Differential roles of PDK1- and PDK2-phosphorylation sites in the yeast AGC kinases Ypk1, Pkc1 and Sch9

Françoise M. Roelants, Pamela D. Torrance† and Jeremy Thorner

Correspondence

Jeremy Thorner
jeremy@socrates.berkeley.edu

Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology,
University of California, Berkeley, CA 94720-3202, USA

Saccharomyces cerevisiae Pkh1 and Pkh2 (orthologues of mammalian protein kinase, PDK1) are functionally redundant. These kinases activate three AGC family kinases involved in the maintenance of cell wall integrity: Ypk1 and Ypk2, two closely related, functionally redundant enzymes (orthologues of mammalian protein kinase SGK), and Pkc1 (orthologue of mammalian protein kinase PRK2). Pkh1 and Pkh2 activate Ypk1, Ypk2 and Pkc1 by phosphorylating a Thr in a conserved sequence motif (PDK1 site) within the activation loop of these proteins. A fourth protein kinase involved in growth control and stress response, Sch9 (orthologue of mammalian protein kinase c-Akt/PKB), also carries the conserved activation loop motif. Like other AGC family kinases, Ypk1, Ypk2, Pkc1 and Sch9 also carry a second conserved sequence motif situated in a region C-terminal to the catalytic domain, called the hydrophobic motif (PDK2 site). Currently, there is still controversy surrounding the identity of the enzyme responsible for phosphorylating this second site and the necessity for phosphorylation at this site for *in vivo* function. Here, genetic and biochemical methods have been used to investigate the physiological consequences of phosphorylation at the PDK1 and PDK2 sites of Ypk1, Pkc1 and Sch9. It was found that phosphorylation at the PDK1 site in the activation loop is indispensable for the essential functions of all three kinases *in vivo*, whereas phosphorylation at the PDK2 motif plays a non-essential and much more subtle role in modulating the ability of these kinases to regulate the downstream processes in which they participate.

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INTRODUCTION

Mammalian PDK1 (3-phosphoinositide-dependent protein kinase-1) phosphorylates and activates many members of the AGC class of protein kinase (named initially for the cAMP- and cGMP-dependent protein kinases and protein kinase C) (reviewed by Leslie *et al.*, 2001; Storz & Toker, 2002). PDK1 was first identified as the activator of the AGC kinase c-Akt/Rac/PKB (reviewed by Chan *et al.*, 1999; Scheid & Woodgett, 2003). Both PDK1 and PKB possess pleckstrin homology domains that bind the lipid second messengers PtdIns-3,4-bisphosphate and PtdIns-3,4,5-trisphosphate (reviewed by Lemmon, 2003; Misra *et al.*, 2001). These 3-phosphoinositides, generated upon stimulation of receptor-tyrosine kinases that associate with and activate phosphatidylinositol 3-kinase, tether PDK1 and PKB to the plasma membrane, increasing the likelihood of their encounter and causing a conformational change (in PKB) that promotes its phosphorylation by PDK1.

PDK1 phosphorylates additional AGC family kinases, including p70 S6K (Alessi *et al.*, 1998; Pullen *et al.*, 1998), p90 RSK (Jensen *et al.*, 1999), serum- and glucocorticoid-inducible kinase (SGK) (Kobayashi & Cohen, 1999; Park *et al.*, 1999), several protein kinase C (PKC) isoforms (Chou *et al.*, 1998; Le Good *et al.*, 1998), PKC-related protein kinases (PRK1/PKN and PRK2) (Dong *et al.*, 2000; Flynn *et al.*, 2000) and p21-activated protein kinase (PAK) (King *et al.*, 2000b). Various mechanisms ensure the translocation of these proteins to the membrane (thereby placing them in proximity to PDK1) and the concomitant induction of the appropriate conformation change to make them accessible to phosphorylation by PDK1. PDK1 activates all of these targets by phosphorylating a Thr in a conserved motif within the activation loop of these enzymes (reviewed by Biondi, 2004). This sequence ('PDK1 site') has the consensus: Thr*-Phe-Cys-Gly-Thr-X-Glu-Tyr (asterisk indicates phosphorylation and X represents any residue).

Full activation of Akt/PKB, SGK and other PDK1 targets also requires phosphorylation at a second site within a hydrophobic motif, Phe-X-X-Aro-Ser/Thr*-Aro (asterisk indicates phosphorylation and Aro represents Phe, Tyr or

†Present address: Pinnacle BIO Consulting, 219 24th St, Del Mar, CA 92014, USA.

Trp), situated C-terminal to the catalytic domain (Alessi *et al.*, 1996; Kobayashi & Cohen, 1999). In PRK1/PKN, PRK2 and the atypical PKC isoforms, the Ser/Thr is replaced by Asp (or Glu), presumably mimicking a permanently phosphorylated state (Le Good *et al.*, 1998; Vincent & Settleman, 1997). Inhibitors of PtdIns 3-kinase prevent phosphorylation at the hydrophobic motif in PKB *in vivo* (Alessi *et al.*, 1996), but neither purified nor recombinant PDK1 phosphorylates PKB at this sequence (Alessi *et al.*, 1997a, b). For this reason, the hydrophobic motif is called a 'PDK2 site'. There is still controversy about the identity of the enzyme(s) responsible for PDK2 site phosphorylation. Some evidence suggests that PDK2 may be a modified form of PDK1; a small C-terminal fragment from PRK2, dubbed PIF (PDK1-interacting fragment), when bound to PDK1, purportedly permits PDK1 to phosphorylate PKB at its PDK2 site *in vitro* (Balendran *et al.*, 1999, 2000). However, other evidence suggests that phosphorylation at the PDK2 site in PKB and PKC occurs via autophosphorylation (Behn-Krappa & Newton, 1999; Toker & Newton, 2000). Also, it has been variously claimed that so-called integrin-linked kinase (ILK) (Dedhar *et al.*, 1999), mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP K2) (Alessi & Cohen, 1998; Rane *et al.*, 2001) or conventional PKC isoforms (Kroner *et al.*, 2000) phosphorylate the PDK2 site in PKB α , depending on the cell type or the conditions (Brazil *et al.*, 2004). For p70 S6K, NIMA family kinases, NEK6/7, have been implicated in PDK2 site phosphorylation (Belham *et al.*, 2001), but others report that mTOR, in a complex with other proteins, directly phosphorylates the PDK2 site (Hara *et al.*, 2002; Nojima *et al.*, 2003; Tokunaga *et al.*, 2004).

Regardless of how PDK2 site phosphorylation occurs, its effect on the physiological functions of these kinases is not well defined. One consequence, revealed by structural studies of PKB, is a phosphorylation-promoted interaction of the hydrophobic motif with a cleft on the N-terminal lobe of the kinase domain, which induces a conformational change that reconfigures the enzyme into a more catalytically competent state (Yang *et al.*, 2002). Another consequence seems to be that the PDK2 sequence – when phosphorylated (as in PKB, SGK, p70 S6K or RSK2) or with an acidic residue in the corresponding position (as in PRK2 and PKC ζ) – serves as a docking site to recruit PDK1 (reviewed by Biondi, 2004). As in PKB, the phosphorylated hydrophobic PDK2 sequence fits into a hydrophobic groove (the 'PIF pocket') on the small lobe of the PDK1 kinase domain adjacent to a cluster of basic residues (Biondi *et al.*, 2002). When associated with a PIF segment, PDK1 activity is stimulated several-fold (Frödin *et al.*, 2002). Thus, PDK2 site phosphorylation may be a necessary prelude to phosphorylation at the PDK1 site because it helps recruit PDK1 to its targets and elevates PDK1 activity. Indeed, in mouse ES cells containing a knock-in mutation that eliminates the PIF pocket in PDK1, p70 S6K, SGK and RSK2 are not activated in response to PtdIns 3-kinase activation by IGF-1 (Collins *et al.*, 2003). In the same cells, PKB is still activated normally,

indicating that association of PDK1 with PKB via its PIF pocket is not obligatory for efficient PKB activation *in vivo*.

We have shown that the budding yeast *Saccharomyces cerevisiae* contains two related protein kinases, Pkh1 and Pkh2, that are the functional homologues of mammalian PDK1 (Casamayor *et al.*, 1999). We also found that the *S. cerevisiae* genome encodes four protein kinases (Ypk1, Ykr2/Ypk2, Pkc1 and Sch9) that possess both the conserved PDK1 site in their activation loop and the conserved PDK2 site C-terminal to their kinase domain (Fig. 1). Ypk1 and Ypk2 are closely related, share an essential function and are the functional homologues of mammalian SGK (Casamayor *et al.*, 1999). Purified PDK1 or purified Pkh1 activate purified Ypk1 *in vitro* and do so by phosphorylating its PDK1 site (Thr504), but PDK1 and Pkh1 fail to phosphorylate the PDK2 site (Thr662) (Casamayor *et al.*, 1999). Pkh1 preferentially activates Ypk1 and Pkh2 preferentially activates Ypk2 (Roelants *et al.*, 2002), and these two modules participate in signalling pathways necessary for maintenance of cell wall integrity (Roelants *et al.*, 2002; Schmelzle *et al.*, 2002) and for efficient endocytosis (deHart *et al.*, 2002). These pathways respond to the level of sphingolipids in the plasma membrane (Momoi *et al.*, 2004; Roelants *et al.*, 2002; Sun *et al.*, 2000). *In vitro*, Pkh1 and Pkh2 activity against physiological substrates is stimulated by nanomolar concentrations of the long-chain sphingoid base present in yeast, phytosphingosine (Friant *et al.*, 2001; Zhang *et al.*, 2004). Mammalian PDK1 can be activated by sphingosine, the long-chain sphingoid base present in animal cells (King *et al.*, 2000a).

Human enzymes					
PKBα	303	<u>GATMKTFCGTPEYLAPE</u>	<u>MITITPPD</u>	<u>FPQFSYSAS</u>	477
SGK	251	<u>NSTTSTFCGTPEYLAPE</u>	<u>SIGKSPDS</u>	<u>FLGFSYAPP</u>	426
p70^{S6K}	247	<u>GTVTHTFCGTIEYMAPE</u>	<u>TPVDSPPD</u>	<u>FLGFTYVAP</u>	416
PKCζ	405	<u>GDTTSTFCGTPEYLAPE</u>	<u>QPQITDDY</u>	<u>DTQFTSEPV</u>	557
Yeast enzymes					
Ypk1	499	<u>DDKTDTFCGTPEYLAPE</u>	<u>KPIDSVVD</u>	<u>FGGWYTVGN</u>	666
Ykr2	496	<u>NDKTDTFCGTPEYLAPE</u>	<u>KPIDSVVD</u>	<u>FGGWYTVGN</u>	663
Pkc1	978	<u>GNRTSTFCGTPEYMAPE</u>	<u>PPTLTPLP</u>	<u>FRGFSFMFD</u>	1147
Sch9	565	<u>KDRTNTFCGTTEYLAPE</u>	<u>QPMMTATP</u>	<u>FAGFTTFVDE</u>	741
Gad8	382	<u>TDRTNTFCGTPEYLAPE</u>	<u>IPMDSVVA</u>	<u>FANWSYQRP</u>	550
		Activation loop	Turn motif	Hydrophobic motif	

Fig. 1. Comparison of conserved phosphorylation site motifs in human and yeast AGC family kinases. Sequences corresponding to the PDK1 site in the activation loop, the turn motif and the PDK2 site in the C-terminal hydrophobic motif are shown with the most conserved residues underlined and the demonstrated or predicted phosphoacceptor residues in bold type. GenBank accession numbers: PKB α , NP 005154.1; SGK, NP 005618.2; p70 S6K, NP 003152.1; PKC ζ , NP 002735.2; Ypk1, CAA81967.1; Ypk2, CAA89740.1; Pkc1, CAA84932.1; Sch9, NP 012075.1; Gad8, CAB76216.1.

Pkc1 is another demonstrated target of Pkh1 and Pkh2 (Friant *et al.*, 2001; Inagaki *et al.*, 1999). Pkc1 is the upstream activator of a MAP kinase cascade central to maintenance of cell wall integrity and is also involved in actin organization and endocytosis (reviewed by Heinisch *et al.*, 1999). Sch9 is a potential substrate for Pkh1 and/or Pkh2. Sch9 was initially identified as a dosage suppressor of the temperature-sensitive lethality of *cdc25^{ts}* cells, which are deficient in the Ras-specific guanine nucleotide exchange factor (Toda *et al.*, 1988). Subsequent studies showed that Sch9 is involved in nutrient-sensing, stress response and life-span control (Fabrizio *et al.*, 2001), and in ribosome biogenesis and cell size regulation (Jorgensen *et al.*, 2002).

In this study, we sought to establish the importance, both *in vivo* and *in vitro*, of phosphorylation at the PDK1 and PDK2 sites in the yeast AGC kinases that possess both of these motifs.

METHODS

Strains and growth conditions. Yeast strains used in this study are listed in Table 1. Standard rich (YP) and defined minimal (SC) media (Sherman *et al.*, 1986), containing 2% glucose (Glc) as the carbon source and supplemented with appropriate nutrients to maintain selection for plasmids, were used for yeast cultivation. For gene expression from the galactose-inducible *GAL1* promoter in liquid media, cells were pre-grown to mid-exponential phase in SC containing 2% raffinose/0.2% sucrose (Raf/Suc) and then galactose was added (2% final concentration) and incubation was continued for 2 h. Cells were grown routinely at 30 °C, but strains carrying temperature-sensitive mutations were propagated at their permissive temperature (26 °C). Caffeine in agar plates was used at a concentration of 6 mM and myriocin (dissolved in methanol) was present at a final concentration of 1 µM (unless otherwise noted).

Recombinant DNA methods. *Escherichia coli* strain DH5α (Hanahan, 1983) was used for the construction and propagation of plasmids. Conventional recombinant DNA methods were used for the construction of plasmids (Sambrook *et al.*, 1989). The sequences of constructs that contained DNA fragments amplified by PCR or site-directed mutations were verified by the dideoxy chain-termination sequencing method (Sanger *et al.*, 1977). Native and Turbo Pfu polymerases (Stratagene) were used for PCR.

Plasmids. Plasmids pYPK1, pYPK1(D488A), pYPK1(DD), pYPK1(T662D), pYPK1(T504D), pGAL-YPK1, pGAL-YPK1(K376A-KD)-myc

(pAM49), YEp352GAL-YPK1 (pAM75) and YEp352GAL-YPK1-myc (pAM76) have been described previously (Casamayor *et al.*, 1999; Maurer, 1988; Roelants *et al.*, 2002).

To create a *CEN*-based plasmid that expresses YPK1 from its endogenous promoter, the entire ~4 kb genomic fragment containing YPK1 was excised from pYPK1 with *Bam*HI and *Hind*III, gel purified and ligated into *Bam*HI/*Hind*III-linearized vector pRS315, yielding a *CEN*-containing, *LEU2*-marked plasmid, pRS315-YPK1 (B/H) (pAM11). A ~4 kb *Xba*I-*Sal*I fragment from pRS315-YPK1 (B/H) was gel-purified and ligated to an *Xba*I/*Sal*I-linearized pRS316 to create *CEN*-containing, *URA3*-marked plasmid, pRS316-YPK1(X/S) (pAM66).

A ~1.1 kb *Clal*-*Kpn*I fragment of YPK1 from a D488A kinase-dead mutant, pYPK1(D488A), or each of the PDK-site mutants, pYPK1(T504D), pYPK1(T662D) and the double mutant pYPK1(T504D T662D), were used to replace the corresponding segment in pRS316-YPK1(X/S) to create plasmids pRS316-YPK1-(D488A) (pAM83), pRS316-YPK1-(T504D) (pAM87), pRS316-YPK1(T662D) (pAM86) and pRS316-YPK1(T504D T662D) (pAM85), respectively. Using pRS316-YPK1(X/S) as the template, Quick-change PCR mutagenesis (Stratagene) was performed with the following oligonucleotides: 5'-CAATTTGGTGGCTGGGCATACGTTGGAAATG-3' (T662A1) and 5'-CATTTCCAACGTATGCCAGCCACCAAATTG-3' (T662A2), which span the Thr662 codon of YPK1 (the underlined nucleotides represent mismatches which substitute Ala for Thr662). The resulting plasmid is pRS316-YPK1(T662A) (pAM97). To create high-copy-number (2 µm) *URA3*-based plasmids that express YPK1 or the mutated forms of YPK1 from the endogenous YPK1 promoter, a *Not*I-*Sal*I fragment from pAM66 and pAM97 or a *Not*I-*Kpn*I fragment from pAM86 and pAM87 were inserted into the equivalent sites in pRS426 (Christianson *et al.*, 1992), creating pRS426-YPK1 (pFR76), pRS426-YPK1(T504D) (pFR77), pRS426-YPK1(T662D) (pFR78) and pRS426-YPK1(T662A) (pFR79).

To assess the enzymic activity of all of the Ypk1 mutants, plasmids were constructed to overexpress derivatives tagged with a c-Myc epitope. Using YEp352GAL-YPK1-myc (pAM76) as the template, Quick-change PCR mutagenesis was performed with the following oligonucleotides: 5'-GATAAGACAGATGATTTTTGTGGGACCCC-3' (YPK1-C) and 5'-GGGGTCCCACAAAATCATCTGTCTTATC-3' (YPK1-D), which span the Thr504 codon of YPK1 (underlined nucleotides represent mismatches which substitute Asp for Thr504). The resulting plasmid is YEp352GAL-YPK1-(T504D)-myc (pAM90). Using the same template and oligonucleotides 5'-CAATTTGGTGCTGGGATTACGTTGGAAATG-3' (YPK1-E) and 5'-CATTTCC-AACGTAATCCCAGCCACCAAATTG-3' (YPK1-F), which span the Thr662 codon of YPK1 (underlined nucleotides represent mismatches which substitute Asp for Thr662), the resulting plasmid was

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
YPH499	<i>MATa ade2-101^{oc} his3-Δ200 leu2-Δ1 lys2-801^{am} trp1-Δ1 ura3-52</i>	Sikorski & Hieter (1989)
YPT40	YPH499 <i>ypk1-1^{ts} :: HIS3 ypk2Δ :: TRP1</i>	Casamayor <i>et al.</i> (1999)
YES3	YPH499 <i>ypk1Δ :: HIS3</i>	Roelants <i>et al.</i> (2002)
<i>PKC1/pkc1</i>	BY4743 <i>PKC1/pkc1Δ :: KanMX</i>	Research Genetics, Inc.
<i>pkc1Δ :: LEU2</i>	YPH499 <i>pkc1Δ :: LEU2</i>	Fields (1991)
YFR55*	YPH499 <i>sch9Δ :: HIS3</i>	This study
MWY347	<i>MATa ura3 leu2 ade8 cdc25-5^{ts}</i>	Toda <i>et al.</i> (1988)

*Derived from YPH499 by transformation with the 4.7 kb *Pvu*II fragment of *psch9 :: HIS3* (Toda *et al.*, 1988).

YEp352GAL-YPK1-(T662D)-myc (pAM91). Using YEp352GAL-YPK1-(T504D)-myc as the template, Quick-change PCR mutagenesis was performed with the oligonucleotides YPK1-E and YPK1-F. The resulting plasmid was YEp352GAL-YPK1-(DD)-Myc (pAM92). Using YEp352GAL-YPK1-myc as the template, Quick-change PCR mutagenesis was performed with oligonucleotides T662A1 and T662A2. The resulting plasmid was YEp352GAL-YPK1-(T662A)-myc (pAM96).

The same site-directed mutagenesis technique was performed using pGAL-YPK1(K376A-KD)-myc (pAM49) as a template to create pGAL-YPK1(K376A-T504A)-myc (pFR61), pGAL-YPK1(K376A-T662A)-myc (pFR62) and pGAL-YPK1(K376A-T504A-T662A)-myc (pFR63). To express *PKC1* from its own promoter on a 2 µm plasmid, a *Pst*I genomic fragment containing full-length *PKC1* plus 500 bp upstream of its initiation codon ATG was cloned into YEp352, yielding p*PKC1*-PN (pFR22). Site-directed mutagenesis using pFR22 as a substrate was used to replace the activation-loop phosphorylation site Thr983 (encoded by ACA) by Ala (GCC), yielding p*PKC1*(T983A) (pFR75). The same strategy was used to replace Ser1143 (codon TCC) with Ala (codon GCC), yielding plasmid p*PKC1*(S1143A) (pFR74). YEp13-SCH9 (AHB8) (gift of Dr Stephen Garrett, Dept of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, NJ, USA) and the pSCH9(T570A) (pFR72) plasmid containing the PDK1 site mutation was made by site-directed mutagenesis using AHB8 as a template. To create a *CEN*-based plasmid that expresses SCH9 or its derivatives from its endogenous promoter, a *Hind*III-*Pst*I genomic fragment containing full-length SCH9 plus 477 bp upstream of the initiation codon was inserted into the *Hind*III/*Pst*I sites of pRS315, yielding pRS315-SCH9 (pAM200), and site-directed mutagenesis was performed to yield pRS315-SCH9(T570A) (pFR82) and pRS315-SCH9(T737A) (pAM202).

To construct a *CEN*-based plasmid expressing *YPK2* from its own promoter, a 2.5 kb genomic insert containing *YPK2* was inserted into the *Eco*RI site of pRS316 (Sikorski & Hieter, 1989), yielding plasmid pRS316-YPK2 (pAM12). The orientation of the insert in this plasmid is such that the *Hind*III site of the polylinker is 5' of the promoter of *YPK2* and the *Bam*HI site of the polylinker is downstream of the stop codon. To construct a 2 µm DNA-based plasmid that expresses *YPK2* from its own promoter, a *Hind*III-*Bam*HI fragment from pAM12 was introduced into YEp352 (Hill *et al.*, 1986) previously linearized with *Hind*III and *Bam*HI, yielding plasmid YEp352-YPK2 (pAM14).

Preparation of cell extracts and immunoblot analysis. Yeast cells were grown at 30 °C to mid-exponential phase ($OD_{600}=0.5-1$), either in SC medium with appropriate supplements for maintenance of plasmids or in rich medium (YPGlc). For galactose induction from the *GAL1* promoter, galactose (2% final concentration) was added and the cultures were incubated at 30 °C for 2 h. Cells were harvested by brief centrifugation, washed twice by resuspension and resedimentation in ice-cold lysis buffer [50 mM Tris/HCl (pH 7.5), 5 mM EDTA, 3 mM DTT and 1 mM PMSF] and resuspended in 200 µl of the same buffer. Pre-chilled glass beads (0.45–0.6 mm diameter) were added to the meniscus of the cell suspension and lysis was achieved by vigorous mixing in a Fast-Prep (Bio 101 ThermoSavant) for three 1 min intervals, with intermittent cooling on ice. To remove the glass beads, the bottom of the Eppendorf tube was punctured with a syringe needle (<0.5 mm diameter) and inserted into another tube; the lysate was collected into the fresh tube by brief centrifugation in a clinical centrifuge. The crude extract was subjected to centrifugation at 30 000 g for 15 min to remove unbroken cells and large debris. The protein concentration of the crude extract was measured using a dye-binding method (Bradford, 1976) with a protein assay kit as instructed by the manufacturer (Bio-Rad) using bovine serum albumin (New England Biolabs) as the standard.

For immunoblot analysis, samples (50 µg total protein) were diluted into SDS-PAGE sample buffer (Laemmli, 1970), subjected to electrophoresis in an 8–12% gel and transferred to nitrocellulose (Towbin *et al.*, 1979). To detect proteins using ascites fluid containing the anti-c-Myc mAb 9E10 (Evan *et al.*, 1985), a dilution of 1:10 000 was used. Immobilized immune complexes were detected using a commercial chemiluminescence detection system (Renaissance; Dupont NEN) and X-ray film (Biomax MR; Kodak).

Immunoprecipitation analysis. Yeast cultures to be used for immunoprecipitation analysis were grown as described above, then rinsed in ice-cold IP buffer [20 mM Tris/HCl (pH 7.5), 125 mM potassium acetate, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 µg leupeptin ml⁻¹, 1 µg pepstatin A ml⁻¹, 0.1% Triton X-100, 12.5% glycerol]. Glass beads were added to the meniscus of the cell suspension and lysis was achieved by vigorous vortex mixing for eight 30 s intervals with intermittent cooling on ice. The lysate was clarified by centrifugation at 14 000 g at 4 °C for 30 min. The clarified extract was assayed for protein concentration and a sample (1 mg total protein) was diluted to a final volume of 200 µl in IP buffer. An aliquot (20 µl) of Protein G/Protein A-agarose beads (30% slurry) (Oncogene Research Products) and a sample of an appropriate control antibody, either 2 µl pre-immune rabbit serum or 1 µg purified mouse anti-T cell receptor antibody (gift of James Allison, University of California, Berkeley, CA, USA), was added. The samples were then incubated on a roller drum for 1 h at 4 °C to adsorb proteins that bound non-specifically to the solid support and to rabbit or mouse IgG (pre-clearing). The beads were removed by centrifugation for 10 min in a microfuge and the supernatant fraction was transferred to a fresh tube containing another aliquot (15 µl) of Protein G/Protein A-agarose beads and 1 µl anti-c-Myc (mAb 9E10) ascites, and incubated on a roller drum for 1–3 h at 4 °C. The beads were sedimented by brief centrifugation in a microfuge, washed three times (1 ml each) with ice-cold IP buffer and collected by centrifugation for 1 min in a microfuge on maximum speed. Bead-bound immune complexes were solubilized in SDS-PAGE sample buffer and immediately boiled for 5 min in a water-bath and then clarified by brief centrifugation in a microfuge prior to resolution by SDS-PAGE. The proteins of interest were visualized as described above.

Immune-complex protein kinase assays. Cells expressing either wild-type or mutant alleles of Ypk1-myc under control of the *GAL1* promoter were grown in SC containing Raf/Suc to an OD_{600} of 0.6, induced by addition of galactose (2% final concentration) and incubated with shaking at 30 °C for 2 h, collected by centrifugation, washed with ice-cold 1 × PBS, resuspended in 0.2 ml ice-cold IP buffer and lysed as described above. The resulting lysates were clarified by centrifugation at 4 °C for 30 min at 30 000 g. Protein concentration in the resulting crude extracts was determined by the method of Bradford (1976). A volume of extract containing 1 mg total protein was immunoprecipitated with mAb 9E10 as described above. The immunoprecipitates were washed once with ice-cold IP buffer, once with ice-cold IP buffer containing 0.5 M NaCl and twice with ice-cold Buffer A [50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol]. As part of the final wash, the slurry of beads was split into two equal portions. For immunoblot analysis, SDS-PAGE sample buffer (~15 µl) was added directly to one sample of each bead suspension. For protein kinase assays, the activity of the Ypk1-myc immune complex was assayed by adding 30 µl of a mixture containing 1 µM microcystin-LR, 10 mM Mg acetate, 100 µM [γ -³²P]ATP (200–400 c.p.m. pmol⁻¹) and 100 µM Cross-tide (GRPTSSFAEG) (Cross *et al.*, 1995), which we have documented previously as an excellent peptide phospho-acceptor substrate for Ypk1 (Casamayor *et al.*, 1999; Roelants *et al.*, 2002). After incubation for 15 min at 30 °C, each reaction was terminated by spotting a portion (45 µl) of the reaction mixture onto

small squares of phosphocellulose paper (Whatman P81), which were washed and analysed as described in detail elsewhere (Alessi *et al.*, 1995). In some experiments, samples of the immunoprecipitates were resuspended in an appropriate buffer [20 mM Tris/HCl (pH 8.8), 10 mM MgCl₂] and treated with shrimp alkaline phosphatase (0.25 units; US Biochemicals) in either the absence or presence of a mixture of inhibitors of this phosphatase (25 μ M Na *orthovanadate* and 100 μ M β -glycerol-phosphate, adjusted to pH 8).

Bioassays for drug sensitivity. Agar diffusion (halo) assays (Reneke *et al.*, 1988) were used to test the relative sensitivity of various strains to rapamycin. Lawns of the strains examined were prepared by mixing $\sim 2 \times 10^6$ exponentially growing cells rapidly with 3 ml molten (55 °C) 1 % agar and pouring immediately onto plates containing an appropriate medium. Equal volumes (10 μ l) of rapamycin stocks (50 ng μ l⁻¹, 500 ng μ l⁻¹) in rapamycin dilution buffer (10 % Tween 20, 90 % EtOH) were spotted onto sterile cellulose filter discs (0.6 cm), which were placed on the lawn. After incubation at 30 °C for 2 days the plates were photographed.

RESULTS

In vitro analysis of PDK1- and PDK2-site mutations in Ypk1

Ypk1 and Ypk2 are two *S. cerevisiae* AGC kinases that contain both the conserved PDK1 and PDK2 motifs (Fig. 1). Because Ypk1 and Ypk2 are activated both *in vivo* and *in vitro* by Pkh1 and Pkh2, respectively, and because the inviability of *pkh1* Δ *pkh2* Δ cells can be rescued by heterologous expression of mammalian PDK1, it was of interest to use mutational analysis to dissect the importance of PDK1- and PDK2-site phosphorylation for the biochemical activity and physiological function of Ypk1 and Ypk2. Since these enzymes are so similar in length (680 versus 677 residues) and sequence (88 % identity in their catalytic domains), we examined Ypk1 as a model for both enzymes.

Several site-directed mutations were introduced: two alterations of the PDK1 site (T504A and T504D); two alterations of the PDK2 site (T662A and T662D); two double mutants (T504A T662A and T504D T662D); and two kinase-dead alleles, one in the essential Lys in conserved kinase domain II (K376A) and one in the essential Asp in the catalytic loop of conserved kinase domain VIb (D488A). As one means to determine the state of activation of these different Ypk1 variants, each was tagged with a c-Myc epitope and expressed in wild-type yeast cells using the same vector. After cell lysis, each enzyme was immunoprecipitated from the cell-free extracts using beads coated with an mAb directed against the tag. Samples of the resulting immunoprecipitates were examined for catalytic activity, using incorporation of radioactivity from [γ -³²P]ATP into a synthetic peptide (Cross-tide) that we have demonstrated previously serves as an excellent phosphoacceptor substrate for purified Ypk1 *in vitro* (Casamayor *et al.*, 1999), and for protein content by SDS-PAGE and immunoblotting (Fig. 2a). As an independent negative control to assess the non-specific background adsorption to the beads of kinases capable of phosphorylating Cross-tide, extracts prepared

from cells expressing untagged Ypk1 from the same vector were also immunoprecipitated. Immune complexes from the cells expressing untagged Ypk1 showed only very low incorporation of radioactivity into Cross-tide, whereas at least 10-fold higher incorporation was reproducibly displayed by the immune complexes from cells expressing wild-type Ypk1-myc (Fig. 2a, top). That all of the observed incorporation was due to the immunoprecipitated Ypk1-myc was confirmed by the fact that incorporation was reduced to the same level as the non-specific background when immune complexes from cells expressing a kinase-dead mutant, Ypk1(K376A)-myc, were examined. Likewise, immune complexes from cells expressing the PDK1-site mutant, Ypk1(T504D)-myc, showed no incorporation above the non-specific background or the negative control. Thus, even though a negatively charged residue could conceivably mimic phospho-Thr at the PDK1 site, and even though the PDK2 site was available for phosphorylation *in vivo* prior to enzyme isolation, Ypk1(T504D) is totally inactive, indicating that phosphorylation at this site is indispensable for Ypk1 activity. This result agrees with our prior finding that, unlike GST-Ypk1, purified GST-Ypk1(T504D) was inactive *in vitro* and could not be phosphorylated or activated *in vitro* by purified Pkh1 (Casamayor *et al.*, 1999).

In contrast, immune complexes from cells expressing the PDK2-site mutant, Ypk1(T662D)-myc, showed a level of activity that was reproducibly ~ 50 % higher than that observed for normal Ypk1-myc. Immunoblotting, especially after phosphatase treatment, indicated that all of the myc-tagged proteins were recovered with equivalent efficiency (Fig. 2a, bottom), suggesting that such differences in incorporation are not attributable to changes in the levels of these proteins, but reflect changes in specific activity. The fact that Ypk1(T662D)-myc seemed reproducibly more active than Ypk1-myc raised the possibility that an Asp at the PDK2 site may indeed mimic the phosphorylated state, either activating the enzyme by itself or enhancing the ability of Pkh1 to phosphorylate the activation loop. Indirect support for the latter conclusion was provided by the fact that immune complexes from cells expressing the double mutant, Ypk1(T504D T662D)-myc, were totally inactive. This view is also consistent with our prior finding that purified GST-Ypk1(T662D) is inactive *in vitro*, but like GST-Ypk1, could be phosphorylated and activated *in vitro* by purified Pkh1 (Casamayor *et al.*, 1999). Thus, in the absence of P-Thr at the PDK1 site, the T662D mutation alone is unable to activate the enzyme.

To determine whether phosphorylation (or a negative charge) at the PDK2 site is necessary for Ypk1 activity, immune complexes from cells expressing Ypk1(T662A)-myc were examined. We found that the activity displayed by the immune complexes containing Ypk1(T662A)-myc were indistinguishable from those containing wild-type Ypk1-myc. Thus, phosphorylation at the PDK2 site is dispensable for the catalytic activity of Ypk1.

Fig. 2. Catalytic activity and electrophoretic behaviour of Ypk1 and various Ypk1 mutants. (a) Top, wild-type cells (YPH499) overexpressing from the *GAL1* promoter on plasmids either untagged Ypk1 (pAM75) or Myc-epitope tagged alleles of Ypk1 – wild-type Ypk1-Myc (pAM76); catalytically inactive Ypk1(K376A)-Myc (pAM49); PDK1 site mutant, Ypk1(T504D)-Myc (pAM90); PDK2 site mutant, Ypk1(T662D)-Myc (pAM91); double mutant, Ypk1(T504D T662D) (pAM92); or PDK2 site mutant, Ypk1(T662A) (pAM96) – were grown to mid-exponential phase and induced with galactose for 3 h. Extracts were prepared and identical amounts of total protein (1 mg) were immunoprecipitated with the anti-Myc mAb 9E10. The immune complexes were washed extensively, then incubated with a specific peptide substrate (Cross-tide) and [γ - 32 P]ATP, and the resulting product was measured as described in Methods. Bottom, samples of each immunoprecipitate were treated with phosphatase in the absence or presence of phosphatase inhibitors, resolved by SDS-PAGE and visualized by immunoblotting with anti-Myc mAb 9E10. (b) *ypk1Δ* cells (YES3) overexpressing from the *GAL1* promoter on plasmids either untagged Ypk1 (pAM75) or Myc-epitope tagged alleles of Ypk1 – wild-type Ypk1-Myc (pAM76); catalytically inactive Ypk1(K376A)-Myc (pAM49); or a combination of catalytically inactive mutation with mutations in the PDK1 site, Ypk1(K376A T504A)-Myc (pFR61), the PDK2 site, Ypk1(K376A T662A)-Myc (pFR62), or both Ypk1(K376A T504A T662A)-Myc (pFR63) – were grown to mid-exponential phase and induced with galactose for 3 h. Extracts were prepared; identical amounts of total protein (1 mg) were immunoprecipitated with the anti-Myc mAb 9E10. The immune complexes were resolved by electrophoresis on an SDS-slab gel and analysed by immunoblotting with anti-Myc mAb 9E10.

Ypk1 is phosphorylated at multiple sites

We have noted previously that Ypk1 migrates upon SDS-PAGE as a series of poorly resolved isoforms. To determine whether these species could be correlated with PDK1- and/or PDK2-site phosphorylation, samples of each of the immunoprecipitates described above were examined by SDS-PAGE and immunoblotting before and after treatment with phosphatase (Fig. 2a, bottom). To control for the potential presence of contaminating protease activity in the commercial phosphatase preparation, the 'before' sample was actually phosphatase-treated, but in the presence of phosphatase inhibitors. All of the mutant alleles, including Ypk1(T504D T662D)-myc, displayed the same pattern of multiple isoforms as immunoprecipitated wild-type Ypk1-myc, and these multiple bands were all collapsed into the same apparent band of faster mobility by treatment with phosphatase. The fact that even the double mutant did not eliminate any of the bands observed

indicated that none of the isoforms could be correlated with phosphorylation at either the PDK1 or the PDK2 site. Moreover, these findings indicate that the multiple electrophoretic species are derived from phosphorylation of Ypk1 at several positions other than the PDK1 and PDK2 sites. These additional sites do not arise from auto-phosphorylation because, when expressed in *ypk1Δ* cells, there is no alteration in the pattern of these poorly resolved bands, comparing Ypk1-myc to a kinase-dead allele, Ypk1(K376A)-myc, or the same catalytically inactive variant in which the PDK1 site (T504A) or the PDK2 site (T662A), or both (T504A T662A), have been rendered uncharged and unphosphorylatable by mutation (Fig. 2b). Mapping of these additional sites by mass spectrometry is in progress, so that corresponding mutations can be made to assess whether these modifications are critical for any aspect of Ypk1 activity, stability, localization or substrate specificity.

PDK2 site phosphorylation is dispensable for yeast AGC kinase function *in vivo*

The results presented above show that phosphorylation at the PDK1 site is necessary for Ypk1 activity *in vitro*, but phosphorylation at the PDK2 site is not. To determine whether these biochemical properties accurately reflect the functional state of the enzyme *in vivo*, we tested first the ability of the different Ypk1 mutants to rescue the temperature-sensitive lethality of a *ypk1-1^{ts} ypk2Δ* strain at the non-permissive temperature (Fig. 3a). For this purpose, each of the different Ypk1 alleles was cloned into a low-copy-number vector from which they were expressed from their endogenous *YPK1* promoter. The *ypk1-1^{ts} ypk2Δ* cells carrying the vector expressing normal *YPK1* grew robustly as single isolated colonies at all temperatures examined, whereas the same cells carrying the vector alone were unable to grow at 35 °C or above. A *bona fide* kinase-dead mutant, Ypk1(D488A) (Torrance, 2000), the single PDK1 site mutant, Ypk1(T504D), and the double PDK1-site PDK2-site mutant, Ypk1(T504D T662D), were not able to support the growth of the *ypk1-1^{ts} ypk2Δ* cells at restrictive temperature. Thus, lack of P-Thr at the PDK1 site completely abolishes the function of Ypk1 *in vivo*. In striking contrast, both the Ypk1(T662A) and Ypk1(T662D) mutants supported growth of the mutant cells at the non-permissive temperature and did so just as well as wild-type Ypk1. Thus, lack of phosphorylation at the PDK2 site has no detectably detrimental effect on the ability of Ypk1 to promote cell growth in this assay. Thus, the ability of a given mutant to display Ypk1 function *in vivo* was strictly correlated with its catalytic activity *in vitro*.

To determine whether the same relationship holds for other yeast AGC kinases that possess both the PDK1 and PDK2 consensus motifs, appropriate mutant alleles were constructed, first in Pkc1. Normal Pkc1, or a mutant lacking the PDK1 site, Pkc1(T983A), or a mutant lacking the PDK2 site, Pkc1(S1143A), each expressed under control of the authentic *PKC1* promoter from a high-copy-number (2 μm) plasmid, were introduced into a heterozygous *pkc1Δ/ PKC1* diploid. After sporulation and tetrad dissection, the ability of normal Pkc1, Pkc1(T983A) or Pkc1(S1143A) to rescue the inviability of the *pkc1Δ* spores was compared (Fig. 3b). For diploids transformed with the plasmid expressing normal Pkc1, most of the tetrads displayed three or four viable spores, showing that plasmid-borne *PKC1* readily rescued the inviability of *pkc1Δ* spores (which were also marked by the insertion of a resistance marker, *kanMX*, to the antibiotic G418). For the diploids transformed with the plasmid expressing Pkc1(T983A), only two viable spores were obtained in every tetrad and those spores were always G418-sensitive. Hence, PDK1-site phosphorylation appears to be required for the catalytic competence and physiological function of Pkc1. In marked contrast, in the diploids transformed with the plasmid expressing the PDK2-site mutant, Pkc1(S1143A), nearly all of the tetrads displayed three or four viable spores,

demonstrating that the full biological function of Pkc1 is retained in the absence of PDK2-site phosphorylation.

To determine if the same conclusion was true for Sch9, a similar strategy was used. One phenotype of *sch9Δ* cells is a slow-growth phenotype that is manifest in the production of colonies of very small size. As expected, when expressed from its own promoter on a low-copy-number (*CEN*) plasmid in *sch9Δ* cells, wild-type Sch9 restored more robust growth and a distinctly larger colony size (Fig. 3c, upper panel). The same cells expressing a PDK1-site mutant, Sch9(T570A), from the same vector grew just as poorly as cells transformed with the empty vector. By contrast, the PDK2-site mutant, Sch9(T737A), supported vigorous growth and yielded colonies of large size, like normal Sch9. Thus, as for Ypk1 and Pkc1, the function of Sch9 appears to require phosphorylation at the PDK1 site, but not at its PDK2 site. The conclusion that the PDK1-site mutant is inactive *in vivo* was confirmed in two other ways. Overexpression of wild-type Sch9 from a high-copy-number (2 μm DNA) plasmid rescues the temperature-sensitivity of *cdc25^{ts}* cells (Fig. 3c, middle panel) and confers heat-shock sensitivity to wild-type cells (Fig. 3c, bottom panel); the Sch9(T570A) mutant expressed in the same fashion was unable to do either. Thus, as for Ypk1 and Pkc1, PDK1-site phosphorylation is required for Sch9 function, whereas PDK2-site phosphorylation is not.

A specific role for PDK2-site phosphorylation in sphingolipid signalling

Ypk1 and Ypk2 share a high degree of sequence similarity, have similar biochemical properties and are functionally redundant genetically (Casamayor *et al.*, 1999; Roelants *et al.*, 2002; Torrance, 2000). However, *ypk1Δ* cells display several phenotypes that *ypk2Δ* cells do not, indicating that Ypk1 plays the predominant role in executing the biological functions that these enzymes share. For example, *ypk1Δ* cells grow more slowly the lower the temperature (Chen *et al.*, 1993; Maurer, 1988; Roelants *et al.*, 2002). At 26 °C, *ypk1Δ* cells still grow, but with a very small colony size (Fig. 4a, left side). Introduction of wild-type Ypk1 restored robust growth and large colony size, whereas introduction of the PDK1-site mutant, Ypk1(T504D), did not. As seen for rescue of the inviability of *ypk1^{ts} ypk2Δ* cells at high temperature (Fig. 3a), both of the PDK2-site mutants, Ypk1(T662A) and Ypk1(T662D), restored normal growth to *ypk1Δ* cells at 26 °C and did so just as well as wild-type Ypk1 (Fig. 4a, left side).

Another phenotype unique to *ypk1Δ* cells is hypersensitivity to growth inhibition by caffeine (Roelants *et al.*, 2002). Introduction of wild-type Ypk1 restored resistance to the toxic effects of caffeine, whereas introduction of the PDK1-site mutant, Ypk1(T504D), did not (Fig. 4a, middle). Both of the PDK2-site mutants, Ypk1(T662A) and Ypk1(T662D), restored an equivalent level of growth on caffeine plates (Fig. 4a, middle).

A third unique phenotype shown by *ypk1Δ* cells is hypersensitivity to the growth-inhibitory action of the antibiotic myriocin (also known as ISP-1) (Momoi *et al.*, 2004), which is a demonstrated inhibitor of serine palmitoyltransferase (Ikushiro *et al.*, 2004), the first enzyme unique to the sphingolipid biosynthesis pathway in both animal cells and yeast (Dickson & Lester, 1999a). Overexpression of Ypk1 greatly increases the resistance of yeast cells to myriocin (Sun *et al.*, 2000). Remarkably, in this assay, we found that PDK2-site phosphorylation is required for the ability of Ypk1 to confer myriocin resistance. Only expression of the Ypk1(T662D) mutant, which presumably mimics

permanent phosphorylation of Ypk1 at its PDK2 site, was able (like wild-type Ypk1) to permit growth in the presence of myriocin. By contrast, expression of the other PDK2-site mutant, Ypk1(T662A), in which the PDK2 cannot be phosphorylated, was unable to restore growth in the presence of myriocin, despite the fact that it is catalytically active *in vitro* (Fig. 2a) and retains biological function in all three of the other *in vivo* assays examined (Figs 3a and 4a). Thus, phosphorylation at the PDK2 site is not necessary for growth under most conditions, but becomes indispensable when the sphingolipid content of the cells is greatly diminished (due to the action of myriocin).

Fig. 3. Phosphorylation at the PDK2 site is dispensable for the growth-promoting activities of Ypk1, Pkc1 and Sch9. (a) A temperature-sensitive strain, *ypk1-1^{ts} Ypk2Δ* (YPT40), was transformed with either an empty vector (pRS316, *CEN-URA3*) or the same vector expressing from its own promoter wild-type Ypk1 (pAM66), catalytically inactive Ypk1(D488A) (pAM83), a PDK1 site mutant Ypk1(T504D) (pAM87), a PDK2 site mutant Ypk1(T662D) (pAM86), a different Ypk1(T662A) (pAM97) or the double mutant Ypk1(T504D T662D) (pAM85). Transformants were selected at 26 °C, then representative isolates were streaked to single colonies and growth was assessed after 3 days at the indicated temperature (26, 30, 35 or 37 °C). (b) A heterozygous *pkc1Δ::KanMX/PKC1* diploid strain was transformed with a high-copy-number (2 μm, *URA3*) plasmid expressing from its own promoter wild-type Pkc1 (pFR22), a PDK1 site mutant Pkc1(T983) (pFR75) or a PDK2 site mutant Pkc1(S1143A) (pFR74). The transformants were sporulated and eight tetrads of each were dissected. Viability of the four spores of each tetrad was assessed after 3 days at 30 °C. (c) Upper panel, a *sch9Δ::HIS3* strain (YFR55) was transformed with either an empty *CEN*-based *LEU2*-marked plasmid or with the same plasmid expressing from its own promoter wild-type Sch9 (pAM200), a PDK1 site mutant Sch9(T570A) (pFR82) or a PDK2 site mutant Sch9(T737A) (pAM202). Transformants were selected at 30 °C, representative isolates were grown to late exponential phase, resuspended at an initial OD₆₀₀ of 1 and then serial tenfold dilutions were spotted on SCD-Leu plates and grown for 3 days. Middle panel, a *cdc25ts* strain (MWY347) was transformed with a high-copy-number (2 μm DNA, *LEU2*-marked) plasmid expressing from its own promoter either wild-type Sch9 (AHB8) or a PDK1 site mutant, Sch9(T570A) (pFR72). Transformants were selected at 26 °C, then representative isolates were streaked to single colonies and growth was assessed after 3 days at 26 (left) or 35 °C (right). Bottom panel, a wild-type strain (YPH499) was transformed with the same set of high-copy-number plasmids and the transformants were patched on SCD-Leu. Growth was assessed before (left) or after (right) subjecting the cells to heat shock for 45 min at 55 °C.

Interrelationships between Ypk1- and Ypk2-dependent signalling and Pkc1-dependent signalling

We have shown previously that cells deficient in both Ypk1 and Ypk2 undergo cell lysis due to cell wall defects and that elevated levels of Ypk1, Ypk2 or Pkc1 can ameliorate this

phenotype (Roelants *et al.*, 2002). It was therefore of interest to determine whether overexpression of Ypk2 or Pkc1 could, like Ypk1 itself, confer increased resistance to myriocin. Wild-type cells were transformed with high-copy-number plasmids that expressed from the corresponding endogenous promoter either wild-type *YPK1*, *YPK2* or *PKC1*, and

Fig. 4. Absence of PDK2 site phosphorylation in Ypk1 has discernible phenotypic consequences. (a) A *ypk1Δ::HIS3* strain (YES3) was transformed with either an empty vector (pRS316, *CEN-URA3*) or the same vector expressing from its own promoter wild-type Ypk1 (pAM66), the PDK1 site mutant, Ypk1(T504D) (pAM87) or the PDK2 site mutants Ypk1(T662D) (pAM86) or Ypk1(T662A) (pAM97). Representative transformants were selected on glucose-containing medium lacking Ura and then streaked to single colonies, as indicated, on medium containing glucose to assess the growth rate at 26 °C or on glucose medium plus either 6 mM caffeine or 1 μM myriocin and incubated at 30 °C. (b) A wild-type strain (YPH499) was transformed with a high-copy-number (2 μm) plasmid expressing from its own promoter *PKC1* (pFR22), *YPK1* (pAM36) or *YPK2* (pAM14) and growth was assessed after 3 days at 30 °C on SCD-U plates containing 1 μM myriocin.

growth was assessed on medium containing myriocin. The cells expressing Pkc1, but not those expressing Ypk2, were able to grow as well as the cells expressing Ypk1 (Fig. 4b).

This result suggested that Pkc1 and Ypk1 may act in parallel pathways that can both lead to myriocin resistance, or they may act in a common pathway that achieves this resistance. As one means to distinguish between these possibilities, we used epistasis analysis. As observed before, when over-expressed from high-copy-number plasmids, wild-type Ypk1 and Ypk1(T662D), but not Ypk1(T504D) or Ypk1(T662A), were able to confer myriocin resistance to wild-type cells,

and likewise, when overexpressed, wild-type Pkc1, but not the PDK1-site mutant, Pkc1(T983A), was able to confer elevated myriocin resistance (Fig. 5, top panels). However, the PDK-2 site mutant, Pkc1(S113A), was also able to promote increased myriocin resistance, indicating that PDK2-site phosphorylation of Pkc1 is not required for its ability to enhance resistance to the antibiotic. However, in the absence of Ypk1 (and it should be appreciated that Ypk2 is still present), neither wild-type Pkc1 nor its derivatives could confer increased myriocin resistance, whereas wild-type Ypk1 and Ypk1(T662D) did (Fig. 5, middle panels). Thus, the effects of Pkc1 require that the cells

Fig. 5. Epistasis analysis indicates that Pkc1 acts through Ypk1 to mediate myriocin resistance. Wild-type cells (YPH499), *ypk1*Δ::HIS3 cells (YES3) or *pkc1*Δ::LEU2 cells were transformed with either a high-copy-number (2 μm) empty vector or the same vector expressing from their own promoter wild-type YPK1 (pFR76), a PDK1 site mutant, Ypk1(T504D) (pFR77), a PDK2 site mutant, Ypk1(T662D) (pFR78), a different PDK2 site mutant, Ypk1(T662A) (pFR79), wild-type Pkc1 (pFR22), a PDK1 site mutant, Pkc1(T983A) (pFR75) or a PDK2 site mutant, Pkc1(S1143A) (pFR74). The wild-type and *ypk1*Δ::HIS3 transformants were plated on SCD-U medium or SCD-U containing 1 μM myriocin and growth was assessed after 3 days at 30 °C. The *pkc1*Δ::LEU2 transformants were plated on SCD-U medium containing 1 M sorbitol or SCD-U medium containing 1 M sorbitol and 1 μM myriocin, and growth was assessed after 3 days at 30 °C.

contain functional Ypk1. However, the converse was not true. In cells lacking Pkc1 (which must be maintained on plates containing 1 M sorbitol to prevent cell lysis), overexpression of wild-type Ypk1 or Ypk1(T662D) was still able to promote increased resistance to myriocin (Fig. 5, bottom panels). The most parsimonious interpretation of these results is that Ypk1 acts downstream from Pkc1 in the events required to overcome the effects of myriocin. Of course, other more convoluted explanations are possible. In this regard, it is noteworthy that, in the *pkc1Δ* cells maintained on 1 M sorbitol, even the PDK1-site mutant, Pkc1(T983A), was able to confer significant myriocin resistance, suggesting that the Pkc1 protein itself, rather than its catalytic activity, plays a role in myriocin resistance. We cannot exclude, however, the possibility that the PDK1-site mutant of Pkc1, while inactive *in vitro* and on the substrates necessary for growth *in vivo* (Fig. 3b), still retains some activity toward one or more substrates critical for myriocin resistance (although this scenario seems unlikely).

A role for PDK2-site phosphorylation revealed by the effects of rapamycin

Rapamycin is an antibiotic that inhibits the growth of both animal cells and yeast (reviewed by Jacinto & Hall, 2003; Fingar & Blenis, 2004). This compound acts by forming a complex with a cellular receptor protein (a specific class of peptidyl-prolyl isomerase in the cytosol, called FKBP12, that also binds the immunosuppressant drug FK506). The rapamycin-FKBP complex (but not either molecule alone) binds avidly to a specific site on a large, unconventional serine, threonine-protein kinase, called TOR (for target of rapamycin). *S. cerevisiae* cells have two TOR orthologues, Tor1 and Tor2, with both overlapping and distinct functions (Lorberg & Hall, 2004).

We and others have shown that, compared to wild-type cells, *ypk1Δ* cells (but not *ypk2Δ* cells) are hypersensitive (~ 10 -fold) to the growth-inhibitory effect of rapamycin (Gelperin *et al.*, 2002; Roelants *et al.*, 2002). Furthermore, there is evidence in animal cells that mTOR may be responsible for phosphorylating the PDK2 site, at least in p70 S6K (Long *et al.*, 2004), and similarly in fission yeast it has been reported that *Schizosaccharomyces pombe* Tor1 is responsible for phosphorylating the PDK2 site in Gad8 (apparent *Schizosaccharomyces pombe* orthologue of *S. cerevisiae* Ypk1 and Ypk2) (Matsuo *et al.*, 2003). If PDK2 site phosphorylation of Ypk1 is essential for its function in overcoming the inhibitory effects of rapamycin, then one would predict that the non-phosphorylatable mutant, Ypk1(T662A), might be significantly more sensitive to rapamycin action than wild-type cells and, conversely, that the mutant that mimics phosphorylation at this site, Ypk1(T662D), which appears hyperactive by several criteria both *in vitro* (Fig. 2) and *in vivo* (Figs 4 & 5), might display elevated resistance to rapamycin, compared to cells expressing wild-type Ypk1 or Ypk1(T662A).

To test these ideas, we examined the ability of the different alleles of *YPK1* that we constructed to restore to *ypk1Δ* cells a wild-type degree of rapamycin sensitivity. Expression of wild-type Ypk1 in *ypk1Δ* cells greatly reduced their sensitivity to the toxic effects of rapamycin, as judged by the pronounced reduction in the zone of killing in a halo bioassay (Fig. 6, left side; Table 2). As expected, based on the fact that the PDK1-site mutant is completely inactive both *in vitro* (Fig. 2) and in every physiological assay we applied (Figs 3–5), *ypk1Δ* cells expressing Ypk1(T504D) were just as sensitive to rapamycin-induced growth inhibition as *ypk1Δ* cells carrying the empty vector. Expression of the PDK2-site mutants in *ypk1Δ* cells did not reduce their

Fig. 6. Absence of PDK2 site phosphorylation and rapamycin resistance. A *ypk1Δ::HIS3* strain (YES3) was transformed with either an empty vector (pRS316, *CEN-URA3*) or the same vector expressing from its own promoter wild-type Ypk1 (pAM66), a PDK1 site mutant, Ypk1(T504D) (pAM87), a PDK2 site mutant, Ypk1(T662D) (pAM86) or a different PDK2 site mutant, Ypk1(T662A) (pAM97). The transformants were plated as a lawn on SCD-Ura plates and 10 μ l of a stock solution of rapamycin at either 500 $\text{ng } \mu\text{l}^{-1}$ (left) or 50 $\text{ng } \mu\text{l}^{-1}$ (right) were spotted onto sterile discs and placed on the lawn of cells. The plates were incubated at 30 °C for 3 days and photographed. The same *ypk1Δ::HIS3* strain was transformed with high-copy-number plasmids expressing from their own promoter wild-type Pkc1 (pFR22), a PDK1 site mutant, Pkc1(T983A) (pFR75) or a PDK2 site mutant, Pkc1(S1143A) (pFR74) and tested for rapamycin resistance in the same way.

Table 2. Relative rapamycin sensitivity

Plasmid	Halo diameter (mm)*			
	5.0 µg rapamycin		0.5 µg rapamycin	
Vector	38.0 ± 2	[1]	26.0 ± 2	[1]
YPK1	29.5 ± 1	0.77	18.5 ± 1	0.71
<i>YPK1</i> (T504D)	42.2 ± 4	1.11	27.4 ± 3	1.05
<i>YPK1</i> (T662A)	32.5 ± 1	0.85	20.9 ± 1	0.80
<i>YPK1</i> (T662D)	32.9 ± 1	0.86	20.5 ± 1	0.79
PKC1	35.0 ± 2	0.92	24.0 ± 2	0.92
<i>PKC1</i> (T983A)	37.7 ± 1	0.99	26.1 ± 1	1.00
<i>PKC1</i> (S1143A)	35.3 ± 2	0.93	24.3 ± 1	0.93

*For each of the two rapamycin concentrations tested, the value given in the left-hand column represents the mean of the halo diameter (in mm) determined in three independent experiments (error represents the range) and the value in the right-hand column is the relative sensitivity (normalized to the halo diameter seen for the *ypk1Δ* mutant carrying the empty vector).

rapamycin sensitivity as efficiently as normal Ypk1. Most strikingly, however, the degree of rapamycin sensitivity of the *ypk1Δ* cells expressing Ypk1(T662A) and Ypk1(T662D) was indistinguishable. This result is not consistent with the idea that Tor1- and/or Tor2-dependent phosphorylation at the PDK2 site in Ypk1 is a critical modification. However, the fact that both PDK2-site mutants displayed an intermediate level of sensitivity supports some role for PDK2-site phosphorylation that cannot be mimicked by negative charge alone.

Overexpression of Pkc1 also ameliorates the rapamycin sensitivity of *ypk1Δ* cells, but only rather modestly in comparison to Ypk1, whereas Pkc1 lacking its PDK1 site was unable to reduce the drug sensitivity of *ypk1Δ* cells at all (Fig. 6, right side). By contrast, overexpression of Pkc1 lacking its PDK2 site had an effect not detectably different from that of wild-type Pkc1, indicating that phosphorylation at the PDK2 site of Pkc1 is not essential for its function under these conditions, as also observed in other bioassays (Fig. 5).

DISCUSSION

In this study, we first examined the role of PDK1- and PDK2-site phosphorylation on the catalytic activity of Ypk1 recovered from yeast cell extracts and on its *in vivo* functions. We used the same approach to examine the requirement for the PDK1- and PDK2-phosphorylation sites in two other yeast AGC family members, Pkc1 and Sch9. In all three enzymes, absence of the conserved Thr in the PDK1 motif of the activation loop totally abrogated function. Unlike certain PDK1 targets in animal cells, such as PKB (Alessi *et al.*, 1996; Lawlor & Alessi, 2001), an acidic residue in place of the conserved Thr did not suffice to activate Ypk1. In fact, only authentic phosphorylation at the PDK1

site will suffice for activation of most of the mammalian AGC kinases that are PDK1 targets (Kobayashi & Cohen, 1999; Vanhaesebroeck & Alessi, 2000). Both Ypk1(T504A) and Ypk1(T504D) isolated from yeast were completely inactive *in vitro* and could not complement any of six different phenotypes of Ypk1-deficient cells, in accord with our previous results showing that GST-Ypk1(T504D) expressed in and purified from mammalian cells was not constitutively active and could not be phosphorylated and activated by purified Pkh1 (Casamayor *et al.*, 1999).

In marked contrast, altering the conserved phospho-acceptor residue in the PDK2 motifs of Ypk1, Pkc1 and Sch9 did not prevent any of these kinases from fulfilling its essential function in cell growth and viability. Thus, phosphorylation of the PDK2 site in Ypk1, Pkc1 and Sch9 cannot be required for the docking of Pkh1 (or Pkh2) and their subsequent phosphorylation of the PDK1 site in these enzymes. However, depending on the enzyme examined and the nature of the mutation introduced (Ala or Asp), PDK2-site phosphorylation seemed to have differential effects that reflect more subtle, but detectable, roles for modification at this motif in dictating which specific downstream signalling events can be engaged by these kinases.

The specific activity of Ypk1(T662D) isolated from yeast was reproducibly about twofold higher than that of Ypk1 itself or Ypk1(T662A), suggesting that Asp at this position may mimic phosphate and lead to increased catalytic activity. However, GST-Ypk1(T662D) purified from mammalian cells and activated by incubation with purified Pkh1 displayed only slightly higher activity than that of wild-type Ypk1 (Casamayor *et al.*, 1999), suggesting that a negative charge at the PDK2 site does not stimulate the intrinsic activity of the kinase. Another explanation for the higher activity of Ypk1(T662D) recovered from yeast might be that, *in vivo*, phosphorylation at both the PDK1 and PDK2 sites is undoubtedly reversed by the action of as yet uncharacterized phosphatases. Thus, the higher activity observed for Ypk1(T662D) could simply reflect the fact that the negative charge at the PDK2 motif in this mutant is insensitive to phosphatase action and/or somehow makes the P-Thr at the PDK1 site more resistant to dephosphorylation. If so, then it might be expected that Ypk1(T662D) might remain active for longer or have a higher degree of constitutive activity than wild-type Ypk1, whereas Ypk1(T662A) would not, as was observed. In this regard, Ypk1 isolated from yeast displays a series of isoforms that can sometimes be resolved (albeit poorly) upon standard SDS-PAGE. However, these species do not reflect or correspond to phosphorylation at the PDK1 site, the PDK2 site, or both, since the same pattern of multiple bands was observed even when both sites were mutated (either T504A T662A or T504D T662D). Likewise, these species do not result from autophosphorylation since a kinase-dead derivative (even one lacking both its PDK1 and PDK2 sites) was still multiply phosphorylated in a manner indistinguishable from normal Ypk1. Therefore, Ypk1 is the

target of an additional kinase(s) in the cell that remains to be identified. Among the candidate sites for phosphorylation by other kinases is another conserved sequence element in AGC family kinases, the so-called 'turn motif' (Gao & Newton, 2002), situated upstream of the PDK2 site (Fig. 1). In any event, to provide a means to detect PDK1 site- and PDK2 site-specific modification in Ypk1, we attempted, with the help of Dr Dario Alessi (University of Dundee), to raise sheep antibodies against corresponding phosphopeptides; regrettably, however, these reagents were not of sufficient titre and specificity to be useful (P. D. Torrance, unpublished results).

PDK2 site phosphorylation in Ypk1 cannot be an obligatory prelude to Pkh1-dependent phosphorylation of its PDK1 site because Ypk1(T662A) was just as active as Ypk1 *in vitro* and functioned as efficiently as wild-type Ypk1 in all (but one) of six different bioassays. Nonetheless, another possible explanation for the observed higher specific activity of Ypk1(T662D) compared to Ypk1 or Ypk1(T662A) might be that Pkh1 possesses the equivalent of the PIF pocket found in PDK1 and the negative charge at the PDK2 site in Ypk1(T662D) mimics phosphate and thereby permits it to bind with higher affinity to Pkh1. In fact, all of the residues in mammalian PDK1 that constitute its hydrophobic PIF pocket and the adjacent phosphate-binding site are conserved in yeast Pkh1 and Pkh2. Appropriate mutagenesis of Pkh1 and Pkh2 could address this possibility.

In five out of six physiological assays, Ypk1, Ypk1(T662A) and Ypk1(T662D) behaved equivalently, indicating that the status of phosphorylation at the PDK2 site had no significant effect on function. The one striking exception was under conditions (presence of the antibiotic myriocin) where sphingolipid production was inhibited. As described earlier, Pkh1 and Pkh2 are activated by phytosphingosine (Friant *et al.*, 2001), which is an intermediate in the synthesis of yeast sphingolipids and can be derived from their breakdown (Dickson & Lester, 1999b). In the absence of sphingolipids, Pkh1 and Pkh2 should be less active. Because Pkh1 and Pkh2 are the obligatory upstream activators of Ypk1, there will be less active Ypk1 under these conditions. Hence, the ability of Ypk1(T662D), but not Ypk1(T662A), to permit growth on myriocin-containing medium could merely reflect that its higher activity, observed *in vitro*, is also manifested *in vivo* and is above the threshold necessary for modification of critical Ypk1 substrates. On the other hand, wild-type Ypk1 was not detectably more active *in vitro* than Ypk1(T662A), yet was able to support growth on myriocin-containing plates as well or better than Ypk1(T662D). These observations raise the possibility that the role of phosphorylation at the PDK2 site (or a negatively charged residue as a mimic) may be to allow Ypk1 and Ypk2 to better compete with the recently discovered negative regulators of Pkh1 and Pkh2, Pil1 and Lsp1 (Zhang *et al.*, 2004) and thereby enhance their binding to and activation by Pkh1 and/or Pkh2.

In certain respects, the behaviour of Pkc1 with regard to the

requirement for phosphorylation at its PDK1 and PDK2 sites closely resembled Ypk1. The PDK1-site mutant, Pkc1(T983A), was unable to support the growth of *pkc1Δ* cells, whereas the PDK2-site mutant, Pkc1(S1143A), did. Our finding that PDK1-site phosphorylation is necessary for the essential function of Pkc1 is in accordance with previously published results (Inagaki *et al.*, 1999). Likewise, in wild-type cells, we found that overexpression of wild-type Pkc1 or Pkc1(S1143A), but not Pkc1(T983A), conferred elevated resistance to myriocin. However, in other respects, the roles of these sites in other aspects of Pkc1 action were not so straightforward. As we showed here, in *pkc1Δ* cells maintained on an osmotic support, phosphorylation at the PDK1 site seems to be dispensable for the ability of Pkc1 to confer myriocin resistance. If Ypk1 functions downstream of Pkc1 in this particular process, as suggested by the epistasis analysis we conducted, then even non-activatable Pkc1 somehow enhances Ypk1 activity, perhaps by titrating out negative factors or acting as an adaptor that promotes association of Ypk1 with Pkh1. There is some precedent for the latter suggestion in that the atypical PCKζ cooperates with PDK1 in the activation of p70 S6K (Romanelli *et al.*, 2002); and, similarly, it has been proposed that PCKζ acts as an adaptor by binding PKBγ and enhancing in some manner phosphorylation of its PDK2 site (Hodgkinson *et al.*, 2002).

On the other hand, the apparent epistasis relationship could be misleading. As we have shown previously, Ypk1 and Pkc1 contribute synergistically to the responses required for the maintenance of cell wall integrity (Roelants *et al.*, 2002). Thus, in the responses necessary to survive under conditions of limiting sphingolipids, Ypk1 and Pkc1 may also act in parallel, or Ypk1 may even act upstream of Pkc1. Hence, in cells lacking Ypk1, overexpressed Pkc1 may not be sufficiently potent by itself without the assistance of, or activation by, Ypk1 to confer myriocin resistance, whereas overexpressed Ypk1 itself is sufficient. At the mechanistic level, it is possible, in analogy to activation of mammalian PDK1 via binding of its PIF pocket to the phosphorylated (or otherwise negatively charged) PDK2 site in one of its substrates, that Ypk1 phosphorylated at its PDK2 site recruits and activates Pkh1 and thereby promotes subsequent Pkh1-mediated activation of Pkc1. Alternatively, in analogy to mammalian conventional PKC isozymes where PDK1-site phosphorylation is necessary although not sufficient for activity, but rather 'primes' these enzymes for subsequent activation by other activators (Dutil *et al.*, 1998), the proposed Ypk1-promoted Pkh1-dependent PDK1-site phosphorylation of Pkc1 might make it competent to be stimulated by its physiological activator, GTP-bound Rho1 (Kamada *et al.*, 1996; Nonaka *et al.*, 1995).

We have shown previously that expression of either Ypk1 or Ypk2 at its endogenous level is sufficient to rescue the cell lysis phenotype of *ypk1^{ts} ypk2Δ* cells at restrictive temperature or the inviability of *ypk1Δ ypk2Δ* cells (Roelants *et al.*, 2002; Torrance, 2000). By contrast, a *ypk1Δ* mutation alone

confers myriocin sensitivity, and overexpression of Ypk1 (but not Ypk2) is able to restore myriocin resistance. Thus, sensitivity to myriocin is not simply a reflection of a cell wall defect that increases permeability to this drug.

Unlike sensitivity to myriocin, and more like what we have observed for cell wall integrity maintenance, overexpression of either Ypk1 or Ypk2 restores *ypk1Δ* cells to the level of rapamycin resistance displayed by wild-type cells (F. M. Roelants, unpublished results). In *S. cerevisiae*, the targets of rapamycin are Tor1 and Tor2. Like mTOR, which is found in a complex that includes the scaffold protein, raptor (Kim & Sabatini, 2004), the yeast enzymes are also found in larger protein complexes, dubbed TORC1 and TORC2 (Loewith *et al.*, 2002; Wedaman *et al.*, 2003), and only TORC1, which contains the apparent raptor orthologue, Kog1, seems to be rapamycin-sensitive (Loewith *et al.*, 2002; Reinke *et al.*, 2004). Moreover, it has been reported that Ksg1 (the apparent PDK1 orthologue in *Schizosaccharomyces pombe*) phosphorylates Gad8 (the apparent orthologue of *S. cerevisiae* Ypk1) at the PDK1 site in the activation loop (Thr387), whereas *Schizosaccharomyces pombe* Tor1 is required for phosphorylation of Gad8 at its turn motif (Thr527) and at its PDK2 site (Thr546) *in vivo* (Matsuo *et al.*, 2003). Similarly, mTOR is able to phosphorylate p70 S6K in its turn and PDK2 motifs *in vitro* (Long *et al.*, 2004). Thus, it would be tempting to speculate that the reason why *ypk1Δ* cells are hypersensitive to rapamycin is because Tor1 (or Tor2) in the TORC1 complex may be necessary to phosphorylate the turn and/or PDK2 site in both Ypk1 and Ypk2 and, thus, in the presence of rapamycin, *ypk1Δ* cells behave as if they are deficient in both Ypk1 and Ypk2 activity. However, in the case of Ypk1, it seems improbable that TORC1-dependent modification of the PDK2 site per se is essential for Ypk1 function because Ypk1(T662A) and Ypk1(T662D) restored the same degree of rapamycin resistance. However, these mutants could still require TORC1-dependent phosphorylation at the turn motif for full activity and, hence, in the presence of rapamycin neither is as active as wild-type Ypk1, explaining why neither could confer the same high degree of rapamycin-resistance as wild-type Ypk1. Indeed, our observation that Ypk1 is phosphorylated at multiple sites other than at the PDK1 and PDK2 motifs is at least consistent with the possibility that phosphorylation at the turn motif may also occur and be important for function. We are currently exploring the role of the turn motif in Ypk1 and Ypk2 function via appropriate mutagenesis and other methods.

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