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Cloning and Mutagenesis of the p110α Subunit of Human Phosphoinositide 3′-Hydroxykinase

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Abstract—Activation of phosphoinositide 3′-hydroxykinase (PI3K) is required for mitogenic signal transduction by several growth factors and oncogenes. PI3K is a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit. In the current study, we report the cloning and characterization of the p110α catalytic subunit of human PI3K. This clone is highly homologous (>99% amino acid identity) to bovine brain p110α, but contains 10 amino acid differences from the human p110α sequence previously reported. Comparison of this sequence with known Ser/Thr kinases and p110 homologs highlighted several conserved residues within the putative kinase domain. Mutational analysis of these residues (Asp^{915}, (Asp^{933}+Phe^{934})) yielded PI3K mutants with virtually complete loss of phosphoinositide phosphorylating activity. Expression of the wild-type p110α protein in CHO cells is sufficient to activate the serum response element derived from the promoter of c-*fos*, an immediate early gene product. In contrast, the catalytically impaired p110α mutants as well as the p85α subunit of PI3K were inactive in the *fos* assay. These studies suggest that the mitogenic signal transduction pathway mediated by PI3K is dependent upon the enzymatic activity of the p110α subunit of PI3K.

Introduction

Several growth factor receptors that encode tyrosine kinases interact with a set of downstream effector molecules to activate second messenger signal transduction pathways. Many of these effector molecules interact with the cytoplasmic domains of growth factor receptors through adaptor subunits containing SH2 domains. These SH2 domains bind to specific phosphorylated tyrosine residues that are frequently found on activated growth factor receptors. One potentially important effector molecule that interacts with growth factor receptors via SH2 domains is phosphoinositide 3′-kinase (PI3K). This enzyme has been found associated with several tyrosine kinase growth factor receptors including PDGFr, EGFr, and ErbB2, as well as T-cell receptors, focal adhesion centers, and oncogene products such as polyoma middle T antigen and v-Src (for recent reviews, see refs 1, 2).

PI3K is a lipid kinase which phosphorylates phosphoinositides at the 3′-hydroxyl of the inositol ring. In vitro, PI3K utilizes PI, PI(4)P and $PI(4,5)P_2$ as substrates, converting them to their respective 3′-phosphorylated products.[3] In vivo, activation of PI3K is associated with a burst of $PI(3,4,5)P_3$ production in stimulated fibroblasts and neutrophils, suggesting that PI3K's primary substrate is $PI(4,5)P_2$.[2,4,5] These products are not substrates for phospholipase C,[6] so they appear to represent novel second messengers. $PI(3,4,5)P_3$ has been reported to directly activate certain protein kinase C isoforms, and more recently to serve as a ligand for SH2 domains.[7–9] The direct physiological consequence of $PI(3,4,5)P_3$ production is still uncertain, however.

PI3K is required for mitogenic signaling by at least some tyrosine kinase growth factor receptors and oncogenes.[1,2] It has also been demonstrated that microinjection of anti-p110α antibody blocks mitogenic signaling through the PDGF and EGF, but not the CSF-1, bombesin, or LPA receptors.[10] Taken together these observations suggest that PI3K plays a critical role in some signal transduction pathways, including mitogenesis.

As originally identified, PI3K consists of catalytic (p110) and adapter (p85) subunits. The catalytic function is contained within the p110 subunit while the p85 subunit contains two SH2 domains and one SH3 domain and is thought to function as an adapter subunit linking the catalytic subunit to phosphotyrosine-containing regulatory proteins. To date, four enzymes with demonstrable PI3K catalytic activity have been identified (p110, α, β, γ, and VPS34).[11–15] PI3K γ and human VPS34 do not associate with a p85 regulatory domain, and human VPS34 also shows different substrate preferences compared to the canonical p110 isoforms of PI3K.[13,15]

Biochemical characterization of recombinant PI3K's enzyme activity has been reported from our laboratory and others.[11,16,17] Less information is available concerning the biological consequences of overexpression of PI3K in intact cells.[18] In the current study, we present the cloning and biological characterization of a human PI3K p110α isozyme. The sequence of this clone is similar, but not identical to, previously reported p110α sequences. We also report that overexpression of the catalytic domain itself, in the absence of exogenous p85, is sufficient to activate the

serum response element (SRE) of the *fos* promoter, which is a fundamental step in growth factor induced *mitogenesis*. Specific point mutations of conserved amino acids in the catalytic domain of p110α eliminate its ability to phosphorylate PI in vitro, and also abolish its ability to activate the *fos* SRE.

Methods

Cloning of human p110α

The strategy for cloning the human p110α subunit was to amplify the gene from a human brain cDNA library (Clontech) in two pieces using DNA primers homologous to the bovine p110α sequence. Tails were added to the 5′ ends of the PCR primers to add restriction sites for subcloning and to optimize the ribosome translation initiation sequence (Kozak sequence). The sequences of the PCR primers are provided in Table 1.

The N-terminal half of p110 was amplified using oligo A and oligo C. Oligo A represents the sense strand of the bovine sequence from nucleotides 1–33 and includes the ATG start codon and a tail with a KpnI site and an optimized translation initiation 'Kozak' sequence. Oligo C represents the antisense strand of p110 from nucleotides 1770–1798 and includes a BamHI site in the non-homologous tail section. The C-terminal half was PCR amplified using oligo B and oligo D. Oligo B represents the midgene 'sense' strand of p110 and encompasses bovine sequences 1656–1687 beginning immediately after the KpnI site. Oligo D represents the antisense strand from 3171 to the stop codon. A BamHI site has been incorporated immediately after the stop codon. Both PCR amplified fragments were trimmed with KpnI and BamHI and subcloned into pGem4Z (Promega). The subclones were spliced together using the overlapped XbaI site contained in both the N- and C-terminal subclones. The complete p110α DNA was sequenced in both strands.

Confirmation of 5′ ATG start and PCR amplified sequence

Because the sequence of the primers is incorporated into the amplified fragments and this sequence is a bovine sequence, it was important to determine independently the human p110α sequences overlapping the PCR primers. To obtain independent p110α clones, a λ-Zap human brain cerebellum phage cDNA library (Stratagene) was screened with a random primed C-terminal p110α pGem4Z plasmid (subcloned from PCR cloning above). Several independent p110α clones were obtained which contained sequence from the C-terminal portion of the gene (encompassing amino acids 523–1068). Sequencing of these clones confirmed that the sequences of the bovine based PCR primers were identical to the true human sequence with the exception of one base pair in the 'C' oligonucleotide. This non-homologous base pair was eliminated from the final human p110α clone when the two halves were spliced together. Despite exhaustive λ library screening, clones were not obtained for the extreme N-terminus of the p110α gene. The N-terminus of p110α was confirmed by independently amplifying the N-terminus from a λ-ZapII human fetal brain cDNA library (Stratagene) using an antisense primer internal to the p110 gene and a second primer homologous to sequence in the λ cloning vector (Table 1). The complete p110α sequence has been submitted to GenBank (accession number U79143).

PCR amplification of the λ library DNA yielded several independent clones overlapping the ATG methionine start and including sequences 5′ to the coding region. Stop codons were identified in all three reading frames immediately 5′ to the putative ATG methionine start (Fig. 1).

Because of the error rate inherent in PCR amplification, any amino acid differences between the current human gene and previously reported human and bovine genes could potentially be due to PCR artifacts.

Table 1. PCR primers utilized in cloning of human p110α

Oligo A.	5′	GAGAGGTACC**GCCACC**ATGCCTCCAAGACCATCAGGTGAACTGTGGGGC KpnI Kozak	3′
Oligo B.	5′	GAGAGGTACCGGAGCCACAGACACTATTGTGTAACTATCCCCG KpnI	3′
Oligo C.	5′	GAGAGGATCCCCATAGCCTGTTCAGGCTTGATTGGAGGCC Bam HI	3′
Oligo D.	5′	Stop GAGAGGATCC**TCA**GTTCAAAGCATGCTGCTTAATTGTGTGGAAGATCC BamHI	3′
Oligo E.	5′	GATTCGGATCCCATCTTGAAGAAGTTGATGGAGG	3′
Oligo F.	5′	TCGAGGTCGACGGTATCGATAAG	3′

Oligonucleotides A and C were employed to PCR amplify the N-terminal half of the human p110α coding sequence while B and D were used for the C-terminal half. The oligonucleotides were based on the bovine p110α sequence and incorporated 5′ extensions with restriction enzyme sites to facilitate subcloning. Oligonucleotide A incorporates an optimized 'Kozak' sequence (bold) immediately 5′ to the ATG translation start site. Oligonucleotides E and F were used to confirm the extreme 5′ coding sequence and determine the immediate 5′ untranslated region of p110α. Oligo E represents the human p110α antisense sequence from 193–171 with a 5′ overhang incorporating an EcoRI site. Oligo F is a sequence contained in the λ-Zap vector at the cloning insertion site.

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-80              -60              -40              -20              +1
 .    .    .    .    .    .    .    .    .    .    .    .    .    .
TGATATCGAATTCCTTTGGGACAACCATACATCTAATTCCTTAAAGTAGTTTTATATGTAAAACTTGCAAAGAATCAGAACAATGCCTCCACGACCA
.AspIleGluPheLeuTrpAspAsnHisThrSerAsnSerLeuLysEndPheTyrMetEndAsnLeuGlnArgIleArgThrMetProProArgPro
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Figure 1. Upstream regulatory region of human p110α. A portion of the human p110α sequence 5′ to the ATG translation start site is shown. The sequence contains stop codons in the p110α reading frame which validate the ATG start assigned to the coding sequence.

To eliminate this possibility, the λ-library clones described above were sequenced through the areas where there were differences in amino acid sequence relative to the bovine clone or the previously cloned human sequence. Three of the 10 observed differences in the human clones were contained in the C-terminal clone obtained by λ library screening. The other amino acid differences were verified by re-cloning the N-terminal domain by PCR using alternative PCR primers. All independent clones confirmed that the amino acid differences observed between the bovine and human p110α clones are legitimate. A comparison of the human and bovine p110α nucleotide sequences are presented in Fig. 2.

All oligonucleotides were from Midland Certified Reagent Company. Dideoxy sequencing was performed using Sequenase (U.S. Biochemical Corp.). Template cDNA libraries used in PCR amplification were from Clontech. PCR was performed using Taq polymerase

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                .    .    .    .    .
human α*  MPPRPSSGELWGIHLMPPRILVECLLPNGMIVTLECLREATLVTIKHELF
          ||||||||||||||||||||||||||||||||||||||||||:|||||||
human α   MPPRPSSGELWGIHLMPPRILVECLLPNGMIVTLECLREATLITIKHELF 50
          ||||||||||||||||||||||||||||||||||||||||||||||||||
bovine α  MPPRPSSGELWGIHLMPPRILVECLLPNGMIVTLECLREATLITIKHELF
                .    .    .    .    .
          VDLRDLNSPHSRAMYVYPPHVESSPELPKHIYNKLDRGQIIVVIWVIVSP
          ||||||||||||||||||:||||||||||||||:||||||||||||||
      151 VDLRDLNSPHSRAMYVYPPNVESSPELPKHIYNKLDKGQIIVVIWVIVSP 200
          ||||||||||||||||||:||||||||||||||||||||||||||||||
          VDLRDLNSPHSRAMYVYPPNLESSPELPKHIYNKLDKGQIIVVIWVIVSP
                .    .    .    .    .
          ILKVCGCDEYFLEKYPLSQYKYIRSCIMLGRMPNLKMMAKESLYSQLPMD
          ||||||||||||||||||||||||||||||||||.:||||||||||||||
      251 ILKVCGCDEYFLEKYPLSQYKYIRSCIMLGRMPNLMLMAKESLYSQLPMD 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||
          ILKVCGCDEYFLEKYPLSQYKYIRSCIMLGRMPNLMLMAKESLYSQLPMD
                .    .    .    .    .
          CFTMPSYSRRISTATPYMNGETSTKSLWVINRALRIKILCATYVNLNIRD
          ||||||||||||||||||||||||||||||.||||||||||||||:||||
      301 CFTMPSYSRRISTATPYMNGETSTKSLWVINSALRIKILCATYVNVNIRD 350
          ||||||||||||||||||||||||||||||||||||||||||||||||||
          CFTMPSYSRRISTATPYMNGETSTKSLWVINSALRIKILCATYVNVNIRD
                .    .    .    .    .
          SREAGFSYSHAGLSNRLARDNELRENDKEQLKAISTRDPLSEITEQEKDF
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      501 SREAGFSYSHAGLSNRLARDNELRENDKEQLKAISTRDPLSEITEQEKDF 550
          ||||||||||||||||||||||||||||||||:||:||||||||||||||
          SREAGFSYSHAGLSNRLARDNELRENDKEQLRAICTRDPLSEITEQEKDF
                .    .    .    .    .
          HLNRQVEAMEKLINLTDILKQERKDETQKVQMKFLVEQMRRPDFMDALQG
          ||||||||||||||||||||:||||||||||||||||||||||||||||
      701 HLNRQVEAMEKLINLTDILKQEKKDETQKVQMKFLVEQMRRPDFMDALQG 750
          ||||||||||||||||||||||||||||||||||||||||||||||||||
          HLNRQVEAMEKLINLTDILKQEKKDETQKVQMKFLVEQMRRPDFMDALQG
                .    .    .    .    .
          LLSPLNPAHQLGNLRLKECRIMSSAKRPLWLNWENPDIMSELLFQNNEII
          :||||||||||||||.||||||||||||||||||||||||||||||||||
      751 FLSPLNPAHQLGNLRLEECRIMSSAKRPLWLNWENPDIMSELLFQNNEII 800
          ||||||||||||||||||||||||||||||||||||||||||||||||||
          FLSPLNPAHQLGNLRLEECRIMSSAKRPLWLNWENPDIMSELLFQNNEII
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Figure 2. Comparison of PI3K p110 sequences. The human p110α amino acid sequence is compared with the previously reported human p110α sequence (human α*)[14] and the bovine p110α sequence (bovine α).[11] A discontinuous comparison of the two p110α amino acid sequences is presented for regions where the sequence varies. The sequence comparison was generated using the 'Best Fit' subroutine of the GCG sequence analysis programs. Conserved residues are denoted by the solid vertical line (|) while functionally similar amino acid residues are connected by (:) and (.) symbols.

and reagents from Perkin Elmer Cetus. Phage cDNA libraries used to confirm PCR amplified sequences were from Clontech and Stratagene. The coding sequence of the N-terminal and C-terminal halves of human p110α was PCR amplified from a human brain cerebral cortex 'QUICK clone' cDNA (Clontech).

Expression plasmid construction

To produce a recombinant baculovirus DNA, the human p110α and p85α genes were cloned into the baculovirus shuttle vector pVL1393 (Pharmigen). The p110α coding sequence with the optimized Kozak sequence was cleaved from pSSG110WT with BamHI and Acc65I and the 5′ overhangs blunt ended with DNA pol I Klenow fragment. The blunted fragment was isolated from an agarose gel and ligated into the SmaI site of pVL1393. Clones containing the insert were screened for orientation and sequenced through the restriction fragment junctions to confirm the proper fused sequence. Recombinant baculovirus separately containing p85 and p110 inserts were generated as described by the manufacturer (Baculogold, Pharmigen).

Point mutations in p110α were generated by PCR mutagenesis.[19] The full length p110α wild type clone in pGem4Z (Promega), pSSG110WT, was employed as a template to PCR amplify segments of the gene using PCR primers containing the point mutations. The point mutations substituted alanines for Asp915 (D915A), Asp933 combined with Phe934 (D933A/F934A), Glu970 (E970A) or Arg922 (R922A). Mutated PCR products were trimmed with restriction enzymes and subcloned into pGem4Z. The subcloned fragments were sequenced through their entire length to confirm the desired mutation. Mutated restriction fragments were swapped for the corresponding wild type fragments in the full length p110α clone. The p110α mutants were then cloned into expression vectors.

Human p85α was cloned by PCR amplification of the gene from a human kidney cDNA library (Clonetech). The N-terminal and C-terminal SH2 domains of human p85α were mutated using the pSelect site directed mutagenesis system (Promega). Arg358 and Arg649 in the FLVRES motif of the respective SH2 domains were mutated to alanine. This mutation has been shown to severely reduce the binding affinity of an SH2 domain for its phospho-tyrosine binding site.[20] The individual SH2 mutants were combined in a single p85 molecule by restriction fragment swapping. The SH3 domain was mutated at Trp55 to alanine. The equivalent mutation has been shown to eliminate Src SH3 domain binding to its proline rich binding site in p85α.[21] The final triple mutant clone was sequenced through both SH2 and SH3 domains to verify the sequence integrity. The p85α wild-type and mutant clones were inserted into the vector pVL1393 (Pharmingen) for insect cell expression and a CMV-neo eucaryotic expression plasmid for transient expression studies.

Expression of PI3K heterodimer in baculovirus/insect cell system

SF9 cells were centrifuged at 200 *g* for 5 min and resuspended in fresh TMNFH at a cell density of 6×10^5–1×10^6 cells/ml in a volume of 500–800 ml contained in a 1 L Kontes spinner vessel. For co-expression of heterodimer, cells were infected with p85α virus at a MOI of 1 plaque-forming unit/cell and p110α at a MOI of 5 plaque-forming units/cell. Spinner vessels were stirred at 40 rpm at 27 °C. One millilitre of sample was removed daily to monitor cell density and viability. When viability decreased to 50–65%, cells were harvested by centrifuging at 200 *g* for 10 min. The cell pellet was washed with PBS, centrifuged and the cell pellet stored at −70 °C.

Purification of PI3K heterodimer

Virus infected SF9 cells (1.2 L, 4×10^5 cells) expressing p85 and p110 were resuspended in 60 ml of lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 50 mM NaF, 10 mM benzamidine, 25 μg/ml 4-(2-aminoethyl)benzosulfonyl fluoride, 1 μg/ml Pepstatin, 0.13 U/ml Aprotinin, 4 μg/ml *N*-tosyl-L-lysine chloromethyl ketone, 25 μg/ml leupeptin, and 4 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone). The cells were sonicated on ice. Cellular debris was pelleted at 12,000 *g*. The crude lysate was subjected to DEAE-Sepharose, S-Sepharose and Reactive-Blue 4 agarose chromatography as described.[17]

Recombinant PI3K enzymology

PI3K catalytic activity was determined essentially as described.[17] Briefly, a stock solution of small unilamellar vesicles was prepared by drying chloroform stock solutions of the lipids in a polypropylene tube under a steam of nitrogen. Tris–HCl, pH 7.4 was added and the lipid suspended by vortexing. This solution was sonicated on ice for 30 s for each 100 μl of solution. Vesicles were prepared fresh for each experiment. The assay buffer contained: 50 mM Tris–HCl, pH 7.4, 1.0 mM DTT, 2 mM $MgCl_2$, 50 μM γ-^{32}P-ATP and 600 μM PI. All reactions were run in a large excess of vesicles, as empirically determined from reaction progress curves. Reactions were terminated and the lipid aggregated by the addition of an equal volume of 1.0 N HCl. The aggregated lipid was collected on a positively charged nylon membrane (Wallac Oy, cat. no. 1205-403) in a Betaplate (Wallac Oy, Turku, Finland) manifold.

Assays requiring the identification of the lipid product or increased sensitivity were analyzed by thin-layer chromatography on grooved Whatman Silica Gel 60A thin layer plates. The plates were developed in a filter paper-lined and equilibrated tank using *n*-propanol:2.0 M acetic acid (13:7, by volume).

Northern blot analysis

Multiple human tissue Northern blots (Clontech) were hybridized with a ^{32}P-labeled mRNA transcribed with T7 RNA polymerase (Promega) using clone pSSG110WT cut with BamHI as the template. Hybridization was performed in QuikHyb Hybridization Solution (Stratagene) and washed as recommended by the manufacturer.

fos promoter assay

CHO cells were grown to 90% confluency in six-well dishes in a media of alpha-MEM (Life Technologies), 10% charcoal stripped calf serum (HyClone) and 1 × penicillin/streptomycin. Media was changed to alpha-MEM plus 5% charcoal stripped calf serum and cells were transfected using a $CaPO_4$ precipitation technique.[22] Ten micrograms of co-precipitated plasmid DNA was added dropwise to each cell well. The p110 and p85 genes were contained in an expression plasmid under control of the CMV promoter, while v-*ras* and v-*raf* were under control of a viral LTR promoter. The reporter plasmid contains the gene for secreted alkaline phosphatase under the control of the serum response element of the human c-*fos* promoter.[23] Transfections were incubated for 16 h at 37 °C under a CO_2 atmosphere. DNA/media was removed, the cells were washed twice with PBS and a media of alpha-MEM plus 0.5% charcoal stripped calf serum and 1 × penicillin/streptomycin was added to each well. After 30 h a media aliquot was removed for assay of secreted alkaline phosphatase (SEAP). Media aliquots were incubated for 1 h at 65 °C to inactivate endogenous alkaline phosphatase. SEAP activity in the media was assayed using a CMPD chemiluminescence assay essentially as described by the manufacturer (Tropix). Luminescence was monitored in a ML2200 luminometer (Dynatech).

Results

Human p110α was cloned from a brain cDNA library in two pieces using PCR primers homologous to bovine p110α[11] (see Table 1). The C-terminal sequence was verified by screening a human brain cerebellum phage cDNA library with a random primed C-terminal probe. Because no N-terminal clones were obtained, the sequence of this domain was verified by independent PCR amplification from a human brain cDNA library. The clone was sequenced on both strands and this DNA sequence has been submitted to GenBank. The nucleotide sequence immediately 5′ to the ATG translation start is shown in Figure 1. The amino acid sequence of this human p110α is virtually identical to that of bovine p110α (99.7% amino acid identity).

After the completion of these studies, the sequence of human p110α was published by other investigators.[14] Our p110 clone was found to contain 39 different nucleotides, resulting in 10 different amino acids, compared to the EMBL sequence cited (Fig. 2). Each amino acid difference was verified in independent clones of p110 obtained from screening the primary phage library, and so do not result from PCR artifacts in our sequence. All 10 of the observed amino acid differences are conserved between our clone and bovine p110α (Fig. 2).

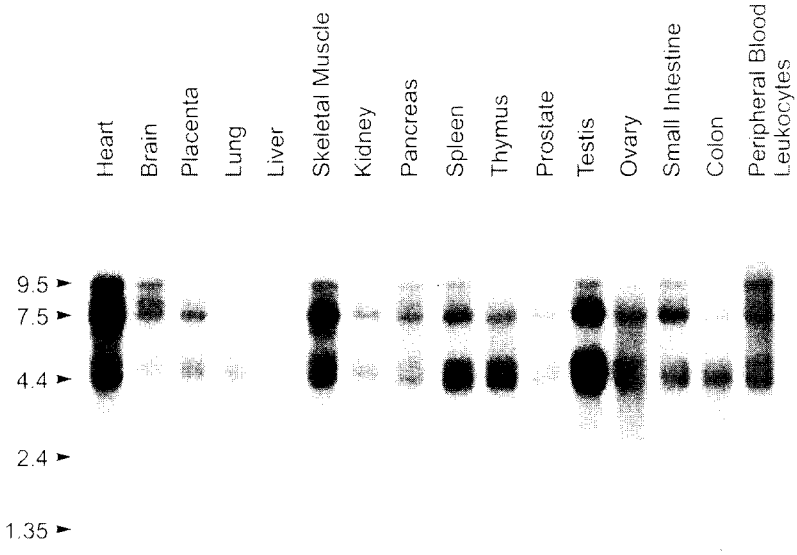

Figure 3. Tissue distribution of human p110α mRNA expression. Poly-(A) selected RNA from multiple human tissue samples (Clontech) was probed with ^{32}P labeled p110α RNA as described in Methods. The RNA probe represents the entire antisense strand of the human p110α coding sequence. The northern blots were washed at a high stringency in 0.1 × SSC at 70 °C. The position of molecular weight markers are indicated to the left.

Northern blot analysis of p110α

Human mRNAs from several organs on a multiple tissue Northern blot were hybridized with an RNA probe encoding the full-length anti-sense p110α clone. Multiple transcripts were observed, with substantial variation in signal intensity between the different tissues examined (Fig. 3). The most abundant expression of human p110 transcripts were detected in heart, skeletal muscle, and testis, with dominant transcript lengths of 4.4, 7.5 and 9.5 kbases. Some tissue-specific distribution between these three different mRNAs was apparent. These results are significantly different than the published patterns for human p110γ or for p110β expression in mouse tissue.[12,13]

Expression and mutagenesis of p110α

SF9 insect cells were infected with baculoviruses separately encoding human p110α and human p85α at a variety of multiplicity of infections (MOI's). In the absence of p85, only trace levels of p110 could be detected by 'Western' blot analysis, and no PI-phosphorylating activity was observed in these lysates (Fig. 4). Optimal expression of p110, as detected by both 'Western' blot and PI-phosphorylating activity, was obtained with a p110/p85 infection ratio of between 1 and 5. Co-immunoprecipitation experiments of infected cell lysates demonstrate that under these conditions, all p110 detected is complexed with p85. This complex was readily purified to apparent homogeneity using standard column chromatography techniques.[17]

The sequence of p110α displays only moderate homology to the consensus sequences of the catalytic domains of common protein kinases. For example, the glycine-rich P loop believed to be important for nucleotide binding in protein kinase A and other protein kinases is not apparent in p110α. Sequence alignments between p110α and consensus kinase sequences does suggest the existence of several key catalytic domain amino acids in the putative nucleotide binding site of p110α. These residues include the catalytic loop ($D^{915}RHNSN^{920}$) and the $D^{933}FG^{935}$ triplet.[24] To test the importance of these and other conserved residues in the catalytic activity of p110, and thus to explore the proposed homology between PI3K and other protein kinases, four mutant p110α genes harboring point mutations were generated. The resulting clones were co-expressed in SF9 cells with wild-type p85α, and the resulting p85α/p110α complexes were immunoprecipi-

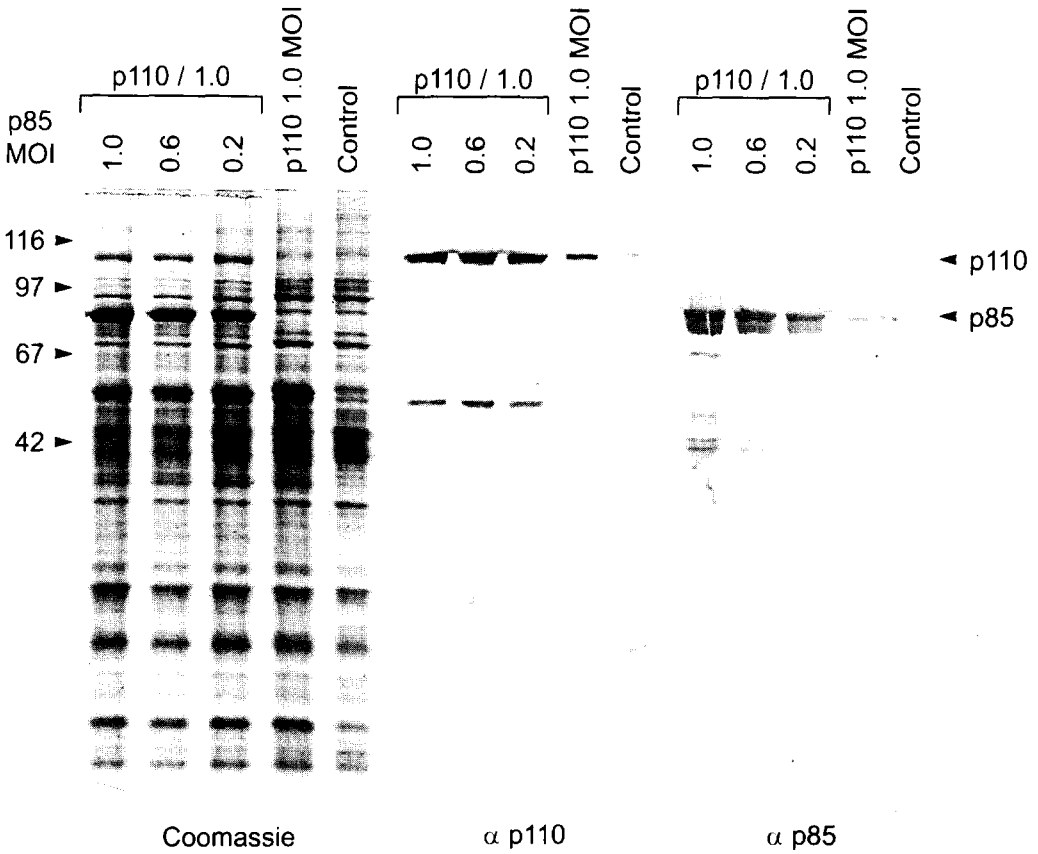

Figure 4. Co-expression of human p110α with human p85α in SF9 insect cells. SF9 insect cells were infected with recombinant baculovirus vectors encoding the human p110α and p85α genes. Cells infected at different multiplicities of infection (MOI) were analysed for expression of the two PI3-kinase subunits in total cell lysates. A Coomassie-stained SDS denaturing gel is shown to the left. Western blots of identically loaded gels are shown to the right. The p110α plot was probed with a polycolonal antibody raised to a peptide containing the 15 C-terminal amino acid of p110α while the p85α blot was probed with a monoclonal antibody (UBI) to p85 amino acids 327–434. Visualization of p110 and p85 bands was obtained using an alkaline phosphatase-conjugated secondary antibody. The p110 lane represents a lysate from SF9 cells infected with just the p110α baculovirus. The control lane is a lysate from cells infected with the non-recombinant virus.

tated and assayed for PI phosphorylating activity. In each case, p85α binding and immunoprecipitation efficiency were not significantly different among the mutants generated (Fig. 5). In two of the mutants, however, catalytic activity was dramatically impaired. The D915A and (D933A, F934A) mutants were essentially inert (Fig. 5). By contrast, the E970A and R922A mutants displayed near wild-type activity. Since protein expression and binding to p85 are unaffected by these mutations, these results imply that the Asp^{915} and Asp^{933}, Phe^{934} amino acids are essential for the catalytic activity of p110α.

Serum response element activation by p110

Numerous studies have demonstrated that PI3K activation is necessary for mitogenic signaling by many growth factors and oncogenes. Less information is available on whether PI3K is sufficient to activate elements of the mitogenic pathway in the absence of other signals. An early step in growth factor-induced mitogenesis is initiation of transcription of several 'immediate early' genes, such as c-*fos*. We explored the role of p110α activity in the mitogenic signal transduction pathway by measuring the ability of p110α to activate the serum response element (SRE) found in the c-*fos* promoter.

Transient transfections of wild-type p110α induced a five-fold activation of the c-*fos* SRE promoter element (Fig. 6). For comparison purposes, v-Ha-*ras* induced a 38-fold activation of this reporter constructed under similar conditions and v-*raf* elicited a 13-fold response. Transfection with the (D933A, F934A) mutant p110 expression plasmid did not activate the SRE *fos* promoter. These data demonstrate that p110α alone is sufficient to activate the serum response element of the c-*fos* promoter, and that the catalytic activity of the protein is required for this activity.

Mitogenic activation of 3T3 fibroblasts by phospholipase C-γ has been shown to be dependent on the SH3-containing region of this lipid-modifying signal transduction protein.[25] Since the p85 adapter subunit of PI3K contains its SH2 and SH3 domains, we antici-

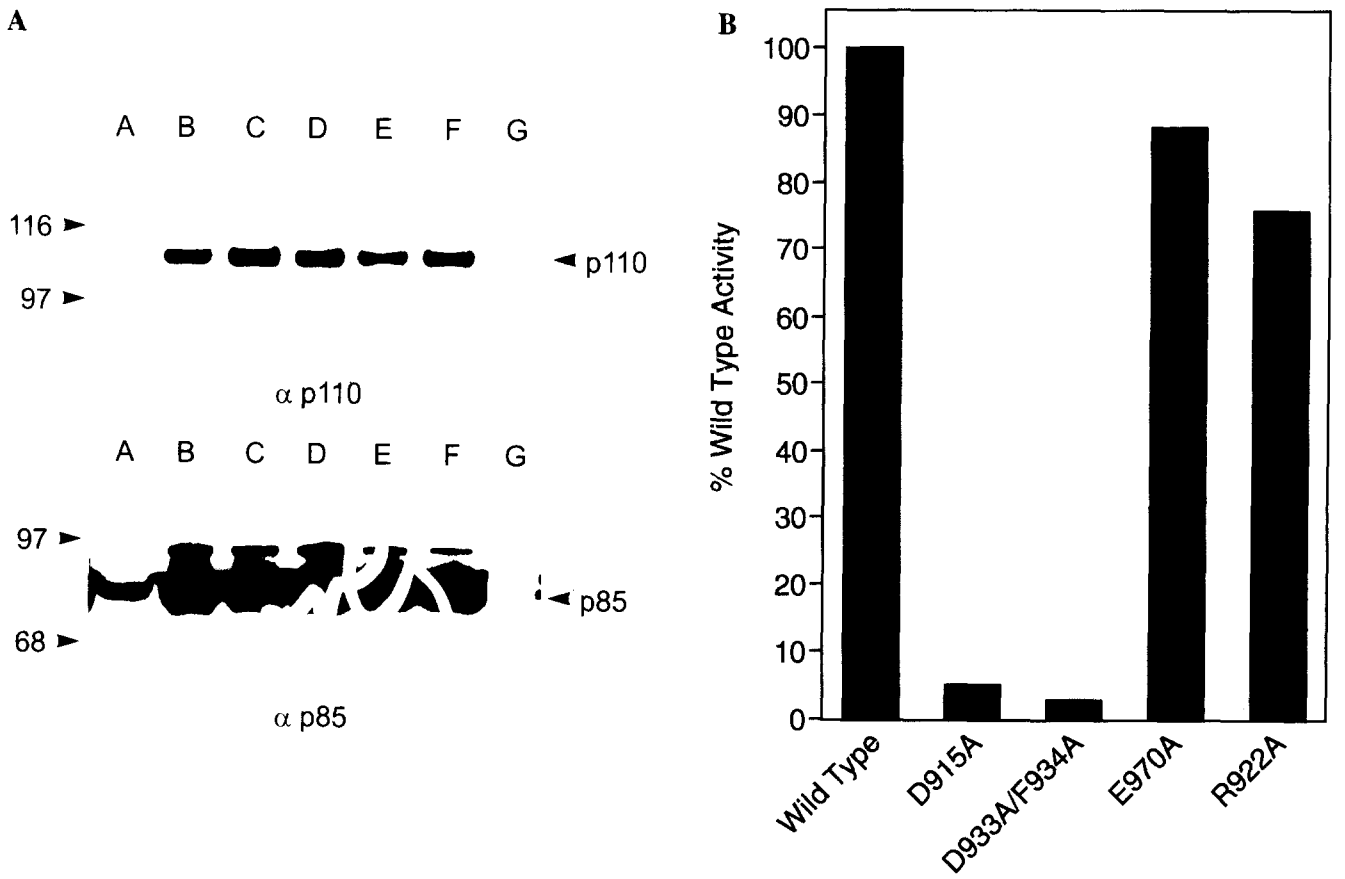

Figure 5. Catalyic activity of p110α mutants. Panel A: mutant p110α subunits were co-expressed with p85α in SF9 insect cells and the heterodimers were immunoprecipitated with an anti-p85α antibody. The precipitated proteins were electrophoresed on SDS denaturing polyacrylamide gels and analysed by Western blot. Westerns were probed with either a polyclonal antibody to bovine p110α (UBI) or a monoclonal antibody to p85α (UBI). Reactive bands were visualized by probing the blots with horseradish peroxidase conjugated secondary antibodies and developed with the ECL chemiluminescence kit (Amersham). The autoradiograph films were scanned by densitometer to normalize activity to the amount of immunoprecipitated protein. Lanes: (A) non-recombinant virus; (B) p110α wild-type; (C) D915A mutant; (D) D933A/F934A mutant; (E) E970A mutant; (F) R922A mutant; (G) p110α wild-type expressed without p85. Panel B: the immune precipitated PI3-kinase heterodimers described in Panel A were assayed for PI3-kinase activity in a filter plate assay as described and the activity normalized to the amount of protein as determined in the Western blots of Panel A. Activity relative to that observed with wild-type enzyme is plotted for the four mutants.

pated that the PI3K p85α domain might also contribute to activation of the SRE *fos* promoter. However, transfection of p85α alone did not activate the c-*fos* promoter (data not shown). Interestingly, co-transfection of wild-type p85α abolished activation of the *fos* reporter construct induced by wild-type p110α, v-*raf*, or v-*ras* (Fig. 6). Co-transfection of the catalytically inert mutant p110α gene had no effect on *ras*-mediated promoter activation (data not shown). Site specific mutations that inactivated the SH2 or the SH3 domains of p85 attenuated the suppressive effect. This experiment suggests that p85 overexpression acts as a dominant negative suppressor of p110 and *ras*-mediated activation of the *fos* promoter by binding to critical cellular proteins through p85's SH2 and SH3 domains.

Discussion

The sequences of several p110 proteins with demonstrated phosphoinositol kinase activity have been reported. In the current study, we report the cloning and characterization of a unique human p110 which is most closely related to bovine p110α (>99% amino acid identity). We therefore refer to our clone as human p110α. This clone contains upstream stop codons in all three reading frames, and a Kozak-like sequence immediately preceding the ATG start codon. However, our sequence harbors 10 amino acid differences compared to the previously reported sequence for human p110α.[14] It is likely that the clones are derived from the same gene, since the 3′ untranslated sequences are identical for over 200 nucleotides. All of

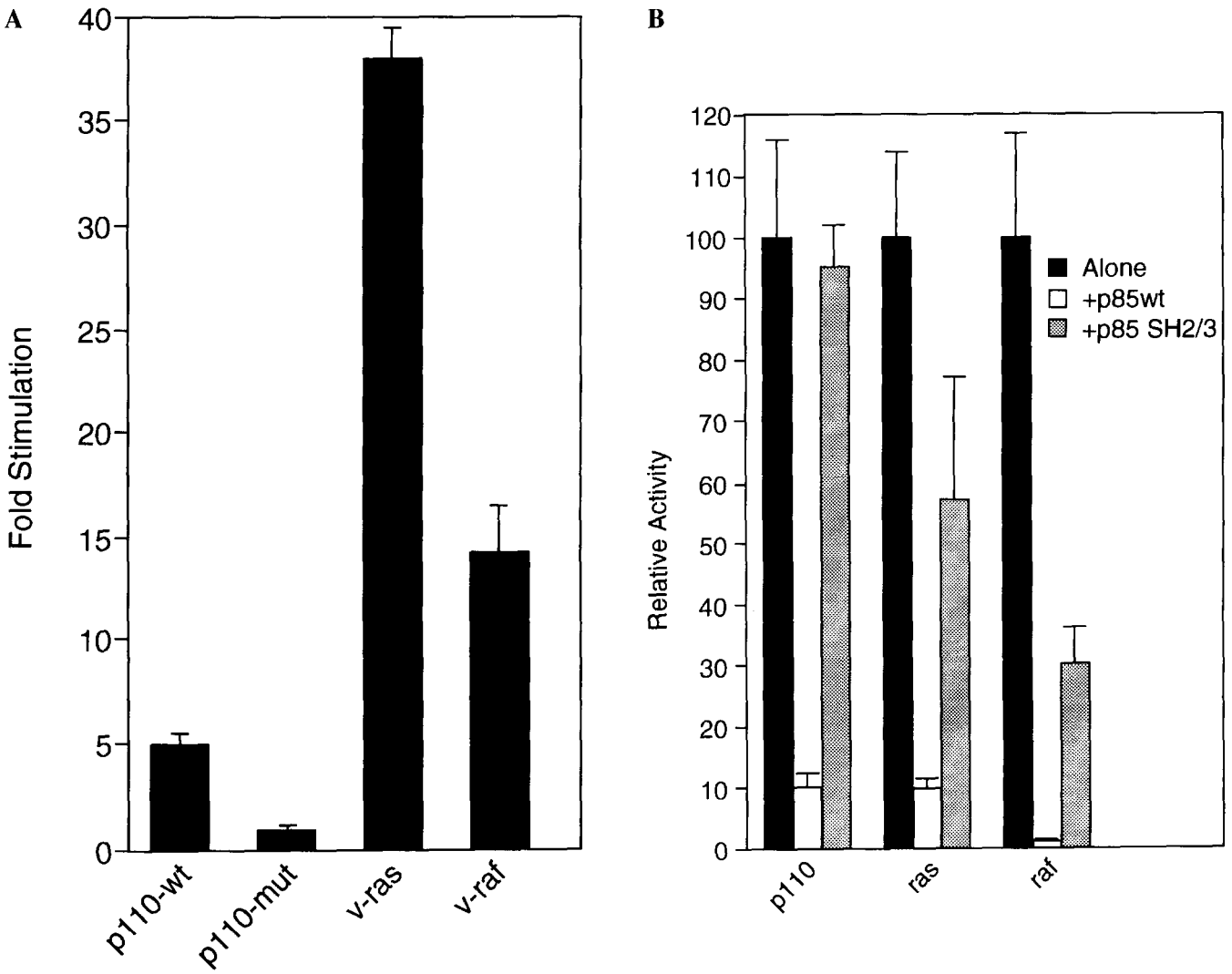

Figure 6. Activation of the *fos* promoter serum response element by p110α. A eucaryotic expression plasmid containing either wild-type p110α or the D933A/F934A p110α mutant was transiently co-transfected into CHO cells with a reporter plasmid containing the *fos* promoter SRE element linked to a secreted alkaline phosphatase (SEAP) coding sequence. The amount of SEAP secreted into the cell culture media was measured in a chemiluminescence assay as described in Methods. Results are presented as the average of three separate transfections within the same experiment with the error bars representing the standard deviation for the three data points. Multiple experiments gave similar results. Panel A: the relative stimulation of SEAP activity generated by expression of the wild-type or mutant p110α, v-*ras* or v-*raf*. Panel B: plasmids expressing p110α, v-*ras* or v-*raf* were transfected alone or with plasmids expressing either p85α wild-type or a p85α triple mutant with both SH2 domains and the SH3 domain mutated. SEAP expression from the SRE reporter plasmid was assayed as described in Methods and plotted relative to the activity obtained when p110α, *ras* or *raf* were expressed alone (Panel A).

the 10 amino acid differences between our clone and the sequence cited by Volinia et al. are conserved between our sequence and the bovine p110α sequence.[11,14] None of these amino acid differences are contained in regions overlapping the PCR primers used to clone p110α. We sequenced multiple clones on both strands, and always identified the same sequence, as indicated in Figure 1.

Comparison of the p110α sequence to those of several protein kinases revealed apparent conservation of key catalytic residues.[13,15,24,26] Selective point mutations of several of these key residues provides information on the universality of ATP binding and enzyme activation mechanisms. Two of the PI3K mutants, D915A and (D933A, F934A), were found to be catalytically inert. Similar results have been reported for the yeast VPS34 gene product.[27] While non-conservative substitutions always run the risk of leading to general denaturation of proteins, we demonstrated that these p110 mutants were still capable of binding p85, suggesting that the overall structure of the p110α subunit of PI3K was not grossly affected by the mutations. Two other point mutations, E970A and R922A, had little or no effect on phosphoinositide phosphorylation activity. These results demonstrate that the highly conserved DFG motif and the conserved upstream aspartic acid in protein kinases are also essential for catalytic activity of p110. C-terminal to the DFG sequence, the homology between protein kinases and p110 is less obvious and the attempt to link key charged amino acids of p110 to conserved amino acids of the protein kinases was unsuccessful. This may suggest that the structure of the catalytic site of the inositol lipid kinases, while partially conserved compared to the protein kinases, may be altered C-terminal to the DFG motif.

The wild-type and catalytically inert p110 derivatives were used to demonstrate that human p110α is sufficient, in the absence of other mitogens or transfected proteins, to activate the SRE of the c-*fos* promoter. This activity is clearly due to the catalytic activity of the protein, since the (D933A, F934A) kinase-defective mutant was inactive in the assay. These results are consistent with the loss of c-*fos* promoter activating activity resulting from a 34 amino acid deletion in the proposed kinase domain of mouse p110α.[18,28]

PI3K is believed to be an essential part of the signal transduction cascade leading to mitogenesis from some activated growth factor receptors and oncogenes.[1,2] PI3K has recently been shown to be a unique dual-function kinase, exhibiting both protein (Ser) and phosphoinositide kinase activity. This catalytic activity resides in the p110 subunit of the PI3K heterodimer, although our data and earlier studies suggest that binding to p85 enhances the catalytic activity of the enzyme.[18,28] Co-expression of the wild-type p85 with p110 or v-*ras* suppressed activation of the SRE driven promoter constructions. These results suggest that while the enzymatic activity of PI3K is enhanced by its association with p85, over-expression of wild-type p85 protein acts as a dominant-negative inhibitor of *fos* promoter activation.[29,30] A dominant negative effect of mammalian p85 on mammalian p110 activity has also been reported in *Schizosaccharomyces pombe* and a negative regulatory effect of p85 on p110 activity has been proposed.[31]

The dominant negative effect of p85 overexpression on *fos* promoter activation could be mediated by binding to p110, or through other interactions mediated by the SH2/SH3 domains. The p110 contact domain of p85 is located between the two SH2 domains.[28,32,33] Therefore, mutations in the SH2 domains might potentially affect the interactions with and activation of the p110 catalytic activity in the heterodimer. Baculovirus coexpression of a p85 mutant mutated in both SH2 domains with p110 indicated that heterodimer formation was unaffected, however (unpublished data). It therefore seems unlikely that the mutational attenuation of the p85 dominant negative effect is due to an altered interaction of the p85 SH2/SH3 triple mutant, although a possible effect of the SH3 domain mutation has not been specifically addressed.

In conclusion, we have cloned the human p110α component of PI3K. Mutational studies indicate significant functional conservation of key amino acids involved in ATP binding between PI3K and protein kinases. Moreover, we found that enzymatically active human p110α is sufficient to activate the *fos* promoter, which is a key step in the mitogenic signal transduction pathway.

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