 3-kinase cDNA. To isolate cDNAs encoding novel PI 3-kinases, we screened a human cDNA library with a probe derived from regions conserved between bovine p110 and yeast Vps34. PCR with degenerate oligonucleotide primers and Jurkat cDNA as a template yielded a 333-bp product. This product was subcloned and sequenced, and conceptual translation yielded a polypeptide sequence that was approximately 50% identical to bovine p110 and 36% identical to yeast Vps34 (data not shown). This probe was labeled by random priming in the presence of [α-^{32}P]dCTP and used to screen a λZAP 293 cDNA library.

An initial screen of approximately 700,000 plaques yielded 11 positive clones. Restriction analysis of the cDNA inserts suggested that four were derived from the same gene. The longest cDNA insert, from clone 7-1, was selected for further analysis.

The 3.4-kb insert from clone 7-1 contained a 2,038-nucleotide open reading frame and a 1,382-nucleotide 3′ untranslated region including a consensus polyadenylation site and a poly(A) tail of 15 residues (data not shown). Since no in-frame stop codons were detected upstream of potential initiation codons, the library was rescreened with a PCR product derived from the 5′ end of clone 7-1. Nine positive clones were identified, and the clone with the largest insert, clone 9-1, was analyzed further.

Clone 9-1 contained a 3.2-kb insert, including 0.4 kb of sequence identical to the 5′ end of clone 7-1 and 1.4 kb of additional 5′ sequence. PCR analysis with primers derived from 7-1 indicated that the 3′ 1.4 kb of the 9-1 insert was not identical to 7-1. Sequencing of the 5′ 1.4-kb portion of 9-1 revealed an 1,184-nucleotide open reading frame contiguous with the open reading frame encoded by 7-1. A 224-nucleotide 5′ untranslated region contained translational stop codons in all three reading frames preceding potential translation initiation codons. Together, clones 7-1 and 9-1 define a 4.8-kb cDNA containing an open reading frame of 3,210 nucleotides.

The open reading frame encoded by clone 9-1 ended with a translational stop codon 1.6 kb downstream from the first potential initiation codon, after which the 9-1 sequence diverges from that of 7-1. In order to determine whether 9-1

and 7-1 represent differentially spliced products of the same gene, Northern analysis was performed on poly(A)$^+$ RNA isolated from 293 cells with both probes used to screen the cDNA library. Both probes identified a major transcript of ~4.8 kb (data not shown), consistent with the size of the full-length cDNA defined by clones 7-1 and 9-1. No other major mRNA species were detected with either probe, suggesting that the termination codon present in 9-1 may be the result of a cloning artifact.

Conceptual translation of the full-length cDNA yields a protein product of 1,070 amino acids (Fig. 1). There are three potential initiation codons, two of which lie in favorable Kozak consensus contexts for translation initiation (30). Comparison of this amino acid sequence with known sequences in the EMBL and GenBank data bases demonstrated that this novel cDNA was most similar to bovine p110 (22), exhibiting 42% identity at the amino acid level (Fig. 2). This relatively low level of similarity in amino acid sequence suggests that this cDNA encodes a novel isoform of p110, which we refer to as p110β. p110β also has significant similarity to Vps34, a PI 3-kinase required for vacuolar protein sorting in *S. cerevisiae* (21, 45); p110β and Vps34 exhibit 28% identity at the amino acid level (data not shown), comparable to the 27% amino acid identity between bovine p110 and Vps34 (22). p110β is less similar to Tor2, an essential protein in *S. cerevisiae* involved in rapamycin sensitivity (32), exhibiting 22% amino acid identity (data not shown). It is noteworthy that Tor2 has not been demonstrated to possess intrinsic PI 3-kinase activity.

The high level of amino acid identity among the carboxy termini of these proteins allows the definition of a putative PI 3-kinase domain (Fig. 3). Within the region shown, p110β is 52% identical to bovine p110 and 33% identical to Vps34 at the amino acid level (excluding the insert present in Vps34 but not in p110β or bovine p110). The 116-amino-acid consensus includes only residues that are identical among all three PI 3-kinases and represents 23% of the 510 amino acids depicted (not including the Vps34 insert).

p110β encodes a PI 3-kinase. To determine whether p110β encodes a PI 3-kinase, the p110β cDNA was tagged with three copies of an epitope derived from influenza virus hemagglutinin (19) and expressed transiently in 293 human embryonic kidney cells. Cell lysates containing tagged p110β were immunoprecipitated with the anti-Tag monoclonal antibody 12CA5 (19) and assayed for PI 3-kinase activity (Fig. 4A).

Anti-p85 antibodies immunoprecipitated endogenous PI 3-kinase activity from untransfected 293 cells (Fig. 4A, lane 1). This activity was inhibited by the presence of 0.5% NP-40 (lane 2), confirming its identity as PI 3-kinase (64). Anti-Tag immunoprecipitates of lysates from untransfected cells or cells transfected with a human p85 cDNA (p85) contained no PI 3-kinase activity (lanes 3 and 4). Transfection of a tagged p110β cDNA (p110Tag), either alone (lane 5) or together with p85 (lane 7), resulted in the immunoprecipitation of PI 3-kinase activity with anti-Tag antibodies. This activity was also inhibited by the presence of NP-40 (lanes 6 and 8). Approximately 10-fold more activity was present in anti-Tag immunoprecipitates of lysates from cells overexpressing both p85 and p110Tag compared with cells overexpressing p110Tag alone (lanes 5 and 7).

Immunoprecipitated p110β was also active on PI-4-P and PI-4,5-P_2 as substrates (Fig. 4B). In this assay, PI 3-kinase appears to be much more active on PI compared with PI-4-P and PI-4,5-P_2 (compare lane 1 with lanes 2 and 3, and lane 4 with lanes 5 and 6). Activity in anti-Tag immunoprecipitates of cells transfected with p85 and p110Tag was comparable in substrate specificity to endogenous activity precipitated by anti-p85 antibodies (compare lanes 1 to 3 with lanes 4 to 6).

PI 3-kinase purified from rat liver consists of two 110-kDa proteins which are separable by one-dimensional SDS-PAGE and contain related but distinct peptide sequences (8). Comparison of peptide sequences from rat liver PI 3-kinase (5a) with the amino acid sequence of p110β demonstrated that four V8 protease peptides derived from the lower-mobility p110 band (upper p110) were present in p110β and preceded in the p110β sequence by a glutamate residue. Of the 48 amino acid residues definitively identified by direct sequencing, 46 were identical in p110β. Taking cross-species sequence heterogeneity into account, it is very likely that the p110β cDNA encodes the human homolog of upper p110. Taken together with the 3′-OH specificity of rat PI 3-kinase (8), this demonstrates that p110β is indeed a PI 3-kinase.

To examine whether endogenous p110β has PI 3-kinase activity, 293 cell lysates were precipitated with either preimmune serum or anti-p110β antibodies. Immunoprecipitates were assayed for PI 3-kinase activity (Fig. 4C). p110β-specific antiserum precipitated PI 3-kinase activity (lane 2) which was not precipitated by preimmune serum (lane 1).

p110β associates with p85 in intact cells. Bovine p110 has been shown to associate with p85 in intact cells (22). To determine whether p110β associates with p85 in intact cells, lysates from cells transfected with p85 and p110Tag cDNAs were immunoprecipitated with anti-p85 or anti-Tag antibodies. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-p85 or anti-Tag antibodies (Fig. 5A).

p85 and p110Tag proteins were overexpressed, as demonstrated by immunoprecipitation and immunoblotting of lysates from transfected cells with anti-Tag (Fig. 5A, lanes 3 and 4) and anti-p85 (lanes 6 and 8) antibodies. Cotransfection of p85 and p110Tag resulted in at least 20-fold overexpression of p110β relative to endogenous levels (data not shown). The protein immunoprecipitated by anti-Tag antibodies from lysates of p110Tag-transfected cells had a mobility on SDS-PAGE consistent with the molecular weight predicted from the p110Tag cDNA (Fig. 1 and Materials and Methods). Approximately 10-fold more p110Tag was expressed when the p110Tag cDNA was cotransfected with p85 than when p110Tag was expressed alone (lanes 3 and 4); this difference in the level of expression is commensurate with the difference in PI 3-kinase activity detected in anti-Tag immunoprecipitates (Fig. 4A, lanes 5 and 7). No proteins were detected in anti-Tag or anti-p85 immunoblots of anti-Tag immunoprecipitates of lysates from untransfected cells or from cells transfected with p85 alone (lanes 1 and 2). However, transfection of p110Tag alone resulted in the coimmunoprecipitation of endogenous p85 with p110Tag (lane 3). Coexpression of p85 and p110Tag resulted in an approximately 10-fold increase in the amount of p85 coimmunoprecipitated with p110Tag (lane 4).

Similarly, no proteins were detected in anti-Tag immunoblots of anti-p85 immunoprecipitates of lysates from untransfected cells or cells transfected with p85 alone (Fig. 5A, lanes 5 and 6). p110Tag was not detected in anti-p85 immunoprecipitates of lysates from cells transfected with p110Tag alone (lane 7). However, upon cotransfection of p85 and p110Tag, p110Tag did coimmunoprecipitate with p85 (lane 8).

To examine the association of endogenous p85 and p110β, 293 cell lysates were precipitated with either preimmune serum or antisera specific for p85 or p110β. Immunoprecip-

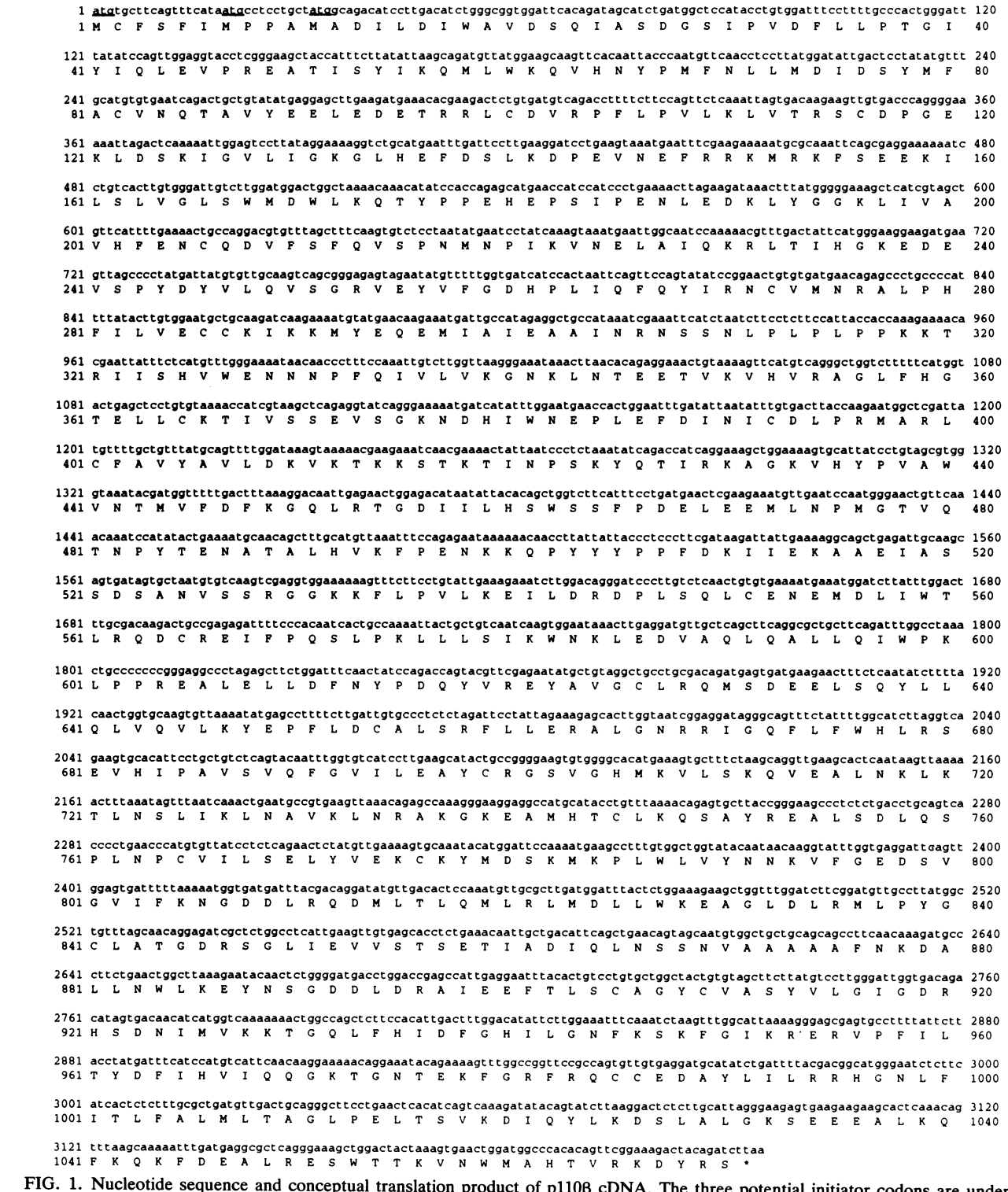

FIG. 1. Nucleotide sequence and conceptual translation product of p110β cDNA. The three potential initiator codons are underlined. Nucleotide and amino acid numbers are indicated to the left and right of the sequence.

itated proteins were resolved by SDS-PAGE and immunoblotted with anti-p85 or anti-p110β antibodies (Fig. 5B). Anti-p110β immunoblotting of anti-p85 immunoprecipitates demonstrated the presence of a ~110-kDa protein not present in preimmune immunoprecipitates (compare lane 2 with lane 1). This protein comigrated with a ~110-kDa protein specifically precipitated by anti-p110β antibodies (compare lane 4 with lane 3). A protein comigrating with p85 was detectable in anti-p85 blots of anti-p110β precipitates (lane 4) upon longer exposure (data not shown).

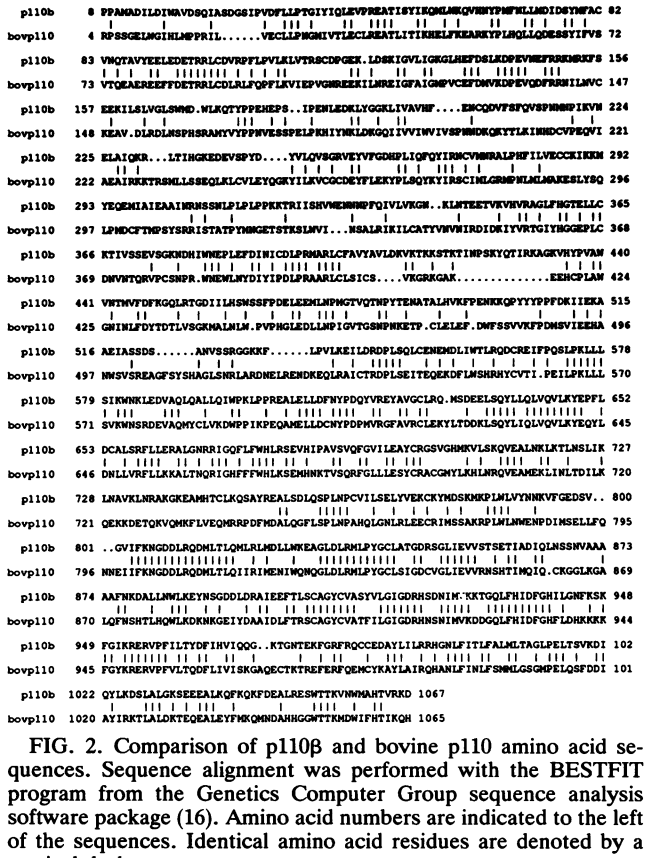

FIG. 2. Comparison of p110β and bovine p110 amino acid sequences. Sequence alignment was performed with the BESTFIT program from the Genetics Computer Group sequence analysis software package (16). Amino acid numbers are indicated to the left of the sequences. Identical amino acid residues are denoted by a vertical dash.

p110β mRNA is widely expressed in mouse tissues. Both PI 3-kinase activity and p85 are detectable in a wide range of tissues and cell lines (11, 50). Although bovine p110 has been shown to associate with p85 (22), its pattern of expression has not been described. Having demonstrated that p110β encodes a novel PI 3-kinase (Fig. 4) that also associates with p85 (Fig. 5), we sought to determine the tissue distribution of p110β mRNA.

Northern analysis was performed on poly(A)$^+$ RNA isolated from mouse tissues and from a variety of cell lines (Fig. 6). The major 4.8-kb species of p110β mRNA was detected in mouse brain, heart, kidney, liver, lung, and spleen (Fig. 6), as well as in fat, intestine, muscle, and thymus (data not shown). p110β mRNA was also detected in testis upon prolonged exposure of the blot (data not shown), as well as in human umbilical vein endothelial cells, Jurkat human leukemic T cells, 293 human embryonic kidney cells, mouse 3T3 fibroblasts (Fig. 6), HeLa cells, and NBT2 rat bladder carcinoma cells (data not shown). Minor larger mRNA species were detected in some tissues and cells (Fig. 6).

The 187-amino-acid inter-SH2 region of p85 mediates association with p110β in vitro. Having demonstrated association between p85 and p110β, we sought to identify domains of p85 involved in this interaction. GST fusion proteins containing different domains of p85 were expressed in *E. coli* and purified by glutathione-agarose affinity chromatography. Immobilized fusion proteins were incubated with lysates from 293 cells transfected with a p110Tag cDNA. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-Tag antibodies.

FIG. 3. PI 3-kinase consensus sequence derived from amino acid sequences of p110β, bovine p110 (22), and yeast Vps34 (21). Sequence alignment was performed with BESTFIT as for Fig. 2 with amino acids 573 to 1070 of p110β, 565 to 1068 of bovine p110, and 341 to 875 of Vps34. The consensus sequence was calculated with PRETTY (16) and consists of amino acid residues identical among all three PI 3-kinases.

A schematic diagram of the fusion proteins is shown in Fig. 7. Equal amounts of each fusion protein (Fig. 8A) were assayed for binding to p110Tag (Fig. 8B). p110Tag was expressed upon transfection, as demonstrated by anti-Tag immunoblotting of total lysates (Fig. 8B, lanes 1 and 2). Neither GST alone (lane 3) nor GST-SH3-bcr, encompassing the SH3 domain and the region of *bcr* homology (40), bound to p110Tag (lane 4). GST-N+C-SH2, containing the two SH2 domains of p85 (23), bound to p110Tag (lane 5). Separation of this portion of p85 into three individual fragments demonstrated that the inter-SH2 domain was necessary and sufficient for binding to p110Tag in vitro (lane 7). Neither SH2 domain of p85 bound to p110Tag (lanes 6 and 8), despite the ability of each to bind to tyrosine-phosphorylated growth factor receptors (reference 23 and data not shown). PI 3-kinase assays of GST fusion proteins that had been incubated with lysates containing p110Tag also dem-

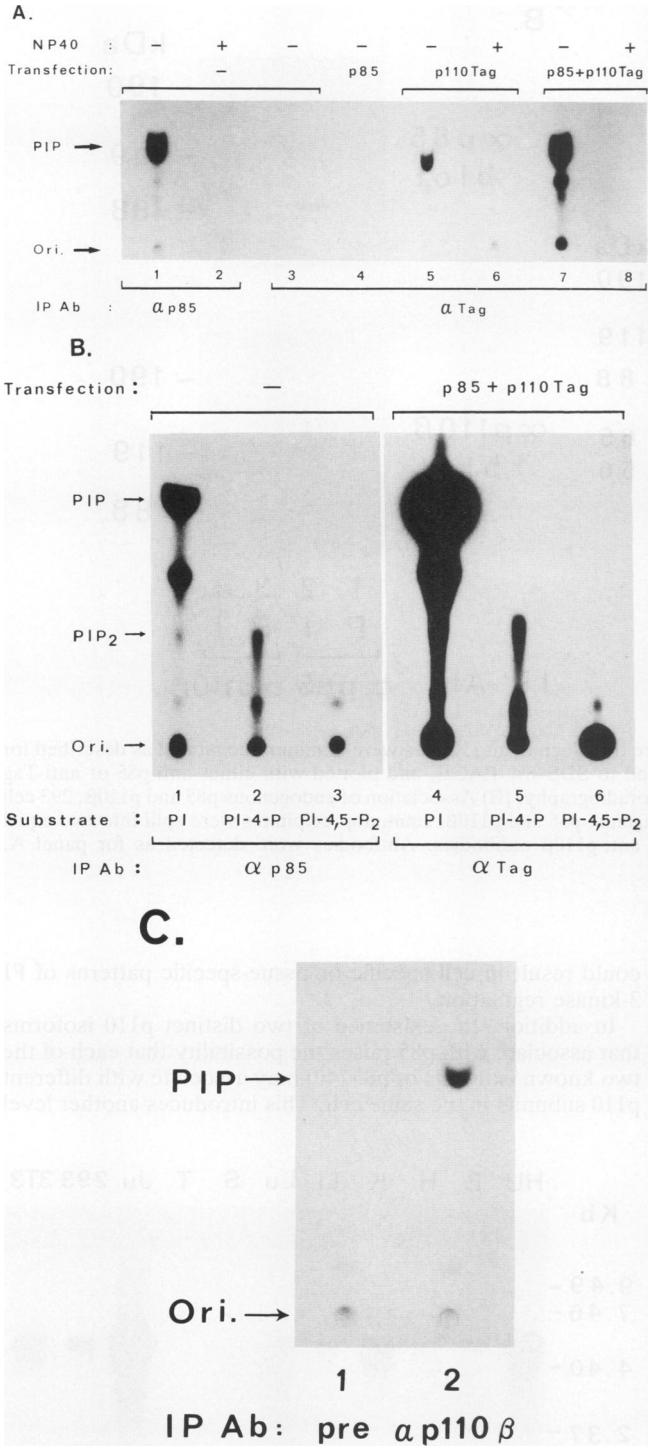

FIG. 4. (A) PI 3-kinase activity in immunoprecipitates of lysates from transiently transfected 293 cells. Cells were transfected with vector alone, p85, epitope-tagged p110β, or both cDNAs. Lysates were precipitated with either anti-p85 (αp85) or anti-Tag (αTag) antibodies. Immunoprecipitates were assayed for PI 3-kinase activity in the absence or presence of 0.5% NP-40. (B) PI 3-kinase activity on various substrates. Cells were transfected and lysates were precipitated as for panel A. Immunoprecipitates were assayed for PI 3-kinase activity on PI, PI-4-P, and PI-4,5-P_2 as substrates. (C) Endogenous PI 3-kinase activity in anti-p110β immunoprecipitates. 293 cell lysates were precipitated with preimmune serum or anti-p110β antibodies, and immunoprecipitates were assayed for PI 3-kinase activity.

onstrated increased activity associated with GST-N+C-SH2 and GST–inter-SH2 but not with the other fusion proteins (data not shown).

The p85 inter-SH2 domain binds PI 3-kinase activity and p110β in intact cells. To corroborate the in vitro binding data, the p85 inter-SH2 domain was tagged with the hemagglutinin epitope and expressed transiently in 293 cells alone or with p110β. Lysates from transiently transfected cells were precipitated with anti-Tag or anti-p110β antibodies and subjected to PI 3-kinase assay (Fig. 9) or SDS-PAGE and immunoblotting (Fig. 10).

Anti-p110β antibodies precipitated endogenous PI 3-kinase activity from untransfected cell lysates and lysates containing overexpressed epitope-tagged p85 inter-SH2 domain (INT) (Fig. 9, lanes 1 and 2). Anti-p110β precipitates of cell lysates containing overexpressed p110β contained elevated PI 3-kinase activity (compare lanes 3 and 4 with lanes 1 and 2). PI 3-kinase activity was not detectable in anti-Tag precipitates of untransfected cell lysates or lysates containing overexpressed p110β alone (lanes 5 and 7). However, overexpression of INT alone resulted in the precipitation of endogenous PI 3-kinase activity by anti-Tag antibodies (lane 6). Cotransfection of INT and p110β resulted in an increase in anti-Tag-precipitable PI 3-kinase activity (compare lanes 6 and 8).

The tagged p85 inter-SH2 domain was detected by anti-Tag immunoblotting as a doublet that migrated on SDS–12% PAGE with an apparent molecular mass of ~34 kDa (Fig. 10, lanes 2 and 4); this is commensurate with the 270-amino-acid protein encoded by the cDNA. Consistent with the ability of INT to associate with PI 3-kinase activity, INT also coimmunoprecipitated with p110β when both were overexpressed in 293 cells (Fig. 10, lanes 4 and 8). Detectable coimmunoprecipitation required cotransfection of INT and p110β; no p110β was detectable in anti-Tag immunoprecipitates of lysates from cells transfected with INT alone (lane 2), despite the observation that endogenous PI 3-kinase activity was detectable in these immunoprecipitates (Fig. 9, lane 6). Anti-Tag and anti-p110β immunoblotting of immunoprecipitates from cell lysates containing overexpressed INT and p110β, respectively, demonstrated appropriate overexpression of the transfected cDNAs (Fig. 10, lanes 2, 4, 7, and 8).

DISCUSSION

We have identified and characterized p110β, a novel isoform of PI 3-kinase that associates with p85 and is widely expressed in tissues and cell lines. Our findings confirm the existence of distinct p110 isoforms and are consistent with biochemical analyses of PI 3-kinase which indicated the presence of different p110 species (8).

PI 3-kinase assays on immunoprecipitates, both of cell lysates containing overexpressed, epitope-tagged p110β and of endogenous p110β (Fig. 4A and C, respectively), clearly demonstrate that p110β is a PI 3-kinase. Comparison of the p110β amino acid sequence with peptide sequences derived from purified rat PI 3-kinase confirmed this finding and strongly suggested that p110β is the human homolog of the upper p110 component of rat PI 3-kinase. p110β phosphorylates PI, PI-4-P, and PI-4,5-P_2 in vitro, and the in vitro substrate specificity of p110β was similar to that of endogenous PI 3-kinase (Fig. 4B). p110β had PI 3-kinase activity when expressed alone or with p85 in 293 cells (Fig. 4A). In contrast, bovine p110 was not active in COS-1 cells unless

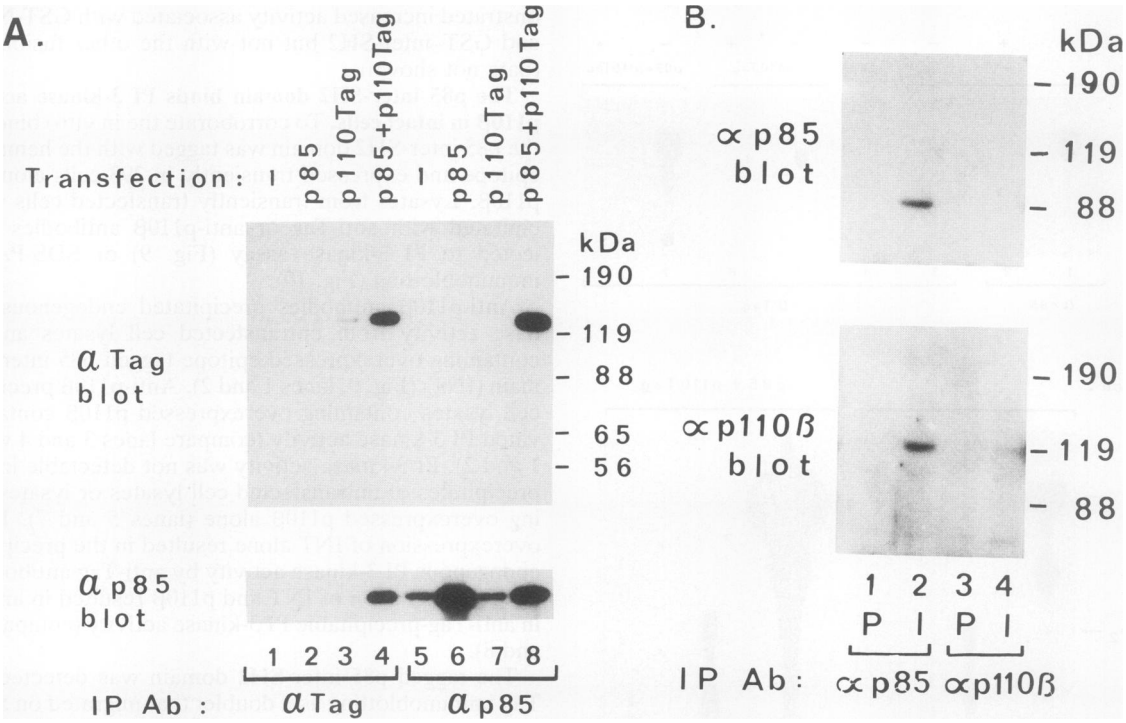

FIG. 5. (A) Association of p85 and p110β in intact cells. 293 cells were transfected, and lysates were immunoprecipitated as described for Fig. 4. Immunoprecipitates were split into two equal portions, subjected to SDS–8% PAGE, and blotted with either anti-p85 or anti-Tag antibodies. Antibodies were detected with ^{125}I-protein A and then by autoradiography. (B) Association of endogenous p85 and p110β. 293 cell lysates were precipitated with preimmune serum or specific antibodies against p85 and p110β. Immunoprecipitates were split into two equal portions, subjected to SDS–8% PAGE, and blotted with anti-p85 or anti-p110β antibodies. Antibodies were detected as for panel A. Abbreviations: P, preimmune; I, immune.

coexpressed with p85 (22). The reasons for this disparity are uncertain.

Coimmunoprecipitation assays on lysates from transiently transfected cells (Fig. 5A) and on endogenous cellular proteins (Fig. 5B) confirm that p85 and p110β associate with each other in intact cells. The observation that endogenous p85 is detectable in anti-Tag immunoprecipitates of cell lysates containing overexpressed p110Tag alone (Fig. 5A, lane 3) whereas p110Tag is not detectable in anti-p85 immunoprecipitates from the same cell lysates (lane 7) can be explained by the fact that the anti-p85 immunoprecipitates, which contain endogenous p85 present in all of the cells, contain only a fraction of expressed p110Tag which depends on the transfection efficiency and the amount of p110Tag expressed relative to endogenous p110 species that compete for binding to p85.

Transient transfection of 293 cells with p110Tag alone consistently yielded lower levels of p110Tag expression compared with those observed in cells cotransfected with p85 and p110Tag (Fig. 5A, compare lanes 3 and 4). One possible explanation for this observation is that free p110 may have a shorter half-life than p110 complexed with p85, resulting in lower steady-state levels of p110 when it is expressed alone.

The ubiquity of p110β expression (Fig. 6) is consistent with the wide distribution of PI 3-kinase activity. Since the distribution of bovine p110 mRNA has not been described, it is possible that the PI 3-kinase activity present in anti-p85 immunoprecipitates could be accounted for at least in part by p110β. Differential expression of distinct p110 isoforms could result in cell-specific or tissue-specific patterns of PI 3-kinase regulation.

In addition, the existence of two distinct p110 isoforms that associate with p85 raises the possibility that each of the two known isoforms of p85 (40) may associate with different p110 subunits in the same cell. This introduces another level

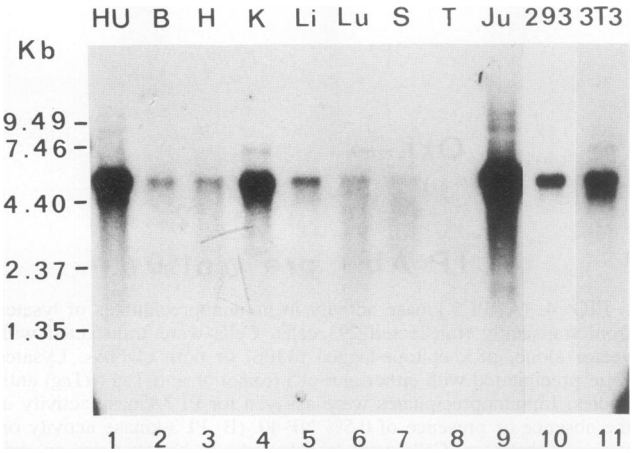

FIG. 6. Northern analysis of p110β mRNA. Two micrograms of poly(A)$^+$ RNA from mouse tissues and various cell lines was loaded in each lane. Abbreviations: HU, human umbilical vein endothelial cells; B, brain; H, heart; K, kidney; Li, liver; Lu, lung; S, spleen; T, testis; Ju, Jurkat; 3T3, NIH 3T3.

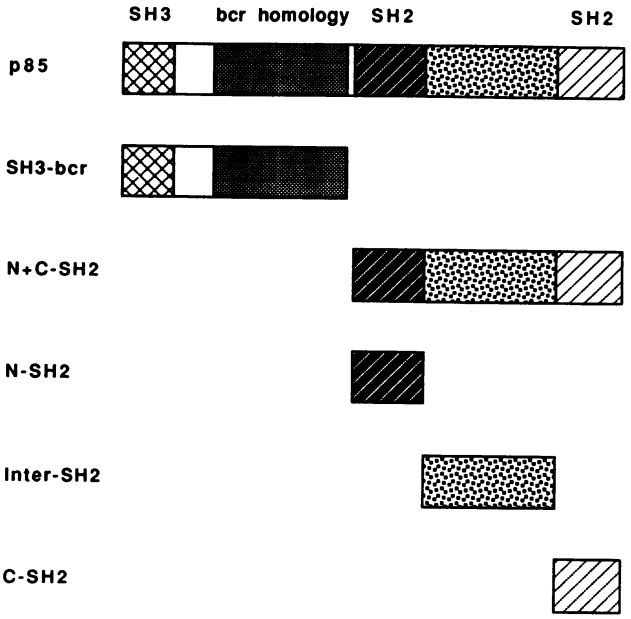

FIG. 7. Schematic diagram of GST-p85 fusion proteins. The domain structure of full-length p85 is shown at the top of the figure. The GST portion of each fusion protein is not shown.

of complexity to studies of PI 3-kinase in cell lines, as different p110 isoforms may be subject to disparate modes of regulation. For example, bovine p110 and p110β may have different substrate specificities in vivo. It is clear that intracellular levels of PI-3,4-P_2 and PI-3,4,5-P_3 rise in response to growth factors, cytokines, and chemotactic agents (2, 9, 13, 20, 24, 31, 43, 44, 52, 53, 55, 56, 61). Studies on the kinetics of PI phosphate synthesis have demonstrated a lag time between the appearance of PI-3,4,5-P_3 and PI-3,4-P_2 in response to fMet-Leu-Phe in neutrophils (53), PDGF in Swiss 3T3 cells (20), and thrombin in platelets (31). This disparity in kinetics suggests a model of PI-3,4-P_2 generation by 5′-dephosphorylation of newly synthesized PI-3,4,5-P_3 (53). The presence in particulate fractions of cell lysates of such a phosphatase activity (20, 53) is consistent with this hypothesis. However, with the discovery of a novel p110 isoform that can associate with p85, this difference in the rates of PI-3,4-P_2 and PI-3,4,5-P_3 synthesis could be accounted for by the simultaneous membrane recruitment through p85 of distinct PI 3-kinase catalytic subunits with different substrate specificities and enzyme kinetics.

Preliminary experiments suggest that p110β is phosphorylated predominantly on serine residues in vitro and in intact cells (data not shown). Recently, a serine kinase activity (PIK kinase) that copurifies with PI 3-kinase and phosphorylates p85 and p110 has been described; phosphorylation of p85 by this kinase is thought to inhibit PI 3-kinase activity (7). In vitro kinase assays with PI 3-kinase purified from rat liver demonstrated that the two forms of p110 are differentially phosphorylated by PIK kinase. Although the in vivo significance of p110 phosphorylation by PIK kinase remains to be determined, it is possible that distinct p110 isoforms may be differentially regulated by phosphorylation. Moreover, the copurification of PIK kinase with PI 3-kinase through several steps and the size of the PIK kinase–PI 3-kinase complex (~200 kDa [7]) are consistent with the possibility that p110 may harbor both serine kinase and PI 3-kinase activities. If this is the case, then distinct isoforms of p110 may possess serine kinase activities that are subject to distinct modes of regulation.

In vitro association assays with bacterial fusion proteins (Fig. 8B) and coimmunoprecipitation assays on lysates from transiently transfected cells (Fig. 9 and 10) implicate the 187-amino-acid inter-SH2 region of p85 as a domain of interaction between p85 and p110β. We do not understand why this domain appears as a doublet on the SDS-PAGE gel when it is expressed in mammalian cells (Fig. 10); it appears as a single band on the SDS-PAGE gel when expressed as a GST fusion protein in bacteria (Fig. 8A). Although INT associated with endogenous PI 3-kinase activity when expressed in 293 cells (Fig. 9, lane 6), endogenous p110β was not detectable in a complex with overexpressed INT (Fig.

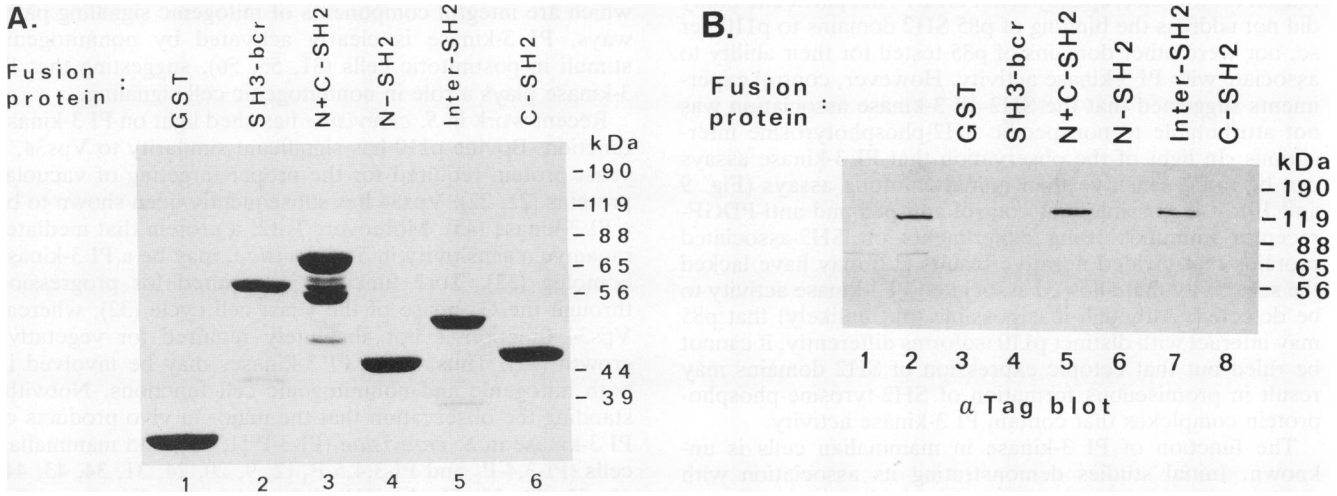

FIG. 8. (A) Coomassie blue-stained GST-p85 fusion proteins analyzed by SDS–10% PAGE. Approximately 5 μg of each fusion protein was loaded in each lane. The same quantity of protein was used for in vitro binding studies. (B) Binding of epitope-tagged p110β to GST-p85 fusion proteins. Lysates from 293 cells transfected with an epitope-tagged p110β cDNA were incubated with immobilized GST fusion proteins. Bound proteins were washed, separated by SDS–10% PAGE, and immunoblotted with anti-Tag antibodies. Antibodies were detected with enhanced chemiluminescence.

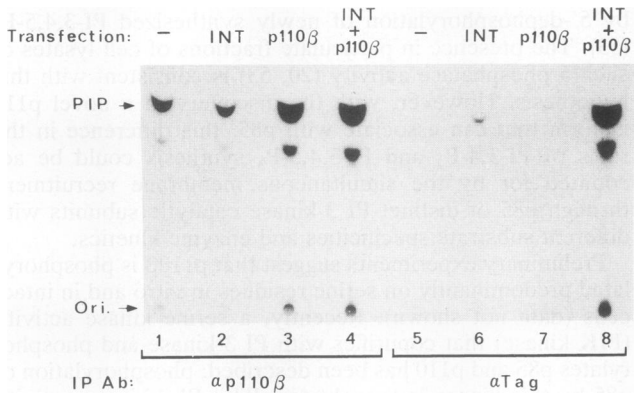

FIG. 9. Association of PI 3-kinase activity with the p85 inter-SH2 domain. 293 cells were transfected with vector alone, epitope-tagged p85 inter-SH2 cDNA, p110β cDNA, or both cDNAs. Lysates were precipitated with anti-Tag or anti-p110β antibodies, and immunoprecipitates were assayed for PI 3-kinase activity.

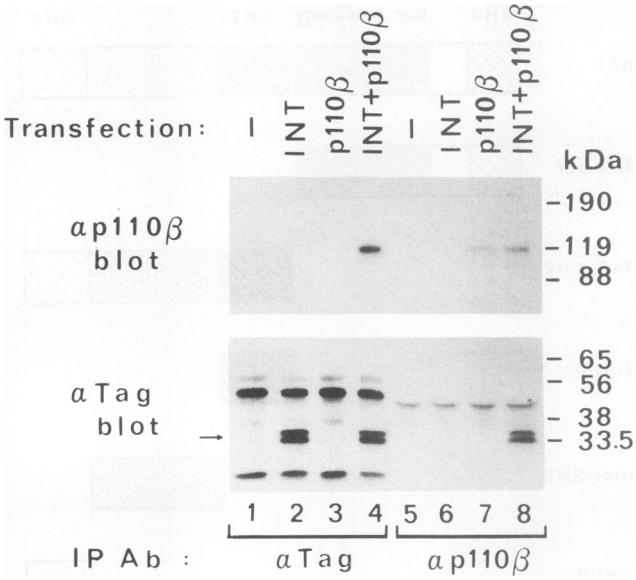

FIG. 10. Association of the p85 inter-SH2 domain and p110β in intact cells. 293 cells were transfected, and cell lysates were precipitated as described for Fig. 9. Immunoprecipitates were split into two equal portions, subjected to SDS–8% (anti-p110β blotting) or –12% (anti-Tag blotting) PAGE, and immunoblotted. Antibodies were detected with ^{125}I-protein A and autoradiography (anti-p110β) or enhanced chemiluminescence (anti-Tag). The arrow denotes the tagged p85 inter-SH2 domain.

10, lane 2). This could be explained by the possibility that the associated endogenous PI 3-kinase activity arises from a different p110 isoform not recognized by anti-p110β blotting antibodies; alternatively, the demonstration that p110β is present in 293 cells (Fig. 5B) and the fact that the PI 3-kinase assay is more sensitive than anti-p110β immunoblotting (compare Fig. 9, lanes 1 and 2, with Fig. 10, lanes 5 and 6) suggest that in this assay INT is associated with amounts of p110β that are detectable by PI 3-kinase assay but beyond the sensitivity of the immunoblotting antibody.

The p85 inter-SH2 domain that mediates binding to p110β is highly conserved between the two known isoforms of p85 (74% identity and 89% similarity between bovine p85α and bovine p85β [40]). Since bovine p85α and p85β both associate with bovine p110 (22), it is likely that this region of p85β also mediates association with p110.

The identification of the p85 inter-SH2 domain as the region mediating interaction with p110β is inconsistent with recent studies suggesting that the SH2 domains of p85 are involved in interactions with PI 3-kinase activity (12). The reason for this discrepancy is not clear. That particular study did not address the binding of p85 SH2 domains to p110 per se, nor were other domains of p85 tested for their ability to associate with PI 3-kinase activity. However, control experiments suggested that the SH2–PI 3-kinase association was not attributable to nonspecific SH2-phosphotyrosine interactions. In light of the observation that PI 3-kinase assays can be more sensitive than immunoblotting assays (Fig. 9 and 10), it is possible that control anti-p85 and anti-PDGF-receptor immunoblotting experiments on SH2-associated proteins that yielded negative results (12) may have lacked the sensitivity that allowed associated PI 3-kinase activity to be detected. Although it is possible (but unlikely) that p85 may interact with distinct p110 isoforms differently, it cannot be ruled out that ectopic expression of SH2 domains may result in promiscuous formation of SH2-tyrosine-phosphoprotein complexes that contain PI 3-kinase activity.

The function of PI 3-kinase in mammalian cells is unknown. Initial studies demonstrating its association with viral oncoproteins and activated growth factor receptor tyrosine kinases suggested that PI 3-kinase might play a general role in mitogenic pathways (reviewed in reference 5). Indeed, analysis of polyomavirus middle T antigen and v-*abl* mutants has revealed a strong correlation between an elevation in 3-phosphoinositide levels and the transformed phenotype (34, 46, 58, 60). Studies with receptor point mutants deficient in PI 3-kinase binding have yielded variable results regarding the role of PI 3-kinase in mitogenesis (18, 28, 59, 66); however, cellular levels of PI 3-kinase metabolites, which correlate better with viral oncoprotein transformation than association with PI 3-kinase activity (34), were not measured in these studies. In addition, these receptor point mutants may also be deficient in binding other molecules which are integral components of mitogenic signaling pathways. PI 3-kinase is clearly activated by nonmitogenic stimuli in postmitotic cells (31, 55, 56), suggesting that PI 3-kinase plays a role in nonmitogenic cell signaling.

Recent work in *S. cerevisiae* has shed light on PI 3-kinase function. Bovine p110 has significant similarity to Vps34, a yeast protein required for the proper targeting of vacuolar proteins (21, 22). Vps34 has subsequently been shown to be a PI 3-kinase (45). Moreover, Tor2, a protein that mediates rapamycin sensitivity in *S. cerevisiae*, may be a PI 3-kinase homolog (32). Tor2 function is required for progression through the G_1 phase of the yeast cell cycle (32), whereas Vps34 function is not absolutely required for vegetative growth (21). Thus, yeast PI 3-kinases may be involved in both mitogenic and nonmitogenic cell functions. Notwithstanding the observation that the major in vivo products of PI 3-kinase in *S. cerevisiae* (PI-3-P [1, 45]) and mammalian cells (PI-3,4-P_2 and PI-3,4,5-P_3 [2, 9, 20, 24, 31, 34, 43, 44, 46, 52, 53, 55, 56, 60, 61]) differ, these studies may offer useful clues to the function of PI 3-kinase in mammalian cells. The cloning of cDNAs encoding novel PI 3-kinases should facilitate the dissection of PI 3-kinase function in higher eukaryotic systems.

ACKNOWLEDGMENTS

We thank Reiner Lammers and Axel Ullrich for the 293 cDNA library; Christopher Carpenter and Lewis Cantley for providing peptide sequence data on purified PI 3-kinase; Jan Sap, Ying-Ping Jiang, and Wei Li for RNA; Ben Margolis for comments on the manuscript; and Kiki Nelson for peptide and oligonucleotide synthesis.

This work was supported by a Physician Scientist Award from the NIH (DK01927; E.Y.S.) and a grant from Sugen, Inc. (J.S.).

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