Reconstitution of the mammalian PI3K/PTEN/Akt pathway in yeast

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The mammalian signalling pathway involving class I PI3K (phosphoinositide 3-kinase), PTEN (phosphatidylinositol 3-phosphatase) and PKB (protein kinase B)/c-Akt has roles in multiple processes, including cell proliferation and apoptosis. To facilitate novel approaches for genetic, molecular and pharmacological analyses of these proteins, we have reconstituted this signalling pathway by heterologous expression in the unicellular eukaryote, *Saccharomyces cerevisiae* (yeast). High-level expression of the p110 catalytic subunit of mammalian PI3K dramatically inhibits yeast cell growth. This effect depends on PI3K kinase activity and is reversed partially by a PI3K inhibitor (LY294002) and reversed fully by co-expression of catalytically active PTEN (but not its purported yeast orthologue, Tep1). Growth arrest by PI3K correlates with loss of PIP_2 (phosphatidylinositol 4,5-bisphosphate) and its conversion into PIP_3 (phosphatidylinositol 3,4,5-trisphosphate). PIP_2 depletion causes severe rearrangements of actin and septin architecture, defects in secretion and endocytosis, and activation of the mitogen-activated protein kinase, Slt2. In yeast producing PIP_3, PKB/c-Akt localizes to the plasma membrane and its phosphorylation is enhanced. Phospho-specific antibodies show that both active and kinase-dead PKB/c-Akt are phosphorylated at Thr^{308} and Ser^{473}. Thr^{308} phosphorylation, but not Ser^{473} phosphorylation, requires the yeast orthologues of mammalian PDK1 (3-phosphoinositide-dependent protein kinase-1): Pkh1 and Pkh2. Elimination of yeast Tor1 and Tor2 function, or of the related kinases (Tel1, Mec1 and Tra1), did not block Ser^{473} phosphorylation, implicating another kinase(s). Reconstruction of the PI3K/PTEN/Akt pathway in yeast permits incisive study of these enzymes and analysis of their functional interactions in a simplified context, establishes a new tool to screen for novel agonists and antagonists and provides a method to deplete PIP_2 uniquely in the yeast cell.

Key words: c-Akt, phosphorylation, phosphoinositide 3-kinase (PI3K), protein kinase B (PKB), PTEN, *Saccharomyces cerevisiae*.

INTRODUCTION

Generation of PIP_3 (phosphatidylinositol 3,4,5-trisphosphate) at the plasma membrane is key to the regulation of mammalian cell proliferation and survival by growth-promoting factors [1]. Many human tumours [2] have alterations in the PI3K (phosphoinositide 3-kinase) that generates this lipid [3], in the PTEN (phosphatidylinositol 3-phosphatase) that degrades this lipid [4] and in a protein kinase [PKB (protein kinase B)/c-Akt] that requires this lipid for its activation [5].

Class IA PI3K, the best studied and most related to oncogenesis, comprises a regulatory/adaptor subunit (p85) and a catalytic subunit (p110). In humans, seven regulatory subunits, generated by alternative splicing from three genes (p85α, p85β and p55γ) and three p110 catalytic subunit isoforms (α, β and γ), have been described. p85 contains an SH2 domain (Src homology 2 domain) that binds phosphotyrosine residues on receptor-tyrosine kinases, simultaneously recruiting p110, stimulating its activity and generating locally PIP_3 from PIP_2 (phosphatidylinositol 4,5-bisphosphate) in the plasma membrane.

The reverse reaction – dephosphorylation of PIP_3 to PIP_2 – is catalysed by PTEN, which is considered a tumour suppressor because loss-of-function mutations are found in overt tumours [6] and in the germline in diseases marked by hereditary predisposition to cancer [7]. PIP_3 is also metabolized to $PI(3,4)P_2$ (phosphatidylinositol 3,4-bisphosphate) by 5-phosphoinositide phosphatases, like SHIP1 and SHIP2 (SH2-containing inositol phosphatase-1 and -2); but PIP_3 and $PI(3,4)P_2$ engage the same effectors, for the most part.

The three isoforms (α, β and γ) of PKB/c-Akt are major targets of PIP_3, which binds to the N-terminal PH domain (pleckstrin homology domain), thereby anchoring the enzyme to the plasma membrane and inducing a conformational change that exposes its catalytic domain to its upstream activators. Full activation of Akt requires phosphorylation of Thr^{308} within the activation loop (or T-loop) and also at a more distal site, Ser^{473}. Phosphorylation of Akt1 at Thr^{308} is mediated by PDK1 (3-phosphoinositide-dependent protein kinase-1), which has a C-terminal PIP_3-specific PH domain that facilitates its recruitment to membranes. Hence, Thr^{308} is called the PDK1 site. The enzyme responsible for phosphorylation of Akt1 at Ser^{473} (termed the PDK2 site) is controversial. Over time, various investigators have implicated several different classes of protein kinase in PDK2 site phosphorylation [8], whereas others suggest that autophosphorylation is the cause [9]. Recently, it has been reported that members of the PIKK (phosphatidylinositol kinase-related kinase) family of atypical protein kinases are responsible for PDK2 site phosphorylation of Akt1, including DNA-PKcs (the catalytic subunit of DNA-dependent protein kinase) [10], the product of the gene mutated in ATM (ataxia telangiectasia) [11], and the mTOR (mammalian target of rapamycin) orthologue [12]. Once activated, Akt phosphorylates substrates that promote cell survival and stimulate cell growth. Thus hyperactivation of Akt is considered a prerequisite for oncogenesis. Consequently, the PI3K/Akt pathway has become

Abbreviations used: ATM, ataxia telangiectasia; CPY, carboxypeptidase Y; DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase; ERK, extracellular-signal-regulated kinase; HA, haemagglutinin; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide-dependent protein kinase-1; PH domain, pleckstrin homology domain; PI3K, phosphoinositide 3-kinase; PIKK, phosphatidylinositol kinase-related kinase; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PLCδ1, phospholipase δ1; PTEN, phosphatidylinositol 3-phosphatase; SH2 domain, Src homology 2 domain.

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Table 1 ***Sacch. cerevisiae*** **strains used in the present study**

Strain	Genotype	Reference or source
YPH499	*MATa ade2-101^{och} his3-Δ200 leu2-Δ1 lys2-801^{amb} trp1-Δ1 ura3-52*	[30]
YFR105	YPH499 *pkh1-Δ1::TRP1*	[17]
YFR106	YPH499 *pkh2-Δ1::HIS3*	[17]
YPT40	YPH499 *ypk1-1^{ts}::HIS3 ykr2-Δ1::TRP1*	[16]
DLY1	*MATa ade1 his2 leu2-3,112 trp1-1 ura3Δ*	Daniel Lew
INA106	DLY1 *pkh1*D398G *pkh2::LEU2*	Kunihiro Matsumoto
SH100	*MATa leu2-3,112 ura3-52 trp1 his3 ade2Δ*	Michael Hall
SH221	SH100 *tor1::HIS3-3 tor2::ADE2-3* YCplac111(*LEU2*)-*tor2-21*	Michael Hall
DL454	*MATa ura3-52 his4 trp1-1 leu2-3,112 canR slt2Δ::TRP1*	David Levin
YFR55	YPH499 *sch9Δ::HIS3*	[57]
BY4741	*MATa; his3Δ1; leu2Δ; met15Δ; ura3Δ*	EUROSCARF*
Y03114	BY4741 *tel1::kanMX4*	EUROSCARF
RCY307	*MATa HO::hisG esr1(mec1)Δ::LEU2, lys2, ura3, leu2::hisG, ade2::LK, his4 arg4N::ESR1-kanMX4*	Rita Cha
RCY426	*MATa HO::hisG esr1(mec1)Δ::LEU2, lys2, ura3, leu2::hisG, ade2::LK, his4 arg4N::esr1-40 kanMX4*	Rita Cha
YCB655	*MATa leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,25 tra1-2*	Jerry Workman

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an important target in attempts to develop more efficacious anti-tumour agents [13].

Although it has been reported that fission yeast (*Schizosaccharomyces pombe*) can generate PIP_3 [14], PIP_3 seems to have no role in the physiology of budding yeast (*Saccharomyces cerevisiae*) [15]. Nonetheless, the *Sacch. cerevisiae* genome encodes: (i) two functional PDK1 orthologues (Pkh1 and Pkh2) involved in cell integrity and endocytosis [16,17]; (ii) an apparent PTEN orthologue (Tep1) of uncharacterized biological function [18,19]; (iii) an Akt-like protein kinase (Sch9), which lacks an apparent PH domain, involved in nutrient sensing, ribosome biogenesis, lifespan and cell-size control [20]; and (iv) clear-cut homologues of the PIKK family, specifically Tor1 and Tor2 (mTOR) [21], Tel1 (ATM) [22], Mec1 (ATR) [23] and Tra1 (most resembles DNA-PKcs) [24].

To address central questions in the biology of PIP_3-dependent signalling and to establish a readily accessible and versatile tool to screen for pharmacological agents that influence this critically important pathway, we devised methods to successfully reconstitute the mammalian PI3K/PTEN/Akt pathway in yeast cells, which is described here. *In vivo* conversion of the essential PIP_2 pool into PIP_3 by expression of PI3K impaired yeast growth by altering morphogenesis and vesicular trafficking. The function of PTEN could be readily assessed by its ability to reverse the growth inhibition caused by PI3K. PIP_3 generation led to membrane translocation and activation of Akt, enhancing its phosphorylation at both Thr^{308} and Ser^{473}. The yeast PDK1 orthologues are required for PDK1 site phosphorylation, whereas none of the yeast PIKK family members seems necessary for PDK2 site phosphorylation, implicating some other endogenous enzyme.

EXPERIMENTAL

Strains, media and growth conditions

The *Sacch. cerevisiae* strains used in the present study are listed in Table 1. *Escherichia coli* DH5α F′[K12Δ(*lacZYA-argF*)U169 *deoR supE44 thi-1 recA1 endA1 hsdR17 gyrA96 relA1* (*Φ80lacZΔM15*)F′] was used for routine molecular biology techniques. YPD [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) dextrose/glucose] broth or agar was the general non-selective medium used for yeast cell growth. Synthetic minimal medium (SGlc) contained 0.17 % yeast nitrogen base without amino acids, 0.5 % ammonium sulphate and 2 % glucose and lacked appropriate amino acids and nucleic acid bases to maintain selection for plasmids. In SGal (synthetic galactose) and SRaf (synthetic raffinose) media, glucose was replaced with 2 % (w/v) galactose or 2 % (w/v) raffinose respectively. Galactose induction in liquid medium was performed by growing cells in SRaf to mid-exponential phase and then adding galactose to 2 % for 6–8 h. Growth assays on plates were performed, typically, by growing transformants overnight in SGlc lacking uracil, leucine or both, adjusting the culture to an absorbance A_{600} 0.5 and spotting samples (5 μl) of the cell suspension and three serial 10-fold dilutions on to the surface of SGlc or SGal plates lacking the appropriate nutrients to maintain selection for plasmids, followed by incubation at 28 °C for 2–3 days. When yeast cells were treated with PI3K inhibitors, transformants were grown overnight in selective medium, adjusted to an A_{600} of 0.1, and then samples (5 μl) of the cell suspension were used to seed the wells of 96-well microtitre plates containing serial dilutions of either wortmannin (Sigma) or LY294002 (Biomol, Hamburg, Germany) suspended in 100 μl of liquid SGal medium lacking the appropriate nutrients to maintain selection for plasmids.

Plasmid construction

Transformation of *E. coli* and yeast and other basic molecular biology methods were carried out using standard procedures. To generate plasmid YCpLG-PI3K, the cDNA encoding PI3K-CAAX was excised from plasmid Psg5/5MycTp110XCAAX [25] (a gift from M. Collado, Spanish National Cancer Centre, Madrid, Spain) with BamHI and cloned into the same site in yeast vector YCpLG [26]. To produce plasmid YCpLG-PI3K^{K802R} (where K802R stands for $Lys^{802} \rightarrow Arg$), bearing a catalytically inactive ('kinase-dead') allele of PI3K-CAAX, site-directed mutagenesis was carried out using a DpnI-based strategy [27] with Turbo PfuI DNA polymerase (Stratagene) and the primers 5′-CAGAACAA-TGAGATCATCTTTCGAAATGGGGATGATTTACGGC-3′ and 5′-GCCGTAAATCATCCCCATTTCGAAAGATGATCTCATT-GTTCTG-3′. Cassettes in which cDNAs encoding either c-Akt, c-Akt^{K179M} or an N-myristoylated c-Akt were fused in frame to an HA (haemagglutinin) epitope-tagged version of eGFP (enhanced

green fluorescence protein) [HA–eGFP-Akt, HA–eGFP-Akt^{K179M} and myr-HA–eGFP-Akt respectively] were excised with HindIII and BamHI from the original Pcefl(X)-derived plasmids that were constructed for expression in mammalian cells [28] (a gift from M. Lorenzo, Universidad Complutense, Madrid, Spain) and cloned into the corresponding sites in yeast vector pYES2 (Invitrogen), yielding plasmids pYES-GFP-c-Akt, pYES-GFP-c-Akt^{K179M} and pYES-myr-GFP-c-Akt respectively. The cDNA encoding PTEN was excised with EcoRI from plasmid Pcmv-pten [29] [a gift from J.M. Paramio, CIEMAT (Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain)] and inserted into the same site in pYES2, generating pYES-PTEN. Plasmid pYES-PTEN^{G129D}, expressing a catalytically inactive ('phosphatase-dead') allele, was generated by site-directed mutagenesis, as above, but using primers 5′-CACTGTAAAGCTGGAAAGGAACGAACTGGTGTAATG-3′ (upper) and 5′-CATTACACCAGTTCGTTCCTTTCCAGCTTTACAGTG-3′ (lower). To construct plasmid pYES-Tep1-Myc, first the *TEP1* coding sequence was amplified by PCR from yeast genomic DNA using the primers 5′-CGGATCCATGAGAGAGGAGGGGAGTG-3′ (upper) and 5′-GGGATCCTATAATTTCCCATTCCAAT-3′ (lower) and cloned into the BamHI site in a yeast vector, pRS306-Myc6, which had been previously generated by inserting a Myc_6 epitope into the polylinker in the integrative *URA3* vector, pRS306 [30]. Secondly, the resulting *TEP1*–myc_6 fusion was excised using EcoRI and cloned into the same site in pYES2. To construct a *PTEN-TEP1* chimaera, we used a now-standard PCR-based method [31] in which *PTEN* was amplified using the 'upper' primer indicated above, *TEP1* was amplified with the 'lower' primer indicated above, with the following primers to generate the desired junction: 5′-GCCTCATTAGAAATTCCTGGTCTATAATCCAGAT-3′ and 5′-ATCTGGATTATAGACCAGGAATTTCTAATGAGGC-3′. To construct a plasmid expressing a Tep1–GFP fusion, the *TEP1* gene was amplified by PCR using the same oligonucleotides described above, which provide BamHI sites at both ends of the amplicon, and cloned in frame into the BamHI site of the polylinker in pGFP-C-FUS [32], yielding pTep1-GFP. All constructs were verified by direct DNA sequencing. Other plasmids used in the present study were pLA10H [33] to express GFP-tagged septin and pRS426-GFP-2xPH(PLCδ) to visualize PIP_2 *in vivo* [34].

Protein detection by immunoblotting

Standard procedures, as described previously [35], were used for yeast growth, cell harvesting and cell breakage, as well as for the preparation of protein-containing cell-free extracts, fractionation by SDS/PAGE and transfer on to nitrocellulose membranes. Polyclonal anti-PTEN antibodies (Upstate Biotechnology, Lake Placid, NY, U.S.A.) were used to detect PTEN or the PTEN-Tep1 chimaera in yeast extracts. Yeast MAPK (mitogen-activated protein kinase) Slt2 [orthologue of mammalian ERK5 (extracellular-signal-regulated kinase 5)] was detected with the polyclonal anti-Slt2 antibodies described previously [36]. Rabbit anti-phospho-p44/p42 MAPK (anti-P-Thr^{202}-P-Tyr^{204}) antibodies (New England Biolabs, Hitchin, Herts., U.K.) were used to detect dually phosphorylated forms of Slt2 and of two other yeast MAPKs, Kss1 and Fus3 (orthologues of mammalian ERK1 and ERK2). Anti-phospho-p38 (Cell Signaling Technology, Beverly, MA, U.S.A.) was used to detect dual phosphorylation of yeast MAPK Hog1 [orthologue of mammalian SAPKα (stress-activated protein kinase α)]. Rabbit anti-phospho-Akt(P-Thr^{308}) and rabbit anti-phospho-Akt(P-Ser^{474}) (Cell Signaling Technology) were used to detect the phosphorylation of the corresponding sites in PKB/c-Akt. Yeast CPY (carboxypeptidase Y) was detected with anti-CPY antiserum (Molecular Probes). Yeast actin was detected by cross-reaction with a mouse anti-actin monoclonal antibody (clone C4; MP Biomedicals, Irvine, CA, U.S.A.). GFP fusion proteins were detected using a mouse anti-GFP antibody (JL-8; BD Biosciences, San Jose, CA, U.S.A.). After washing, bound primary antibodies were revealed using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies, as appropriate, and a chemiluminescence detection system (ECL®; Amersham Biosciences). For quantitative measurements on immunoblots, densitometry [in a GS-800 densitometer, model Power Look 2100xL (Bio-Rad), using the software Quantity One] was applied to films and the extent of Thr^{308} and Ser^{473} phosphorylation was normalized to the amount of protein recovered in the extract as follows. Ratios of c-Akt^{K179M}/c-Akt were calculated by dividing the percentages of the phosphorylation signal of Thr^{308} or Ser^{473} of GFP–c-Akt^{K179M} relative to GFP–c-Akt by the corresponding percentages of the GFP signal of GFP–c-Akt^{K179M} signal relative to GFP–c-Akt. To obtain these ratios, duplicate measurements on at least three different experiments were performed.

Microscopy techniques and immunofluorescence

For fluorescence microscopy of live yeast cells (to visualize GFP), exponentially growing cultures were harvested by brief centrifugation, washed once with sterile water and viewed directly. To observe actin, yeast cells were treated with FITC-conjugated phalloidin (Sigma) as described previously [37]. To monitor vacuolar morphology and bulk fluid-phase endocytosis into the vacuole, staining with FM4-64 [38] and Lucifer Yellow [39] respectively was performed. To obtain reliable and statistically significant data, $\geqslant 200$ cells were examined for each condition or experiment. Indirect immunofluorescence with monoclonal anti-PIP_3 antibodies (Echelon, Salt Lake City, UT, U.S.A.) at a 1:200 dilution was performed, essentially as described for animal cells [40], using Cy3-conjugated monoclonal anti-mouse IgG antibodies (Chemicon, Temecula, CA, U.S.A.) at a 1:500 dilution as the secondary antibodies. Cells were examined under an Eclipse TE2000U microscope (Nikon, Tokyo, Japan) and digital images were acquired with an Orca C4742-95-12ER charge-coupled-device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and Aquacosmos Imaging Systems software.

RESULTS

Expression of PI3K inhibits yeast cell growth and generates PIP_3

For expression in yeast, the cDNA encoding p110α was cloned under the control of the galactose-inducible *GAL1* promoter. To target the enzyme to the plasma membrane in the absence of the p85 subunit, the -CAAX box of H-ras was fused in frame to the C-terminus of p110α. Wild-type *Sacch. cerevisiae* cells (YPH499) transformed with this construct grew normally on glucose medium, but failed to grow on galactose medium (Figure 1A). The growth inhibition caused by PI3K expression was alleviated by a point mutation (K802R, Lys^{802} $\rightarrow$ Arg) in a residue essential for enzyme activity. Hence, the growth inhibition was due to the catalytic function of PI3K and not the result of any non-specific toxic effect of producing this heterologous membrane-targeted polypeptide in yeast.

To determine whether the presence of active PI3K generates PIP_3 at the expense of PIP_2, both phosphoinositides were visualized in the cells. To detect PIP_2, cells were co-transformed with a plasmid expressing a fluorescent PIP_2-specific reporter [GFP fused to two tandem copies of the PH domain of mammalian

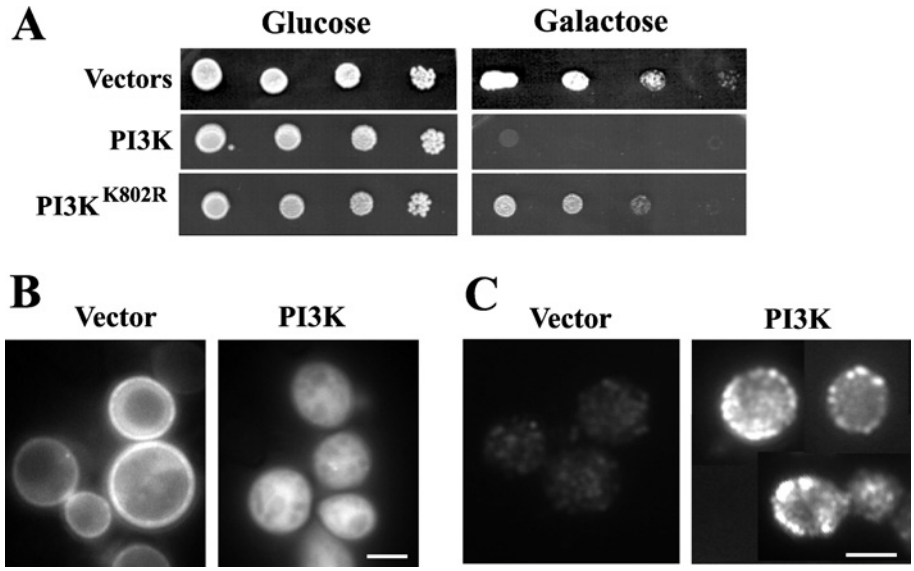

Figure 1 Expression of active PI3K in yeast inhibits growth, depletes PIP_2 and generates PIP_3 at the plasma membrane

(**A**) A wild-type yeast strain (YPH499) carrying a *URA3*-marked vector (pYES2) was transformed with another empty *LEU2*-marked vector (YCpLG) or the same vector harbouring a construct to express, under the control of a galactose-inducible promoter (*GAL1*), either membrane-targeted mammalian PI3K, namely p110α-CAAX (YCpLG-PI3K), or a catalytically inactive mutant (YCpLG-PI3K^{K802R}). Serial 10-fold dilutions of cultures of representative transformants were spotted on minimal synthetic (S) medium lacking Ura and Leu under repressing (glucose) or inducing (galactose) conditions. (**B**) Wild-type cells (YPH499) carrying an empty *LEU2*-marked vector (YCpLG) or the same vector expressing PI3K (YCpLG-PI3K) were transformed with a URA3-marked vector expressing two tandem copies of the PIP_2-specific PH domain of mammalian PLCδ1 fused to GFP [pRS426-GFP-2xPH(PLCδ)]. Representative transformants were propagated on SGlc-Leu-Ura, shifted to SGal-Leu-Ura for 6 h, and then viewed under the fluorescence microscope. (**C**) Wild-type cells (YPH499) were transformed with empty vector (YCpLG) or the same vector expressing PI3K (YCpLG-PI3K). Representative transformants were propagated on SGlc-Leu, shifted to SGal-Leu for 6 h, fixed, permeabilized, stained with specific anti-PIP_3 antibodies (Echelon), counterstained with fluorescently labelled secondary antibodies and then viewed under the fluorescence microscope.

PLCδ1 (phospholipase δ1)]. In yeast, this reporter specifically decorates the plasma membrane [34]. Indeed, in our control cells, the reporter clearly stained the plasma membrane prominently. In contrast, in the cells where catalytically active PI3K was expressed, only cytoplasmic fluorescence was seen, indicating a dramatic depletion of PIP_2 from the plasma membrane (Figure 1B). To confirm that PIP_3 was produced at the expense of PIP_2, the same cells were fixed and stained with an anti-PIP_3 antibody. Similar to other methods that failed to detect PIP_3 in *Sacch. cerevisiae* [14,15], no staining was seen in the control cells. In contrast, large patches of fluorescent staining with the anti-PIP_3 antibodies were found around the perimeter of the cells expressing functional PI3K (Figure 1C). Thus the PI3K produced was catalytically active, and the most likely cause of its observed inhibitory effect on yeast cell growth was efficient conversion of the PIP_2 pool into PIP_3.

Expression of PTEN antagonizes the effect of PI3K in yeast

If the growth inhibitory action of PI3K is due to conversion of PIP_2 into PIP_3, then reversal of this reaction by PTEN should ameliorate growth arrest. Therefore PTEN cDNA was cloned under the control of the *GAL1* promoter in a vector compatible with that used to express PI3K. Expression of PTEN alone at a high level, confirmed by immunoblotting with specific anti-PTEN antibodies (results not shown), had no discernible effect on growth or morphology (Figure 2A). However, in cells expressing PI3K, co-expression of PTEN prevented growth arrest. A point mutation (G219E) reported to abrogate the lipid phosphatase activity of PTEN [41] blocked its ability to reverse PI3K-induced growth inhibition (Figure 2A). Thus the counteracting effect of PTEN on PI3K-initiated signalling was reproduced in yeast cells.

Other authors have suggested that the *Sacch. cerevisiae TEP1* gene encodes a PTEN orthologue [18,19]. Tep1 is similar to

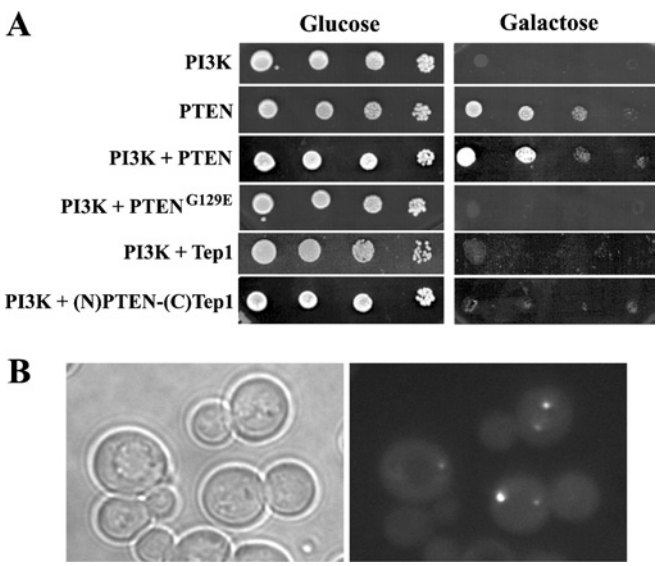

Figure 2 Growth inhibition caused by PI3K expression is relieved by co-expression of active mammalian PTEN, but not by *Sacch. cerevisiae* Tep1

(**A**) Wild-type cells (YPH499) were co-transformed with the following sets of *URA3*- and *LEU2*-marked vectors: pYES2 and YCpLG-PI3K (top row); pYES-PTEN and YCpLG (second row); pYES-PTEN and YCpLG-PI3K (third row); pYES-PTEN^{G129E} (catalytically inactive) and YCpLG-PI3K (fourth row); pYES-TEP1-Myc and YCpLG-PI3KA (fifth row); and pYES-PTEN-TEP1 and YCpLG-PI3K (bottom row). Serial 10-fold dilutions of cultures of representative transformants were spotted on minimal synthetic (S) medium lacking Ura and Leu under repressing (glucose) or inducing (galactose) conditions. (**B**) Wild-type cells (YPH499) carrying a *URA3*-marked plasmid (pTep1-GFP) expressing a Tep1–GFP fusion under the control of the methionine-repressible *MET25* promoter were grown on complete SGlc-Ura-Met medium for 8 h, and observed by transmitted light (left panel) or by fluorescence (right panel) microscopy.

PTEN through its N-terminal catalytic region (27 % identity from residues 1–260), but contains a large insert (residues 104–160) and also differs completely from PTEN over its C-terminal segment. Although Ptn1, the apparent PTEN homologue in fission yeast, hydrolyses 3-phosphoinositides *in vitro* [14], no such activity for budding yeast Tep1 has been detected [42]. Normally, *TEP1* is expressed only in *MAT*a/*MAT*α diploids undergoing meiosis and sporulation [19]. Therefore *TEP1* (fused in frame at its C-terminus to six tandem c-Myc epitopes for immunodetection) was cloned in a vector under *GAL1* promoter control. This Tep1 derivative rescues the phenotypes of *tep1*Δ/*tep1*Δ cells and has no detectably deleterious effect on haploid cells (results not shown). However, Tep1 expression was unable to alleviate PI3K-induced growth inhibition (Figure 2A), even though immunoblotting with specific anti-c-Myc antibodies confirmed that Tep1 was expressed at a high level (results not shown). Thus, as observed *in vitro*, Tep1 may be incapable of dephosphorylating PIP_3 *in vivo*. However, a Tep1–GFP fusion was localized exclusively as discrete cytoplasmic spots (Figure 2B), reminiscent of the cytosolic puncta reported for *Schizo. pombe* Ptn1 [19], and was never detected at the cell periphery (or even as a diffuse cytoplasmic signal) in any of the $\geqslant$200 cells examined. Therefore, even if Tep1 is a functional 3-phosphoinositide phosphatase, it may be incapable of gaining access to the plasma membrane-localized pool of PIP_3 generated by PI3K. In contrast, PTEN displayed a diffuse cytoplasmic pattern in yeast, revealed by indirect imunofluorescence using anti-PTEN antibodies (results not shown) and, operationally, must gain access to the cytosolic face of the plasma membrane. Unlike PTEN itself, a $PTEN^{1-190}$-$Tep1^{261-434}$ chimaera, in which the unique C-terminal extension of Tep1 was fused in frame to the N-terminal catalytic domain of PTEN, was unable to rescue the growth inhibition caused by PI3K (Figure 2A), even though expression of the chimaera in yeast lysates (detected with anti-PTEN antibodies) was as efficient as that of PTEN itself (results not shown). This result suggests that a localization signal in the C-terminal extension of Tep1 prevents the protein from reaching the plasma membrane.

Sublethal concentrations of LY294002 partially alleviate PI3K-induced growth arrest

Wortmannin and LY294002 are two relatively specific inhibitors of mammalian PI3K, effective over the nanomolar range [1]. The sole PI3K in *Sacch. cerevisiae*, Vps34, converts phosphatidylinositol into phosphatidylinositol 3-phosphate, but cannot use PIP_2; at the nanomolar level, neither wortmannin nor LY294002 has any effect on Vps34 or on protein trafficking or cell growth, largely due to their poor penetration through the yeast cell wall [43]. At much higher doses, wortmannin shows some toxicity against yeast [44]. Likewise, over the millimolar range, LY294002 was inhibitory to the growth of our strain ($IC_{50} \sim 1.5$ mM). However, at this concentration, LY294002 actually stimulated the growth of cells expressing mammalian PI3K by 3–5-fold (Figure 3). Therefore compounds that inhibit human PI3K, but are generally non-toxic, could be selected from chemical libraries by screening for those that fully restore growth to yeast cells expressing p110α. Moreover, any compound that acts on yeast will surely enter an animal cell.

PI3K expression disrupts the cytoskeleton and alters secretory and endocytic trafficking

Because p110α expression efficiently converted plasma-membrane PIP_2 into PIP_3 and prevented growth, it provided a novel means to examine the physiological consequences of this perturbation and thereby shed light on the essential functions of PIP_2

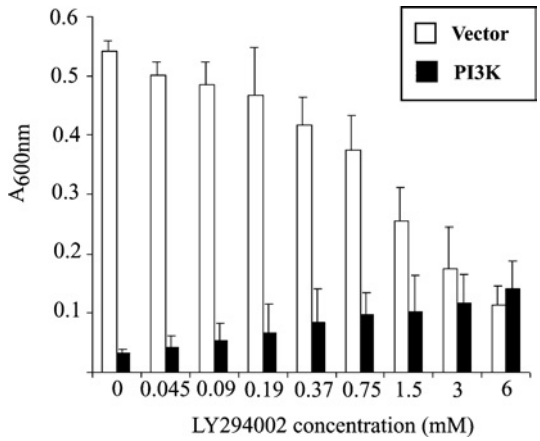

Figure 3 Sublethal doses of a specific PI3K inhibitor partially relieve PI3K-imposed growth inhibition

Wild-type cells (YPH499) were transformed with an empty vector (YCpLG; white bars) or with the same vector expressing PI3K (YCpLG-PI3K; black bars). Representative transformants were propagated in SGlc-Leu medium, then shifted to SGal-Leu medium containing the indicated concentrations of LY294002 (Biomol), and cell growth after 24 h in the SGal-Leu medium was determined by measuring *A* at 600 nm. Values shown are the means for three independent trials (error bars represent S.D. values).

in yeast. Upon shift to galactose, asynchronous cultures of PI3K-expressing cells did not arrest with a uniform cellular or nuclear morphology, assessed by microscopy and flow cytometry (results not shown), indicating that loss of plasma membrane PIP_2 does not block cell-division cycle progression at a specific stage.

PIP_2 has been implicated as a regulator of both the actin [45] and septin [46] cytoskeletons. Our system provided a unique means to confirm and extend those conclusions. In control cells, the actin cytoskeleton showed a highly polarized distribution, with actin filaments in the mother cell and actin patches restricted to the bud (Figure 4A, left panel). In the same strain expressing PI3K, this asymmetry was lost because actin patches and diffuse actin staining were found in more than 80 % of the mother cells (Figure 4A, right). Similarly, in control cells, a fluorescently tagged septin was incorporated exclusively into the collar-like structure found at the mother-bud neck (Figure 4B, left panel), whereas in the same strain expressing PI3K, abnormal septin-containing structures were seen in >50 % of cells (Figure 4B, right panel). Thus PIP_2 in the plasma membrane is important for maintaining proper architecture of both the actin and septin cytoskeletons. Since the functions of both structures are essential for yeast cell viability, their disruption is sufficient to explain the growth inhibitory effect of PI3K expression.

Specific phosphoinositides have been implicated in both secretory and endocytic transport in yeast [47]. To examine secretion, we monitored the precursor forms of vacuolar CPY because their productive processing and maturation depends on their efficient delivery by Golgi-derived vesicles. In control cells, both the p1 and p2 precursor forms, and also the mature (m) form, were observed by immunoblotting (Figure 4C, left panel), whereas in cells expressing PI3K, the ER (endoplasmic reticulum)-specific precursor (p1) accumulated at the expense of the p2 form (Figure 4C, right), although the effect was rather mild and the amount of mature CPY generated was little affected. Using similar means, maturation of other vacuolar (alkaline phosphatase Pho8) and exocytic (cell-wall protein Gas1) marker proteins was unaffected (results not shown).

Endocytic transfer of plasma-membrane material to the yeast vacuole membrane can be monitored by staining with a lipophilic

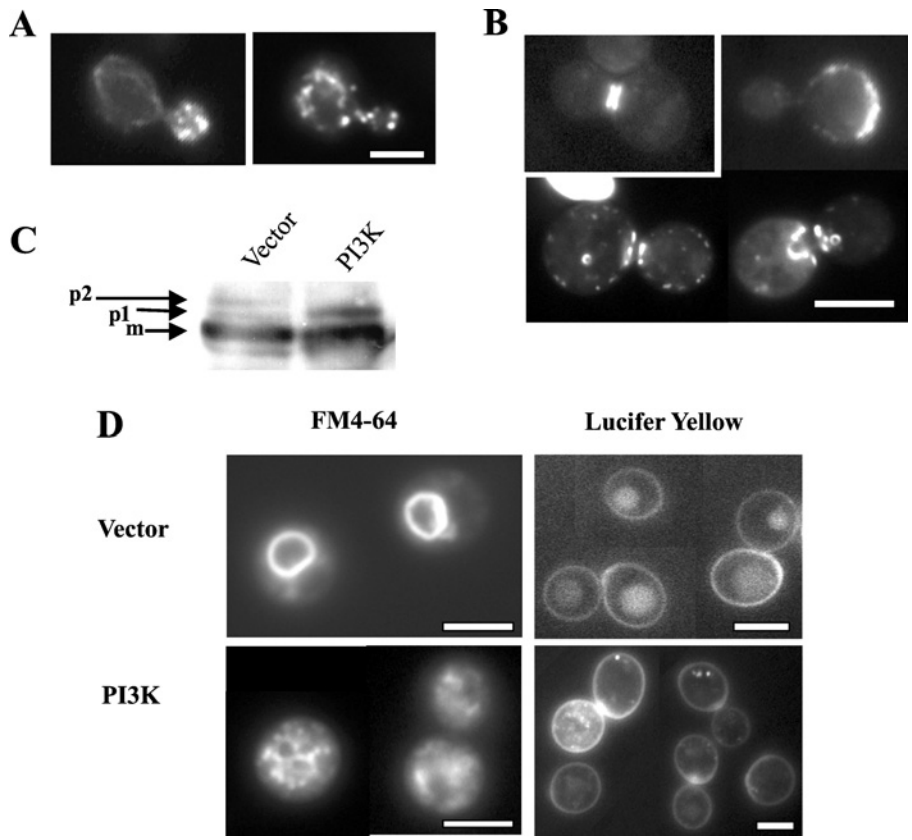

Figure 4 Expression of PI3K perturbs the actin and septin cytoskeletons and secretory and endocytic transport

(**A**) Cells (YPH499) transformed with an empty vector (YCpLG; left panel) or the same vector expressing PI3K (YCpLG-PI3K; right panel) were propagated on SGlc-Leu, shifted to SGal-Leu for 6 h, fixed and stained with rhodamine-conjugated phalloidin to visualize the actin cytoskeleton. A representative cell from each population is shown. (**B**) Cells (YPH499) carrying a *URA3*-marked vector (pLA10) that expresses, from the native *CDC10* promoter, a septin–GFP chimaera (Cdc10–GFP) were co-transformed with an empty *LEU2*-marked vector (YCpLG; inset) or the same vector expressing PI3K (YCpLG-PI3K; remaining images), propagated in SGlc-Ura-Leu, shifted to SGal-Ura-Leu for 6 h, and then viewed under the fluorescence microscope. (**C**) Yeast cells (YPH499) carrying vector alone (YCpLG) or the same vector expressing PI3K (YCpLG-PI3K) were propagated on SRaf-Leu medium, and then shifted to SGal-Leu for 6 h. The cells were chilled, harvested and lysed, and the resulting extracts were resolved by SDS/PAGE and analysed by immunoblotting with anti-CPY antibodies. Precursor-1 (p1), ER form; precursor-2 (p2), Golgi form; m, mature vacuolar CPY. (**D**) Yeast cells (YPH499) carrying vector alone (YCpLG) or the same vector expressing PI3K (YCpLG-PI3K) were propagated in SGlc-Leu, and then shifted to SGal-Leu for 6 h in the presence of either the lipophilic dye FM4-64 (left panels) or the water-soluble dye Lucifer Yellow (right panels). To examine internalization of these fluorochromes, the cells were examined by fluorescence microscopy, and representative cells are shown for each condition.

fluorescent dye, FM4-64, and fluid-phase endocytosis into the vacuole can be evaluated by uptake of a water-soluble fluorescent dye, Lucifer Yellow. In control cells, both fluorochromes selectively stained the membrane and lumen of the vacuoles respectively (Figure 4D, upper panels). In the same cells expressing PI3K, no distinctive vacuolar membranes were seen and no dye import was observed (Figure 4D, lower panels), indicating that endocytosis was severely impaired.

PI3K expression activates the cell-integrity MAPK, Slt2

Cell-membrane biogenesis and cell-wall biogenesis require the transport of secretory vesicles along actin cables to the bud tip [48]. Damage to the cell envelope promotes the expression of genes necessary to co-ordinate appropriate repair of the cell wall via the cell-integrity pathway, which activates the MAPK, Slt2/Mpk1 (yeast orthologue of mammalian Erk5) [49]. Hence, as an independent and sensitive means to assess whether PI3K expression affects actin-mediated secretory vesicle transport, activation (dual phosphorylation) of Slt2 was examined using phospho-specific antibodies. In control cells (carrying two empty expression vectors), Slt2 was present largely in its unactivated (unphosphorylated) state, whereas in cells expressing plasmid-borne PI3K, a marked increase in activated (dually phosphorylated) Slt2 was readily detected (Figure 5). Moreover, just as co-expression of PTEN ameliorated PI3K-induced growth inhibition, Slt2 activation in cells expressing PI3K was greatly reduced by

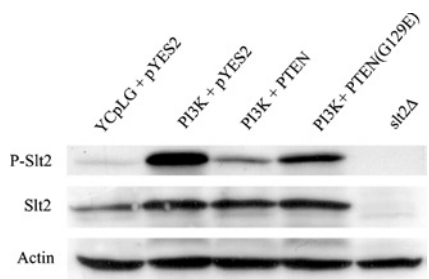

Figure 5 PI3K expression activates the MAPK of the cell-integrity signalling pathway

Wild-type cells (YPH499) carrying two empty expression vectors (YCpLG and pYES2; left-most lane), YCpLG-PI3K and pYES2 (second lane), YCpLG-PI3K and pYES-PTEN (third lane), YCpLG-PI3K and pYES-PTEN^{G129E} (fourth lane) or a DL454 strain (*slt2*Δ) in which the *SLT2* gene was deleted (right-most lane) were propagated in SGlc-Leu-Ura, then shifted to SGal-Leu-Ura for 6 h. The cells were chilled, harvested and lysed, and the resulting extracts were resolved by SDS/PAGE and analysed by immunoblotting with the indicated antibodies (anti-phospho-MAPK antibodies, anti-Slt2 antibodies and anti-actin antibodies, as a control for equivalent protein loading).

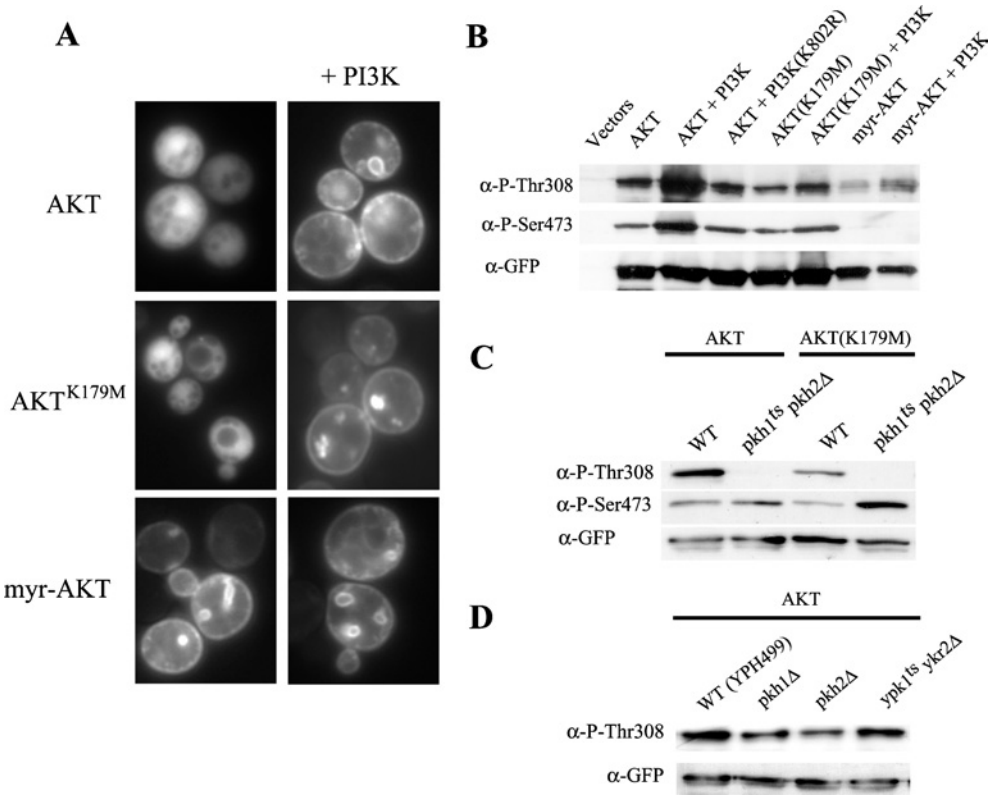

Figure 6 PI3K-dependent PIP_3 generation promotes translocation of c-Akt to the plasma membrane and stimulates its phosphorylation by the yeast PDK1 orthologues Pkh1 and Pkh2

(**A**) Yeast cells (YPH499) carrying either an empty vector (YCpLG; left panels) or the same vector expressing PI3K (YCpLG-PI3K; right panels) were transformed with pYES2-derived plasmids expressing, as indicated, GFP–c-Akt, catalytically inactive GFP–c-Akt and constitutively membrane-targeted myr-GFP–c-Akt. Then, the cells were propagated on SGlc-Leu-Ura, shifted to SGal-Leu-Ura for 6 h and observed under the fluorescence microscope. Representative images are shown. (**B**) Yeast cells (YPH499) were co-transformed, as indicated, with the following pairs of plasmids: pYES2 and YCpLG (left-most lane); pYES-GFP-c-Akt and YCpLG (second lane); pYES-GFP-c-Akt and YCpLG-PI3K (third lane); pYES-GFP-c-Akt and YCpLG-PI3K^{K802R} (fourth lane); pYES-GFP-c-Akt^{K179M} and YCpLG (fifth lane); pYES-GFP-c-Akt^{K179M} and YCpLG-PI3K (sixth lane); pYES-myr-GFP-c-Akt and YCpLG (seventh lane); and pYES-myr-GFP-c-Akt and YCpLG-PI3K (right-most lane). Representative transformants were propagated in SGlc-Leu-Ura, shifted to SGal-Leu-Ura for 6 h, and then cell-free lysates were resolved by SDS/PAGE and analysed by immunoblotting with commercial phospho-specific anti-c-Akt1 antibodies (Cell Signaling Technology) directed against P-Thr^{308} and P-Ser^{473} and with anti-GFP antibodies (BD Biosciences) to indicate the amount of c-Akt recovered in each lane. (**C**) Wild-type cells (DLY1) or an otherwise isogenic *pkh1^{ts} pkh2*Δ derivative (INA106) were transformed with pYES-GFP-c-Akt or h pYES-GFP-c-Akt^{K179M}, as indicated, then propagated in SGlc-Ura, shifted to SGal-Ura for 5 h at 28 °C, and incubated at 37 °C for an additional 3 h. Cell-free extracts from each culture were examined as in (**B**). (**D**) Wild-type cells (YPH499) and otherwise isogenic *pkh1*Δ (YFR105), *pkh2*Δ (YFR106) and *ypk1^{ts} ykr2*Δ (YPT40) derivatives were transformed with pYES-GFP-c-Akt, propagated in SGlc-Ura, shifted to SGal-Ura for 5 h at 28 °C, and then incubated at 37 °C for an additional 3 h. Cell-free lysates of these cultures were examined as in (**B**), but using only anti-phospho-Akt1(Thr^{308}) and anti-GFP antibodies, as indicated.

the co-expression of active PTEN, but not catalytically inactive PTEN (Figure 5). In contrast, PI3K expression did not activate the MAPKs of the pheromone response pathway, Fus3 (Erk1 orthologue), or the filamentous growth response pathway, Kss1 (Erk2 orthologue), as judged by immunoblotting with anti-phospho-Erk antibodies, nor the hyperosmotic stress response pathway, Hog1 (p38 SAPK orthologue), monitored with anti-phospho-p38 antibodies (results not shown).

PI3K expression causes relocalization and activation of c-Akt in yeast

In animal cells, receptor-induced activation of PI3K produces PIP_3, thereby stimulating membrane recruitment and PDK1-mediated phosphorylation of PKB/c-Akt [50]. To determine whether PI3K-induced PIP_3 production in yeast has similar effects, cDNAs expressing GFP–c-Akt1, a catalytically inactive derivative, GFP–c-Akt1^{K179M}, and a constitutively membrane-targeted (N-myristoylated) variant, myr-GFP–c-Akt1, were cloned under the control of the *GAL1* promoter in a vector compatible with that used to express PI3K. None of these fusions alone caused any morphological abnormality or growth impairment on galactose medium, nor was any MAPK activated (results not shown). Both active and kinase-dead GFP–c-Akt1 displayed diffuse nuclear and cytoplasmic fluorescence (Figure 6A, left panels). In cells co-expressing PI3K, however, both fusions showed a distinct rim of fluorescence around the periphery of every cell (Figure 6A, right panels). This pattern was indistinguishable from that displayed by the membrane-targeted myr-c-Akt–GFP, in the absence or presence of PI3K (Figure 6A, bottom panels). Thus, as in animal cells, PIP_3 production recruits c-Akt to the plasma membrane (and, to a lesser extent, to some, as yet unidentified, membrane-bound cytoplasmic organelles).

To correlate membrane translocation of c-Akt1 with its phosphorylation state, extracts of cells carrying the three different c-Akt1 fusions, in the absence and presence of PI3K, were resolved by SDS/PAGE and analysed by immunoblotting with phospho-specific antibodies.

First, readily detectable basal phosphorylation at the PDK1 site (Thr^{308}) and at the PDK2 site (Ser^{473}) was observed in cells expressing c-Akt1 alone. For kinase-dead c-Akt1^{K179M} expressed under the same conditions, phosphorylation at Thr^{308} was reduced

only moderately (ratio of c-Akt^{K179M}/c-Akt was 0.75 ± 0.22), whereas phosphorylation at Ser^{473} was not significantly different (ratio of c-Akt^{K179M}/c-Akt was 1.11 ± 0.25), indicating that autophosphorylation is not required for either of these modifications (Figure 6B). Secondly, in the presence of active (but not catalytically inactive) PI3K, we reproducibly observed that phosphorylation at both Thr^{308} and Ser^{473} was substantially elevated in both active and kinase-dead c-Akt1. The myr-c-Akt1 derivative was always expressed at a level significantly lower than that of either active or kinase-dead c-Akt1, perhaps explaining the observed reduction in Thr^{308} phosphorylation, and phosphorylation at Thr^{308} was only moderately increased upon PI3K expression. No Ser^{473} phosphorylation of myr-c-Akt1 was detectable in the absence or presence of PI3K, suggesting perhaps that the kinase responsible for Ser^{473} phosphorylation cannot attack c-Akt restricted to the plasma membrane.

Yeast PDK1 orthologues are responsible for phosphorylation of c-Akt at Thr^{308}

To determine whether modification of c-Akt1 at Thr^{308} requires Pkh1 and Pkh2, the phosphorylation state of GFP–c-Akt1 and GFP–c-Akt1^{K179M} was examined in wild-type cells and in otherwise isogenic *pkh1^{ts} pkh2*Δ cells after shift to a restrictive temperature. Inactivation of Pkh1 in cells lacking Pkh2 abolished phosphorylation at Thr^{308} in both proteins, and the effect was specific because phosphorylation at Ser^{473} was unaffected (Figure 6C). Either Pkh1 or Pkh2 are competent to phosphorylate c-Akt1 at Thr^{308} because deletion of either *PKH1* or *PKH2* alone did not eliminate Thr^{308} phosphorylation (Figure 6D). Inactivation of two endogenous targets of Pkh1 and Pkh2, the Ypk1 and Ykr2/Ypk2 protein kinases (functional orthologues of mammalian SGK) [16,17] had no effect on the state of c-Akt modification because phosphorylation at Thr^{308} (Figure 6D) and Ser^{473} (results not shown) was unaffected when *ypk1^{ts} ykr2*Δ cells were shifted to restrictive temperature.

The PDK2 site-directed kinase is not Tor2 nor any other PIKK family member

We found that kinase-dead c-Akt1^{K179M} is still phosphorylated at Ser^{473} (Figures 6B and 6C), ruling out autophosphorylation as the sole basis for PDK2 site phosphorylation. The possibility that the most closely related, endogenous yeast AGC kinase, Sch9 [20], cross-phosphorylates c-Akt1 at the PDK2 site was ruled out by the fact that there was no discernible difference in the level of c-Akt1 phosphorylation at Ser^{473} in *sch9*Δ cells compared with otherwise isogenic *SCH9*$^{+}$ cells (results not shown).

It has been suggested that various members of the PIKK family of atypical protein kinases (DNA-PKcs, ATM and mTOR) are responsible for PDK2 site phosphorylation in c-Akt1. To address the role of Tor1 and Tor2 in phosphorylation of Ser^{473} in c-Akt1 in yeast, cells were treated with the TOR-specific inhibitor rapamycin at concentrations that inhibit growth, but no reduction in the level of Ser^{473} (or Thr^{308}) phosphorylation in either c-Akt1 or c-Akt1^{K179M} was seen (results not shown). It has been appreciated recently, however, that Tor1 and Tor2 can each be found in discrete complexes, so-called TORC1 and TORC2, and that only TORC1 is rapamycin-sensitive [51]. Hence, as a more definitive approach, we examined the state of Ser^{473} phosphorylation in *tor1*Δ *tor2*Δ cells carrying a plasmid expressing a Tor2^{ts} mutant, which were shifted to restrictive temperature for periods of time more than sufficient to inactive the thermolabile Tor2 and prevent cell growth. By 9 h after shift, 100 % of the mutant cells had lost cortical actin polarization (a sensitive measure of Tor2 function [45]), whereas all of the control *TOR1*$^{+}$ *TOR2*$^{+}$ cells retained normal actin polarization (results not shown). In the mutant cells, there was no detectable reduction in Ser^{473} phosphorylation in either c-Akt1 or c-Akt1^{K179M}, even 9 h after shift to restrictive temperature, compared with *TOR1*$^{+}$ *TOR2*$^{+}$ cells under the same conditions (Figure 7A). A null mutation (*tel1*Δ) in the yeast ATM orthologue, a non-essential gene, did not reduce Ser^{473} phosphorylation of c-Akt1 (Figure 7 B) or c-Akt1^{K179M} (results not shown). Similarly, a shift of temperature-sensitive mutations in the yeast ATR orthologue (*mec1^{ts}*) or the apparent yeast DNA-PKcs orthologue (*tra1^{ts}*), both essential genes, did not significantly reduce Ser^{473} phosphorylation in c-Akt1 (Figure 7B) or c-Akt1^{K179M} (results not shown). Thus, although a kinase capable of phosphorylating efficiently the PDK2 site in c-Akt1 clearly exists in yeast, it does not appear to correspond to any member of the PIKK family encoded in the *Sacch. cerevisiae* genome.

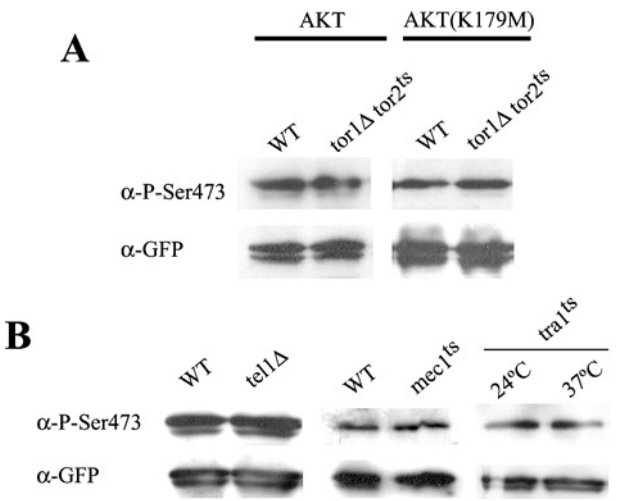

Figure 7 Yeast PIKK homologues are not responsible for phosphorylation of c-Akt at its PDK2 site

(**A**) Wild-type cells (SH100) and an otherwise isogenic *tor1*Δ *tor2^{ts}* derivative (SH221) were transformed with either pYES-GFP-c-Akt or pYES-GFP-c-Akt^{K179M}, as indicated, propagated in SGlc-Ura, shifted to SGal-Ura at 28 °C for 5 h and then incubated at 37 °C for an additional 9 h. Cell-free extracts were then examined as in Figure 6(B). (**B**) The following strains were transformed with pYES-GFP-c-Akt: wild-type (WT) cells (BY4741) and an otherwise isogenic *tel1*Δ derivative (Y03114); wild-type cells (RCY307) and an otherwise isogenic *mec1^{ts}* derivative (RCY426); and a strain carrying a *tra1^{ts}* allele (YCB655). Representative transformants were propagated in SGlc-Ura and either shifted to SGal-Ura for 5 h at 28 °C (left-most pair of lanes) and then incubated at 37 °C for an additional 3 h (middle pair of lanes) or shifted to SGal-Ura for 5 h and then incubated for an additional 3 h at either 24 or 37 °C (right-most pair of lanes). Cell-free lysates of these cultures were examined as in (**A**), but using only anti-phospho-Akt1(Ser^{473}) and anti-GFP antibodies, as indicated.

DISCUSSION

Using appropriate expression vectors, we reconstructed the mammalian PI3K/PTEN/PKB signalling pathway in *Sacch. cerevisiae*. The ability of PTEN to reverse the toxic effect of PIP_3 production by PI3K could be convincingly recapitulated in yeast. Thus the strains constructed provide a simplified model for genetic analysis of these enzymes and to screen for compounds that are PI3K antagonists and PTEN agonists. Our approach also provided a novel means to deplete specifically the plasma-membrane PIP_2 pool without blocking the production of other phosphoinositides. Finally, this system offered unequivocal confirmation that membrane recruitment of PKB/c-Akt is PIP_3-dependent.

High-level expression of membrane-targeted p110α caused severe growth arrest, whereas a catalytically inactive mutant did not. Before the present study, two other groups expressed mammalian PI3K in yeast. In one study, expression of class I PI3K from the *GAL1* promoter did not significantly interfere with growth [52]. In the other [53], heterologously expressed PI3K was somewhat deleterious. In our work, we used a prenylatable C-terminal CAAX box to direct p110α to its substrate in the plasma membrane, most likely explaining its more potent growth-inhibitory action. Restoration of growth to these cells provides a straightforward positive selection to identify new inhibitors of PI3K that are efficacious *in vivo*. Co-expression of PTEN, but not a catalytically inactive mutant, relieved PI3K-induced growth arrest. Such cells provide a tool for testing the activity of various PTEN alleles *in vivo* and another positive growth-based assay to screen for compounds that enhance the function of crippled PTEN variants. Therefore this inexpensive yeast-based system may be useful in developing anti-cancer chemotherapeutics.

Using a PIP_2-specific fluorescent reporter and PIP_3-specific antibodies, we showed that PI3K expression caused a dramatic reduction in the PIP_2 pool and a concomitant marked increase in the PIP_3 content of the plasma membrane. Despite the presence of other elements in the p110α polypeptide that might perturb cell function (its Ras-binding domain, its lipid-binding C2 domain, the engineered CAAX box etc.), production of catalytically inactive p110α at the same level was innocuous. Therefore depletion of PIP_2 appears to be the sole cause of the growth-inhibitory effects of PI3K expression, indicating that the plasma-membrane pool of PIP_2 is essential for cell viability. Our results confirm, and significantly extend, conclusions drawn from globally eliminating all cellular PIP_2 by shifting temperature-sensitive phosphatidylinositol 4-phosphate 5-kinase (*mss4*) mutants to restrictive temperature [45].

PI3K-mediated reduction in plasma-membrane PIP_2 content altered both actin and septin architecture. The actin perturbation, by itself, could explain the growth arrest, based on prior reports showing that Mss4 is critical for proper actin organization [45] and that correct functioning of the actin cytoskeleton is essential for yeast cell viability [48]. However, proper septin assembly is also essential [54]. Our data provide the first demonstration that PIP_2 itself is critical for the maintenance of septin structure *in vivo*, despite evidence to the contrary about the phosphoinositide binding specificity of yeast septins *in vitro* [46]. The block to endocytosis caused by PI3K-induced PIP_2 depletion was much more dramatic than the moderate impairment we found in secretory transport. This result is again consistent with, but confirms in a unique way, the cumulative evidence that efficient endocytosis requires PIP_2 at the plasma membrane [55], whereas secretory transport requires phosphatidylinositol 4-phosphate on the Golgi membrane [47].

PI3K expression uniquely activated Slt2/Mpk1, the MAPK of the cell-integrity signalling pathway [49], but none of the three other MAPKs present. This pathway is initiated by the protein kinase, Pkc1 (yeast orthologue of mammalian PRK2), which is activated by GTP-bound Rho1, a small GTPase. Rom2, the guanine nucleotide-exchange factor for Rho1, is recruited to the plasma membrane, in part, via its PH domain, which has been shown to bind PIP_2 and PIP_3 equally well [56].

Overall, our results indicate that, for at least some essential cellular functions (actin assembly, septin assembly, endocytosis etc.), PIP_3 cannot functionally substitute for PIP_2. Indeed, PIP_3 is normally never made in budding yeast (*Sacch. cerevisiae*) [15,47]. In contrast, PIP_3 is present in fission yeast (*Schizo. pombe*), but kept at a low level, by action of a PTEN-like phosphatase, Ptn1 [14]. The purported *Sacch. cerevisiae* PTEN orthologue, Tep1, could not alleviate the toxicity of PIP_3 production, even when overexpressed. Moreover, we failed to detect PIP_3 in *tep1*Δ mutants using the same anti-PIP_3 antibodies that readily detected this lipid in Tep1-containing cells expressing PI3K (I. Rodríguez-Escudero, M. Molina and V.J. Cid, unpublished results). Thus Tep1 is probably not a functional 3-phosphoinositide phosphatase *in vivo*, in agreement with its lack of detectable enzyme activity *in vitro* [42]. However, given that Tep1–GFP is restricted to cytoplasmic puncta, the possibility that Tep1 cannot gain access to the PIP_3 generated upon PI3K expression has not been ruled out.

c-Akt expressed in yeast displayed a strictly PI3K-dependent translocation from a diffuse nucleocytoplasmic distribution to a distinct pattern of rim-staining around the plasma membrane. Given the vast body of evidence that the PH domain of c-Akt binds preferentially to PIP_3 [and $PI(3,4)P_2$] [5], this result provides independent proof that PIP_3 was generated at the plasma membrane in yeast upon PI3K expression, corroborating the analysis we performed with the PIP_3-specific antibodies. Immunoblotting with phospho-specific antibodies against P-Thr^{308} and P-Ser^{473} showed that phosphorylation at both sites was substantially stimulated upon PI3K expression. Even in the absence of PI3K expression, there was readily detectable basal phosphorylation at both sites; nonetheless, c-Akt1 overexpression was innocuous to the yeast cell. Using commercial antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) directed against the consensus phosphoacceptor motif preferred by c-Akt1, we found no difference in the pattern of bands when immunoblots of extracts of control yeast cells were compared with yeast cells expressing c-Akt1 (I. Rodríguez-Escudero, F. Roelants, J. Thorner, M. Molina, V. J. Cid, unpublished results). These findings suggest that high-level expression of c-Akt1 alone does not perturb yeast cell physiology because efficient substrates are absent. In this regard, among the important downstream targets of c-Akt in animal cells are members of the FOXO (Forkhead box O) family of transcription factors [5]. However, the residues directly phosphorylated by c-Akt1 in their metazoan counterparts are not conserved in any of the four forkhead transcription factors encoded by the *Sacch. cerevisiae* genome (Fkh1, Fkh2, Fhl1 and Hcm1), at least as we align these proteins.

Sch9 is the *Sacch. cerevisiae* protein kinase that most closely resembles c-Akt1. Like AGC kinases that are targets of PDK1 in animal cells [50], Sch9 contains recognizable matches to the consensus PDK1 site (corresponding to Thr^{308} in c-Akt) and to the consensus PDK2 site (corresponding to Ser^{473} in c-Akt) [16,57]. Despite the similarities, the phospho-specific antibodies directed against c-Akt that we used did not detect any background band in lysates of control yeast cells. Apparently, limited sequence divergence within the motifs and in the immediately flanking residues is sufficient to prevent cross-reaction with Sch9. Moreover, all of the behaviours we report here for c-Akt were identical in *sch9*Δ cells (I. Rodríguez-Escudero, F. Roelants, J. Thorner, M. Molina, V. J. Cid, unpublished results) and, hence, none of our findings can be attributed to endogenous Sch9.

In addition to its PIP_3-dependent translocation to the plasma membrane, other aspects of c-Akt regulation were also recapitulated in yeast cells. Phosphorylation of the PDK1 site (Thr^{308}) in c-Akt *in vivo* was strictly dependent on the *Sacch. cerevisiae* PDK1 orthologues Phk1 and Phk2, as we showed previously *in vitro* [16]. Moreover, mammalian PDK1 rescues the lethality of *pkh1*Δ *pkh2*Δ cells [16]. Thus our current findings provide a further demonstration that PDK1 function has been highly conserved. We also found robust phosphorylation of the PDK2 site (Ser^{473}), indicating that a PDK2-like activity exists in *Sacch. cerevisiae*. This modification was not due to obligatory autophosphorylation

because Ser^{473} phosphorylation was still prominent in catalytically inactive c-Akt. Despite recent claims in animal cells that the major PDK2-site kinases are members of the atypical PIKK family, we found that phosphorylation of Ser^{473} in c-Akt was not reduced in yeast cells in which the mTOR-like (Tor1 and Tor2) or the other PIKK-like proteins (Tel1, Mec1 or Tra1) were inactivated using appropriate temperature-sensitive or null mutations. If, like PDK1, the function of the PDK2-site kinase has been highly conserved, then our results suggest that PDK2-site phosphorylation may not be a physiologically relevant function of mTOR, ATR or DNA-PKcs. Given that the number of protein kinases in *Sacch. cerevisiae* is substantially lower than that in animal cells, our findings may help narrow the search for the still elusive PDK2-site kinase.

We are grateful to C. Rivas, M. Collado, M. Lorenzo, J. Paramio, N. Hashash, R. Cha, C. Kiesecker, J. Workman, K. Matsumoto, M. Hall, D. Lew and D. Alessi for providing plasmids and strains. This work was supported by grant BIO2004-02019 from the Comisión Interministerial de Ciencia y Tecnología (CICYT, Spain) to M. M., by a fellowship from Comunidad Autónoma de Madrid (CAM, Spain) to I. R.-E., by a grant from the University of California–University of Madrid collaborative programme titled Complutense New Del Amo Program (NDAP) to V. J. C. and J. T., and by the NIH (National Institutes of Health) research grant GM21841 to J. T.

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Received 7 April 2005/11 May 2005; accepted 25 May 2005
Published as BJ Immediate Publication 25 May 2005, doi:10.1042/BJ20050574