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PI3K is negatively regulated by PIK3IP1, a novel p110 interacting protein

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Abstract

Signaling initiated by Class Ia phosphatidylinositol-3-kinases (PI3Ks) is essential for cell proliferation and survival. We discovered a novel protein we call PI3K interacting protein 1 (PIK3IP1) that shares homology with the p85 regulatory PI3K subunit. Using a variety of in vitro and cell based assays, we demonstrate that PIK3IP1 directly binds to the p110 catalytic subunit and down modulates PI3K activity. Our studies suggest that PIK3IP1 is a new type of PI3K regulator.
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Class Ia phosphatidylinositol-3-kinases (PI3K) are lipid kinases that generate pro-growth and survival signals in cells. They are heterodimers composed of a regulatory subunit (p85) and a catalytic subunit (p110) (for review, see [1]). When cells are exposed to growth factors or other stimuli, the p85/p110 complex is recruited to tyrosine-phosphorylated proteins [2] resulting in catalytic activation of the PI3K heterodimer. Active PI3K phosphorylates the phospholipid PI(4,5)P_2 causing a rise in PI(3,4,5)P_3 levels. PI(3,4,5)P_3 in turn recruits and stimulates 3-phosphoinositide dependent protein kinase-1 (PDK1), Akt [1] and other downstream PI3K effectors [3].

Kringles are triple looped amino acid motifs that mediate protein–protein interactions [4]. Only a few proteins such as hepatocyte growth factor (HGF) possess kringle domains. Searching GenBank for novel proteins that harbor kringles, we identified a unique transmembrane protein that, in addition to having a kringle motif, possesses a domain sharing homology to the PI3K regulatory subunit p85. We provide evidence herein showing that this newly discovered protein binds to and negatively regulates the activity of the p110 PI3K subunit through its p85-like domain. We propose to name this new protein PI3K interacting protein 1 (PIK3IP1).

Materials and methods

Antibodies. A polyclonal antibody was made in a rabbit against a PIK3IP1 peptide (NH2-CHTSQTPVDPQEGST-COOH = amino acids [AA] 289–252) (Genemed Synthesis, Inc., San Francisco, CA) and affinity purified. Anti-p110 antibodies—Santa Cruz Biotechnology (Santa Cruz, CA) (sc-7174/sc-602), Upstate Cell Signaling Solutions (Lake Placid, NY) (#06-567/#06-568), and BD Biosciences (Mountain View, CA) (#611399). Anti-p85 antibody—Upstate Cell Signaling Solutions (#06-195). Phospho-Akt (Ser-473 [#9271] or Thr-308 [#9275]) and Akt (#9272) antibodies—Cell Signaling Technology (Beverly, MA).

Cell lines. C33A (human uterine cervical epithelial carcinoma) and 293 (human embryonic kidney epithelial) cells were purchased from ATCC

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(Manassas, VA). p85 alpha/beta double knock out mouse embryonic fibroblasts (DKO-MEFs) and control fibroblasts (CT-MEFs) were kindly provided by Dr. Lewis Cantley (Beth Israel Hospital, Boston, MA). Cells were grown in EMEM (C33A), MEM (293) or DMEM (DKO- and CT-MEF) containing 10% fetal bovine serum typically to 80% confluence. For some studies, cells were serum starved for 16–24 h before treatment.

Protein isolation, SDS–PAGE, immunoprecipitation and Western blot analysis. Protein isolation, SDS–PAGE, immunoprecipitation (IP) and Western blot (WB) analysis were carried out using standard procedures. Generally, 40 μg of protein lysate made in RIPA buffer was subjected to SDS–PAGE. For IP, 1 mg of total protein was incubated with the indicated antibodies. Membranes were probed with primary antibodies (as indicated at the manufacturer's recommended concentrations). Signals were illuminated by the Western Lightning Chemiluminescence Reagent PLUS (Perkin-Elmer Inc., Boston, MA) and captured on X-ray film. Densitometry of signals was carried out using Scion Image 1.63 software (Scion Corporation, Frederick, MD).

In vitro PI3K assay. PI3K assays were performed as described [5] with minor modifications. Briefly, protein lysates (1 mg) were immunoprecipitated with anti-p110 antibodies. The immunoprecipitates were washed 3× with RIPA buffer, and 3× with PI3K reaction buffer (RB) (20 mM Tris–HCl, pH 7.4, 100 mM NaCl, and 0.5 mM EGTA). Immunoprecipitates or purified recombinant active human p110 alpha/p85 alpha or p110 beta/p85 alpha complex (200 ng) (Upstate Cell Signaling Solutions) were suspended in 50 μl PI3K-RB containing 0.2 mg/ml phosphatidylinositol (PI, Sigma) and incubated for 10 min at RT. Then, 440 μl PI3K-RB containing 30 μCi [^{32}P-γ]ATP, 0.88 mM ATP and 20 mM $MgCl_2$ was added and incubated for an additional 10 min at RT. In some experiments, PIK3IP1 36-mer peptide (NH2-YSYKRGKDLKEQHDQKVCEREMQRITLPL SAFTNPT-COOH) or 36-mer mutant scrambled peptide (NH2-QKDGK YSRPHDTNPTQEMQAKEYITLLKVCERLFSR-COOH) were added to the reaction at a final concentration of 0, 1, 10, 50, 100 μM. The reaction was stopped using 100 μl of chloroform:methanol:HCl (200:100:2) and products were separated by thin layer chromatography in chloroform:methanol:ammonium hydroxide:water (86:76:10:14) running buffer. Lysates were routinely assessed for p110 abundance by IP and WB analysis.

siRNA-mediated knockdown. 293 cells were plated at 2×10^6 cells in a 100 mm cell culture dish. To knock down (KD) endogenous PIK3IP1 expression, 21 basepair double-stranded RNA oligonucleotides specific for PIK3IP1 (Sense: 5′-GGAUUUGAAAGAACAGCAUtt; Antisense: 5′-AUGCUGUUCUUUCAAAUCCtt) were obtained from Ambion, Inc. (Austin, TX; Catalog#: 16704 and siRNA ID#: 35518) and transfected into cells (60 ng/well) using Silencer™ siPORT Amine siRNA Transfection kit (Ambion, Inc.). Negative control siRNAs purchased from Ambion (#4637) were also transfected (60 ng/well). They consist of 19 bp nontargeting sequences with 3′ dT overhangs and have no significant homology to any known gene sequences in mouse, rat, or human. The transfected cells were incubated at 37 °C for 48 h and then harvested for IP, WB and PI3K assay.

Results and discussion

Identification, chromosomal localization and structural analysis of a novel gene product, PIK3IP1

We utilized the signature amino acid sequence found in kringle motifs (NYCRNPD) and the cDNA sequence of a known kringle-bearing protein called HGF as baits to screen for new kringle containing proteins through search of GenBank databases. Our examination of the human nucleotide database uncovered several expressed sequence tags (ests) encoding for the same gene product. We obtained and sequenced some of these human ests from ATCC. One (GenBank I.D.: AA122030) yielded a full-length open reading frame (ORF) (Supplemental Figure 1), the sequence of which we utilized to localize the corresponding gene to human chromosome 22q12.2 (~30 MB) using the USC Genome Browser (http://genome.ucsc.edu/) According to this database, the gene structure contains 6 exons and 5 introns spanning about 12,000 bp. We confirmed the chromosomal localization of the novel gene using a BAC clone (CTD-3108G17) and FISH in normal human metaphase lymphocytes (data not shown). In addition, we identified its ortholog in several species such as mouse (chromosome 11A1; GenBank I.D.: NP_835362), rat (chromosome 14q1; GenBank I.D.: XP_223593), chicken (GenBank I.D.: XP_415257) and zebrafish (chromosome 5; GenBank I.D.: CAM14081). They are highly homologous at the mRNA and protein levels. Using the ClustalW webtool (http://www.ebi.ac.uk/clustalw/) [6], we determined that human and mouse proteins are approximately 80% identical and 93% similar overall.

Next, we carried out a motif search on the amino acid sequence of this novel protein by probing web-based search engines. The Blocks search engine (http://blocks.fhcrc.org/blocks/blocks_search.html) [7] predicted that the new protein bears a single kringle domain (Supp. Figure 1: red box—signature NYCRNPD AA underlined in red) and a sequence towards the C-terminus with homology to the iSH2 domain of the regulatory subunit (p85 beta) of bovine PI3K (Supp. Figure 1: blue box—p85-like domain). The Blocks webtool uses a different set of parameters than BLAST to identify homologous regions and searches for the most highly conserved regions in clusters of proteins. Employing the ClustalW webtool, alignment of the regions in human p85 beta and PIK3IP1 that share homology was performed (Fig. 1). Overall similarity reaches 70% and 65% between PIK3IP1 (AA 197–219) and p85 beta (AA 502–524) or alpha (AA 505–527), respectively. An amino acid stretch (AA 210–218: NH2-EREMQRITL-COOH) within the p85-like domain of PIK3IP1 is 78% identical to p85 beta (AA 515–523). Using other analytical web tools such as SMART (Simple Modular Architecture Research Tool located at http://smart.embl-heidelberg.de/ [8,9]), a signal peptide (green box) and a transmembrane domain (orange box) (Supp. Figure 1) were identified. Based on the structural features and functional characterization described below, we named the novel protein phosphatidylinositol-3-kinase interacting protein 1 (PIK3IP1).

Expression patterns of PIK3IP1

To determine whether *pik3ip1* is an expressed gene, we performed Northern blot analysis of adult and fetal human

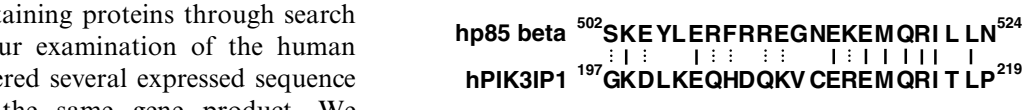

Fig. 1. Amino acid comparison of human PIK3IP1's p85-like domain to the homologous region of human p85 beta. Alignment of the homologous regions of hPIK3IP1 and hp85 beta is shown. Solid lines indicate identical AA; dotted lines indicate similar AA.

tissues. A major mRNA transcript of approximately 2.4 Kb for *pik3ip1* was identified in several human tissues with the most abundant expression seen in heart, brain and lung in the adult and kidney in the fetus (Supplemental Fig. 2A). We found a similar expression pattern in mouse tissues using a full-length mouse cDNA (GenBank I.D.: AA754893) as probe; a major transcript measuring approximately 2.4 Kb and a minor transcript of 1.35 Kb were identified in many murine tissues such as heart and skeletal muscle (Supplemental Fig. 2B). We prepared an affinity purified polyclonal antibody corresponding to a peptide (black underline—Supp. Figure 1) in the C-terminal end of PIK3IP1 and used it to confirm that the predicted open reading frame of the *pik3ip1* cDNA is authentic by performing IP and WB of in vitro translated PIK3IP1 protein. In these experiments a specific band with Mr of 37 kDa was detected in reactions from *pik3ip1* input cDNA in sense but not anti-sense orientation (data not shown). We used the antibody to analyze human adult tissues for PIK3IP1 protein expression by WB and found that its protein expression (Supp. Fig. 2C) paralleled for the most part the *pik3ip1* mRNA abundances seen in adult human tissues observed in Supplemental Fig. 2A.

PIK3IP1 and p110 associate in vitro

Since the portion of p85 (i.e. the interSH2 or iSH2 domain) that the p85-like domain of PIK3IP1 resembles is critical to the association of p85 and p110 [10,11], we wanted to determine if PIK3IP1, like p85, interacts with p110. Accordingly, we performed binding assays using myc- or hemagglutinin (HA)-tagged-PIK3IP1, p85 or p110 as described in the Supplemental materials and methods. In these experiments we subjected the expression vectors to in vitro transcription and translation in the presence of ^{35}S-methionine and cysteine; products were assessed for size by SDS–PAGE with or without IP using anti-myc or -HA antibodies. As expected, p110 alpha co-immunoprecipitated with p85 alpha (Fig. 2, lane 14). Interestingly, p110 alpha also interacted with the portion of PIK3IP1 harboring the p85-like domain (C-PIK3IP1; Fig. 2, lane 12) as well as full length PIK3IP1 (FL-PIK3IP1; Fig. 2, lane 7). p110 alpha did not associate with the negative control C-terminally deleted PIK3IP1 lacking the p85-like domain (CD-PIK3IP1; Fig. 2, lane 9). Similar results were obtained with p110 beta (data not shown). We also confirmed these observations using a yeast-two hybrid approach (data not shown).

PIK3IP1 and p110 associate in vivo in the absence of p85

We next assessed whether an interaction between PIK3IP1 and p110 occurs in vivo and if this interaction affects p85–p110 association. We first carried out co-IP experiments in p85 alpha/beta double knock out mouse embryonic fibroblasts (DKO-MEFs) and the controls (CT-MEFs). In WB analysis, the DKO-MEF protein lysates expressed PIK3IP1 and p110 alpha and beta but not p85 as expected (Supplemental Figure 3). In IP/WB studies of p85 DKO-MEFs, PIK3IP1 was found to pull down p110 alpha (or beta—data not shown) while p110 co-immunoprecipitated PIK3IP1 in the absence of p85. Antibody to p85 or control IgG did not precipitate proteins as expected (Fig. 3A). These data indicate that PIK3IP1 associates with p110 in cells lacking p85.

PIK3IP1, p110 and p85 associate in vivo in a complex

Protein lysates were prepared from CT-MEFs, and we confirmed that PIK3IP1, p110 alpha, p110 beta, and p85

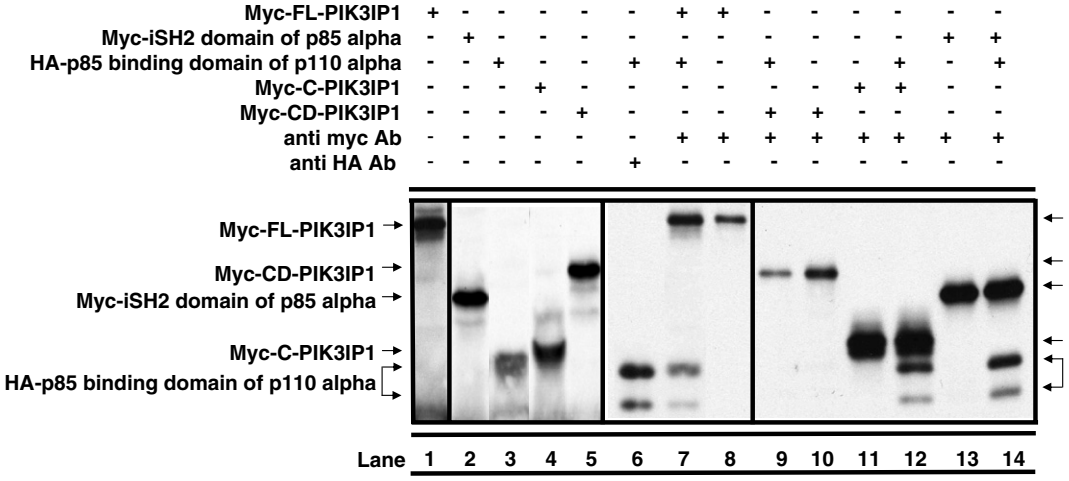

Fig. 2. PIK3IP1 and p110 associate in vitro. The entire PIK3IP1 protein (FL-PIK3IP1—myc tagged), the C-terminal portion of human PIK3IP1 including the p85-like domain (C-PIK3IP1—myc tagged), the iSH2 domain of human p85 alpha (iSH2-p85 alpha—myc tagged; positive control), the C-terminally deleted PIK3IP1 lacking the p85-like domain (CD-PIK3IP1—myc tagged; negative control), and the p85 binding domain of human p110 alpha (p85BD-p110 alpha—hemagglutinin tagged) were radiolabeled during in vitro translation. Products were assessed for size by SDS–PAGE (lanes 1–5). Co-IP experiments were then carried out. Arrows depict protein bands corresponding to the descriptors on the left.

proteins are expressed endogenously (Supplemental Figure 3). To examine protein–protein interactions in these cells, we assessed the lysates by co-IP/WB for interaction of PIK3IP1 with p110 alpha, p110 beta, or p85 proteins using anti-PIK3IP1, anti-p110, anti-p85 antibodies or rabbit IgG as a control antibody. Fig. 3B shows that specific interac-

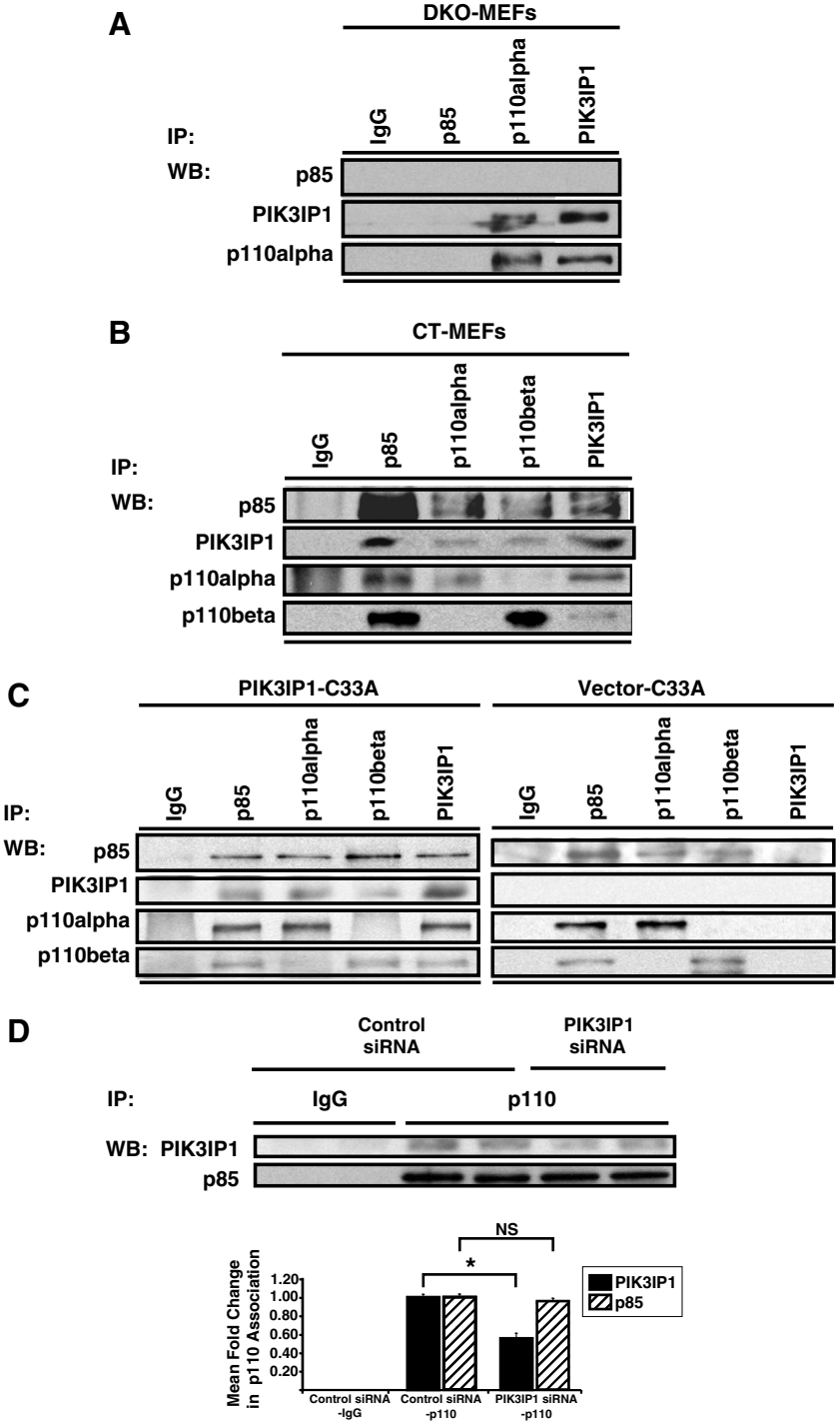

Fig. 3. PIK3IP1, p110 and p85 associate in a complex. (A) PIK3IP1 and p110 associate in the absence of p85 in DKO-MEFs. Co-IP of PIK3IP1 with p110 alpha using anti-PIK3IP1, anti-p110, anti-p85 antibodies or rabbit IgG as a control antibody was performed in DKO-MEFs, and immunoprecipitated proteins were subjected to WB using the same antibodies. (B) PIK3IP1, p110 and p85 associate in a complex in CT-MEFs. Co-IP experiments # were carried out in CT-MEFs as described in (A). (C) Overexpression of PIK3IP1 does not alter the association of p85 with p110 in cells. Co-IP experiments # were carried out in PIK3IP1-C33A and Vector-C33A cells as described in (A). # non-specific signal is seen in some lanes following IP with control IgG. (D) Suppressed expression of PIK3IP1 does not alter the association of p85 with p110 in cells. A reduction of PIK3IP1 protein by siRNA mediated KD resulted in a concomitant decrease in PIK3IP1 association with p110, as expected, but no significant change in p85 interaction with p110 (and data not shown). Densitometric readings of the western signals for PIK3IP1 and p85 following co-IP with anti-p110 were averaged and normalized; the results are shown graphically $^{*}p = 0.043$; NS, not significant.

tions between PIK3IP1 or p85 protein with p110 alpha or p110 beta occur. Notably, p85 also co-immunoprecipitated PIK3IP1 protein and vice versa suggesting that p85, p110 and PIK3IP1 exist in a complex. We performed similar experiments in 293 human kidney epithelial cells and observed a similar pattern of complex formation (data not shown).

PIK3IP1 does not prevent the interaction of p85 and p110

We next assessed C33A human uterine cervical carcinoma cells stably overexpressing PIK3IP1 to determine if overexpression of PIK3IP1 disrupts the association of p85 with p110. We chose to carry out our experiments in C33A cells since it is known that this cell line harbors amplification of the *pik3ca* (p110 alpha) gene [12], that it has robust p110 activity, and because it expresses very low levels of endogenous PIK3IP1 mRNA and protein. Overexpression of PIK3IP1 in C33A cells did not lead to alterations in p85 or p110 protein abundance as compared to controls (Supplemental Figure 4A and B). We confirmed that complex formation between p85 or PIK3IP1 and the p110s occurs in the PIK3IP1-C33A cells as we observed in CT-MEFs and 293 cells. We did not notice a substantial change in p85/p110 association in the PIK3IP1 overexpressing cells as compared to controls (Fig. 3C). We next performed siRNA-mediated knock down (KD) of endogenous PIK3IP1 in 293 cells to examine whether reduced levels of PIK3IP1 protein alter p85/p110 interaction. Typically, we achieved a PIK3IP1 KD efficiency rate of 40–70%. We found that a reduction of PIK3IP1 in these cells did not affect p85 or p110 total protein abundance (Supplemental Figure 4C) nor did it affect association of p85 and p110 via IP/WB assays (Fig. 3D). However, as

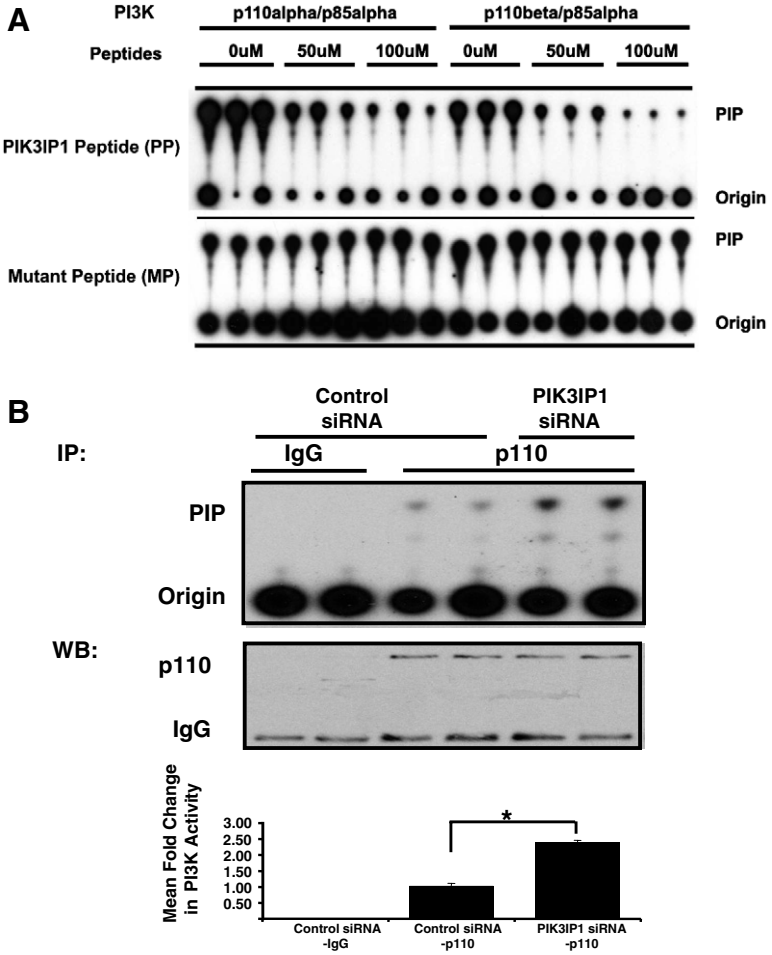

Fig. 4. PIK3IP1 negatively regulates PI3K activity. (A) PIK3IP1 peptide suppresses PI3K activity of recombinant p110 alpha/p85 alpha or p110 beta/p85 alpha complexes in a dose-dependent manner. 36-mer PIK3IP1 peptide (PP) or control mutant peptide (MP) was added to recombinant human PI3K (p110 alpha/p85 alpha or p110 beta/p85 alpha complex) and conventional PI3K assays were performed. p110a–p110 alpha/p85 alpha complex; p110b–p110 beta/p85 alpha. (B) siRNA mediated KD of endogenous PIK3IP1 in 293 cells causes an increase in PI3K activity. Cell lysates prepared following siRNA mediated KD of PIK3IP1 were subjected to IP using anti-p110 or Ig antibody, and PI3K activity was measured by conventional PI3K assay. WB analysis was also performed on the protein lysate to assess for p110 pulldown. The average PIP level was calculated, normalized and graphed following densitometry for each treatment. $^{*}p = 0.006$. Control siRNA-IgG—cells treated with control siRNA and immunoprecipitated with Ig antibody; control siRNA-p110—cells treated with control siRNA and immunoprecipitated with anti-p110 antibody; and PIK3IP1 siRNA-p110—cells treated with PIK3IP1 siRNA and immunoprecipitated with anti-p110 antibody.

expected, a significant decrease in PIK3IP1/p110 association was noted in cells treated with PIK3IP1 siRNA as compared to controls (Fig. 3D). Altogether, these data indicate that PIK3IP1 does not prevent the interaction of p85 and p110 in cells that either have enhanced or reduced PIK3IP1 protein expression suggesting that the binding of p85 and PIK3IP1 to p110 is not mutually exclusive; rather, it appears that they exist in a ternary complex. The fact that PIK3IP1 does not disrupt p85–p110 coupling to any substantial degree is not surprising since p85 and p110 associate with very high affinity [13] through multiple contact sites [10,14].

PIK3IP1 negatively regulates PI3K activity and suppresses activation of Akt

Since we determined that a specific interaction occurs between PIK3IP1, p85 and p110 proteins in vitro and in cells, we wanted to determine the functional consequence of this interaction on PI3K activity. We generated a 36-mer peptide (PP) corresponding to the p85-like domain of human PIK3IP1 (blue underline—Supp. Fig. 1) and a 36-mer mutant peptide (MP). Then, we tested whether PP altered PI3K activity; MP was used as a negative control. We observed that addition of PP to purified recombinant human PI3K (either p110 alpha/p85 alpha or p110 beta/p85 alpha complex) at concentrations of 0–100 μM led to a dramatic decrease in PI3K activity in a dose-dependent manner (Fig. 4A—lowest concentration of PP showing measurable inhibition was 10 μM [data not shown]), while addition of MP at the same concentrations did not. It should be noted that the concentrations of PP that altered PI3K activity in our experiments lie within the effective range published for phosphopeptides derived from IRS-1 that bind to p85 and activate PI3K activity [15].

Next we examined stable clones of C33A cells overexpressing PIK3IP1 for PI3K activity as compared to vector controls. We observed a diminution in the average PI3K activity in the PIK3IP1 overexpressing C33A cells by more than 60% as compared to vector controls (Supp. Fig. 5A). The level of phospho-Akt was also reduced in WB (Supp. Fig. 5B) In other experiments, we knocked down endogenous PIK3IP1 in 293 cells using PIK3IP1-specific siRNA and found that a reduction in PIK3IP1 in these cells led to a significant rise in PI3K activity as compared to control siRNA treated cells (Fig. 4B). An increase in the levels of phospho-Akt was also noted in WB (data not shown).

PIK3IP1 overexpression augments staurosporine induced apoptosis

Wan et al. [16] found that co-administration of wortmannin (a PI3K inhibitor) and the protein kinase inhibitor staurosporine potentiated the apoptotic effect of staurosporine on Ishikawa human endometrial adenocarcinoma cells. We treated PIK3IP1 overexpressing and vector control C33A stable transfectants with staurosporine and found a significant increase in apoptotic parameters such as a higher percentage of cells in sub-G_0 phase of the cell cycle in flow cytometric analyses (Supplemental Fig. 6), a greater release of lactate dehydrogenase (LDH) into the culture medium (data not shown), and a higher level of caspase 3 activation (data not shown) in the PIK3IP1 overexpressing cells as compared to vector controls. These data suggest that, like wortmannin, PIK3IP1 promotes apoptosis under conditions of cellular stress such as staurosporine treatment.

Together, our results show that complex formation between PIK3IP1, p85 and p110 occurs and that p85 and PIK3IP1 both independently bind to p110's 'p85 binding domain' to alter p110's activity with functional consequences on cell survival. It should be noted that while numerous adapter-like proteins are known to associate with the PI3K holoenzyme to activate its function, to our knowledge only two proteins have been identified that suppress PI3K activity: our protein PIK3IP1 and the negative regulator Ruk [17]. Unlike Ruk which binds to the SH3 domain of p85 alpha to reduce PI3K activity [17], PIK3IP1 is unique in that it binds directly to p110 to down modulate PI3K.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.096.

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