

# The Phosphatidylinositol 3'-kinase p85 $\alpha$ Gene Is an Oncogene in Human Ovarian and Colon Tumors<sup>1</sup>

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## Abstract

Phosphatidylinositol 3'-kinases (PI3ks) are a family of lipid kinases that play a crucial role in a wide range of important cellular processes associated with malignant behavior including cell growth, migration, and survival. We have used single-strand conformational polymorphism/heteroduplex analysis to demonstrate the presence of somatic mutations in the gene for the p85 $\alpha$  regulatory subunit of PI3k (*PIK3RI*) in primary human colon and ovarian tumors and cancer cell lines. All of the mutations lead to deletions in the inter-SH2 region of the molecule proximal to the serine<sup>608</sup> autoregulatory site. Expression of a mutant protein with a 23 amino acid deletion leads to constitutive activation of PI3k providing the first direct evidence that p85 $\alpha$  is a new oncogene involved in human tumorigenesis.

## Introduction

The class IA (PI3ks)<sup>4</sup> are a family of widely expressed lipid kinases that exist as heterodimers consisting of a unique catalytic subunit (p110 $\alpha$ ,  $\beta$ , or  $\delta$ ) along with one of a number of shared regulatory subunits (p85 $\alpha$ , p85 $\beta$ , or p55 $\gamma$ ; Ref. 1). PI3k is known to be involved in a wide range of cellular processes associated with malignant behavior including proliferation, adherence, transformation, and survival (2). Increased PI3k activity has been reported in human colon, bladder, and ovarian tumors (3–5). The gene coding for p110 $\alpha$  (*PIK3CA*) is amplified (increased copy number) in a proportion of ovarian (6) and cervical (7) tumors implicating *PIK3CA* as a potential oncogene in these cancers. This and the regulation of the PI3k pathway by the tumor suppressor gene *PTEN* (8) have provided indirect evidence for the importance of PI3k activation in human tumorigenesis. Here we report the presence of somatic mutations in the gene for the p85 $\alpha$  regulatory subunit of PI3k (*PIK3RI*) in primary human colon and ovarian tumors and cancer cells providing the first direct evidence that p85 $\alpha$  is a new oncogene involved in human tumorigenesis.

## Materials and Methods

**Cell Lines and Tumor Samples.** The LIM2537 colon carcinoma cell line was derived at the Ludwig Institute Melbourne from a primary colonic carcinoma from a 73-year-old male using the methods described previously (9). The

cells grew as small spindle-shaped cells in monolayer culture. The cells formed tumors in nude mice when 10<sup>6</sup> cells were injected s.c. The tumors that formed were undifferentiated. The cells were positive for keratin 8 and 18 staining but were negative when stained with antibodies to dipeptidyl peptidase IV or sucrase (9). The other colon carcinoma cell lines studied were LIM1215 (10), LIM1863 (11), LIM1899 (9), LIM2405 (9), LIM2412 (9), SK-CO1 (ATCC HTB-77), HT29 (ATCC HTB38), LoVo (ATCC CCL-229), SW1222 (ATCC HB-11028), and SW480 (ATCC CCL-228). The ovarian cancer lines OVCAR-3 (ATCC HTB-161) and SKOV-3 (ATCC HTB-77) and the gastric cancer line LIM1839 (12) were also investigated. All of the cell lines were cultured in RPMI 1640 containing 10% (v/v) FBS. In addition, insulin (10  $\mu$ g/ml) was added to all of the cultures of the OVCAR-3 cell line.

Fresh surgical specimens of colon carcinomas were obtained from patients undergoing elective surgery at Western Hospital (Victoria, Australia). Samples of primary tumor and normal-appearing mucosa (>5 cm from the margins of the carcinoma) from the same patients were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Epithelial ovarian cancer samples were obtained from the Peter MacCallum Cancer Institute Tissue Bank. The collection and/or use of tissues for this study were approved by the appropriate institutional ethics committees.

**Screening for Mutations.** Exons 12, 13, 14, and 15 of p85 $\alpha$  were individually amplified from genomic DNA using primers complementary to surrounding intronic sequences. Details of these primers are available from the authors. Samples were prepared for SSCP/HD analysis and separated on 0.5 $\times$  MDE gel matrix (BioWhittaker Molecular Applications Inc., Rockland, ME) as described previously (13). Cases showing aberrant band shifts were repeated and compared with the matching normal DNA (where available) to determine whether the change was germ line or somatic. Cases showing consistent band shifts were sequenced using a Thermo Sequenase Cycle Sequencing kit (Amersham Pharmacia Biotech, Sydney, Australia).

**Constructs and Antibodies.** Wild type p85 (p85<sup>WT</sup>) and the p85 $\alpha$ -exon 13 deletion mutant (p85 <sup>$\Delta$ exon13</sup>) were cloned by reverse transcription-PCR from LIM2537 poly A+ RNA using the oligonucleotide 5'-GCAAACATGATG-GCTGAGGG-3' as the forward primer and 5'-TCGCCTCTGCTGTG-CATATACTGG-3' as the reverse primer and inserted into pcDNA3.1 using the pcDNA3.1/V5-His TOPO TA Cloning kit (Invitrogen Corporation, Carlsbad, CA) as per the manufacturer's instructions. GFP-AH and GFP-AH<sup>R25C</sup> plasmids were kindly provided by Julian Downward (Imperial Cancer Research Fund, London, United Kingdom). Primary antibodies were obtained from the following sources: anti-p85 polyclonal antibody (06-195) from Upstate Biotechnology (Lake Placid, NY), anti-p85 $\alpha$  monoclonal antibody (sc-1637) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), anti-V5 antibody from Invitrogen, and anti-Akt and anti-phospho-Akt(Ser473) antibodies from New England Biolabs (Beverly, MA).

**Transfections.** Human embryonic kidney 293 cells (ATCC CRL-1573) cultured in RPMI 1640 containing 5% FBS were transiently transfected with 1  $\mu$ g of plasmid DNA/10<sup>5</sup> cells using FuGENE 6 transfection reagent (Roche Diagnostics, Castle Hill, Australia) according to the manufacturer's protocol. Transfections were allowed to proceed for 24 h, and then the cells were washed with PBS and replaced in medium.

**Western Blotting and Immunoprecipitation.** Cells were lysed in 10 mM Tris (pH 7.5) containing 100 mM NaCl, 2 mM EDTA, 0.5% deoxycholate, and 1% Triton X-100. For Western blots, 50  $\mu$ g of total protein was resolved on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The

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<sup>4</sup> The abbreviations used are: PI3k, phosphatidylinositol 3'-kinase; GFP, green fluorescent protein; SSCP/HD, single-strand conformational polymorphism/heteroduplex; nt, nucleotides; FBS, fetal bovine serum.

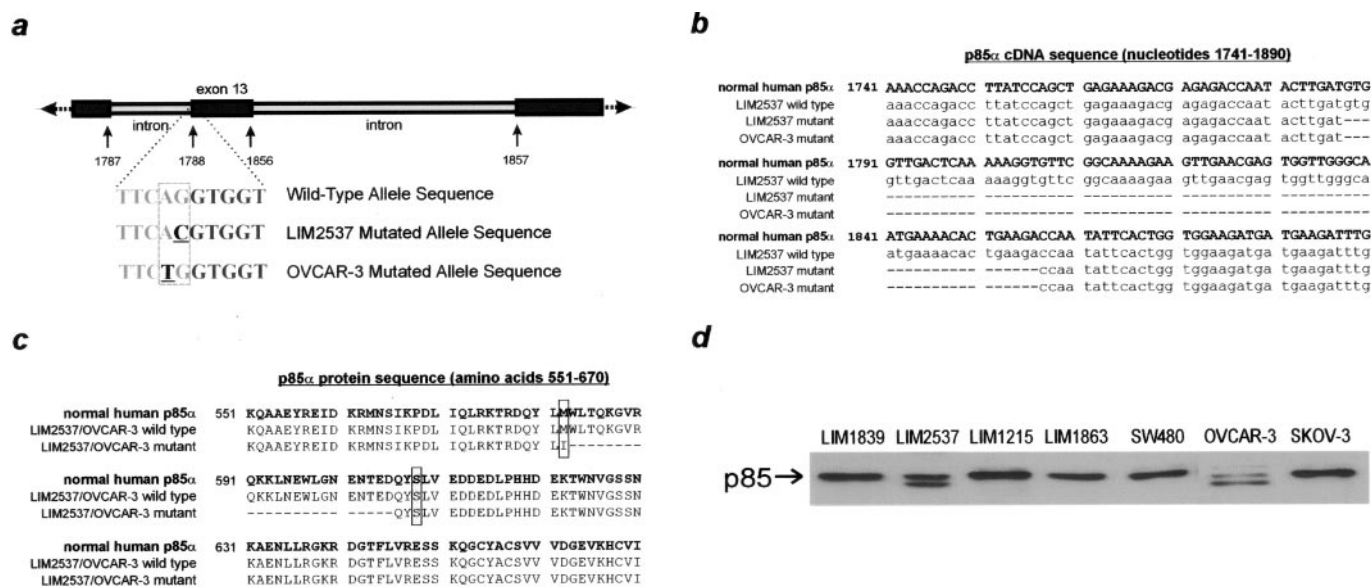


Fig. 1. Characterization of *p85 $\alpha$*  mutations in the LIM2537 and OVCAR-3 cell lines. *a*, a diagrammatic representation of the genomic structure of the *p85 $\alpha$*  gene surrounding exon 13 and illustrating the genomic sequence of the 3' intron/exon boundary of wild-type *p85 $\alpha$*  and the mutated alleles of the LIM2537 and OVCAR-3 cell lines. Intronic sequence is shown in *light font* and exonic sequence in *dark font*. The splice acceptor site is *boxed* and the mutated bases *underlined*. *b*, cDNA sequence of *p85 $\alpha$*  (nt 1741–2041) for normal *p85 $\alpha$*  as taken from GenBank (accession no. M61906; *bolded*), LIM2537 wild type and mutant, and OVCAR-3 mutant. *c*, predicted amino acid sequence of wild-type human *p85 $\alpha$*  and the mutant *p85 $\alpha$*  proteins from LIM2537 and OVCAR-3 cell lines. The normal *p85 $\alpha$*  sequence derived from GenBank is shown in *bold*. *Open box* highlights the Met<sup>582</sup>→Ile change in the mutated protein, and *shaded box* indicates the position of Ser<sup>608</sup>. *d*, expression of p85 in selected cancer cell lines. Cell protein (50  $\mu$ g) was resolved on a 10% polyacrylamide gel and probed with a polyclonal anti-p85 antibody. Shown are the results for the colon cancer cell lines LIM2537, LIM1215, LIM1863, and SW480, the ovarian cell lines OVCAR-3 and SKOV-3, and the gastric cell line LIM1839. Similar results were obtained using a monoclonal anti-p85 $\alpha$  antibody (data not shown).

membrane was blocked by incubation with 5% non-fat powdered milk in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (Tris-buffered saline). Proteins of interest were detected with specific antibodies and visualised using a chemiluminescence detection system (Amersham). For immunoprecipitation, 200  $\mu$ g of cell protein was incubated (4°C overnight) with 2.5  $\mu$ l of anti-p85, 5  $\mu$ l of anti-p110 $\alpha$ , or 2  $\mu$ l of anti-V5 antibody. Immune complexes were then precipitated with a 50% slurry of protein A (for anti-p85 and anti-p110 $\alpha$ ) or protein G (for anti-V5) and washed with Tris-buffered saline.

**PI3k Activity.** HEK293 cells were transfected with V5-tagged wild-type or mutant PI3k (p85 $\Delta$ exon13) constructs together with a p110 $\alpha$  expression vector, and the PI3k activity of anti-V5-immunoprecipitated proteins was assessed using the method described by Phillips *et al.* (3). *In vivo* PI3k activity was monitored using the membrane localization of GFP-AH as an indicator of the production of 3-phosphorylated lipids. HEK293 cells were grown on 20-mm diameter glass coverslips and were cotransfected with PI3k expression vectors and the GFP-AH plasmid (or the GFP-AH<sup>R25C</sup> control) as described above. Transfected cells were washed with PBS and placed in RPMI 1640 without serum. The cells were incubated for 24 h and then the coverslips were inverted on slides and the cells analysed for GFP fluorescence using a Bio-Rad MRC 1000 confocal microscope (Bio-Rad Laboratories, Hercules, CA).

## Results and Discussion

Phosphorylation of the serine<sup>608</sup> residue of the p85 $\alpha$  regulatory subunit of PI3k suppresses the activity of the p110 catalytic subunit. Dephosphorylation of this site results in constitutive activity of PI3k (14). We hypothesized that mutational disruption of the serine<sup>608</sup> autoregulatory site might contribute to the increased PI3k activity detected in tumors. Therefore, we undertook a search for mutational events that might compromise the integrity of this site. We used SSCP/HD analysis to screen genomic DNA for mutations affecting exons 12–15 of the *p85 $\alpha$*  gene in selected colon and ovarian cancer cell lines. Mutations detected by SSCP/HD were confirmed by sequencing.

We detected a G→C transversion in 3 of 12 colon cancer cell lines (LIM2537, LIM1899, and LIM2414) and an A→T transversion in one of two ovarian cancer cell lines (OVCAR-3). In each case, the

base changed was situated in the intronic sequence of the exon 13 splice acceptor site of one allele of the *p85 $\alpha$*  gene (Fig. 1*a*). These mutations were predicted to disrupt the splice site leading to the skipping of exon 13 during transcription. This was confirmed by sequencing of cDNA, which demonstrated loss of 69 bp encoded by exon 13 (Fig. 1*b*). Because the loss was in-frame, we predicted the mutations would give rise to a smaller protein ( $M_r$  82,400) with an internal deletion of 23 amino acids (amino acids Met<sup>582</sup>–Asp<sup>605</sup> are deleted and replaced with a single Ile residue; Fig. 1*c*). Western blot analysis of proteins extracted from these cell lines demonstrated the expression of both the  $M_r$  85,000 wild-type protein and a smaller immunoreactive protein of a molecular weight ( $M_r$  ~82,000) consistent with the expression of the mutant allele (Fig. 1*d*).

Splice variants of *p85 $\alpha$*  have been described previously (15, 16). However, genetic alterations detected here are unlikely to be naturally occurring polymorphisms. For one cell line (LIM2537) we had access to matching normal DNA, and this did not harbor the variant (data not shown) indicating that, for this cell line at least, the substitution was somatic. Whereas we cannot exclude the possibility that the mutation arose during passage of the cell line, we have found that it was present in the cells at a very early passage number (<20).

The serine kinase Akt, a downstream effector of PI3k, is known to be phosphorylated in a PI3k-dependent manner (17). Therefore, we measured phosphorylation of Akt as an indicator of endogenous PI3k activity in a range of colon and ovarian cell lines. Phosphorylated Akt was detected in all of the four cell lines that express the mutant p85 $\alpha$  (Fig. 2), a result consistent with the mutation increasing endogenous PI3k activity. Interestingly, some of the other cell lines also had increased levels of phosphorylated Akt, suggesting that there may be other mutations or alternative mechanisms increasing PI3k and/or Akt activity in these cells.

In order to separate the wild-type and mutant p85 $\alpha$  to enable analysis of the intrinsic activity of the individual proteins, we generated V5-tagged proteins by cloning the wild-type and exon 13-deleted

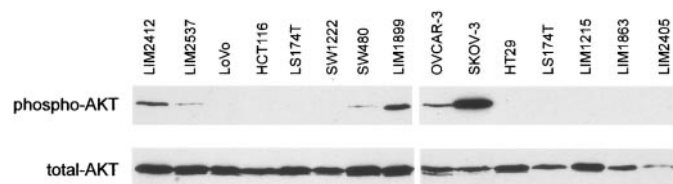


Fig. 2. Phosphorylation of the Akt in selected cancer cell lines. Cells were lysed and Western blotted as described in "Materials and Methods" and probed with an antibody recognizing phospho-Akt(Ser473). The same blots were then reprobed with an antibody for total Akt. All cell lines shown are colon cancer cell lines with the exception of OVCAR-3 and SKOV-3, which are ovarian cancer cell lines.

(p85 <sup>$\Delta$ exon13</sup>) cDNAs from LIM2537 cells into a mammalian expression vector. The COOH-terminal V5-tagged proteins were individually expressed in HEK293 cells, and the *in vitro* PI3k activity of anti-V5 immunoprecipitates assessed. The exon-13-deleted mutant was found to exhibit increased PI3k activity compared with the wild-type control ( $1.8 \pm 0.3$ -fold, mean  $\pm$  SE,  $n = 3$ ;  $P < 0.05$ , two-way ANOVA).

To confirm that the expression of the mutant p85 $\alpha$  increased endogenous PI3k activity, we monitored the production of 3-phosphorylated lipids using a PI(3,4,5)P<sub>3</sub>-binding probe consisting of the NH<sub>2</sub>-terminal region of Akt (aa 1–147, which includes the 3-phosphorylated lipid-binding PH domain) fused to (GFP-AH). In the absence of PI3k activity the probe remains cytosolic, but when PI3k is activated, the formation of 3-phosphorylated lipids in the membrane results in the relocation of the probe to the membrane (18). Stimulation of the cells with serum was used as a positive control for PI3k activation. A second probe (GFP-AH<sup>R25C</sup>) in which the Akt PH domain has been mutated so that it no longer binds to 3-phosphorylated lipids was used to control for nonspecific effects. Transfection of HEK293 cells with mutant protein resulted in relocation of the GFP-AH to the plasma membrane (Fig. 3). Transfection with wild-type p85 $\alpha$  did not translocate the GFP-AH, and the mutant was unable to relocate the GFP-AH<sup>R25C</sup> control probe. These data demonstrate that deletion of exon 13 of p85 $\alpha$  results in a constitutively active protein. The specific mechanism underlying the induction of constitutive activity in the mutant protein is yet to be determined. One possibility, given the close proximity of the deletion to the serine<sup>608</sup> autoregulatory site, is that phosphorylation of this site may be impaired, perhaps because of a disruption of the kinase recognition sequence or a conformation change preventing access of the kinase to this serine residue.

We extended our studies to primary tumors by using SSCP/HD analysis to screen genomic DNA from primary human colon and ovarian carcinomas for mutations in exons 12–15 of the p85 $\alpha$  gene. Somatic mutations were identified in 3 of 80 ovarian carcinomas and 1 of 60 colon carcinomas. Of the ovarian mutations, one is a 12-bp deletion (bases tcagtggttga) across the same splice acceptor site disrupted in the cell lines discussed above, and we predict this deletion will also lead to skipping of exon 13. Another is a 4-bp deletion (agta) across the splice donor site of exon 13, which creates a premature stop codon in the mRNA that we predict will produce a protein truncated at Asp<sup>605</sup>.

The remaining two mutations detected are deletions spanning the same region of exon 12 of p85 $\alpha$ . One, detected in an ovarian tumor, is a 27-bp deletion (nt 1751–1777; Fig. 4) that results in the deletion of nine amino acids (Leu<sup>570</sup>-Asp<sup>578</sup>) including a potential phosphorylation site (Thr<sup>576</sup>). The second mutation is a 9-bp deletion (nt 1751–1759) leading to the loss of three amino acids (Leu<sup>570</sup>-Gln<sup>572</sup>) in a colon tumor. We do not have appropriate material from the primary tumor samples to enable confirmation of the expression of the predicted mutant proteins. However, it is interesting to note that

the site of these exon 12 deletions corresponds to the point of truncation of the p85 $\beta$  protein in the oncogenic p85 $\beta$ -HUMORF8 chimeric protein described by Janssen *et al.* (19). Similarly, p65, an oncogenic form of murine p85 $\alpha$  described by Jiminez *et al.* (20), is truncated at the site of the exon 13 deletion. The apparent clustering of mutations at these sites suggests an important functional role for these regions of the gene.

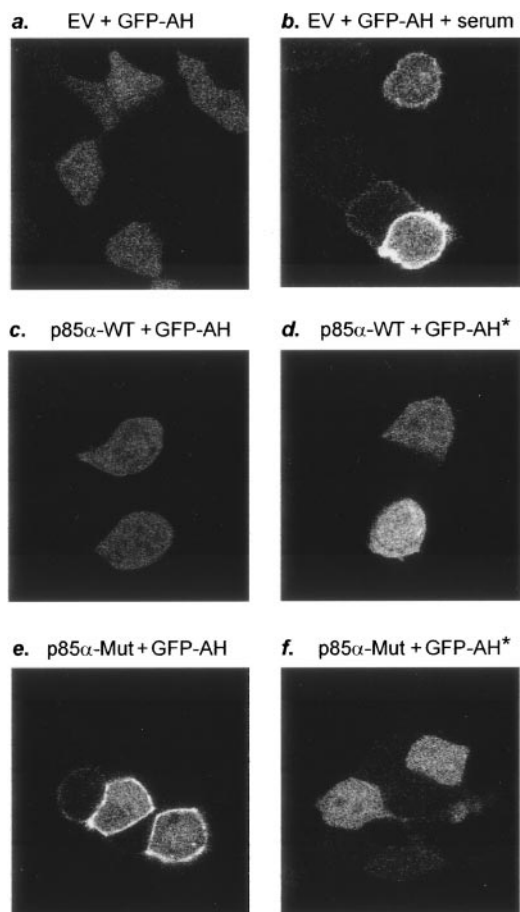


Fig. 3. Redistribution of GFP-AH in cells transfected with mutant p85 $\alpha$ . HEK293 cells were transfected with 1  $\mu$ g of pcDNA3.1 empty vector (EV; a and b), wild type p85 $\alpha$  (p85 $\alpha$ -WT; c and d), or the p85 <sup>$\Delta$ exon13</sup> mutant (p85 $\alpha$ -mut; e and f) constructs together with 1  $\mu$ g of GFP-AH (a, b, c, and e) or the GFP-AH<sup>R25C</sup> control construct (GFP-AH\*; d and f) for 24 h. Cells were washed and cultured for an additional 24 h in medium without serum. FBS (5%) was added to b as a positive control. Confocal microscopy was used to visualize the subcellular location of the GFP.

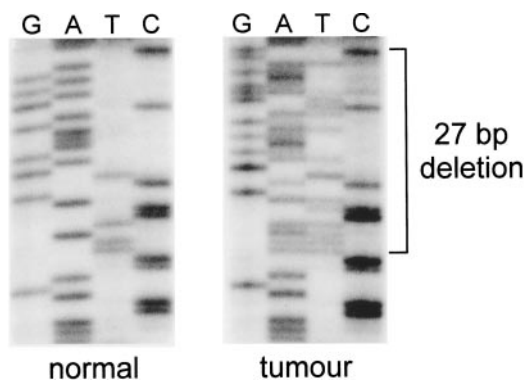


Fig. 4. Sequence analysis of a p85 $\alpha$  deletion mutant. Genomic DNA was extracted from normal-appearing and tumor tissue from a patient with ovarian carcinoma and sequenced using a primer specific for p85 $\alpha$ . Shown is the region of the sequencing gel demonstrating the deletion of 27 bp in the tumor sample.

In summary, we have described the first somatic mutations in any of the human PI3k genes and demonstrated that at least one of these results in a constitutively active enzyme. Our results indicate that the PI3k p85 $\alpha$  gene (*PIK3R1*) is a new human oncogene and underscores an important role for PI3k in human tumorigenesis. We believe it likely that human tumors will also harbor somatic alterations in other PI3k genes and suggest that the PI3k family can be considered a new class of human oncogenes.

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