Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase

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p85/p110 phosphoinositide 3-kinase (PI3K) is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit, which is involved in a variety of cellular responses including cytoskeletal organization, cell survival and proliferation. We describe here the cloning and characterization of p65-PI3K, a mutant of the regulatory subunit of PI3K, which includes the initial 571 residues of the wild type p85α-protein linked to a region conserved in the eph tyrosine kinase receptor family. We demonstrate that this mutation, obtained from a transformed cell, unlike previously engineered mutations of the regulatory subunit, induces the constitutive activation of PI3K and contributes to cellular transformation. This report links the PI3K enzyme to mammalian tumor development for the first time.

Keywords: mutants/phosphatidylinositol 3-kinase/transformation

Introduction

Phosphoinositide-3-kinases (PI3K) are lipid kinases that phosphorylate phosphoinositides (PtdIns) at position 3 of the inositol ring (Panayotou *et al.*, 1992). The cloning of PI3K from several species, including mammals, *Drosophila*, yeast, soybean and *Dictyostelium*, has allowed subdivision of the PI3K superfamily into three classes, depending on their primary structure and substrate specificity (MacDougall *et al.*, 1995; Zvelebil *et al.*, 1996). The first characterized form of PI3K, isolated from mammals was a heterodimer composed of a catalytic (p110) and a regulatory (p85) subunit (Escobedo *et al.*, 1991; Otsu

et al., 1991; Skolnik *et al.*, 1991; Hiles *et al.*, 1992; Hu *et al.*, 1993). Sequence analysis of the p110 primary sequence allows the definition of several regions including a p85-binding region, a ras-binding domain, a region homologous to PI4-kinases and the catalytic core (Dhand *et al.*, 1994; Leevers *et al.*, 1996; Zvelebil *et al.*, 1996). Similarly, p85 includes a SH3 domain, a bcr-homologous region flanked by two proline-rich regions, and two SH2 domains, separated by an inter-SH2 region (Dhand *et al.*, 1994; Holt *et al.*, 1994; Klippel *et al.*, 1994). p85:p110 mammalian PI3K, corresponding to class I PI3K, is regulated by cell surface receptors and phosphorylates PtdIns, PtdIns (4)P and PtdIns (4,5)P_2 *in vitro*, although PtdIns (4)P and PtdIns (4,5)P_2 are thought to be their major *in vivo* substrates (Stephens *et al.*, 1993). Regarding the mechanism by which the enzyme is activated upon receptor stimulation, it is known that p110 can associate with, and be activated by, the GTP-bound form of the small GTP-binding protein ras (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996). The p85 regulatory subunit also induces p110 activation through the binding of specific phosphotyrosine residues to p85-SH2 domains (Otsu *et al.*, 1991). This binding results in p85:p110 recruitment by activated receptor Tyr kinases, thereby facilitating PI3K activation (Skolnik *et al.*, 1991). Class I PI3K can also be regulated by diverse mechanisms involving the association of regulatory proteins to the SH3 domain, the Pro-rich regions, or to the bcr-homologous region of p85 (Pleinman *et al.*, 1994).

Activated mammalian p85:p110 PI3K controls several important cellular functions, including cytoskeletal organization, cell division and survival maintenance (Roche *et al.*, 1994; Wennstrom *et al.*, 1994; Yao *et al.*, 1996). The exact mechanism by which p85:p110 PI3K regulates so many different cellular responses is not known. No phospholipase has been described to cleave 3-phosphorylated PtdIns, making it feasible that the 3-phosphorylated lipids themselves act as second messengers (Serunian *et al.*, 1989). These lipids bind PH and SH2 domains directly (Rameh *et al.*, 1995; Klippel *et al.*, 1997), a feature that contributes to the induction of some PI3K effectors. Not only may 3-phosphorylated poly-PtdIns act as second messengers, but p85 could contribute to cellular stimulation by recruiting signaling molecules to the stimulated receptors via its SH3, SH2 and bcr-homologous domains. Finally, p110 has been described to phosphorylate protein substrates (Hunter, 1995); this activity may also contribute to the stimulation of downstream cellular effectors. In spite of the limited knowledge regarding the mechanism of PI3K action, several of its cellular effectors have been recently identified, and include the small GTP binding protein rac, the Ser/Thr kinase AKT, Bruton tyrosine kinase, 3-phosphoinositide dependent-kinase 1, certain isoforms of PKC, and p70-S6 kinase (Chung *et al.*,

1994; Toker *et al.*, 1994; Boudewijn *et al.*, 1995; Franke *et al.*, 1995; Hawkins *et al.*, 1995; Alessi *et al.*, 1997).

We sought to analyze signaling molecules involved in lymphoid cell transformation. For this purpose, murine lymphoid tumors were obtained by X-ray irradiation of BALB/c congenic-CB17 *scid/scid* mice (Lieberman *et al.*, 1992). These mice are genetically identical to normal BALB/c animals, except for the defective expression of an enzyme involved in DNA repair (DNA-PK, Blunt *et al.*, 1995), which renders these animals incapable of lymphoid-specific receptor rearrangement and increases their tumor susceptibility. A number of thymic lymphomas were generated from which we derived transformed thymic cell lines. These cell lines were analyzed systematically and one, CMN-5, contained no wild type alleles for the p85 regulatory subunit. Instead, these cells expressed p65-PI3K, a mutant whose cloning and characterization we report here. Expression of this mutant in unrelated cells allowed us to conclude that p65-PI3K induces the constitutive activation of PI3K and contributes to cellular transformation. These results suggest that PI3K mutation can be linked to mammalian tumor development.

Results

Identification of p65-PI3K

Murine lymphomas were generated by X-ray irradiation of BALB/c congenic-CB17 *scid/scid* mice. In the process of characterizing one cell line derived from a thymic lymphoma (CMN-5) which displayed an immature thymocyte phenotype (CD3⁻CD4⁻CD8⁻), we examined the expression of p85, the regulatory subunit of PI3K (Figure 1A). These assays revealed, that rather than p85, CMN-5 cells expressed a novel 65 kDa protein that was not observed in BALB/c thymocytes, purified CD3⁻CD4⁻CD8⁻ thymocytes, cells from *scid/scid* mice or other transformed T cell lines (Figure 1A and B), all of which clearly express p85-PI3K. Two p85 isoforms (α and β) are known to interact with p110 (Otsu *et al.*, 1991). Isoform-specific antibodies (Ab) (against bovine p85α and p85β) (Reif *et al.*, 1993) allowed us to conclude that p65 reacts with anti-p85α Ab, but does not react with anti-p85β Ab (not shown). We were, in any case, unable to detect p85β in murine T cells; this has also been described for other cell types of murine origin (Balstensperger *et al.*, 1994).

The lack of p85α expression in CMN-5 cells and the novel appearance of p65 suggested that this protein may represent a mutant of p85α. To test whether p65 is able to associate with p110, as does p85α, we examined p85, p65 and p110α content in either p110α immunoprecipitates (Figure 1C and D) or p85 immunoprecipitates (not shown) obtained from CMN-5 and T14 cells. The T14 cell line was obtained similarly to CMN-5, but expresses p85α (Figure 1B). These analyses indicated that p65 associates with p110 to a similar extent as p85, a result that was confirmed by comparing the autoradiographic patterns of anti-p85 immunoprecipitates from ³⁵S-methionine-labeled CMN-5 or control cells (not shown). Immunoprecipitates containing similar amounts of p85 (or p65) and p110α protein (not shown) were also assayed *in vitro* using PtdIns or a mixture of PtdIns(4)P plus PtdIns(4,5)P₂ as substrates (Figure 1E). These assays revealed that p65,
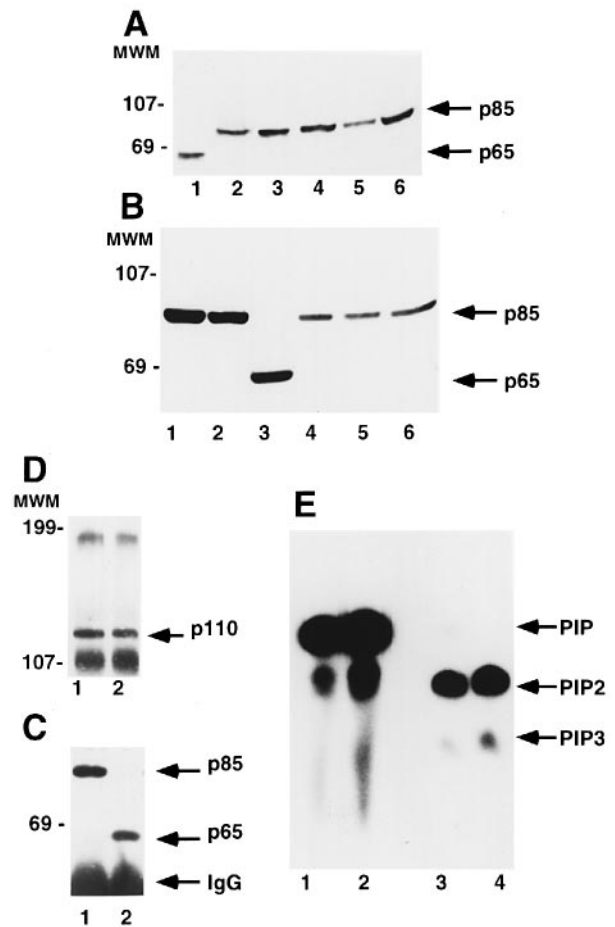

Fig. 1. Expression and biological function of p65. (**A**) Total cell proteins (50 µg/lane) from the different cell lysates were resolved in 7% SDS–PAGE. Proteins were transferred onto nitrocellulose and membranes blotted with anti-p85 Ab. CMN-5 cells, lane 1; BALB/c thymocytes, lane 2; CD3⁻CD4⁻CD8⁻ immunopurified BALB/c thymocytes, lane 3; LSTRA cells, lane 4; CTLL-2 cells, lane 5; and Sci cells, lane 6. (**B**) Total cellular protein (100 µg/lane for lanes 1–3 or 50 µg/lane for lanes 4–6) from the different cell lysates were resolved and analyzed as in (A). *scid/scid* hepatocytes, lane 1; BALB/c hepatocytes, lane 2; CMN-5 cells, lane 3; and three independently generated thymomas (CMN-1, CMN-2 and T14) in lanes 4, 5 and 6, respectively. (C and D) Total cellular protein (800 µg) from the different cell lysates were immunoprecipitated with anti-p110α Ab and resolved in 7% SDS–PAGE. Proteins were then transferred onto nitrocellulose and membranes blotted with anti-p85 Ab (**C**) or anti-p110 Ab (**D**). T14 cells, lane 1 and CMN-5 cells, lane 2. (**E**) Total cellular protein (200 µg) from the different cell lysates were immunoprecipitated with anti-p85 Ab and associated phospholipid kinase activity estimated *in vitro* using PtdIns or PtdIns(4)P plus PtdIns(4,5)P₂ as substrate. T14 cells, lanes 1 and 3; CMN-5 cells, lanes 2 and 4. The figure illustrates a representative experiment of five with similar results.

obtained from CMN-5 cells, associates PI3K activity being this activity 1.7-fold higher (mean of five assays) than p85-associated PI3K activity, obtained from T14 cells. These results suggest that p65 interacts with p110α, associates lipid kinase activity, and displays a substrate preference similar to that of p85:p110.

Cloning of p65-PI3K

The results obtained in CMN-5 cells prompted further characterization of the p65 molecule. p65 and p85α expressing cells were first compared by Northern blotting
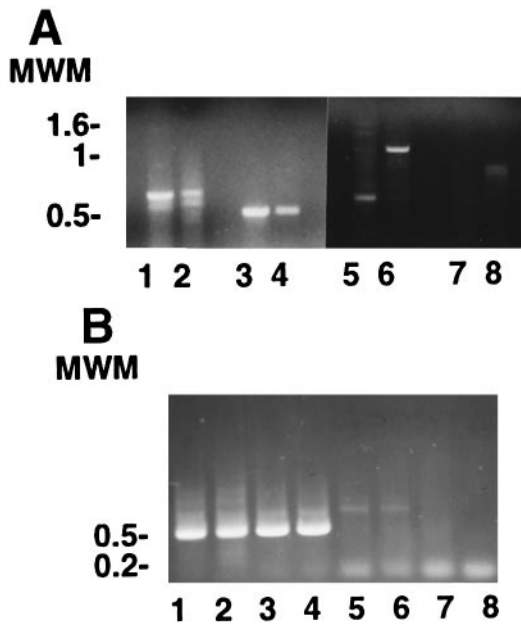
Fig. 2. PCR analysis of CMN-5 DNA. (**A**) RT–PCR analysis was carried out using cDNA obtained from CMN-5 cells (lanes 1, 3, 5 and 7) or from BALB/c thymocytes (lanes 2, 4, 6 and 8). For the reactions, the following combinations of 20 bp oligonucleotides were used: oligonucleotide at bp 1601 (5′) plus oligonucleotide at bp 2215 (lanes 1 and 2); oligonucleotide at bp 1210 (5′) plus oligonucleotide at bp 1648 (lanes 3 and 4); oligonucleotide at bp 1601 (5′) plus oligonucleotide at bp 2722 (lanes 5 and 6) and oligonucleotide at bp 1601 (5′) plus oligonucleotide at bp 2487 (lanes 7 and 8). (**B**) PCR analysis was carried out using genomic DNA obtained from BALB/c hepatocytes (lanes 1 and 5); scid/scid hepatocytes (lanes 2 and 6) and CMN-5 cells (lanes 3 and 7); a control CMN-5-cDNA sample was also included (lanes 4 and 8). For the reactions, oligonucleotides amplifying actin DNA (lanes 1–4) or oligonucleotides corresponding to bp 2030(5′) and bp 2345 of p85α cDNA were used. The figure illustrates a representative experiment of five with similar results.
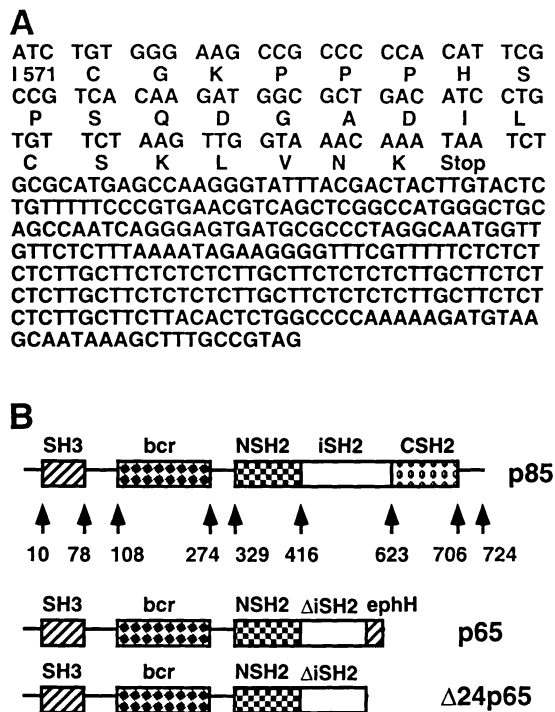
using a probe (bp 657–1719 of p85α) that included the inter-SH2 domain region required for p110 association (Dhand *et al.*, 1994; Holt *et al.*, 1994; Klippel *et al.*, 1994). This analysis revealed the presence of a 2.7 kb mRNA species in CMN-5 cells different from the 4.4 kb species found in normal congenic BALB/c thymocytes (Skolnik *et al.*, 1991). Subsequent RT–PCR analysis (performed as described in Gonzalez-Garcia *et al.*, 1997) of cDNAs prepared from CMN-5 cells or from BALB/c thymocytes revealed the presence of cDNA encoding the N-terminus but not the C-terminus of p85α (Figure 2A). Accordingly, PCR analysis of genomic DNA from CMN-5 cells also failed to amplify the region encoding the C-terminus of p85α (Figure 2B). This suggests that no wild type allele encoding p85α is present in CMN-5 cells. In agreement, Southern blotting comparison of the genomic DNA from CMN-5 cells with that from scid/scid and BALB/c mouse cells showed that while these congenic mice exhibited a similar pattern of genomic DNA fragments hybridizing with the full-length p85α probe, a distinct pattern was obtained in CMN-5 samples (Figure 3). These results demonstrate that no wild type p85α allele is present in CMN-5 cells. Two possibilities could account for the lack of wild type alleles. First, the two alleles could be identically mutated, a statistically unlikely occurrence, since it implies that the same recombination event has occurred twice in a single cell. It is also possible that one
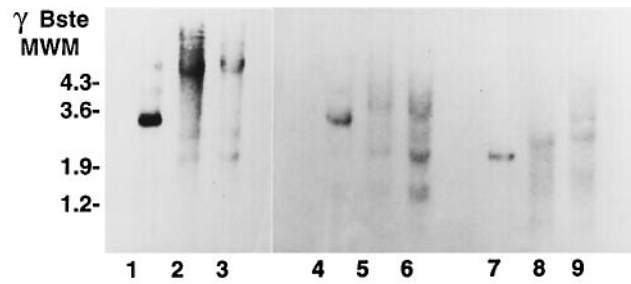
Fig. 3. Southern blotting analysis of p65- and p85-expressing cells. Genomic DNA (15 µg) extracted from CMN-5 cells (lanes 1, 4 and 7), *scid/scid* hepatocytes (lanes 2, 5 and 8) or BALB/c hepatocytes (lanes 3, 6 and 9) were digested with *Bam*HI (lanes 1–3), *Pst*I (lanes 4–6) or *Stu*I plus *Ssp*I (lanes 7–9) restriction enzymes. DNA was analyzed by Southern blotting.

allele has been mutated and the other has been lost. This possibility is frequently observed in cells lacking tumor suppressor gene expression and concurs with our preliminary results in FISH staining of metaphase CMN-5 cells (not shown). The genomic reorganization of the p85α gene in CMN-5 cells is probably responsible for the appearance of the smaller mRNA species and the shorter ORF for p85α in these cells.

The primary sequence of p65 was then obtained by molecular cloning. A λZAPII-cDNA library was prepared using mRNA extracted from CMN-5 cells (Clontech, Palo Alto, CA). The library was screened with a full-length murine p85α cDNA (Escobedo *et al.*, 1991) and 10 positive clones were purified and sequenced. Two of the clones contained an entire ORF encoding a 595 amino acid protein with a predicted molecular weight of 65.4 kDa. This sequence was also partially represented in the remaining eight clones. Up to residue 571, the sequence was identical to the first published murine p85α sequence (Escobedo *et al.*, 1991), with the exception of Pro46. In this position, p65 clones (derived from a library of a genetic background similar to that used by Escobedo *et al.*) contain a Gln residue, which is found by other authors in murine p85α clones (Fruman *et al.*, 1996); this position is also Gln in human and bovine p85α (Otsu *et al.*,1991; Skolnik *et al.*, 1991). Therefore, differences at residue 46 probably do not represent polymorphism, but rather a sequencing error. After residue 571 of p85α, p65 contained a distinct 24 amino acid COOH-terminal tail (Figure 4A), which displayed sequence similarity (58–61% conservation) to part of the Cys-rich domain found in members of the eph Tyr kinase receptor family (Fox *et al.*, 1995). A homologous region was also found in the sequence of the IgE binding factor (Martens *et al.*, 1985). The 5′ end untranslated sequences of p65 and p85α cDNAs were identical (Escobedo *et al.*, 1991). In contrast, the 3′ end untranslated sequences of p65 and p85α cDNAs were different, although both contained a long CT-rich sequence (Figure 4A and Escobedo *et al.*, 1991) shown to favor genomic instability (Htun *et al.*, 1984); this region may have led to the generation of the mutant. Alternatively, the presence of a bcr-homologous region in p85α (Dhand *et al.*, 1994; Holt *et al.*, 1994) may have facilitated its mutation. Sequence analysis has allowed definition of several regions in p85α (Escobedo *et al.*, 1991; Skolnik *et al.*, 1991), represented in Figure 4B. From all these regions, p65-PI3K encompasses the N-terminus of

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A

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ATC TGT GGG AAG CCG CCC CCA CAT TCG
I 571  C   G   K   P   P   P   H   S
CCG TCA CAA GAT GGC GCT GAC ATC CTG
 P   S   Q   D   G   A   D   I   L
TGT TCT AAG TTG GTA AAC AAA TAA TCT
 C   S   K   L   V   N   K  Stop
GCGCATGAGCCAAGGGTATTTACGACTACTTGTACTC
TGTTTTTCCCGTGAACGTCAGCTCGGCCATGGGCTGC
AGCCAATCAGGGAGTGATGCGCCCTAGGCAATGGTT
GTTCTCTTTAAAATAGAAGGGGTTTCGTTTTTCTCTCT
CTCTTGCTTCTCTCTCTTGCTTCTCTCTCTTGCTTCTCT
CTCTTGCTTCTCTCTCTTGCTTCTCTCTCTTGCTTCTCT
CTCTTGCTTCTTACACTCTGGCCCCAAAAAGATGTAA
GCAATAAAGCTTTGCCGTAG
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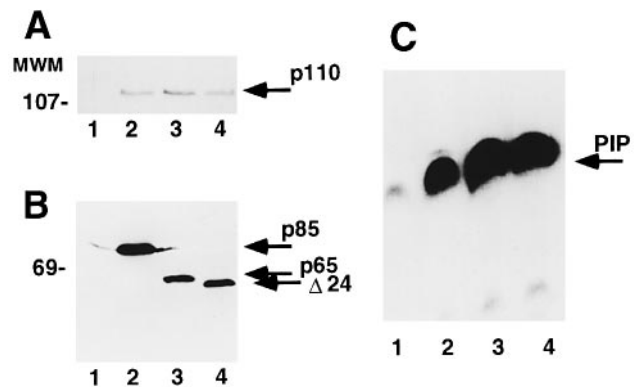
B

Fig. 4. Primary sequence of p65. (**A**) C-terminal primary sequence of p65 (starting from residue 571). From residues 1–571, the sequence is identical to that of p85α. (**B**) Schematic representation of the different domains of p85α, p65 and Δ24p65.

Fig. 5. p65 expression in CTLL-2 cells results in *in vitro* PI3K activation. (A and B) CTLL-2 cells (2×10^7) were electroporated with 100 µg of the different cDNAs; cells were lysed 24 h later and 300 µg of total cellular protein from the different cell lysates immunoprecipitated using anti-HA Ab. Proteins were resolved in 7% SDS–PAGE and gels analyzed as in Figure 1C and D. Anti-p110 Western blot (**A**) and anti-p85 Ab (**B**). PSG5-empty vector, lane 1; pCG-HA-p85, lane 2; PSG5-HA-p65, lane 3; and PSG5-HA-Δ24p65, lane 4. (**C**) Anti-HA immunoprecipitates [obtained as in (A)] were analyzed for their *in vitro* phospholipid kinase activity as in Figure 1. PSG5-empty vector, lane 1; pCG-HA-p85, lane 2; PSG5-HA-p65, lane 3; and PSG5-HA-Δ24p65, lane 4. The figure illustrates a representative experiment of four with similar results.

p85α, including its SH3 domain, bcr-homologous region, N-terminal SH2 domain and part of the inter-SH2 domain, but lacks the final part of the inter-SH2 domain and the C-terminal SH2 domain. In addition, p65-PI3K contains a C-terminal eph-related domain (Figure 4).

Increased in vitro activity of p65 PI3K

The cloning of p65 permitted the use of a genetic approach to characterize its biological function. Based on the p65 sequence, we engineered another mutant, Δ24p65, encompassing the first 571 residues of p65 (homologous to p85α), but lacking its final eph-related 24 amino acid tail (Figure 4B). We first tested whether p65 expression resulted in p110 activation. An unrelated cell line (CTLL-2, Merida *et al.*, 1991) was electroporated with influenza virus hemagglutinin epitope (HA)-tagged constructs and expression of the corresponding proteins was analyzed by immunoprecipitation using anti-HA Ab, followed by Western blotting (Figure 5A and B). These analyses indicated that HA-p85α, HA-p65 and HA-Δ24p65 were expressed to a similar extent (Figure 5B). Endogenous p110α was found to associate to a similar degree with HA-p85α, HA-p65 and HA-Δ24p65 (Figure 5A). Its detection however, was impeded by the low affinity of anti-p110α Ab and by the low amount of endogenous p110α associating with transfected regulatory subunits. Parallel examination of the anti-HA immunoprecipitates by *in vitro* phospholipid kinase assays indicated that both HA-p65 and HA-Δ24p65 contained higher associated PI3K activity than did HA-p85α (between 1.5- and 2-fold increase in four different experiments, Figure 5C). HA-Δ24p65 consistently yielded an *in vitro* kinase activity similar than HA-p65 indicating that the presence of the

eph-related domain does not affect the activity associated with p65. Finally, considering that transfected regulatory proteins had to compete with endogenous p85 for association with endogenous p110, we performed similar assays (as in Figure 5) using CTLL-2 cells cotransfected with p110α and either p65 or p85α. These experiments yielded a similar 1.5- to 2-fold higher activity in p65:p110α transfected cells compared to cells transfected with p85α:p110α (not shown).
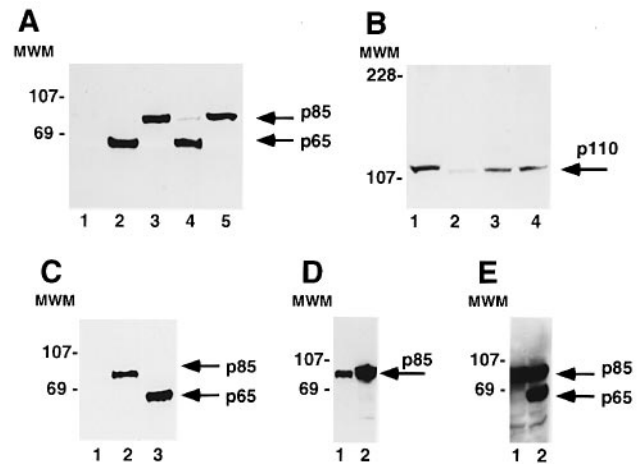
AKT induction by p65-PI3K

As a first approach to examine the activation of PI3K by p65 *in vivo*, we analyzed the activation of the Ser/Thr kinase AKT, a downstream effector of PI3K (Franke *et al.*, 1995). The cDNA encoding HA-AKT was transfected into CMN-5 cells, in a control congenic thymoma and in an unrelated cell line. All cell lines expressed similar HA-AKT levels as detected by immunoprecipitation with anti-HA Ab, followed by Western blotting with anti-AKT Ab (Figure 6A). Parallel examination of the anti-HA immunoprecipitates in an *in vitro* AKT kinase assay demonstrated, however, that HA-AKT displayed higher kinase activity when transfected in p65-expressing cells (CMN-5 cells, 2.8-fold induction, mean of three experiments) than in other cell types expressing p85α (Figure 6B). A genetic approach was also used to show *in vivo* PI3K activation upon p65 expression. For these assays, the HA-AKT expression plasmid was cotransfected with p65, Δ24p65, p85α (non-tagged constructs) or a constitutively active mutant of p110 (c-myc-tagged p110-CAAX) in CTLL-2 cells. Expression of p65, Δ24p65, p85α and p110-CAAX was determined by Western blotting (not shown). HA-AKT expression and kinase activity was analyzed in the lysates (Figure 6C and D) as above. HA-AKT kinase activity was low when transfected alone and even lower when cotransfected with p85α, as expected from the fact that overexpressed p85α sequesters endogen-
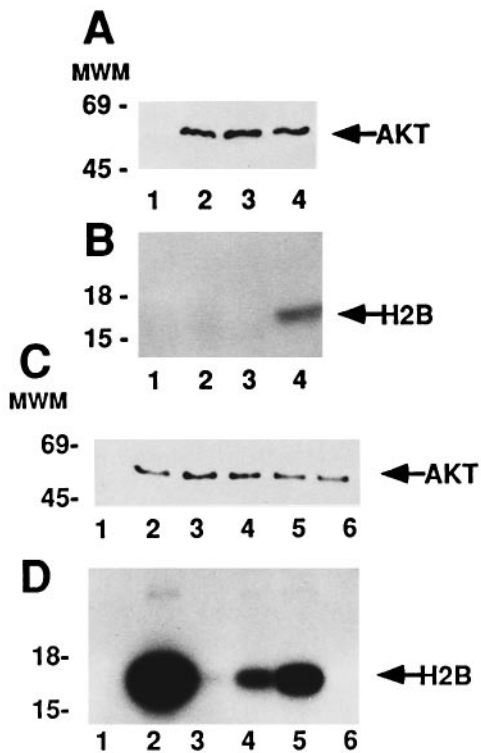
Fig. 6. Activation of AKT in cells expressing p65. (A and B) CMN-5 cells (10^7, lanes 1 and 4), CTLL-2 cells (10^7, lane 2) and Sci thymoma cells (10^7, lane 4) were electroporated with 50 μg of PSG5 empty vector (lane 1) or PSG5-HA-AKT (lanes 2–4). Cells were lysed 24 h after electroporation and 150 μg of cellular protein from the different lysates were immunoprecipitated using anti-HA Ab. Immunoprecipitates were either resolved in 7% SDS–PAGE and analyzed by Western blotting with anti-AKT Ab (**A**), or assayed for *in vitro* AKT kinase activity (**B**). AKT kinase reactions were resolved in 15% SDS–PAGE and gels analyzed by autoradiography. (C and D) CTLL-2 cells (10^7) were electroporated with PSG5 empty vector (50 μg, lane 1) or with PSG5-HA-AKT (25 μg) plus one of the following: c-myc-p110-CAAX (25 μg, lane 2); PSG5 empty vector (25 μg, lane 3); PSG5-p65 (25 μg, lane 4); PSG5-Δ24p65 (25 μg, lane 5) or PSG5-p85 (25 μg, lane 6). Electroporated cells were collected and lysed 24 h later; lysates were immunoprecipitated with anti-HA Ab and analyzed by Western blotting (**C**) as in (A), or assayed for their AKT *in vitro* kinase activity (**D**) as in (B). The figure illustrates a representative experiment of three with similar results.

Fig. 7. Expression of PI3K subunits in transiently transfected COS cells and in stably transfected 3T3 and CTLL-2 cells. (**A**) COS (2×10^5) cells were transfected using one of the following cDNAs: PSG5-empty vector (1 μg, lane 1); PSG5-empty vector (0.5 μg) plus PSG5-p65 (0.5 μg, lane 2); PSG5-empty vector (0.5 μg) plus PSG5-p85 (0.5 μg, lane 3); PSG5-p65 (0.5 μg) plus c-myc-wt-p110 (0.5 μg, lane 4) or PSG5-p85 (0.5 μg) plus c-myc-wt-p110 (0.5 μg, lane 5). Cells were lysed and 40 μg of total cellular protein were resolved in 7% SDS–PAGE. Gels were analyzed by Western blotting using anti-p85 Ab. (**B**) Cells were transfected as above using PSG5 (0.5 μg) plus c-myc-p110-CAAX (0.5 μg, lane 1); PSG5-empty vector (1 μg, lane 2); PSG5-p65 (0.5 μg) plus c-myc-wt-p110 (0.5 μg, lane 3) or PSG5-p85 (0.5 μg) plus c-myc-wt-p110 (0.5 μg, lane 4). Samples were prepared as in (A) and gels analyzed by western blotting using anti-p110 Ab. (**C**) 3T3 stable cell lines were obtained upon transfection using pSV-Neo (1 μg) combined with: PSG5-empty vector (3 μg, lane 1); PSG5-HAp65 (3 μg, lane 2) or PSG5-HAp85 (3 μg, lane 3). Cell lines were obtained upon selection in medium containing G418, lysed and lysates immunoprecipitated using anti-HA Ab. Gels were analyzed as in (A). (D and E) CTLL-2 cells electroporated with pSV-Neo vector (10 μg) combined with 40 μg of PSG5 empty vector (lanes 1), or 40 μg of PSG5-HA-p85 (lane 2, **D**), or 40 μg of PSG5-HA-p65 (lane 2, **E**) were selected in G418-containing medium, lysed and total cellular lysates resolved in 7% SDS–PAGE. Gels were analyzed as in (A). The figure illustrates a representative experiment of three with similar results.

ous p110 (Yamauchi *et al.*, 1993). p110-CAAX expression yielded maximal AKT activation (28-fold over basal AKT kinase activity, mean of three experiments) and p65 expression, as well as expression of Δ24p65, activated AKT ~3-fold over basal AKT activity (3-fold and 3.6-fold, respectively, mean of three experiments). As endogenous p110 may be limiting for the association with transfected p65, we also performed AKT assays from cells transfected with HA-AKT plus wild type p110α and either p85α or p65. AKT induction by p65:p110α was 22-fold over control levels (mean of three assays), in the same range as that induced by p110-CAAX. Transfection of p85α:p110α also resulted in AKT activation (7.5-fold over control, mean of three assays), indicating that increased p85α:p110α expression also resulted in increased *in vivo* PI3K activity, although significantly lower than that mediated by p65:p110. Therefore, while the induction of PI3K by p65 *in vitro* (Figure 5) was not particularly favored by cotransfection with p110, the activation of AKT *in vivo*

is more clearly demonstrated when the catalytic subunit, associating with p65, is not limiting.

In vivo generation of 3-phosphoinositides by p65
To confirm that p65 activates PI3K *in vivo*, 3-phosphorylated inositol lipid levels were measured in different cells expressing p65 or p85α. We first performed transient transfection assays, followed by orthophosphate metabolic radiolabeling. Using CTLL-2 cells, this protocol resulted in a significant degree of cell death. Transient transfections were therefore performed in COS cells, which maintained high cellular viability throughout the protocol. For COS cell transfection, cDNAs encoding p65 or p85α were transfected alone or cotransfected with p110α. Cells were also transfected with cDNA encoding p110-CAAX as a positive control. The transfected proteins were expressed to a similar extent, and were ~15-fold more abundant than endogenous p85α and p110α (Figure 7A and B). CMN-5 and T14 cells which express p65 or p85α, respectively (see Figure 1), were also analyzed. Finally, to complement these studies stable transfectants of 3T3 and CTLL-2 cells were also subjected to this analysis. In these cells, the different transfected proteins were expressed to a similar extent (3T3 cells, Figure 7C; data not shown for CTLL-
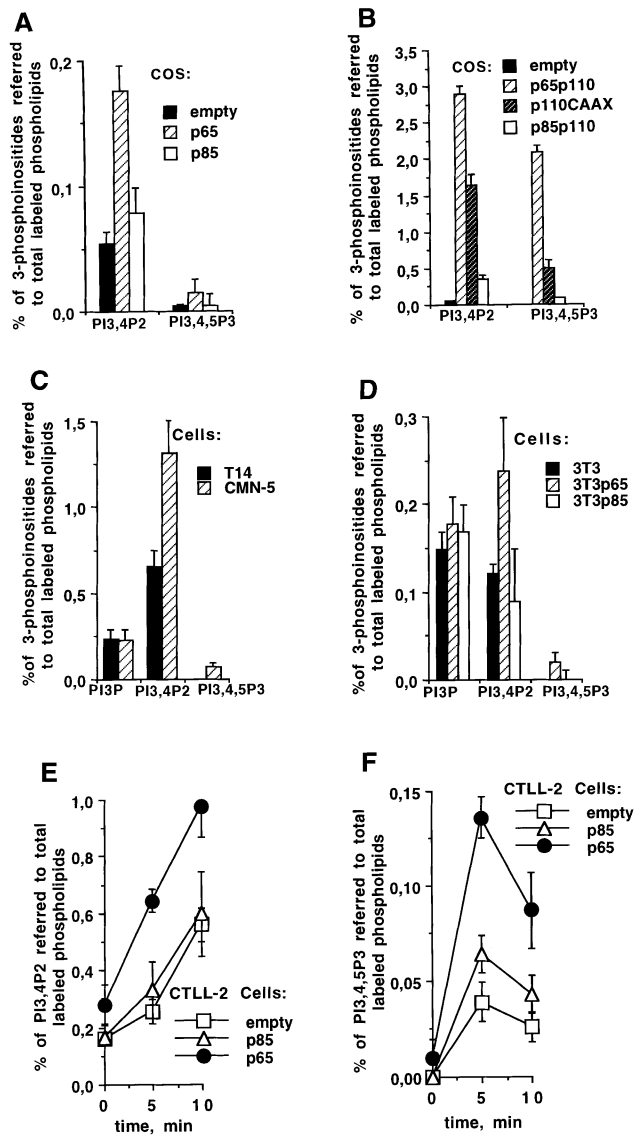
Fig. 8. Percentage of 3-phosphoinositides referred to total labeled phospholipids in transiently transfected COS cells and in stably transfected 3T3 and CTLL-2 cells. The cell samples were prepared as in Figure 7. Cells were labeled with ^{32}P-orthophosphate for 4 h, lipids extracted, deacylated and separated by HPLC. (**A** and **B**) Percentage of PtdIns $(3,4)P_2$ and PtdIns$(3,4,5)P_3$ in transiently transfected COS cells expressing the appropriate wild type or mutated PI3K subunits, as indicated in the figure. (**C**) Percentage of PtdIns(3)P, PtdIns $(3,4)P_2$ and PtdIns$(3,4,5)P_3$ in T14 cells and CMN-5 cells. (**D**) Percentage of PtdIns(3)P, PtdIns $(3,4)P_2$ and PtdIns$(3,4,5)P_3$ in stable transfectants of 3T3 cells expressing the appropriate wild type or mutated PI3K subunits, as indicated in the figure. (E and F) Percentage of PtdIns $(3,4)P_2$ (**E**) and PtdIns$(3,4,5)P_3$ (**F**) in wild type CTLL-2 cells or stable transfectants of CTLL-2 cells expressing p65 or p85 (indicated) prior to and following addition of 500 units/ml of recombinant IL–2 for the indicated periods of time. The figure represents the mean +SD of three different experiments.

2 cells). The amount of transfected regulatory subunit was found to be similar to the amount of endogenous p85 (CTLL-2 cells, Figure 7D and E; data not shown for 3T3 cells), and both associated endogenous p110 (not shown).

Transient expression of p65 alone in COS cells yielded a production of 3-phosphoinositides ~2.3-fold higher than those produced in p85α-transfected cells (Figure 8A, mean of three assays). In contrast, co-expression of p110 and

p65 induced a dramatic increase in the amount of 3-phosphoinositides formed *in vivo* (Figure 8B, mean of three assays). While a high production of 3-phosphoinositides was expected from the fact that both subunits were expressed to a high extent (Figure 7), it was surprising to find that the amount of PtdIns$(3,4,5)P_3$ in cells expressing p65:p110α was ~23-fold higher than in cells expressing p85α:p110α, particularly considering that *in vitro* (see above), the ~1.7-fold-induction in p65 samples did not vary by cotransfection with p110α (both in CTLL-2 cells, Figure 5 and COS cells, not shown). It therefore seems that, *in vivo*, the increased availability of p110α to associate with p65 leads to a proportional increase in the formation of 3-phosphoinositides that is not observed in cells transfected with p85α plus p110α, which only slightly induced 3-phosphoinositide formation (Figure 8B). This is consistent with the involvement of p65 in mediating a particular subcellular localization (see below), multimolecular complex formation or a conformational change in PI3K that is neither maintained *in vitro* nor observed in p85:p110 complexes.

We subsequently compared 3-phosphoinositide formation in CMN-5 and T14 cells expressing only p65 or p85α, respectively. CMN-5 cells contained approximately twice the amount of PtdIns$(3,4)P_2$ compared to T14 cells, and contained PtdIns$(3,4,5)P_3$, which was not detectable in T14 cells (Figure 8C). The observation that CMN-5 cells contain only twice the amount of PtdIns$(3,4)P_2$ found in T14 cells prompted us to perform *in vivo* lipid measurements in stable 3T3 transfected cells. 3T3 cells expressing p65 contained ~2.6-fold higher amounts of PtdIns$(3,4)P_2$ than did 3T3-p85α cells, and contained detectable levels of PtdIns$(3,4,5)P_3$ (Figure 8D). Consistently, examination of the lymphoid cell line CTLL-2, stably expressing either p65 or p85α, yielded a similar ~2-fold induction in 3-phosphoinositide production in p65-expressing CTLL-2 cells versus p85-expressing cells (see Figure 8E and F at time 0). The levels of 3-phosphoinositides formed in cells expressing p65 stably is in contrast with the much higher p65-mediated 3-phosphoinositide formation detected in transiently cotransfected COS cells (Figure 8B). Accordingly, AKT activity was significantly higher in cells transiently expressing p65:p110 (22-fold, see above) than in cells expressing p65 stably, such as 3T3-p65 cells (~3.3-fold, mean of three experiments). Similarly, the AKT kinase activity was significantly higher in cells transiently transfected with p110-CAAX (28-fold, see above) than in cells stably expressing p110-CAAX, such us 3T3-p110-CAAX cells (~5.1-fold, mean of three assays, not shown). These results indicate that high levels of 3-phosphoinositides, such as those found in cells transiently co-transfected with p65 and p110 (Figure 8B), are not maintained in cells expressing constitutive active PI3K mutants stably.

These measurements were performed under conditions of serum starvation. In an attempt to analyze whether the constitutive activation of p65:p110 complexes is regulated by other cellular mechanisms following transmembrane receptor stimulation, we measured the 3-phosphoinositides formed upon addition of interleukin–2 to serum-starved, radiolabeled CTLL-2 cells expressing either p85α or p65. These experiments (Figure 8E and F) allow us to conclude that, in addition to the level of induction of PI3K by p65
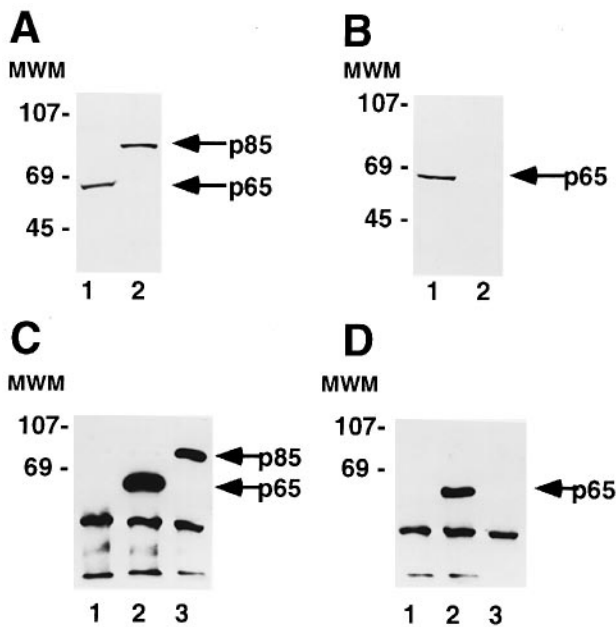
Fig. 9. Subcellular localization of p65 and p85α. (A and B) CMN-5 cells and T14 cells (2×10⁶) were centrifugated and resuspended in cytosol extraction buffer, cells were frozen and thawed twice, and supernatants containing the cytosolic fraction were resolved in 7% SDS–PAGE (**A**). Subsequently pellets were resuspended in 1% T-X100 lysis buffer and supernatants containing the membrane fraction were resolved in 7% SDS–PAGE (**B**). Gels were analyzed by western blotting using anti-p85 Ab. (C and D) CTLL-2 cells were transfected as for Figure 5 using PSG5 (lane 1); PSG5-HAp65 (lane 2) and PSG5-HAp85α (lane 3). Cells were fractionated into cytosolic (**C**) and membrane-enriched (**D**) fractions as in (A) and (B), and lysates immunoprecipitated using anti-HA Abs and resolved in 7% SDS–PAGE. Gels were analyzed as in (A). The figure illustrates a representative experiment of three with similar results.

found in these cells under starvation conditions, IL-2 addition increases the formation of 3-phosphoinositides. These results indicate that some of the mechanisms of PI3K regulation are intact in p65:p110 complexes, as expected from the fact that some of these mechanisms act directly on the catalytic subunit or through domains conserved in p65 (see introduction). Overall, these analyses suggest that p65 drives the *in vivo* induction of PI3K.

### Intracellular localization of p65 and p85α

p85:p110 is a cytosolic heterodimer that localizes proximal to the plasma membrane following cellular stimulation (Otsu *et al.*, 1991; Skolnik *et al.*, 1991; Kodaki *et al.*, 1994; Pleinman *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996). The fact that transient transfection of p65 plus p110α yielded a much higher *in vivo* than *in vitro* kinase activity prompted us to analyze whether truncation of the p85 molecule would modify its subcellular localization. To study this, CMN-5 and T14 cells were fractionated into a cytosolic and a membrane-enriched fraction, as previously described (Carrera *et al.*, 1991). As shown in Figure 9A and B, while p85 appeared almost entirely in the cytosolic fraction, a significant proportion of p65 appeared in the membrane-enriched fraction, suggesting that in fact, truncation of the C-terminal part of p85α changes its subcellular localization. We also analyzed CTLL-2 cells transiently transfected with p85α or p65, and obtained a similar result (Figure 9C and D). In

addition, using transfected COS cells, we found that p65 expression was enriched in the membrane fraction; however, probably due to the high level of transfected protein expression, p65 and p85α were detectable in both fractions (not shown). Fractionation assays suggest that a proportion of p65 localizes to the plasma membrane more stably than does p85, thereby increasing the opportunities of associated p110 to phosphorylate lipid substrates. The membrane localization of p65, but not of p85α, was confirmed by immunofluorescence (our data, not shown). Association of p65 to the plasma membrane may contribute to the greater *in vivo* activity of p65:p110 compared with p85:p110.

Transforming activity of p65-PI3K

Since p65 had been generated in a transformed cell, we next examined its ability to induce cell transformation in NIH-3T3 fibroblasts using v-raf and v-ras expression vectors as positive controls. A representative experiment is shown in Figure 10A, and the results of seven experiments are plotted in Figure 10B. While p85α-transfected cell plates did not differ significantly from control plates, p65 alone, as well as v-raf and p110-CAAX, exhibited a low but consistent ability to form small foci in 3T3 cells (Figure 10A and B). For these assays, parallel samples were cotransfected with each of the expression vectors and a vector encoding the neomycin resistance gene, allowing confirmation throughout the experiments that the different constructs displayed similar efficiency in the generation of drug-resistant colonies (not shown). As v-ras induces the activation of both PI3K and c-raf (Wood *et al.*, 1992; Rodriguez-Viciana *et al.*, 1996), we next examined whether p65 transforming activity could be enhanced in combination with v-raf. While p85α did not affect the low transforming activity of v-raf (Figure 10A and B), co-expression of p65 and v-raf resulted in a synergistic transforming efficiency, inducing an increased number of foci that appeared earlier and were larger than those produced by either oncogene independently. A similar result was obtained using p110-CAAX (Figure 10A and B). Both p85α mutants and wild type p85α have been described to block v-ras-induced transformation (Rodriguez-Viciana *et al.*, 1997), suggesting that overexpression of p85α titrates out the PI3K effectors. In contrast, expression of either p65 or p110-CAAX in combination with v-ras did not block, but rather enhanced, v-ras-induced transformation (Figure 10C). p65 thus mimics PI3K activation by v-ras rather than inhibiting it. Comparison of p65 and Δ24p65 in these assays (Figure 10B and C) indicated that the eph-homologous region of p65 plays no role in cellular transformation.

Constitutive active p110 mutants participate in cellular transformation via a rac-dependent pathway (Rodriguez-Viciana *et al.*, 1997). We subsequently analyzed whether the p65-PI3K transforming activity would rely on the same pathway as rac, as is the case for p110-CAAX. A number of oncogene combinations were tested in the focus formation assay (Figure 10D; controls shown in Figure 10B). While v-raf cooperated with v-rac to transform cells to an extent similar to that of v-ras, p65 did not cooperate with v-rac, as was the case with constitutive active mutants of p110 (Rodriguez-Viciana *et al.*, 1997). These results indicate that both p110-CAAX and p65 act on the same
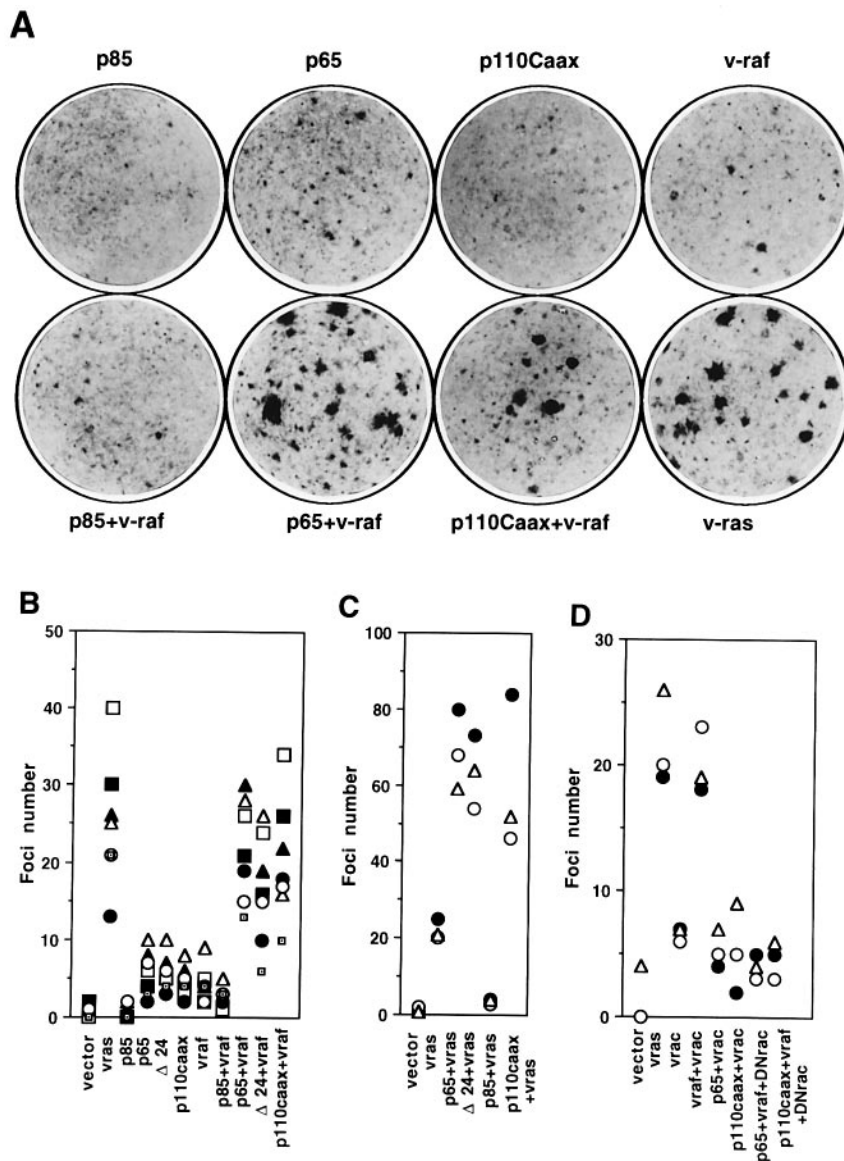
Fig. 10. Transforming activity of p65. (**A**) 3T3 cells (2×10^5) were transfected with 3 µg of PSG5-p85α, PSG5-p65, c-myc-p110-CAAX, or LXV3Δraf-myc (indicated). Other samples were transfected with 1.5 µg of PSG5-p85α plus 1.5 µg of LXV3Δraf-myc, 1.5 µg of PSG5-p65 plus 1.5 µg of LXV3Δraf-myc, 1.5 µg of c-myc-p110-CAAX plus 1.5 µg of LXV3Δraf-myc, or 200 ng of PSG5-vras plus 3 µg of PSG5 (indicated). Transfected cell plates were cultured for 3 to 4 weeks prior to methylene blue staining. (**B**) Focus number obtained in seven different experiments as in (A). For transfections with PSG5 alone or PSG5-Δ24p65 alone, 3 µg cDNA were used. For transfections with Δ24p65 plus LXV3Δraf-myc, 1.5 µg of each cDNA were used. The remainder of the samples were as in A. (**C**) Focus number obtained in three different experiments [as in (A)] after transfection of 3T3 cells with PSG5 (3 µg); PSG5 (3 µg) plus PSG5-vras (200 ng); PSG5-p65 (3 µg) plus PSG5-vras (200 ng); PSG5-Δ24p65 (3 µg) plus PSG5-vras (200 ng); PSG5-p85α (3 µg) plus PSG5-vras (200 ng); or c-myc-p110-CAAX (3 µg) plus PSG5-vras (200 ng) (indicated). Control samples using a single cDNA (PSG5-p65, PSG5-Δ24p65, PSG5-p85α or c-myc-p110-CAAX) were as in (A). (**D**) Focus number obtained in three different experiments [as in (A)] after transfection of 3T3 cells with PSG5 (3 µg); PSG5 (3 µg) plus PSG5-vras (200 ng); PSG5 (1.5 µg) plus LXV3V12rac-myc (1.5 µg); LXV3Δraf-myc (1.5 µg) plus LXV3V12rac-myc (1.5 µg); PSG5-p65 (1.5 µg) plus LXV3V12rac-myc (1.5 µg); c-myc-p110-CAAX (1.5 µg) plus LXV3V12rac-myc (1.5 µg); or LXVN17rac-myc (1µg) plus LXV3Δraf-myc (1 µg) plus either PSG5-p65 (1 µg) or c-myc-p110-CAAX (1 µg) (indicated).

pathway of v-rac. Accordingly, the triple transfection of a dominant negative mutant of rac with p65 plus v-raf inhibited the transforming activity of v-raf plus p65 (Figure 1D), as expected from the fact that dominant negative-rac blocks PI3K effect on cellular rac (Rodriguez-Viciana *et al.*, 1997).

In conclusion, both p65-PI3K and p110-CAAX act alone as weak oncogenes; however, in combination with another weak oncogene, v-raf, the mutants induce a fully-transformed cellular phenotype. The similarity of the results obtained with either p110-CAAX or p65-PI3K suggest that the transforming potential of p65-PI3K, a mutant of p85α generated in CMN-5-transformed cells, is related to its ability to increase p110 kinase activity.

Discussion

Here we describe the identification and characterization of p65-PI3K, a mutant of the regulatory subunit of PI3K generated in a thymic lymphoma cell line. Evidence is

provided indicating that the tumor does not contain wild type p85α gene alleles, but rather contains a reorganized version of the gene, which is responsible for the generation of p65-PI3K. Five p85-related proteins have been previously described (Pons *et al.*, 1995; Antonetti *et al.*, 1996; Fruman *et al.*, 1996; Inukai *et al.*, 1996, 1997). One of them, p55^PIK, is encoded by a different gene and shows similarity to the N-terminal SH2, C-terminal SH2 and inter-SH2 domains of p85α. The other proteins are described as alternative splice forms of p85α, lacking its SH3 and bcr-homologous regions. In contrast to p65-PI3K, therefore, none of these p85-related proteins represents p85α genomic alterations. Analysis of the p65-PI3K cDNA sequence indicated that the protein encompasses the first 571 residues of the p85α regulatory subunit linked to a domain conserved in the eph tyrosine kinase receptor family. Using the cloned mutant, we demonstrated that transfection of this protein results in *in vivo* PI3K activation and contributes to induction of cell transformation.

The higher *in vitro* kinase activity of p65:p110 compared with p85:p110 (Figure 5) suggests that p65 may, by interacting with p110, induce a conformational change resembling the molecular changes that occur in p85:p110 complexes following cellular stimulation. p65 lacks the final part of the inter-SH2 region of p85 required for the association to, and the activation of, p110 (Dhand *et al.*, 1994; Holt *et al.*, 1994; Klippel *et al.*, 1994). It is therefore possible that the mutation found in p65, affecting the region of association with p110, alters the conformation of the complex. Prediction of tertiary structure of the inter-SH2 domain, with its second half folding over its N-terminus (Dhand *et al.*, 1994), supports that mutation of part of the inter-SH2 domain may affect the structure of p85:p110 complex.

Regarding the *in vivo* PI3K kinase activity analysis (Figure 8), the high lipid production detected in COS cells expressing p65:p110 was expected, in view of the very large amounts of both subunits expressed in these cells (Figure 7). It was surprising, however, to find that cells expressing p65:p110α contain ~23-fold higher amounts of PtdIns(3,4,5)P$_3$ (and similar AKT induction) than cells expressing p85α:p110α; particularly considering that *in vitro*, we detected only a 1.7-fold activity induction in p65 samples. The different magnitude of the detected activity induction *in vitro* and *in vivo* have been reported for other mechanisms of PI3K activation (Rodriguez-Viciana *et al.*, 1996). This could be due to the constraints of the *in vitro* system, or in addition to some property of p65 that confers an advantage exclusively *in vivo*, such as a differential subcellular localization. We have shown that a significant proportion of p65 appears in the membrane-enriched-fraction, suggesting that truncation of the C-terminal part of p85α in fact affects its subcellular localization. Association of p65 to the plasma membrane, increasing the opportunities of associated p110 to phosphorylate lipid substrates, thus possibly contributes to the greater *in vivo* activity of p65:p110 compared to p85:p110. Finally, the different induction levels detected in transiently transfected cells versus stable cell lines suggests that high 3-phosphoinositide levels, such as those found in transiently cotransfected cells (Figure 8B), may compromise cellular viability in long term culture. Cells may thus develop mechanisms for downregulating PI3K activity,

such as increasing specific phosphatase activity (Damen *et al.*, 1996). Inducible vectors are being prepared to examine this observation in detail.

In conclusion, we favor the hypothesis that the p65:p110 complex differs from the p85:p110 complex in its conformation or in its associated proteins, which lead to a differential ability to phosphorylate substrates *in vitro*. In addition, the different subcellular localization of p65 and p85 favors the *in vivo* activity of p65:p110. The high production of lipids catalyzed by p65:p110 (or by p110-CAAX, see above), appears, however, to be subject to other downregulatory mechanisms in cells expressing these mutants stably. Similarly, the observation that IL-2 increases p65:p110 activity (Figure 8E and F), indicates that the activity of this complex is also subject to upregulation by other cellular mechanisms.

The presented data does not exclude that other mechanisms could contribute to increase p65:p110 activity. These mechanisms could include the selective activation by p65 of other catalytic subunits (such us p110 β or δ in T cells, Hu *et al.*, 1993; Chantry *et al.*, 1997; Vanhaesebroek *et al.*, 1997); mechanisms based on the lack of the C-SH2 domain, such as SH2-based downregulatory mechanisms (Rameh *et al.*, 1995) or acquisition of a different activation mechanism in the p65 molecule. Finally, a possible mechanism that may be used in cells expressing p65:p110 is based on the observation that p110 phosphorylates Ser 608 of p85 and is, in turn, downregulated (Carpenter *et al.*, 1993). The absence of Ser 608 in p65 may result in p110 being in a constitutively activated state.

In spite of the open questions regarding the complete understanding of p65 action in cells, it is clear that p65 upregulates PI3K activity, being this property independent of the presence of the eph-related domain. Truncation of p85 at residue 571 is thus responsible for the p65 activating properties. This property appears specific to this mutation, since none of the previously described p85-related proteins (Pons *et al.*, 1995; Antonetti *et al.*, 1996; Fruman *et al.*, 1996; Inukai *et al.*, 1997) or p85 mutants (Dhand *et al.*, 1994; Holt *et al.*, 1994; Klippel *et al.*, 1994) induces constitutive p110 activation.

With regard to the mechanism by which p65 contributes to cellular transformation, the data presented on PI3K activation by p65 suggest that it probably affects cell growth by a mechanism similar to that utilized by genetically engineered, constitutively active p110 mutants (Rodriguez-Viciana *et al.*, 1997). Focus formation assays also support this view. In fact, neither activated p110 nor p65 can induce transformation alone, but both cooperate with v-raf and with suboptimal doses of v-ras to transform cells (Figure 10, Rodriguez-Viciana *et al.*, 1997). Moreover, p110-CAAX, like p65-PI3K, activates AKT (Figure 6) and lie on the same signaling pathway of rac (Figure 10). Of the effectors that v-ras triggers to induce cell transformation, which include c-raf and PI3K (Wood *et al.*, 1992; Rodriguez-Viciana *et al.*, 1996), the pathway used by p65 and p110-CAAX is different from that of c-raf but seems to be the same as that used by v-AKT (Boudewijn *et al.*, 1995), possibly affecting p70-S6 kinase activation (Chung *et al.*, 1994; McIlroy *et al.*, 1997). Induction of this pathway has been postulated to affect survival maintenance and/or cell cycle progression (Kennedy *et al.*, 1997), possibly contributing to the transforming activity

of constitutive active PI3K mutants. Considering that cellular rac is a downstream effector of PI3K (Figure 10, Hawkins *et al.*, 1995), and its involvement in cytoskeletal organization (Nobes *et al.*, 1995), it is also possible that active PI3K mutants by inducing c-rac contribute to mediate the cytoskeletal changes required for the anchorage-independent cell growth, characteristic of trans-formed cells.

Regarding the implication of these results in the generation of mammalian tumors, the possibility that p85 can be naturally mutated appears to be favored by the presence of two regions in the p85 gene that facilitate genomic instability: the bcr-homologous region and the 3′ end untranslated CT-rich sequences (Figure 4, Htun *et al.*, 1984; Dhand *et al.*, 1994; Holt *et al.*, 1994). The fact that activation of this pathway is required for v-ras-induced transformation suggests that enzymes in this pathway could be targets for inhibition of tumor cell growth.

The data presented show for the first time that a mutation of the PI3K regulatory subunit promotes the constitutive activation of the catalytic subunit and contributes to cell transformation. Based on these observations, we strongly favor the possibility that p65 induces transformation due to its ability to activate PI3K. The fact that p65-PI3K has been generated in a transformed mammalian cell, and not genetically engineered, together with its ability to contribute to fibroblast transformation, suggest that similar mutations may account for the generation or progression of other mammalian tumors.

Materials and methods

Antibodies
Anti-p85, anti-HA (12CA5) and anti-AKT Ab were from Upstate Biotechnology, Babco, and Santa Cruz Biotechnology, respectively. Anti-p110 Ab were prepared by immunizing rabbits with the CT-peptide CKMDWIFHTIKQHALN, as described (Hiles *et al.*, 1992).

Biochemistry
Cell lysis, immunoprecipitations and Western blotting were performed as described (Carrera *et al.*, 1995). Phospholipid *in vitro* kinase assays were performed as described (Merida *et al.*, 1991). *In vivo* measurement of PtdIns was performed essentially as described (Rodriguez-Viciana *et al.*, 1997), involving ^{32}P metabolic labeling and HPLC analysis of the extracted deacylated phospholipids ($1–5 \times 10^6$ c.p.m. per analysis). For AKT kinase assays, anti-AKT immunoprecipitates were resuspended in 25 µl of kinase buffer (50 mM Tris pH 7.5, 10 mM MgCl$_2$, 1 mM DTT) mixed with 2.5 µl of histone H2B at 1 mg/ml and 0.4 µl of 2.5 mM ATP containing 3 µCi of γ^{32}P-ATP (Amersham). Reactions were incubated for 20 min at room temperature. Subcellular fractionation was performed essentially as described (Carrera *et al.*, 1991), using 20 mM Tris–HCl pH 8.0, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10% glycerol, 137 mM NaCl, 0.002% CHAPS, as cytosolic extraction buffer, and using 1% TX100 lysis buffer (Carrera *et al.*, 1995) as membrane extraction buffer.

Plasmid construction
p65, originally cloned in pBluescript, was subcloned into the *Eco*RI site of PSG5 using the restriction enzymes *Hin*dII (at the 5′ end) and *Nco*I (at the 3′ end). In all constructions, overhanging ends were blunted using Klenow enzyme. Δ24p65 was obtained from pBluescript-p65 by replacing the *Nsi*I–*Nco*I restriction fragment of p65 with a similar cDNA fragment containing a stop codon immediately after residue 571. This fragment was obtained by PCR, using AAGGATTATGCATAACCATG-ATA as 5′ oligonucleotide and CGAATGTGGGGGCGGCCATGGTCA-GATGAGGTCCG as 3′ oligonucleotide. pBluescript-Δ24p65 was then subcloned into PSG5 exactly as described for PSG5-p65. PSG5-p85α was generated by replacing the *Bss*HII–*Bam*HI restriction fragment of PSG5-p65 with the corresponding fragment of α-p85 (obtained from pGem-p85α, kindly donated by Dr L.Williams, Escobedo *et al.*, 1991).

pCG-HA-p85 was donated by Dr L.Williams (Klippel *et al.*, 1994). PSG5-p65 was tagged with the HA epitope by inserting at its 5′ end *Xcm*I site the oligonucleotide (annealed with the corresponding complementary oligonucleotide) CGAATTCACCATGTACCCGTACG-ATGTCCCGGATTACGCGGC. PSG5-HAΔ24p65 was obtained by repla-cing the appropriate *Bss*HII–*Bsa*BI restriction fragment of PSG5-Δ24p65 with the corresponding fragment of PSG5-HA-p65. PSG5-HA-AKT was prepared by inserting the appropriate *Xba*I–*Bam*HI restriction fragment of SVL-PKB^{tag} (Boudewijn *et al.*, 1995) into the *Bam*HI site of PSG5. c-myc-p110-CAAX was prepared by inserting the Myc-T epitope at the 5′ end of αp110, and the p21-ras CAAX Box (CKCVLS-Stop) at the 3′ end of p110α (Hiles *et al.*, 1992). PSG5-vras, LXV3Δraf-myc, LXV12rac-myc and LXN17rac-myc were previously described (Rodrig-uez-Viciana *et al.*, 1997).

Transfections
CTLL-2 cells were electroporated using 50 µg of cDNA as described (Carrera *et al.*, 1995). NIH-3T3 cells were transfected using 3–4 µg of cDNA and Tfx–50 reagent (Promega, Madison, WI) as indicated by the manufacturer. COS cells were transfected using 1 µg of cDNA and lipofectamine reagent (Gibco-BRL) as indicated by the manufacturer. For stable cell lines, cells were transfected as above combining the appropriate expression vector with pSVNeo (5:1) and cells were selected in medium containing G418. 3T3 focus formation assays were performed as described (Rodriguez-Viciana *et al.*, 1997).

Acknowledgements

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