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.

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
Trp), situated C-terminal to the catalytic domain (Alessi et al., 1996; Kobayashi & Cohen, 1999). In PRK1/PKN, PRK2 and the atypical PKC isozymes, 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 (Balendra 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 isozymes (Kroner et al., 2000) phosphorylate the PDK2 site in PKBα, 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 PKCζ) – 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; Schmelzel 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 GATMCTGCTTFPEYLAPE MITCTPPD FQFESYAS 477 |
| SGK | 251 HSTTTTGCTTFPEYLAPE SIKRSYSS FLGSFYAPP 456 |
| P70S6K | 247 GTYHTTCTTGCTTFPEYLAPE TPDSSPDQ FLGSFYAP 416 |
| PKCζ | 405 GOTTTCTGCTTFPEYLAPE QDTOYSOY DPYGTPQR 557 |

### Yeast enzymes

| Ypk1 | 499 DDKDTTFCTGCTTFPEYLAPE KPIDSRVD PGWVTGVS 666 |
| Ykr2 | 496 DDKDTTFCTGCTTFPEYLAPE KPIDSRVD PGWVTI0D 663 |
| Pkc1 | 978 GNTTFFCTGCTTFPEYLAPE PFTLTPVP PFHFI0PD 1147 |
| Sch9 | 565 KORTNTFTGCTTFPEYLAPE QDMTATT FASFRVDV 741 |
| Gad8 | 382 TDKNTTFCTGCTTFPEYLAPE KPIDSRVD PAKYGRPR 550 |

**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: PKBα, NP 005154.1; SGK, NP 005618.2; p70 S6K, NP 003152.1; PKCζ, NP 002735.2; Ypk1, CAA1967.1; Ypk2, CAA89740.1; Pkc1, CAA84932.1; Sch9, NP 012075.1; Gad8, CAB76216.1.
Pkcl is another demonstrated target of Pkh1 and Pkh2 (Friant et al., 2001; Inagaki et al., 1999). Pkcl 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 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 mirosin (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 Fpu 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), YEp352 GAL-YPK1 (pAM75) and YEp352 GAL-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 BamHI and HindIII, gel purified and ligated into BamHI/HindIII-linearized vector pHRS351, yielding a CEN-containing, LEU2-marked plasmid, pHRS315-YPK1 (B/H) (pAM11). A ~4 kb Xbal–SalI fragment from pHRS315-YPK1 (B/H) was gel-purified and ligated to an Xbal/SalI-linearized pRS316 to create CEN-containing, URA3-marked plasmid, pRS316-YPK1(X/S) (pAM66).

A ~1.1 kb ClaI–Kpn1 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′-CAATTTGTTGCTGGCACTGGAAAATG-3′ (Toda et al., 1999) and 5′-CTTTTTCAACGATTGGCCACCACAATTTG-3′ (Toda2 et al., 1999), 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 Norl–SalI fragment from pAM66 and pAM97 or a Norl–Kpn1 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 YEp352 GAL-YPK1-myc (pAM76) as the template, Quick-change PCR mutagenesis was performed with the following oligonucleotides: 5′-GATAAGACAGATGATTACGTTGGAAATG-3′ (YPK1-C) and 5′-GGGGTCCCACAAAAAATCATCTGCTTATCAT-3′ (YPK1-D), which span the Thr504 codon of YPK1 (underlined nucleotides represent mismatches which substitute Asp for Thr504). The resulting plasmid is YEp352 GAL-YPK1-(T504D)-myc (pAM90). Using the same template and oligonucleotides 5′-CAATTTGTTGCTGGCACTGGAAAATG-3′ and 5′-CAATTTCAACGATTGGCCACCACAATTTG-3′ (YPK1-E), which span the Thr662 codon of YPK1 (underlined nucleotides represent mismatches which substitute Asp for Thr662), the resulting plasmid was constructed to overexpress derivatives tagged with a c-Myc epitope.

**Table 1.** _S. cerevisiae_ strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPH499</td>
<td>MATα ade2-101α his3-D200 leu2-Δ1 lys2-801α trp1-Δ1 ura3-52</td>
<td>Sikorski &amp; Hieter (1989)</td>
</tr>
<tr>
<td>YPT40</td>
<td>YPH499 ypk1-1α::HIS3 ypk2Δ::TRP1</td>
<td>Casamayor et al. (1999)</td>
</tr>
<tr>
<td>YES3</td>
<td>YPH499 ypk1Δ::HIS3</td>
<td>Roelants et al. (2002)</td>
</tr>
<tr>
<td>PKC1/pkc1</td>
<td>BY4743 PKC1/pkc1Δ::KanMX</td>
<td>Research Genetics, Inc.</td>
</tr>
<tr>
<td>pck1α::LEU2</td>
<td>YPH499 pck1α::LEU2</td>
<td>Fields (1991)</td>
</tr>
<tr>
<td>YFR55+</td>
<td>YPH499 schρ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>MWY347</td>
<td>MATα ura3 leu2 ade8 ck25-5*</td>
<td>Toda et al. (1988)</td>
</tr>
</tbody>
</table>

*Derived from YPH499 by transformation with the 4.7 kb _PvuII_ fragment of _psch9::HIS3_ (Toda et al., 1988).
Yepl352GAL-YPK1-(T662D)-myc (pAM91). Using Yepl352GAL-
YPK1-(T504D)-myc as the template, Quick-change PCR was
performed with the oligonucleotides YPK1-E and YPK1-F. The
resulting plasmid was Yepl352GAL-YPK1-(DD)-myc (pAM92). Using
Yepl352GAL-YPK1-myck as the template, Quick-change PCR muta-
genesis was performed with oligonucleotides T662A1 and T662A2. The
resulting plasmid was Yepl352GAL-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 PstI
non-specifically to the solid support and

Preparation of cell extracts and immunoblot analysis. Yeast cells were grown at 30°C to mid-exponential phase (OD600 = 0.5–1),
either in SC medium with appropriate supplements for maintenance
of plasmids or in rich medium (YPGlc). For galactose induction
of plasmids or in rich medium (YPGlc). For galactose induction
was used to centrifuge at 30,000 × g for 10 min in a microfuge and the supernatant frac-
tion was washed with ice-cold 1 % (v/v) 2-mercaptoethanol,
1 % Triton X-100, 5 mM EDTA, 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

For immunoblot analysis, samples (50 µg total protein) were diluted
into SDS-PAGE sample buffer (Laemmli, 1970), subjected to elec-
phoresis in an 8–12 % gel and transferred to nitrocellulose (Towbin
et al., 1979). To detect proteins using ascs fluid containing the anti-
c-Myc mAb 9E10 (Evan et al., 1985), a dilution of 1 : 10,000 was used.

Immuno precipitation 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 frac-
tion 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 micro-
fuge, washed three times (1 ml each) with ice-cold IP buffer and
collected by centrifugation for 1 min in a microfuge on maximum
speed. 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 Raffinose 0-6, 
induced by addition of galactose (2 % final concentration) and incu-
bated 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 mM [γ⁻³²P]ATP (200–400 C.P.M. pmol⁻¹)
and 100 µM Cross-tide (GRPRTSSFAEG) (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
Because Ypk1 and Ypk2 are activated both contain both the conserved PDK1 and PDK2 motifs (Fig. 1). of by Pkh1 and Pkh2, respectively, and because the inviability bation at 30°C for 2 days the plates were photographed.

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 ~2×10^6 exponentially growing cells rapidly with 3 ml molten (55°C) 1% agar and pouring immediately onto plates containing an appropriate medium. Equal volumes (10 µl) of rapamycin stocks (50 ng µl⁻¹, 500 ng µl⁻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 pkh1Δ pkh2Δ 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 Vιb (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.
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 autophosphorylation because, when expressed in ypkl Δ 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−/− 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−/− 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−/− 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 Pck1. Normal Pck1, or a mutant lacking the PDK1 site, Pck1(T983A), or a mutant lacking the PDK2 site, Pck1(S1143A), each expressed under control of the authentic PKC1 promoter from a high-copy-number (2 μm DNA) plasmid, were introduced into a heterozygous pck1Δ/ PKC1 diploid. After sporulation and tetrad dissection, the ability of normal Pck1, Pck1(T983A) or Pck1(S1143A) to rescue the inviability of the pck1Δ spores was compared (Fig. 3b). For diploids transformed with the plasmid expressing normal Pck1, most of the tetrads displayed three or four viable spores, showing that plasmid-borne PKC1 readily rescued the inviability of pck1Δ spores (which were also marked by the insertion of a resistance marker, kbmX, to the antibiotic G418). For the diploids transformed with the plasmid expressing Pck1(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 Pck1. In marked contrast, in the diploids transformed with the plasmid expressing the PDK2-site mutant, Pck1(S1143A), nearly all of the tetrads displayed three or four viable spores, demonstrating that the full biological function of Pck1 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 Pck1, 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 cdc25Δ 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 Pck1, 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−/− 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).
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 the effect on myriocin resistance was assayed.

Fig. 3. Phosphorylation at the PDK2 site is dispensable for the growth-promoting activities of Ypk1, Pkc1 and Sch9. (a) A temperature-sensitive strain, ypk1ΔYpk2Δ (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 pck1Δ::KanMX/PKC1 diploid strain was transformed with either an empty CEN-based LEU2-marked plasmid or with the same 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.

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 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), ypk1Δ::HIS3 cells (YES3) or pkc1Δ::LEU2 cells were transformed with either a high-copy-number (2 \( \mu \)m) 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 ypk1Δ::HIS3 transformants were plated on SCD-U medium or SCD-U containing 1 mM myriocin and growth was assessed after 3 days at 30 °C. The pkc1Δ::LEU2 transformants were plated on SCD-U medium containing 1 M sorbitol or SCD-U medium containing 1 M sorbitol and 1 mM 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 pck1Δ 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) (pAM66) or a different PDK2 site mutant, Ypk1(T662A) (pAM97). The transformants were plated as a lawn on SCD-Ura plates and 10 μl of a stock solution of rapamycin at either 500 ng ml⁻¹ (left) or 50 ng ml⁻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.
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 phosphoacceptor 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Halo diameter (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0 μg rapamycin</td>
</tr>
<tr>
<td>Vector</td>
<td>38.0±2</td>
</tr>
<tr>
<td>YPK1</td>
<td>29.5±1</td>
</tr>
<tr>
<td>YPK1(T504D)</td>
<td>32.5±1</td>
</tr>
<tr>
<td>YPK1(T662A)</td>
<td>32.9±1</td>
</tr>
<tr>
<td>YPK1(T662D)</td>
<td>32.9±1</td>
</tr>
<tr>
<td>PKC1</td>
<td>35.0±2</td>
</tr>
<tr>
<td>PKC1(T983A)</td>
<td>37.7±1</td>
</tr>
<tr>
<td>PKC1(S1143A)</td>
<td>35.3±2</td>
</tr>
</tbody>
</table>

*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 Ypk1Δ mutant carrying the empty vector).
Phosphorylation of yeast AGC protein kinases

merely reflect that its higher activity, observed to permit growth on myriocin-containing medium could hence, the ability of Ypk1(T662D), but not Ypk1(T662A), Ypk1, there will be less active Ypk1 under these conditions. Pkh1 and Pkh2 are the obligatory upstream activators of breakdown (Dickson & Lester, 1999b). In the absence of wild-type Ypk1 was not detectably more activity in vivo manifested significant effect on function. The one striking exception was status of phosphorylation at the PDK2 site had no signifi-
cation at the PDK2 site (or a negatively charged residue as a 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 signifi-
cant 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 isoforms where PDK1-site phosphorylation is necessary although not sufficient for activity, but rather ‘primed’ 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 ypk1Δ 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 (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 PDKen site in the activation loop (Thr387), whereas Schizosaccharomyces pombe Tor1 is required for phosphorylation of Gad8 at its turn motif (Thr527) and at its PDKen site (Thr546) in vivo (Matsuo et al., 2003). Similarly, mTOR is able to phosphorylate p70 S6K in its turn and PDKen 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 PDKen 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 PDKen 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 PDKen and PDKen 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.

ACKNOWLEDGEMENTS

This work was supported by NCI Postdoctoral Traineeship CA09041 (to F.M.R.), by National Institutes of Health Predoctoral Traineeship GM07232 and a National Science Foundation Predoctoral Fellowship (to P.D.T.), by NIH Research Grant GM21841 (to J.T.) and by facilities provided the Berkeley campus Cancer Research Laboratory.

REFERENCES


phosphoinositide 3-kinase through the protein kinase PDK1. Science 281, 2042–2045.


Nojima, H., Tokunaga, C., Eguchi, S. & 7 other authors (2003). The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates, p70 S6 kinase and 4E-BP1, through their TOR signaling (TOS) motif. J Biol Chem 278, 15461–15464.


